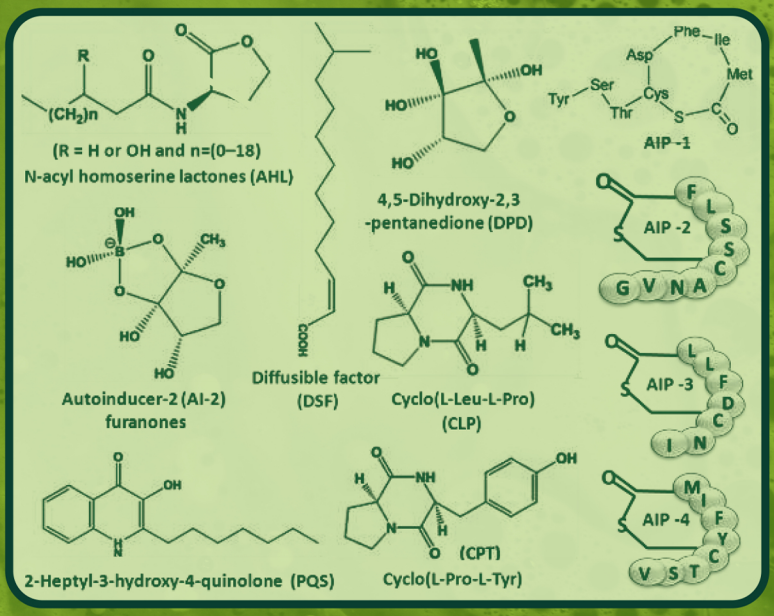
ENVIRONMENTAL TOXICITY OF NANOMATERIALS



EDITED BY
Vineet Kumar • Nandita Dasgupta
Shivendu Ranjan

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Environmental Toxicity of Nanomaterials



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Preface

This book is a comprehensive reference book containing in-depth information on nanoecotoxicity and its implication in various disciplines of sciences. The chapters focus on the causes and prevention of toxicity induced by various nanomaterials. This book foresights the safe utilization of nanotechnology, so that the tremendous prospective of nanotechnology does not harm living beings and environment. Nanomaterials leach from nanomaterial-containing products and contaminate the basic components of environment, air, water, and soil. Every living organism, including terrestrial, aquatic, and amphibians, is in continuous contact with the physical components of environment. Further, advances in the synthesis of nanomaterials leading to desired size, shape, and surface properties will increase their burden on the environment.

At present there is complete uncertainty regarding toxicity behavior of nanomaterials. There is no clarity how nanomaterials will behave once in complex environment. The future of nanomaterials in various industries depends upon their impact on environment and ecosystem. This book critically describes all these aspects of nanotoxicity in detail. The book includes an introduction to nanoecotoxicity, various factors affecting toxicity of nanomaterials, various factors that can impart nanoecotoxicity, various studies in the area of nanoecotoxicity evaluation, and the future risk assessment strategies.

The book contains contribution from international experts and will be a valuable resource for undergraduate and graduate students, doctoral and postdoctoral scholars, industrial personnel, academicians, scientists, researchers, and policy makers from different nanotechnology-associated industries. The book will be beneficial for graduate students to understand the detailed concept of nanoecotoxicology. The book will be beneficial to doctoral and postdoctoral scholars as they can learn the basics of techniques, recent advancements, challenges, and opportunities in this field. This book will provide critical and comparative data to nanoecotoxicologists, and thus it will be beneficial for scientists and researchers working in this field. This book will also be beneficial for academicians to give the basics of nanoecotoxicology as many universities throughout the world have nanobiotechnology as a subject that cannot be completed without discussing nanoecotoxicology.

Once in environment, nanomaterials will affect you.

—Vineet Kumar

Dedicated to those who are suffering because of hazardous materials.

—Dr. Nandita Dasgupta and Dr. Shivendu Ranjan



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1

Toxic Effects of Nanomaterials on Environment

Rajeev Kumar, Moondeep Chauhan, Neha Sharma, and Ganga Ram Chaudhary

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1.1 Introduction

According to the definition given by the US National Nanotechnology Initiative, nanotechnology may be defined as understanding and control of matter at dimensions of roughly 1–100 nm, where unique phenomena enable novel applications. At this level, the physical, chemical, and biological properties of materials differ in fundamental and valuable ways from the properties of individual atoms and molecules or bulk matter. This means that at least one dimension in the approximate range of 1–100 nm and difference in the properties of matter from that of its bulk form are the two fundamental criteria which must be satisfied in order to consider a material as nanomaterial. This definition is extensively broad under which different materials are covered, and undoubtedly nanotechnology has origins, significance, and application in different fields such as agriculture, aerogels, aerospace, automotive, catalysts, coatings, paints and pigments, composites, construction, cosmetics, electronics, optics, energy, environmental remediation, filtration and purification, food products, medical, packaging, paper and board, plastics, security, sensors, and textiles, and research is underway on

many new applications. Hence, nanotechnology is generally defined as a cross disciplinary technology (Foss Hansen et al. 2007).

Similar to conventional substances, it is now known that some nanomaterials may be hazardous, and thus demand for standardization of the term *nanomaterial* and various other terms related to nanotechnology has increased. Many countries and standardization organizations have developed working definitions to identify nanomaterials based on the size of the material, its novel properties, or a combination of both, depending on their scope and the type of applications. For example, according to the International Organization for Standardization (ISO 2010), nanomaterial may be defined as "material with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale," where nanoscale is "length ranging from approximately 1 nm to 100 nm" (Saner and Stoklosa 2013). The European Union defines nanomaterial as a "natural, incidental or manufactured material containing particles, in an unbound state or as aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1nm–100 nm. In specific cases where concerns exist for environment, health, safety or competitiveness they provide exception that number size distribution threshold of 50% may be replaced by a threshold between 1% and 50%." The emphasis in the definition on external dimensions may exclude materials with an internal structure (e.g., porous materials with relatively large internal surface area) or materials with a surface structure at the nanoscale. Thus, it is becoming clear that many parameters other than size modulate risk, including particle shape, porosity, surface area, and chemistry. Some of these parameters become more relevant at smaller scales—but not always. The transition from "conventional" to "unconventional" behavior, when it does occur, depends critically on the particular material and the context. A "one size fits all" definition of nanomaterials will fail to capture what is important for addressing risk (Maynard 2011).

Nanomaterials can be classified into different types on the basis of their source, dimensions, and chemical composition and their potential toxicity level (Dolez 2015). Erupting volcanoes, breaking sea waves, forest fires, sand storms, and soils are some of the major natural sources of inorganic nanomaterials. Some nanomaterials such as ferritin, calcium hydroxyapatite, biogenic magnetite, and ferromagnetic crystalline are naturally found in living organisms and thus are an organic source of nanomaterials. Some nanomaterials are unintentionally produced as by-products of human activity such as internal combustion engines, power plants, incinerators, jet engines, metal fumes (smelting, welding, etc.), polymer fumes, heated surfaces, food transformation processes (baking, frying, broiling, grilling, etc.), and electric motors. Finally, nanomaterials are now manufactured using a large diversity of chemical constituents, for example, metals, semiconductors, metal oxides, carbon, and polymers. There are some nanomaterials designed for specific functionalities and can be surface treated or coated. They come in a large variety of forms, such as spheres, wires, fibers, needles, rods, shells, rings, plates, and coatings, as well as in more exotic flower-like designs. Compared to natural and incidental nanomaterials, manufactured nanomaterials are characterized by their controlled dimension, shape, and composition.

On the basis of dimensionality, nanomaterials can be categorized as zero-dimensional (0D), one-dimensional (1D), two-dimensional (2D), and three-dimensional nanomaterials (3D). Zero-dimensional nanomaterials have all the three external dimensions at the nanoscale (i.e., between 1 and 100 nm), for example quantum dots and metal

oxide nanoparticles (NPs). 1D nanomaterials have two external dimensions at the nanoscale, the third one being usually at the microscale such as nanofibers, nanotubes, nanowires, and nanorods. With only one external dimension at the nanoscale, 2D nanomaterials comprise thin films, nanocoatings, and nanoplates. The last dimensional category of nanomaterials, 3D nanomaterials, also termed as bulk nanomaterials, display internal nanoscale features but no external dimensions at the nanoscale. This includes nanocomposites and nanostructured materials.

On the basis of potential toxicity, nanomaterials can be categorized as fiber-like NPs; biopersistent granular NPs; CMAR NPs (carcinogenic, mutagenic, asthmagenic, reproductive toxin); and liquid and soluble NPs. On the basis of chemical composition, nanomaterials can be classified as carbon-based nanomaterials, metal-based nanomaterials, dendrimers, and composite nanomaterials. Carbon-based nanomaterials are composed mostly of carbon. This classification includes fullerenes, carbon nanotubes, graphene, and the like. Metal-based nanomaterials are materials made of metallic NPs such as gold, silver, and metal oxides; for example, titanium dioxide (TiO_2) NPs are extensively used in applications such as paint, sunscreen, and toothpaste. Dendrimers are nanosized polymers built from branched units. They can be functionalized at the surface and can hide molecules in their cavities. A direct application of dendrimers is for drug delivery. Composite nanomaterials contain a mixture of simple NPs or compounds such as nanosized clays within a bulk material. The NPs give better physical, mechanical, and/or chemical properties to the initial bulk material. Nanotechnology is one of fastest developing business sectors, as 380 billion dollars of worldwide market was reported for year 2013, which is expected to reach 950 billion dollars by 2020 (Dolez 2014). Approximately $2.6 \times 10^5 - 3.09 \times 10^5$ metric tons of global nanomaterials was estimated to be produced in 2010 (Keller 2013). The nanotechnology Consumer Products Inventory (CPI), which documents the marketing and distribution of nanotechnology-related products into the commercial market place, currently lists 1814 user products (30 times increase in number of nano-enabled products in relation to 54 products which were listed originally in 2005) from 622 firms located in 32 different countries. Although, according to CPI, an increase in number may not completely represent market growth as methodology evolved over time, a stable progress of the registered nanotechnology-related products indicated that the popularity of nanotechnology has increased constantly. The Health and Fitness category was reported to have the largest listed products (762), followed by automotive (152), cross cutting (95), food and beverages (72), electronics (70), appliances (39), and goods for children (23). Within the health and fitness category, personal care products (e.g., toothbrushes, lotions, and hairstyling tools and products) were reported to include the biggest subcategory (39% of products).

In the nanomaterial composition group, metals and metal oxides were maximum advertised and were reported to be registered in 37% of products. On a mass basis, TiO_2, silicon dioxide, and zinc oxide were the most produced nanomaterials. However, silver NPs which were only 2% of TiO_2 (on a mass basis) were present in 438 products (24%), thus the most popular advertised nanomaterials in the CPI. About 29% of the CPI (528 products) contained nanomaterials were suspended in a variety of liquid media (e.g., water, skin lotion, oil, car lubricant), and solid products with surface-bound NPs (e.g., hair curling and flat irons, textiles) was the second largest group with 307 products (Vance et al. 2015). The Nanodatabase, which is another inventory of nano-enabled products in the European market, presently enlists 2231 products.

According to the Nanodatabase, majority of the products belong to the health and fitness category (55%), followed by home and garden (21%) and automotive (12%). One thousand two hundred twelve nano-enabled products were originally reported in 2012 which increased to more than 2200 in 2015. As per the Nanodatabase report, 10–25 products are added per week, the reason being increased marketing and uses and applications of nanomaterials (Hansen et al. 2016).

Thus, as the applications of nanomaterials are continuously increasing, their quantity in the environment keeps on increasing. In spite of the innumerable number of evident benefits of nanomaterials, there are some serious concerns about how the nanomaterials used in various applications may interact with the environment. There are significant arguments regarding the adverse effects of nanomaterials on the environment with the potential to cause toxicity to humans and other living organisms. Thus, it is important for nanotoxicology to investigate the effect of nanomaterials to the environment, so the potential damage could be avoided.

1.2 Risk and Hazard of Exposure to Nanomaterials

According to the US Environmental Protection Agency (USEPA), *hazard* may be defined as the "inherent toxicity of a compound." According to this definition, if a chemical substance has the property of being toxic, it is therefore hazardous. Any exposure to a hazardous substance will consequently lead to an adverse health effect or even death for the individual. Hence, hazard may be thought of as the consequence of an event occurring, such as the consequence for an individual being exposed to a toxic or hazardous substance. USEPA defines risk as "a measure of the probability that damage to life, health, property, and/or the environment will occur as a result of a given hazard." If the probability of an event occurring is high and the consequences are significant, the risk is considered to be high. However, human health risks are considered to be high, if the hazard or consequence is adverse health effects, even though the probability of occurrence is low. It is therefore important to consider both the frequency of the event and the degree of severity of the consequences, if the event were to take place. Risks, unlike hazards, can be managed and minimized. Risk may be classified into two categories, known/identified risks and hypothetical/potential risks, depending upon a cause-and-effect relationship. When the relation between a cause and an effect is established, we talk of known or identified risks. The responsibility of such risk can generally be attributed. When the causal relation is established, prevention can be applied. When the relation between a cause and a damage is not well established, we talk of hypothetical or potential risks (Helland 2004; Hristozov and Malsch 2009).

Exposure is a combination of the concentration of a substance in a medium multiplied by the duration of contact. Dose is the amount of a substance that enters a biological system and can be measured as systemic dose, the total amount taken up by the biological system, or the amount in a specific organ (skin, lung, liver, etc.). The likelihood that a hazardous substance will cause harm (the risk) is the determinant of how cautious one should be and what preventative or precautionary measures should be taken. Assessing the risks imposed by the use of nanomaterials in commercial products and environmental applications requires a better understanding of their mobility, bioavailability, and toxicity. For nanomaterials to comprise a risk,

there must be both a potential for exposure and a hazard that results after exposure (Nowack and Bucheli 2007).

As more products containing nanomaterials are developed, there is greater potential risk for exposure of human and the environment to nanomaterials. The environment and humans may be exposed to nanomaterials throughout all stages of their life cycle, starting from production, storage, and transport to use and disposal. Releases of nanomaterials to the environment can be purposeful or deliberate such as remediation of contaminated lands or use of iron NPs to remediate groundwater as well as unintentional release due to wear and tear of materials containing nanomaterials. Emissions of nanomaterials to the environment may also occur by accidental spills during production or transportation and when products are disposed of at the end of their use phase. Release of NPs may come from point sources such as production facilities, landfills, or wastewater treatment plants or from nonpoint sources such as wear from materials containing nanomaterials.

Regardless of whether nanomaterials are released intentionally or unintentionally, deliberately or accidentally, directly or indirectly, they all will end up in air, water, or soil and may result in direct exposure to humans via skin contact, inhalation, and direct ingestion of contaminated drinking water or plants or animals which have accumulated nanomaterials (Brook 2002). Upon emission into the environment, the behavior and distribution of nanomaterials will be determined by the intrinsic properties of the nanomaterial as well as the specific environmental conditions. Assessing the risks imposed by the use of nanomaterials in commercial products and environmental applications requires a better understanding of their mobility, bioavailability, and toxicity. Therefore, in order to determine the extent of environmental exposure to nanomaterials, it is necessary to understand their behavior in the environment.

1.3 Fate and Behavior of Nanomaterials in the Environment

The fundamental properties concerning the environmental fate of nanomaterials are not well understood, as there are few available studies on the environmental fate of nanomaterials. The following sections discuss about the fate of nanomaterials in air, soil, and water (USEPA 2007).

1.3.1 Fate and Behavior of Nanomaterials in Air

The natural sources of nanomaterials in the atmosphere include volcanic eruptions, forest fires, hydrothermal vent systems, physical and chemical weathering of rocks, precipitation reactions, and biological processes. However, the natural background of nanomaterials in the atmosphere is low compared to the levels caused by releases of nanomaterials in the ambient air resulting from the manufacture of nanomaterials, the handling of NPs as aerosols (such as nanotubes), cleaning and conditioning of production chambers (compression, coating, and composition), road traffic, and stationary combustion sources (Biswas and Wu 2005). It has been assessed that the amount of incidental nanomaterials in the atmosphere due to human activity is more than 36% of the total particulate concentrations, and the forecast for the years ahead is that there will be a strong increase in atmospheric nanomaterials due to the activity in industries related to the use of nanomaterials (Farré et al. 2011). Atmospheric

nanomaterials have three major sources: (1) primary emission, which refers to those that are openly released from road traffic exhaust and industrial combustion; (2) secondary emission, which refers to those that are produced in the atmosphere from the compression of low volatility vapors produced from the oxidation of atmospheric gases; and (3) formation at the time of diesel exhaust dilution. A large number of nanomaterials present in the urban environment can be attributed to urban vehicular traffic and emissions from stationary sources. These are essentially primary pollutants with distinct source-related properties. However, once released, nanomaterials, because of their very large surface areas, chemically interact with other pollutants already present in the ambient air or with solar radiation, thereby creating secondary nanomaterials with properties significantly different from those of the primary pollutants (Shi et al. 2001). It is this ever-changing nature of nanomaterials that makes them difficult to identify and quantify. Daughton (2004) eloquently referred to both the parent NPs and their transformation products as "structurally undefinable ubiquitous xenobiotics." The higher mobility of nanomaterials in the environment indicates greater potential for exposure because these particles are dispersed over longer distances from their origin (Wiesner et al. 2008). As a result, they may pose respiratory hazards on inhalation exposure.

The fate of nanomaterials in the air is determined by the duration of time particles remain airborne, their interaction with other particles or molecules in the atmosphere, and the distance they are able to travel in the air before deposition. The processes important to understanding the dynamics of nanomaterials in the atmosphere are diffusion, agglomeration, wet and dry deposition, and gravitational settling. These processes are relatively well understood for ultrafine particles (aerosols); knowledge can be applied to nanomaterials as well (Wiesner et al. 2006). In some cases, however, intentionally produced nanomaterials may behave quite differently from incidental ultrafine particles, especially when the latter cannot agglomerate because they are coated. In addition, there may be differences between freshly generated and aged nanomaterials.

Particles in the lower end of the size range of 1–100 nm will be governed by other transport processes than those in the higher end (Mädler and Friedlander 2007). For particles in the micrometer scale, inertial and gravitational forces dominate. With decreasing particle size, diffusional forces dominate and particle behavior is more like a gas or vapor. The particle diffusion in air is governed by Brownian motion, and the rate of diffusion is inversely proportional to particle diameter, while the rate of gravitational settling is proportional to particle diameter. Particles with high diffusion coefficients (such as those in the nanoscale) therefore have high mobility and will mix rapidly in aerosol systems. This increased particle mobility in air at the nanoscale is important for the transformation processes since the rate of agglomeration is governed primarily by particle mobility and number concentration, both of which increase as particle size decreases. Thus, "aerosolized" NPs may agglomerate rapidly, even at a low mass concentration (Aitken 2004). With respect to the period that particles remain airborne, particles can generally be classified into three groups: Small particles (diameters <80 nm) are described as being in the agglomeration mode; they are short-lived because they rapidly agglomerate to form larger particles. Large particles (>2000 nm, beyond the discussed <100 nm nanoscale range) are described as being in the coarse mode and are subject to gravitational settling. Intermediate-sized particles (>80 and <2000 nm, which include particle sizes outside the discussed

<100 nm nanoscale range) are described as being in the accumulation mode and can remain suspended in air for the longest time, days to weeks, and can be removed from air via dry or wet deposition (Bidleman 1988; Preining 1998; Spurny 1998).

The deposition of particles is dependent on the gravitational settling velocity, which is proportional to the diameter of the particle. As a consequence hereof, smaller NPs in air will deposit at a much slower rate than will larger particles. Agglomeration will therefore significantly increase the deposition of nanomaterials. Note that these generalizations apply to environmental conditions and do not preclude the possibility that humans and other organisms may be exposed to large as well as smaller particles by inhalation. Deposited NPs are typically not easily resuspended in the air or reaerosolized (Colvin 2003).

Nanomaterials suspended in air will most likely be exposed to sunlight, especially to ultraviolet (UV) wavelengths of light, to a much larger degree than for the other environmental compartments. This increases the possibility for photochemical transformations depending on the nanomaterial in question. Nanomaterials are also known to readily adsorb a variety of materials, and many act as catalysts (Wiesner et al. 2006). Therefore, the processes of the highest relevance to include in the air compartment of a fate model for nanomaterials are photochemical reactions, agglomeration, and deposition. Even though the fate processes for ultrafine particles in air are well described, there are still some major issues to be addressed with regard to disclosing the processes governing behavior, transport, and fate of airborne nanomaterials (Stone et al. 2010).

1.3.2 Fate and Behavior of Nanomaterials in Water

Nanomaterials are introduced into the aquatic environment by both point sources such as direct discharge of wastewater containing nanomaterials into surface water and nonpoint sources such as surface runoff and atmospheric deposition (Weinberg et al. 2011). Interestingly, nanotechnology can pollute water resources and can be used for wastewater treatment by employing various techniques such as nanoadsorption, nanodegradation, and nanofiltration. Understanding the environmental implications of nanomaterials is an evolving process, and their direct application in the environment should be undertaken with utmost caution. Consumer products, such as various household appliances, containing nanomaterials can potentially leach out their nanomaterials in water (Kägi et al. 2008; Mueller and Nowack 2008). Nanomaterials used in environmental remediation operations generate waste containing residual NPs that ends up as wastewater and also reaches other environmental compartments such as soil, sediment, and surface water (Brar 2010).

Fate of nanomaterials in aquatic environments is controlled by aqueous solubility, reactivity of the nanomaterials with the chemical environment, and their interaction with certain biotic and abiotic processes. Solubility of nanomaterials in water is more than their bulk counterpart due to the bigger surface area. This effect is described by the Thomson–Freundlich relation (Müller 2007). Even nanomaterials which are insoluble in aqueous media such as TiO_2 and carbon nanotubes may act like a solution because of their ability to form metastable aqueous suspensions and which significantly increases the transport and distribution of the material within the environment (Mackay et al. 2006). Hyung et al. (2007) reported in their study that aqueous stability of multiwalled carbon nanotubes were enhanced due its interaction with natural

organic matter and multiwalled carbon nanotubes were readily dispersed as an aqueous suspension. Sea surface microlayers consisting of lipid, carbohydrate, and proteinaceous components along with naturally occurring colloids made up of humic acids, may have the potential to sorb NPs and transport them in aquatic environments over long distances (Moore 2006). These interactions will delay the NPs removal from the water column.

The presence (quantity as well as quality) of natural organic matter may have a high impact on the transformation processes governing the fate and behavior of nanomaterials in water. Due to their high surface-area-to mass ratios, nanomaterials have the potential to readily sorb to soil and sediment particles, where these soil and sediment particles are subject to sedimentation and consequently are more liable to removal from the water column (Oberdörster et al. 2005). Nanomaterials can undergo biotic and abiotic degradation such as hydrolysis and photocatalysis, which can remove them from the aquatic environment. Nanomaterials in the upper layers of aquatic environments are exposed to sunlight and can undergo light-induced photoreactions. These reactions in aquatic environments may change the physical and chemical properties of nanomaterials and can account for the removal of certain nanomaterials, thus altering their behavior.

Certain organic and metallic nanomaterials may possibly be transformed under anaerobic conditions, such as in aquatic (benthic) sediments. From past studies, it is known that several types of organic compounds are generally susceptible to reduction under such conditions (Nurmi et al. 2005). Therefore, in the aquatic environment nanomaterials may interact with natural organic matter, natural colloids, and suspended particulate matter (PM), resulting in aggregation and potentially sedimentation from solution. Sedimentation and aggregation may represent a pathway for the carrying of NPs from the water column to benthic sediments (Tiede et al. 2016). Complexation by natural organic materials such as humic colloids can facilitate reactions that transform metals in anaerobic sediments. NPs in the aquatic environment are bioaccumulated by deposit and filter feeding organisms. Thus, fate of nanomaterials in the aquatic environment can be influenced by different processes, such as aggregation and disaggregation, diffusion, interaction between NPs and natural water components, transformation, biotic and abiotic degradation, and photoreaction.

For almost all processes in water previously mentioned, the general water chemistry plays a major role for the extent and rate at which they participate in transforming the nanomaterials. In general, the pH of the water is of high importance for processes such as redox reactions, dissolution, sorption, and agglomeration/aggregation. If the pH is close to the so-called point of zero charge, the zeta potential approaches zero and the particles will not be stable in suspension. This will typically mean that an agglomeration process, leading to sedimentation of larger agglomerates, has begun. Also, the ionic strength has a huge influence on processes such as dissolution, speciation, aggregation, and sorption. For example, the higher ionic strength of salt water compared to fresh water may lead to higher agglomeration which in turn can be an onset to limited dissolution and increased sedimentation. However, not only will the magnitude of the ionic strength influence agglomeration, but also the identity of the individual ions contributing to the ionic strength has been shown to influence agglomeration. For example, the presence of divalent cations such as Ca^{2+} and Mg^{2+} will have a stronger impact on agglomeration than the presence of Na^{+} and K^{+}. Also, the identity of anions present in water may influence the transformation processes

dependent on the engineered nanomaterial in question. For silver NPs, the presence or absence of chloride will determine the formation of complexes and precipitates in water (Hartmann et al. 2014).

1.3.3 Environmental Fate of Nanomaterials in Soil

Soil is the matrix of a multilayer food web structure, and it is a complex interface between gases–solid–water–organic/inorganic matter and organisms (Mukhopadhyay 2014). Not much information is available on the behavior of nanomaterials in the soil. Soil conditions are complex and variable; therefore, generic predictions on the environmental fate of nanomaterials are extremely difficult to make. The properties of the soil matrix may influence the diffusion and mobility of NPs. It is reported that transport is moderately fast and that fate of nanomaterials released to soil is influenced by physical and chemical characteristics of the nanomaterial as well as their interaction with natural colloidal materials and on the soil properties (Li et al. 2006; Boxall et al. 2007). Nanomaterials are small enough to pass through soil pores. They can strongly adhere to the soil matrix due to their high surface area and thus accumulate and become inert and completely immobilized. The possibility to sorb to soil and the respective sorption strength of NPs are influenced by their size, chemical composition, and surface characteristics. Large aggregates of nanomaterials can be immobilized by sedimentation, filtration, or straining in smaller pores. On the other hand, it is also possible that NPs travel farther than larger particles (e.g., washed out with the rain) as their small size might allow them to travel easily through the pore spaces between the soil particles.

The properties of soil, such as porosity, charge, and grain size, further influence the mobility of the particles. Surface photoreactions might induce photochemical transformations on the particles that stay on the soil surface (Muller 2007). The nanomaterial behavior in soil is generally similar to the one in natural colloids (Grolimund et al. 1998). The strength of sorption will vary, depending on their size, chemical properties, and surface coatings and the conditions under which it is applied (USEPA 2007). Studies by various authors have demonstrated the differences in the mobility of a variety of insoluble nanosized materials in a porous medium (Lecoanet et al. 2004; Lecoanet and Wiesner 2004; Zhang 2003). Just like mineral colloids, the mobility of nanomaterials agglomerated in colloid-like structures might be strongly affected by electrical charge differences in soils and sediments. The mobility of NPs in soils depends on the NP's physical–chemical properties, the characteristics of the soil and environment, and the interaction of NPs with natural colloidal materials (Viswanath and Kim 2017).

1.4 Human Exposure

To analyze the risks from nanomaterials in the environment, it is essential to understand potential exposure routes of various forms of nanomaterials. Exposure means to expose an object to a particular influence: in the case of nanomaterials, in particular, the contact of humans, animals, or the environment with the possibility of incorporating nanomaterials. Both the quantity and the period of ingestion are of concern. The biological effect of materials or substances depends on their ability of reaching

the body or rather the organs and cells inside the body. Detection of the uptake in the respective organism is an essential factor in evaluating nanomaterials and NPs. Like in the case of other substances, nanomaterials are taken up depending on how they occur in the environment: as free particles, bound in another substance, e.g., as reinforcements in plastics; or distributed in a liquid, e.g., as constituents of lubricants or oils. The exposure of NPs to the environment and humans can be described through different mechanisms. Primarily, occupational exposures occur to workers (including engineers, scientists, and technicians) during the research-scale synthesis and commercial production of nanomaterial-based products. This exposure mainly results from handling of raw materials while carrying out reactions through the equipment. Characterization of resulting material, packing, and transportation can be other sources of this type of exposure. At the second stage, consumers are exposed to such nanomaterials during usage and application and it may lead to harmful and toxic effects (Tsuji et al. 2006).

Basically, there are three pathways for all substances, including nanomaterials, to get into the human body:

1. Exposure through the respiratory system via inhalation (inhalative uptake)
2. Exposure through skin (dermal uptake)
3. Exposure through digestive system via ingestion (oral uptake)

1.4.1 Exposure through Inhalation

The adult human lung has a huge surface area of around 120–140 m^2 for gas exchange of oxygen and carbon dioxide. Anatomically, it is made of a cascade of conducting airways, starting from the trachea, via the bronchi and bronchioli down to the gas exchange zone of the alveoli. These very small "air bubbles" are formed by epithelial cells, which are directly in contact on their interior side with endothelial cells forming the blood vessels. This alveolar barrier separates the blood within the blood vessels from the air inside the lung and can be very thin down to 200–500 nm (Gehr et al. 2006).

As the breathing air contains a variety of different substances, such as germs, dust particles, or other contaminants, the lung has special clearance mechanisms in place to handle such contaminations. Normal air contains 1,000 up to 10,000 microbes/germs and 10–50 μg of fine and ultrafine dust particles per cubic meter. This means that an adult human who inhales 10,000–15,000 L of air per day is in fact inhaling more than 10,000 microbes and more than 10 billion particles each day. The clearance mechanisms of the body consist of two different principles, namely, the alveolar macrophages and the ciliated mucociliary clearance of the upper airways. Depending on their size, particles deposit in the different regions of the lung where they are engulfed by macrophages in the alveolar region, which then move upward to the bronchioles and bronchi. Large particles are directly transported upward via the mucociliary clearance, also called the mucociliary escalator. Afterward, this mucus, containing the foreign substance, is removed from the lung by coughing, swallowing, or spitting (Nanoparticles and the lungs 2017).

The size of the particulate matter (PM) is important not only for the deposition within the respiratory tract but also for the potential transfer into the blood stream.

In this relationship, the dust definitions of the World Health Organization distinguish between inhalable, thoracic, and respirable dust. Whereas inhalable dust is the fraction that can reach the upper airways such as the mouth, nose, and throat (PM_{10} <10 μm), the thoracic fraction is much smaller and can penetrate into the airways of the lung (bronchi and upper bronchioles) ($PM_{2.5}$ <2.5 μm). The respirable particulate dust fraction contains the smallest particles (PM_1 <1 μm), which are able to enter the alveoli, the gas exchange region, and there potentially cross the cell layers to penetrate into the blood stream. Since NPs fall into the same size category as the smallest PM fraction (PM_1), they should be able to cross the air–blood barrier in a similar manner. However, the majority of an applied dose will be recognized by macrophages and transported out of the lung (Krug and Wick 2011; Kreyling et al. 2012). Kreyling et al. (2013) reported in their study that the probability of gold NPs to cross the air–blood barrier was dependent on their specific surface area (i.e., inverse diameter of their gold core). Gold NPs (1.4 nm) with the highest specific surface area were reported to translocate the most. Their study showed that both translocation and accumulation depend specifically on particle characteristics such as specific surface area and surface charge.

There are studies that a small portion of the overall inhaled dose reaches the blood stream and ends up in secondary organs such as the kidneys, liver, heart, spleen, and others. It is discussed that particles may contribute to respiratory, inflammatory, and cardiovascular diseases (Braakhuis et al. 2014; Saber et al. 2014). Miller et al. (2017) reported in their study that translocation of inhaled NPs into systemic circulation and accumulation at sites of vascular inflammation provides a direct mechanism that can explain the link between environmental NPs and cardiovascular disease and has major implications for risk management in the use of engineered nanomaterials.

Many studies have described that inhalation of high concentrations of metal oxides or other nanomaterials, which belong to the granular biopersistent particle fraction, could lead to lung inflammation (Donaldson et al. 2012). Moreover, the geometric shape/structure of particles is also a crucial factor that determines lung toxicity. The structural similarities between mineral fibers and manufactured nanofibers including nanotubes, nanorods, and nanowires have been mentioned as a concern to have the same effects as asbestos fibers under specific circumstances. It has been proven that only long and thin nanofibers and long asbestos fibers are responsible for long-term inflammation and extensive fibrosis in lung tissues (Donaldson et al. 2013; Schinwald et al. 2012).

1.4.2 Exposure through Dermal Deposition

The significance of dermal exposure to dangerous substances continues to rise (Mackevica and Hansen 2016). Either detrimental effects arising from skin exposure may happen locally within the skin or alternatively the substance may be absorbed through the skin and circulated via the bloodstream, probably causing systemic effects (Warheit and Donner 2015). The exposure of human skin to nanomaterials can occur via intentional and unintentional means. Intentional exposure to nanomaterials could be the result of applications of cosmetic products such as creams, lotions, and sunscreens containing coated NPs of TiO_2 and ZnO. These particles are thought to be activity enhancers for cosmetics. Skin exposure to engineered nanomaterials can also occur during the purposeful application of topical creams and other drug treatments (Hagens et al. 2007). Unintentional exposure of nanomaterials to human skin occurs

through directly generated NPs during manufacturing, combustion, and disposal of used nanomaterial-based products. Other sources of unintentional human and environmental exposure to NPs can be through vehicle tailpipe emissions, natural gas/powdered equipment (Rundell 2003), ultrafine particle generation during waxing of skin, welding fume emissions (Zimmer et al. 2002), and emissions from coal, natural gas, and oil-fired power plants.

There are two possible mechanisms of nanomaterial penetration into the skin: intercellular transepidermal mechanism or diffusion through skin pores and hair cavities (Bennat and Muller-Goymann 2000). The main absorption of NPs may occur through several routes, including lipid-soluble particles which penetrate through intercellular lipid mechanisms by stratum corneum cells, through the transcellular cell pathway, hair follicle, and sweat ducts (Monteiro-riviere and Inman 2006). The concerns about penetration of nanomaterials through skin and resulting toxic effects are highly debatable topics among researchers and scientists. These concerns include cytotoxicity of skin, toxicity during accumulation in skin for long time, metabolism with potential of toxicity, and photoactivation of nanomaterials when present in skin (Tsuji et al. 2006). Human skin is an effective barrier toward nanomaterials and other toxic chemicals; however, the presence of hair follicles and sweat glands makes this barrier susceptible by facilitating the penetration of small-sized NPs (Teow et al. 2011).

Mostly nanomaterials are less detected through viable skin while more penetration occurs in hair follicles when the protective layer of the skin is removed, damaged, or wounded (Mavon et al. 2007). TiO_2 nanomaterial surface coating may indirectly damage the skin which results in NPs penetration into the skin. The application of NPs in treating wounds and damage of the skin accelerates penetration (Teow et al. 2011). Antimicrobial properties of AgNPs have made these particles one of the most frequently utilized nanomaterials in skin care products (Miethling-graff et al. 2014). The toxicity of AgNPs has been reported to be mediated by the induction of oxidative stress that is associated with decreased viability, the inhibition of mitochondrial activity, and the initiation of apoptosis and cell death (Foldbjerg et al. 2009). Many products often contain substances which enhance the penetration of material to skin (Finnin and Morgan 1999). Carbon nanotubes when incubated with keratinocytes in tissue culture caused mitochondrial dysfunction, oxidative phosphorylation, and generation of reactive oxygen species (Shvedova et al. 2003). Nanomaterials can also induce an injury response inside the skin leading to inflammation. They can denature proteins and unmask epitopes; for example, soot NPs from diesel exhaust promote antigen uptake by dendritic cells (Barlow et al. 2005). It has been proved that engineered NPs such as quantum dots, single or multiwalled carbon nanotubes with nanoscale titania, and surface coating have lethal effects on fibroblasts and epidermal keratinocytes and are competent of altering their gene or protein expression (Haliullin et al. 2015).

1.4.3 Exposure through Ingestion

The gastrointestinal tract acts as an organ system responsible for consuming and digesting foodstuffs, absorbing nutrients and expelling waste. Gastrointestinal exposure may occur through direct ingestion of water, vegetables and foodstuffs, cosmetics, and drugs with adsorbed NPs deposited on them, from where they enter in lymphatic cell tissues (Mann et al. 2012; Som et al. 2011; Teow et al. 2011; Daughton 2004).

Many factors are involved in controlling the absorption of NPs in the gastrointestinal tract including size of particles, geometry, surface charge, ligand type, and attachment potential to ligand (Li et al.). Besides, NPs cleared from the respiratory tract via the mucociliary escalator (coughs and swallow) can then be ingested into the gastrointestinal tract. Thus, the gastrointestinal tract is considered as vital target for NPs exposure (Li et al. 2006). NPs may accumulate in marine food from waste disposed into water bodies, and this polluted food may act as one possible source of ingestion (Ward and Kach 2009). Toxicity induced by ingested TiO_2 nanomaterials results in damage of the digestive gland cell membrane through an oxidative stress mechanism (Valant et al. 2012).

1.5 Bioaccumulation of Nanomaterials

One of the important aspects in analyzing the potential toxicity of any compound, including a nanomaterial, is the magnitude to which these particles reach tissues within an organism and accumulate. The toxicity of nanomaterials to food chain members has been reported for various bacteria, plants, and multicellular aquatic and terrestrial organisms (Liu et al. 2014; Melissa et al. 2013). Yeo and Nam (2013) studied the entries of nanomaterials in aquatic organisms and found that TiO_2 NPs and nanotubes were largely transported from low-trophic-level organisms such as biofilm and water dropwort to high-trophic-level organisms such as nematodes and mud snails.

Application of biosolids to agricultural lands as well as nano-enabled agricultural products (such as pesticides, fertilizers, plant protectives, soil additives, and growth regulators) and soil remediation nanotechnologies are some of major pathways for plant exposure to nanomaterials. In addition, atmospheric deposition, spillage, discharge, surface runoff, and wastewater reuse for food production can also lead to nanomaterial exposure to plants (Gardea-Torresdey et al. 2014). In addition, the adsorptive capabilities of some nanomaterials and their ability to permeate across membranes raise concerns regarding the transport of toxic chemicals in tissues and cells. The unique ability of certain nontoxic nanomaterials is that, if the nanowaste mixes/interacts with other conventional waste streams containing toxic chemicals, the former may act as a virus such as Trojan horse to transport the latter into the cell (Musee 2011). However, the quantity of nanomaterials which can act as a Trojan horse for other contaminants, after their transformation, will depend on the competition between nanomaterial surfaces and other surfaces (Auffan et al. 2012).

Carbon-based NPs are lipophilic, which means that they can penetrate and react with different kinds of cell membranes (Zhu et al. 2006). Nanomaterials with low solubility (such as C60) could potentially accumulate in biological organisms. Fortner et al. estimate that it is likely that nanomaterials can move up through the food chain via sediment-consuming organisms, which is confirmed by unpublished studies performed at Rice University, United States (Brumfi et al. 2003).

Gardea-Torresdey et al. (2014) have analyzed data from full life cycle studies published in the last 2 years to explain nanomaterial transfer and biomagnification within trophic levels, the uptake and bioaccumulation of nanomaterials in edible plants and impact on food chain. They mentioned that the uptake and translocation of carbon nanomaterials such as C60 fullerenes, multiwalled/single-walled carbon nanotubes,

or graphene remain largely unexplored. Due to very limited literature and contradictory findings, the fate of carbon nanomaterials within food crops and the resulting impact on organisms that consume those tissues remain unknown. Gardea-Torresdey et al. (2014) also mentioned that several phytotoxicity studies involving exposure of plants during a complete life cycle revealed the accumulation of metals in fruits/grains/seeds. However, there is very limited understanding on the extent of nanomaterial entry into the food supply and the resulting implications on environmental and human health.

Hernandez-Viezcas et al. (2013) studied the entire life cycle of soybean (glycine max) plants grown in ZnO and CeO_2 NP-contaminated soil. They reported the translocation of Zn NPs from ZnO NPs in soil, bioaccumulation of Ce as CeO_2 NPs in the soybean pods, and small percentage biotransformation of C(IV) oxidation state to C(III) oxidation. The results of their study showed that CeO_2 NPs in soil can be absorbed by plants and NPs can reach the food chain and the next soybean plant generation.

1.6 Effect of Nanomaterials on Agriculture and Food

Food processing is related to the practice adopted by the food and beverage industries to change raw plant and animal materials into "ready-to-eat form." Nanomaterials are applied to food technology with respect to their properties and predetermined set goals such as taste, flavor, shelf life, appearance, and likes and dislikes of the consumers. Nanomaterials have specific potentials depending on their physicochemical properties such as surface effect, small size effect, quantum size effect, and quantum tunneling effect. These properties regulate their behavior in the biosystem for they may either be tolerated or be the cause of disturbance to biochemical and/or physiological homeostasis. Mostly, nanomaterials have the ability to reach target tissues or organs where their counterparts fail to reach in the organism. Nanoparticles such as zinc, calcium, and silver are found to be biocompatible and antimicrobial in nature. Hence, these are used in the form of edible film incorporated with cinnamon or oregano oil in the packaging of food. Generally, polymers are incorporated with the nanomaterials and are used in food packaging and food processing. Food processing is aimed at good food quality and safe evaluation by improving food sensing and better nanostructured ingredients. These nanomaterials improve the flexibility and durability of the food contents. The nanomaterials enter the body along with the food products, beverages, and other drinks for consumption. Understanding the mechanism involved in toxicity due to nanomaterials may provide necessary information about the nanomaterials which will act as guidelines for their appropriate use in food technology and its related aspects. This will also help to develop more innovative devises to meet the future challenges in food technology (Kumar 2015).

1.7 Conclusion

As more products containing nanomaterials are developed, there is greater potential risk for exposure of humans and the environment to nanomaterials. The environment and humans may be exposed to nanomaterials throughout all stages of their life cycle,

starting from production, storage, and transport to use and disposal. In spite of the numerous benefits of nanomaterials, there are some serious concerns about how the nanomaterials used in various applications may interact with the environment. There are significant arguments regarding the adverse effects of nanomaterials on the environment with the potential to cause toxicity to humans and other living organisms. Thus, it is important for nanotoxicology to investigate the effect of nanomaterials to the environment, so potential damage could be avoided. This chapter discusses the risk and hazard of exposure to nanomaterials—i.e., their fate, behavior, and toxicity in the environment—but there remains much more to be investigated regarding their toxicity. To improve this situation in the future, we need to enhance the quality and reliability of the studies.

REFERENCES

Aitken, R. J., Creely, K. S., and Tran, C. L. 2004. *Nanoparticles: An occupational hygiene review*. Research Report 274, Institute of Occupational Medicine, Health and Safety Executive, UK. http://www.hse.gov.uk/rrpdf/rr274.pdf (accessed on December 27, 2017).

Auffan, M., Rose, J., Proux, O., Masion, A., Liu, W., Benameur, L., and Bottero, J. Y. 2012. Is there a Trojan-horse effect during magnetic nanoparticles and metalloid contamination of human dermal fibroblasts? *Environmental science & technology* 46(19):10789–10796.

Barlow, P. G., Donaldson, K., and MacCallum, J. 2005. Serum exposed to nanoparticle carbon black displays increased potential to induce macrophage migration. *Toxicology letters* 155:397–401.

Bennat, C. and Muller-Goymann, C. C. 2000. Skin penetration and stabilization of formulations containing microfine titanium dioxide as physical UV filter. *International journal of cosmetic science* 22(4):271–284.

Bidleman, T. F. 1988. Atmospheric processes. *Environmental science & technology* 22(4):361–367.

Biswas, P. and Wu, C. Y. 2005. Nanoparticles and the environment. *Journal of the air & waste management association* 55(6):708–746.

Boxall, A. B., Tiede, K., and Chaudhry, Q. 2007. Engineered nanomaterials in soils and water: How do they behave and could they pose a risk to human health? *Nanomedicine* 2(6):919–927

Braakhuis, H. M., Park, M. V., Gosens, I., De Jong, W. H., and Cassee, F. R. 2014. Physicochemical characteristics of nanomaterials that affect pulmonary inflammation. *Particle and fibre toxicology* 11(1):18.

Brar, S. K., Verma, M., Tyagi, R. D., and Surampalli, R. Y. 2010. Engineered nanoparticles in wastewater and wastewater sludge—Evidence and impacts. *Waste management* 30(3):504–520.

Brook, R. D., Brook, J. R., Urch, B., Vincent, R., Rajagopalan, S., and Silverman, F. 2002. Inhalation of fine particulate air pollution and ozone causes acute arterial vasoconstriction in healthy adults. *Circulation* 105(13):1534–1536.

Brumfiel, G. 2003. Nanotechnology: A little knowledge. *Nature* 424(6946):246–248.

Colvin, V. L. 2003. The potential environmental impact of engineered nanomaterials. *Nature biotechnology* 21:1166–1170.

Daughton, C. G. 2004. Non-regulated water contaminants: Emerging research. *Environmental impact assessment review* 24(7):711–732.

Dolez, P. I. (Ed.). 2015. *Nanoengineering: Global approaches to health and safety issues*. Elsevier, Amsterdam.

Donaldson, K., Poland, C. A., Murphy, F. A., MacFarlane, M., Chernova, T., and Schinwald, A. 2013. Pulmonary toxicity of carbon nanotubes and asbestos—Similarities and differences. *Advanced drug delivery reviews* 65(15):2078–2086.

Donaldson, K., Schinwald, A., Murphy, F., Cho, W. S., Duffin, R., Tran, L., and Poland, C. 2012. The biologically effective dose in inhalation nanotoxicology. *Accounts of chemical research* 46(3):723–732.

Farré, M., Sanchís, J., and Barceló, D. 2011. Analysis and assessment of the occurrence, the fate and the behavior of nanomaterials in the environment. *TrAC: Trends in analytical chemistry* 30(3):517–527.

Finnin, B. C. and Morgan, T. M. 1999. Transdermal penetration enhancers: Applications, limitations, and potential. *Journal of pharmaceutical sciences* 88:955–958.

Foldbjerg, R., Olesen, P., and Hougaard, M. 2009. PVP-coated silver nanoparticles and silver ions induce reactive oxygen species, apoptosis and necrosis in THP-1 monocytes. *Toxicology letters* 190:156–162.

Foss Hansen, S., Larsen, B. H., Olsen, S. I., and Baun, A. 2007. Categorization framework to aid hazard identification of nanomaterials. *Nanotoxicology* 1(3):243–250.

Gardea-Torresdey, J. L., Rico, C. M., and White, J. C. 2014. Trophic transfer, transformation, and impact of engineered nanomaterials in terrestrial environments. *Environmental science & technology* 48(5):2526–2540.

Gehr, P., Blank, F., and Rothen-Rutishauser, B. M. 2006. Fate of inhaled particles after interaction with the lung surface. *Paediatric respiratory reviews* 7:S73–S75.

Grolimund, D., Elimelech, M., Borkovec, M., Barmettler, K., Kretzschmar, R., and Sticher, H. 1998. Transport of in situ mobilized colloidal particles in packed soil columns. *Environmental science & technology* 32(22):3562–3569.

Hagens, W. I., Oomen, A. G., de Jong, W. H., Cassee, F. R., and Sips, A. J. 2007. What do we (need to) know about the kinetic properties of nanoparticles in the body? *Regulatory toxicology and pharmacology* 49(3):217–229.

Haliullin, T. O., Zalyalov, R. R., Shvedova, A. A., and Tkachov, A. G. 2015. Hygienic evaluation of multilayer carbon nanotubes. *Meditsina truda i promyshlennaia ekologiia* 7:37–42.

Hansen, S. F., Heggelund, L. R., Besora, P. R., Mackevica, A., Boldrin, A., and Baun, A. 2016. Nanoproducts—What is actually available to European consumers? *Environmental science: Nano* 3(1):169–180.

Hartmann, N. I. B., Skjolding, L. M., Hansen, S. F., Baun, A., Kjølholt, J., and Gottschalk, F. 2014. *Environmental fate and behaviour of nanomaterials: New knowledge on important transformation processes*. Danish Environmental Protection Agency, Copenhagen.

Helland, Å. 2004. *Nanoparticles: A closer look at the risks to human health and the environment perceptions and precautionary measures of industry and regulatory bodies in Europe*. Lund University, Lund, Sweden.

Hernandez-Viezcas, J. A., Castillo-Michel, H., Andrews, J. C., Cotte, M., Rico, C., Peralta-Videa, J. R., Ge, Y., Priester, J. H., Holden, P. A., and Gardea-Torresdey, J. L. 2013. In situ synchrotron X-ray fluorescence mapping and speciation of CeO_2 and ZnO nanoparticles in soil cultivated soybean (*Glycine max*). *ACS nano* 7(2):1415–1423.

Hristozov, D. and Malsch, I. 2009. Hazards and risks of engineered nanoparticles for the environment and human health. *Sustainability* 1(4):1161–1194.

Hyung, H., Fortner, J. D., Hughes, J. B., and Kim, J. H. 2007. Natural organic matter stabilizes carbon nanotubes in the aqueous phase. *Environmental science & technology* 41(1):179–184.

ISO (International Organization for Standardization). 2010. *Nanotechnologies vocabulary part 1: Core terms*. ISO/TS 80004-1:2010. ISO, Geneva.

Kägi, R., Ulrich, A., Sinnet, B., Vonbank, R., Wichser, A., Zuleeg, S., Simmler, H. et al. 2008. Synthetic TiO_2 nanoparticle emission from exterior facades into the aquatic environment. *Environmental pollution* 156(2):233–239.

Keller, A. A., McFerran, S., Lazareva, A., and Suh, S. 2013. Global life cycle releases of engineered nanomaterials. *Journal of nanoparticle research* 15(6):1692.

Kreyling, W. G., Hirn, S., Möller, W., Schleh, C., Wenk, A., Celik, G., Lipka, J. et al. 2013. Air–blood barrier translocation of tracheally instilled gold nanoparticles inversely depends on particle size. *ACS nano* 8(1):222–233.

Kreyling, W. G., Semmler-Behnke, M., Takenaka, S., and Möller, W. 2012. Differences in the biokinetics of inhaled nano- versus micrometer-sized particles. *Accounts of chemical research* 46(3):714–722.

Krug, H. F. and Wick, P. 2011. Nanotoxicology: An interdisciplinary challenge. *Angewandte chemie international edition* 50(6):1260–1278.

Kumar, L. Y. 2015. Role and adverse effects of nanomaterials in food technology. *Journal of toxicology and health* 2(1):2.

Lecoanet, H. F., Bottero, J. Y., and Wiesner, M. R. 2004. Laboratory assessment of the mobility of nanomaterials in porous media. *Environmental science & technology* 38(19):5164–5169.

Lecoanet, H. F. and Wiesner, M. R. 2004. Velocity effects on fullerene and oxide nanoparticle deposition in porous media. *Environmental science & technology* 38(16):4377–4382.

Li, X. Q., Elliott, D. W., and Zhang, W. X. 2006. Zero-valent iron nanoparticles for abatement of environmental pollutants: Materials and engineering aspects. *Critical reviews in solid state and materials sciences* 31(4):111–122.

Liu, Y., Tourbin, M., Lachaize, S., and Guiraud, P. 2014. Nanoparticles in wastewaters: Hazards, fate and remediation. *Powder technology* 255:149–156.

Mackay, C. E., Johns, M., Salatas, J. H., Bessinger, B., and Perri, M. 2006. Stochastic probability modeling to predict the environmental stability of nanoparticles in aqueous suspension. *Integrated environmental assessment and management* 2(3):293–298.

Mackevica, A. and Foss Hansen, S. 2016. Release of nanomaterials from solid nanocomposites and consumer exposure assessment—A forward-looking review. *Nanotoxicology* 10(6):641–653.

Mädler, L. and Friedlander, S. K. 2007. Transport of nanoparticles in gases: Overview and recent advances. *Aerosol and air quality research* 7(3):304–342.

Mann, E. E., Thompson, L. C., Shannahan, J. H., and Wingard, C. J. 2012. Changes in cardiopulmonary function induced by nanoparticles. *Wiley interdisciplinary reviews: Nanomedicine and nanobiotechnology* 4:691–702.

Mavon, A., Miquel, C., and Lejeune, O. 2007. In vitro percutaneous absorption and in vivo stratum corneum distribution of an organic and a mineral sunscreen. *Skin pharmacology and physiology* 20:10–20.

Maynard, A. D. 2011. Don't define nanomaterials. *Nature* 475(7354):31.

Melissa, A. M. J., Ian, L. G., Catherine, J. M., and Christy, L. H. 2013. Toxicity of engineered NPs in the environment. *Analytical chemistry* 85:3036–3049.

Miethling-graff, R., Rumpker, R., and Richter, M. 2014. Toxicology in vitro exposure to silver nanoparticles induces size- and dose-dependent oxidative stress and cytotoxicity in human colon carcinoma cells. *Toxicology in vitro* 28:1280–1289.

Miller, M. R., Raftis, J. B., Langrish, J. P., McLean, S. G., Samutrtai, P., Connell, S. P., Wilson, S. et al. 2017. Inhaled nanoparticles accumulate at sites of vascular disease. *ACS nano* 11(5):4542–4552.

Monteiro-riviere, N. A. and Inman, A. O. 2006. Challenges for assessing carbon nanomaterial toxicity to the skin. *Carbon N Y* 44:1070–1078.

Moore, M. N. 2006. Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? *Environment international* 32(8):967–976.

Mueller, N. C. and Nowack, B. 2008. Exposure modelling of engineered nanoparticles in the environment. *Environmental science & technology* 42(12):4447–4453.

Mukhopadhyay S. S. 2014 Nanotechnology in agriculture: Prospects and constraints. *Nanotechnology, science and applications* 7:63–71.

Muller, N. 2007. Nanoparticles in the environment risk assessment based on exposure-modelling. Diploma thesis, Swiss Federal Institute of Technology, Zurich.

Müller, N. 2007. *Nanoparticles in the environment: Risk assessment based on exposure-modelling: What concentrations of nano titanium dioxide, carbon nanotubes and nano silver are we exposed to?* Doctoral dissertation, Department of Environmental Sciences, ETH Zurich, Zurich.

Musee, N. 2011. Nanotechnology risk assessment from a waste management perspective: Are the current tools adequate? *Human & experimental toxicology* 30(8):820–835.

Nanoparticles and the lungs http://www.nanopartikel.info/en/nanoinfo/body-barriers/2388-nanoparticles-and-lungs#literature (accessed on July 2017).

Nowack, B. and Bucheli, T. D. 2007. Occurrence, behaviour and effects of nanoparticles in the environment. *Environmental pollution* 150(1):5–22.

Nurmi, J. T., Tratnyek, P. G., Sarathy, V., Baer, D. R., Amonette, J. E., Pecher, K., Wang, C. et al. 2005. Characterization and properties of metallic iron nanoparticles: Spectroscopy, electrochemistry, and kinetics. *Environmental science & technology* 39(5):1221–1230.

Oberdörster, G., Oberdörster, E., and Oberdörster, J. 2005. Nanotoxicology: An emerging discipline evolving from studies of ultrafine particles. *Environmental health perspectives* 113(7):823–839.

Preining, O. 1998. The physical nature of very, very small particles and its impact on their behaviour. *Journal of aerosol science* 29(5):481–495.

Rundell, K. W. 2003. High levels of airborne ultrafine and fine particulate matter in indoor ice arenas. *Inhalation toxicology* 15(3):237–250.

Saber, A. T., Jacobsen, N. R., Jackson, P., Poulsen, S. S., Kyjovska, Z. O., Halappanavar, S., Yauk, C. L., Wallin, H., and Vogel, U. 2014. Particle-induced pulmonary acute phase response may be the causal link between particle inhalation and cardiovascular disease. *Wiley interdisciplinary reviews: Nanomedicine and nanobiotechnology* 6(6):517–531.

Saner, M. and Stoklosa, A. 2013. Commercial, societal and administrative benefits from the analysis and clarification of definitions: The case of nanomaterials. *Creativity and innovation management* 22(1):26–36.

Schinwald, A., Murphy, F. A., Prina-Mello, A., Poland, C. A., Byrne, F., Movia, D., Glass, J. R. et al. 2012. The threshold length for fiber-induced acute pleural inflammation: Shedding light on the early events in asbestos-induced mesothelioma. *Toxicological sciences* 128(2):461–470.

Shi, J. P., Evans, D. E., Khan, A. A., and Harrison, R. M. 2001. Sources and concentration of nanoparticles (< 10nm diameter) in the urban atmosphere. *Atmospheric environment* 35(7):1193–1202.

Shvedova, A. A., Castranova, V., and Kisin, E. R. 2003. Exposure to carbon nanotube material: Assessment of nanotube cytotoxicity using human keratinocyte cells. *Journal of toxicology and environmental health, part A* 66:1909–1926.

Som, C., Wick, P., Krug, H., and Nowack, B. 2011. Environmental and health effects of nanomaterials in nanotextiles and facade coatings. *Environment international* 37:1131–1142.

Spurny, K. R. 1998. On the physics, chemistry and toxicology of ultrafine anthropogenic, atmospheric aerosols (UAAA): New advances. *Toxicology letters* 96:253–261.

Stone, V., Nowack, B., Baun, A., van den Brink, N., von der Kammer, F., Dusinska, M., Handy, R. et al. 2010. Nanomaterials for environmental studies: Classification, reference material issues, and strategies for physico-chemical characterisation. *Science of the total environment* 408(7):1745–1754.

Teow, Y., Asharani, P. V., Hande, M. P., and Valiyaveettil, S. 2011. Health impact and safety of engineered nanomaterials. *Chemical communications (Cambridge)* 47:7025–7038.

Tiede, K., Hanssen, S. F., Westerhoff, P., Fern, G. J., Hankin, S. M., Aitken, R. J., Chaudhry, Q., and Boxall, A. B. 2016. How important is drinking water exposure for the risks of engineered nanoparticles to consumers? *Nanotoxicology* 10(1):102–110.

Tsuji, J. S., Maynard, A. D., and Howard, P. C. 2006. Research strategies for safety evaluation of nanomaterials, part IV: Risk assessment of nanoparticles. *Toxicological sciences* 89:42–50.

USEPA (US Environmental Protection Agency) 2007. US Environmental Protection Agency Nanotechnology White Paper. USEPA, Washington, DC.

Valant, J., Drobne, D., and Novak, S. 2012. Effect of ingested titanium dioxide nanoparticles on the digestive gland cell membrane of terrestrial isopods. *Chemosphere* 87:19–25.

Vance, M. E., Kuiken, T., Vejerano, E. P., McGinnis, S. P., Hochella Jr., M. F., Rejeski, D., and Hull, M. S. 2015. Nanotechnology in the real world: Redeveloping the nanomaterial consumer products inventory. *Beilstein journal of nanotechnology* 6(1):1769–1780.

Viswanath, B. and Kim, S. 2017. Influence of nanotoxicity on human health and environment: The alternative strategies. *Reviews of environmental contamination and toxicology volume* 242:61–104.

Ward, J. E. and Kach, D. J. 2009. Marine aggregates facilitate ingestion of nanoparticles by suspension-feeding bivalves. *Marine environmental research* 68:137–142.

Warheit, D. B. and Donner, E. M. 2015. Risk assessment strategies for nanoscale and fine-sized titanium dioxide particles: Recognizing hazard and exposure issues. *Food and chemical toxicology* 85:138–147.

Weinberg, H., Galyean, A., and Leopold, M. 2011. Evaluating engineered nanoparticles in natural waters. *TrAC: trends in analytical chemistry* 30(1):72–83.

Wiesner, M. R., Hotze, E. M., Brant, J. A., and Espinasse, B. 2008. Nanomaterials as possible contaminants: The fullerene example. *Water science and technology* 57(3):305–310.

Wiesner, M. R., Lowry, G. V., Alvarez, P., Dionysiou, D., and Biswas, P. 2006. Assessing the risks of manufactured nanomaterials. *Environmental science & technology* 40(14):4336–4345.

Yeo, M. K. and Nam, D. H. 2013. Influence of different types of nanomaterials on their bioaccumulation in a paddy microcosm: A comparison of TiO_2 nanoparticles and nanotubes. *Environmental pollution* 178:166–172.

Zhang, W. X. 2003. Nanoscale iron particles for environmental remediation: An overview. *Journal of nanoparticle research* 5(3):323–332.

Zhu, S., Oberdörster, E., and Haasch, M. L. 2006. Toxicity of an engineered nanoparticle (fullerene, C60) in two aquatic species, Daphnia and fathead minnow. *Marine environmental research* 62:S5–S9.

Zimmer, A. T., Baron, P. A., and Biswas, P. 2002. The influence of operating parameters on number-weighted aerosol size distribution generated from a gas metal arc welding process. *Journal of aerosol science* 33(3):519–531.

2

Nanotoxicity: Impact on Health and Environment

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2.1 Introduction

Environmental pollutants are released in several forms. These include toxic gases (carbon monoxide, chlorofluorocarbons, hydrocarbons, sulfur oxides, nitrogen oxides); volatile organic compounds; suspended particulates (pesticides, chlorinated compounds, fertilizers); and metals (arsenic, lead, mercury, cadmium, chromium, zinc etc.); oil spills; and microbes (both in water and soil). These pollutants enter and get accumulated in the food chain through skin contact or in the respiratory systems

of higher animals, thereby threatening humans, wildlife, and overall environmental health.

The advent of nanotechnology has brought new opportunities for engineering materials and advanced sophisticated instruments with hitherto unknown precision and performance. Nanotechnology is a boon to today's society plagued with diseases and resource crunch. It has ushered a new age of technology revolution to evolve intelligent materials and smart nanobots, also brought in a new era in biomedical technology. Its application is widespread ranging from diagnostics to drug delivery, from smart materials and robotics to textiles, cosmetics, water treatment, automobile engineering, etc.

There have been numerous instances where paradigm-changing technologies have ended up having serious repercussions on health and environment. Nanotechnology seems to be no exception either. Nanotechnology-generated waste discards and particles have been seen to have a negative impact on the environment (Zhang et al., 2012). The benefits of nanotechnology cannot be ignored, but at the same time, waste discards and some products tend to pose potential risks to environmental and human health. Very little is known about the nature and intensity of such risks. Therefore, it is necessary to have a proper risk assessment for the toxicity generated out of nanotechnology application, i.e., nanotoxicity. A comprehensive review is thus very essential covering all threats of environmental and human health. It is also very important to have in place a regulation to manage nanowaste discharge in the environment and its control.

Toxicity impact of nanomaterials (i.e., nanotoxicity) should be carried out in the early state of product development (Hansen et al., 2007). Such assessment helps in reducing and avoiding environmental threats. Physical, chemical, and biological properties of nanomaterials differ from each other in many ways including route of exposure and effects, which make it necessary for detailed assessment. In order to manage the risks of nanotoxicity, regulations need to be modified to accommodate the characteristic behavior of the nanomaterials or it should be considered as a separate toxic agent. The lawmakers in general and the public at large at present are still not much acquainted with the gravity of the complexities of nanotoxicity risk assessment and management. A new platform is required to assess, monitor, and control the risk associated with nanotoxicity.

2.2 Nanotoxicity Generated by Industrial Processes and Products

Nanosized particles have been present on earth since its creation. Humans have been using it throughout generations unknowingly until recently. Nanoparticles are explicitly produced in several natural processes such as volcanic eruptions, forest fires, sand dunes in deserts, combustion of diesel and petrol, and other geological processes. For the first time in the history of humankind, the nano-based industry is one of the fastest growing industries due to the burgeoning production of various nanomaterials, such as nanoparticles, nanotubes, nanofibers, nanowires, nanocomposites, and nanofilms (Patolla et al., 2012; Ivask et al., 2014). These nanoparticles used and emitted by different commercial and research activities related to the fields of science, industry, pharmacy, medicine, textile, cosmetics, consumer products, electronics, and communication are eventually being dumped in nature (Ivask et al., 2014). Within these

limitless sources and varieties of nanomaterials, few happen to be toxic to the living systems. It is very important to realize that all nanoparticles are not toxic. Toxicity depends on the chemical composition and size of the particles (Nowack & Bucheli, 2007). The human skin, lungs, and gastrointestinal tract are constantly exposed to the environment. The skin generally acts as an effective barrier to foreign substances. The reticuloendothelial system in particular actively neutralizes and eliminates foreign matter in the body (Buzea et al., 2007). In this section, we categorize the sources of different nanoparticles from different industrial processes and products as well as their toxicity.

2.2.1 Textile Industry

In textile industries, engineered nanomaterials (ENMs) are intended to improve various properties of fabric such as self-cleaning; water and dirt repellency; antimicrobial, conductive, and antistatic properties; ultraviolet, solvent, and abrasion resistance; and decreased gas permeability and flammability. Nanoparticles with antimicrobial activities have been widely used in sports wears, medical consumables (surgical gowns and dressing bandages), defense applications, etc. (Rezić, 2011). Heavily used engineered nanomaterials by the textile industry are mainly nanosilver (nano-Ag), nano-titanium dioxide (nano-TiO_2), nanosilica (nano-SiO_2), nano-zinc oxide (nano-ZnO), nanoalumina (nano-Al_2O_3), layered silica (e.g., montmorillonite, $Al_2[(OH)_2/Si_4O_{10}]\cdot nH_2O$), carbon black, and carbon nanotubes.

After-effects on human health from textile-based nanoparticle exposure can cause acute toxicity, chronic toxicity, impairment of deoxyribonucleic acid (DNA), crossing and damaging of tissue barriers, brain impairment and translocation, and also affect the skin and gastrointestinal or respiratory tracts.

Risk from engineered nanomaterials is primarily due to exposure and the hazard associated with the material, and both of these parameters are influenced by product design and product life cycle. Textiles and textile industries are important sources of nanoparticles which are emitted into water and soil by biosolid applications. For example, cloth comes into direct contact with humans and its textile material is affected by the regular washing process (abrasion, detergents and water). Weakly (agglomerated) and strongly (aggregated) bound nanoscale particles released from the textiles end up on human skin or affect other terrestrial organisms (Som et al., 2011). When the engineered nanomaterial attaches itself into the biological system, it shows few changes (physical, chemical or biological) to form a special structure and allows them to perform exceptionally. Sometimes, these changes might affect their behavior, persistency, bioavailability and toxicity (Lowry & Casman, 2009).

Silver nanoparticles that are mostly used as antimicrobial agent are the most extensively used commercial nanoparticles in textile industries. Silver nanoparticles incorporated in textiles mostly release in ionic form (Mueller & Nowack, 2008). A study on commercial textile product wastewater shows presence of both colloidal and ionic silver nanoparticles with diameter ranging from 10 to 500 nm (Benn et al., 2008). Particularly, ionic silver nanoparticles are more toxic than its other forms (e.g., silver sulfide). The chronic exposure to silver causes adverse effects such as long-lasting bluish-gray discoloration of the skin (argyria) and eyes (argyrosis). Besides these, exposure to soluble silver compounds may result in kidney and liver damage; cause irritation of the eyes, skin, intestinal and respiratory tracts, and cause changes in

blood cells (Panyala et al., 2008). Exposure of silver nanoparticles showed clear accumulation in various organs including liver, kidney, testis, lungs, and brain (Kim et al., 2008). Studies on rats show that the inhalation of silver nanoparticles due to release in air causes adverse effects to the liver, kidney and lungs (Kulthong et al., 2010). Silver nanoparticles incorporated in textiles mostly release into the environment through body sweat and dermal exposure. Nanoparticles of ZnO_2, titanium, and carbon nanotubes commonly incorporated into textile items are released into the environment due to abrasion during use, washing, tailoring and finishing, disposal, recycling, export and dissolution (Mueller and Nowack, 2008).

2.2.2 Pharmaceuticals

The use of nanotechnology in pharmaceutical applications and research is increasing in recent times. Nanosized materials are extensively used in active pharmaceutical ingredients for better absorption in the gastrointestinal track and as carrier material in targeted delivery of drug molecules and controlled release. In the pharmaceutical industry, the use of nanotechnology with the aid of molecular knowledge is gradually providing a boost for developing more advanced therapy. Nanoalumina, carbon nanoparticles, nanoceria, gold nanoparticles, magnetite nanoparticles, platinum nanoparticles, silver nanoparticles, and titanium dioxide are the most used nanoparticles by pharmaceutical companies (Narayanan et al., 2013; Som et al., 2011). Due to the smaller size, greater permeability, increased circulation ability and biocompatibility, these nanoparticles have become vital agents for controlling aging and other pathologies, including skin disease, neurodegenerative diseases, cardiovascular diseases, pulmonary diseases and gastrointestinal diseases (Narayanan & Park, 2013; Saraceno et al., 2013; Brakmane et al., 2012). Nanomedicines get within the body via oral exposure, dermal exposure, inhalation, and intravascular injection. Toxicity caused by the nanoparticle depends on the route of exposure (El-Ansary and Al-Daihan, 2009). Gold nanoparticle is an ideal drug carrier because of its inertness and biocompatibility. Other lipid-based, polymer-based, and biological nanoparticles have found application in drug delivery (Yildirimer et al., 2011). Solid nanolipids and nanolipid carriers are widely used in drug delivery. Biocompatibility of these nanoparticles makes them almost nontoxic.

Nanoparticles such as carbon nanotubes and fullerene were initially studied for drug delivery but their nonbiodegradable nature made them potentially toxic. A pulmonary cytotoxicity study of solid nanolipids showed that it reduced the cell viability in a concentration-dependent manner (Pardeike et al., 2009). Poly-lactic-*co*-glycolic acid–polyethylene oxide copolymer (PLGA–PEO) nanoparticles are a drug delivery carrier material found to have no cytotoxicity, no DNA strand break induction, or no oxidative DNA lesion development (Kazimirova et al., 2012). Nanocarriers with positive charge tend to have a more toxic effect than those with negative charge. Studies carried out with positively and negatively charged polystyrene nanoparticles have shown that positively charged nanoparticles are taken up by HeLa and NIH 3T3 cells and cause DNA damage whereas negatively charged nanoparticles have no such effect (Liu et al., 2011). Dendrimers are another promising drug delivery carriers but in many toxicological studies it has been found to have concentration- and generation-dependent cytotoxicity (Duncan & Izzo, 2005). Magnetic nanoparticles are also being tried for drug delivery. Studies have shown that superparamagnetic nanoparticles

(2–30 nm) functionalized with citric acid or methyl carboxyl dextrin cause diarrhea in rats. Nanoparticles have the advantage of crossing the blood–brain barrier, however, high concentrations of cationic nanoparticles have a toxic effect on the system (Ai et al. 2011). Silver nanoparticles have been used in dressing and surgical sutures which received clinical application approval but cytotoxicity of nanosilver materials has been demonstrated in many recent studies (El-Ansary & Al-Daihan, 2009).

Metallic nanoparticle TiO_2 is used extensively in pharmaceutics as coloring agent for capsules, films, tablets, coated pills and medical sticky tapes. Generally, nano-TiO_2 is stable in the environment, however, surface coating is reoriented under environmental pressure which is able to change the behavior of the engineered nanoparticles (Auffan et al., 2010). Although the translocation of nano-TiO_2 across healthy skin remains to be established, it has been found that high concentrations of airborne nano-TiO_2 particles have been found to increase pulmonary and cardiovascular mortality due to the crossing of the blood–brain barrier and transporting to all over the body by the bloodstream (Geiser et al., 2005).

Several transition metal nanoparticles are used for detection of biological molecules or events. The scaffold of nanoparticles can be equipped with a pro-drug, targeting moiety or an imaging agent. This type of nanoparticle has multifunctional activities for a more effective cancer treatment that could be positively translated into clinics (Murphy et al., 2008). Like gold nanoparticles, it belongs to the zerovalent metal category, useful for the delivery of low-molecular-weight drugs as well as cellular/tissue delivery of proteins, peptides or genes (Byrne et al., 2016; Arvizo et al., 2010). A different study shows that physiochemical surface properties of gold nanoparticles can change substantially after coming into contact with biological media. Such changes should be taken into consideration when examining the biological properties or environmental impact of nanoparticles (Alkilany et al., 2009).

2.2.3 Cosmetics

The new-age cosmetic formulations use various nanomaterials such as silver nanoparticles, titanium oxide nanoparticles, zinc oxide nanoparticles and lipid nanoparticles to impart advanced functionalities in human skin care and beautification. For instance, nanoscale titanium oxide is used in cosmetic formulations as an ultraviolet ray absorber for skin protection (Mueller and Nowack, 2008). Fifty percent of the available cosmetics such as sunscreens available in markets use titanium nanoparticles making it the most extensively used nanoparticles in the cosmetic industry (Fujishima et al., 2007). Toxicological studies on titanium nanoparticles show that it poses impending risk of percutaneous absorption and reactive oxygen species-mediated skin aging due to general skin exposure during cosmetic application (Zhang et al., 2012). Studies carried out on pig and hairless mouse showed that titanium nanoparticles can penetrate into the stratum granulosum layers of epidermis (Lu et al., 2015). However, majority of the studies imply that titanium nanoparticles usually remain at the outermost layer of the stratum corneum, while only a little amount of the nanoparticle penetrates to the living epidermis layers. Besides, no irritation or sensitization is seen in mouse models during acute dermal irritation studies and local lymph node assays (Zhang et al., 2012). For cosmetics, systematic absorption is not acceptable; the cosmetic effect is generally induced by little penetration into the skin (Pardeike et al., 2009).

ZnO nanoparticles also got wide usage in cosmetics for providing the same benefits as titanium nanoparticles (Wang et al., 2008). Generally, nanoparticles get inside the body through inhalation, ingestion and dermal exposure. The dermal exposure toxicity data of ZnO nanoparticles through cosmetic applications are very limited in literature. No evidence of ZnO nanoparticle penetration into the human skin equivalent model was found during 24 hours of ZnO nanoparticle exposure (Kim et al., 2011). Oral exposure of 20 nm ZnO was found to have damaging effects on the liver, spleen, and pancreas of adult CD-ICR mice (Wang et al., 2008).

Solid lipid nanoparticles and their improved type nanostructured lipid carriers have brought much promise in cosmetic applications such as enhancement of chemical stability of active ingredients, film formation, controlled occlusion, skin hydration and enhanced skin bioavailability (Müller et al., 2007). These nanoparticles are found to be safe in many toxicology studies carried out in *in vitro* and animal models (Madureira et al., 2016). Talc powder, a magnesium silicate mineral, has extensive use in talc-based cosmetics. Talc nanoparticles that are used as gloss enhancer have been found to have concentration-dependent cytotoxicity. Talc nanoparticles increase the production of reactive oxygen species and malondialdehyde. Reactive oxygen species targets biomolecules such as proteins, membrane lipids, enzymes and even DNA molecules making them oxidized, modified and destructed (Akhtar et al., 2010).

2.2.4 Electronics and Household Appliances

Nanoparticles have many applications in household appliances and consumer goods such as paint and coatings on surfaces, and advanced nanoparticle-based electronic components. Electronic industries have known to be notorious for continuous proliferation of nanomaterials in the environment. Silicon, the main workhorse of the modern semiconductor industry, coupled with nanotechnology, has further broadened their area of applications. Commercially available power generators use silicon for photovoltaic cells in solar panel, converting light to electrical energy (Nelson et al., 2003). Nanostructured silicon shows thermoelectric and piezoelectric properties through smart surface modification which has provided an alternative way for electricity generation through thermal or mechanical energy, respectively (Sun et al., 2016). Nanoparticles are set to find wide applications in various electronic components such as resistors, transistors and supercapacitors; optoelectronic devices; sensors; fuel and energy production and storage systems; water purifiers; electrodes in battery; conductive coatings; and adhesives that are used in household appliances. Carbon nanotubes, graphene nanoparticles, fullerene, etc. have shown much promise for application into various household devices (Klaine et al., 2008). A concern arises about the release of nanoparticles from such articles where the nanoparticles have been incorporated. The nanoparticles release not only during the production step but also during usages and disposal stages. The unique property of nanoparticles responsible for quality enhancement of products can become the reason for ill health and ecological toxicity. Carbon nanotubes on inhalation reach the lungs and may cause inflammation and granulomatosis when the quantity is sufficiently high (Köhler et al., 2008). Graphene is already used in many products such as graphene-based transistors,

graphene photonics and optoelectronics, energy production and storage, and biosensors. Although graphene toxicity data are very limited in literature, the potential toxicity of 5–250 nm graphene nanoparticles towards lungs and pleural space has been reported in a few cases (Ou et al., 2016). A 28-day graphene inhalation exposure study on rats showed no significant toxicological change but significant weight loss was observed in a 90-day graphene exposure study (Kim et al., 2016). Engineered zinc oxide nanoparticles have also found numerous applications in electronics, optoelectronics and gas sensors. Studies show that the particulate nature of ZnO nanoparticles causes acute inflammation of bronchiole alveolar junctions in lungs after 24 hours post inhalation exposure (Chen et al., 2015). ZnO nanoparticles can cause eosinophilia, proliferation of airway epithelial cells, goblet cell hyperplasia, and pulmonary fibrosis in accidental occupational inhalation and in a consumer setting as well (Cho et al., 2011). Very few studies have been performed on environmental behavior and effects of nanosilica. However, few studies on wastewater discharge of electronic industries have shown that nanosilica can pass through wastewater treatment plants which makes it more dangerous for the environment (Chang et al., 2007; Jarvie et al., 2009). Occupational exposure to crystalline silica dust is related with the risk of pulmonary diseases such as silicosis, chronic bronchitis, tuberculosis, chronic obstructive pulmonary disease and lung cancer (Merget et al., 2002).

2.2.5 Construction

The extraordinary chemical and physical properties of nanomaterials (carbon nanotubes, SiO_2 nanoparticles, TiO_2 nanoparticles, C60 fullerenes) enable novel applications and the preprocess efficacy in the construction industry including compressive strength and durability (heat-insulating, self-cleaning, antifogging). It also functions as a key sensing component to monitor structural health and construction safety. Despite these benefits, manufactured nanomaterials are an offset, if their harmful impact on human health is to be considered (Lee et al., 2010; Ge and Gao, 2008). Demolition activities could only result in the release of a huge amount of construction-engineered nanomaterials including hazardous materials (asbestos cement, lead-based paint, and some persistent residues) (Kourmpanis et al., 2008). Sequentially, land farming, land filling and incineration could be the frequent routes for the environmental dispersal of engineered nanomaterials. Subsequently, abrasion and corrosion of buildings and infrastructures could also result in the release of engineered nanomaterials to the environment (Lee et al., 2010).

Carbon nanotubes which are present in ceramics, concrete, nanoelectromechanical systems/microelectromechanical systems or solar cells with respirable size are one of the potential hazards for pulmonary diseases such as inflammation, fibrosis and epithelioid granulomas in mammals (Lee et al., 2010; Lam et al., 2006). A study on C60 fullerene indicates that fullerene or its derivative can generate reactive oxygen species on photoexcitation and can induce significant lipid peroxidation/protein oxidation in biomembranes (Kamat et al., 2000). TiO_2 and SiO_2 are also extensively used in concrete and ceramics for increasing mechanical strength, fire resistance, etc. and they are also equally noted for their impact on human health (Lee et al., 2010).

2.2.6 Automobiles

Automobile exhaust is one of the most prominent sources for urban air pollution which has been found to be related with increased risk for childhood and adult asthma (Oberdorster et al., 1995). Nanoparticles emitted by automobile engines are typically hydrocarbons or sulfates with sizes below 50 nm and they form by nucleation during dilution and cooling of the exhaust (Kittelson 1998). In different studies, it has been seen that nanoparticles less than 50 nm in diameter may contribute to morbidity such as adverse respiratory and cardiovascular effects or increased rate of mortality (Oberdörster et al., 2004, 1995).

2.2.7 Pesticides

Nanoparticles for their advantageous properties such as smaller size and antimicrobial activities have found wide application in agriculture as pesticides, insecticides and fertilizers. Apart from being used as active ingredients in pesticide formulations, nanoparticles have got other uses in pesticides as nanocarriers such as nanoscale shells and capsules, as coatings, and as particulate materials such as nanoclays, inorganic additives, and others (Meredith et al., 2016). Mostly, metal oxide nanoparticles such as ZnO, TiO_2, Cu, and SiO_2 are used in pesticides and fungicides for imparting antimicrobial activities against plant pathogens (Dubey & Mailapalli, 2016). Due to smaller size of these particles, toxic effects are generated by them when used in increased doses. The smaller sizes of TiO_2 (7 nm) and CeO_2 (15 nm) nanoparticles are found to be more toxic than the larger sizes of TiO_2 (20 nm) and CeO_2 (45 nm) nanoparticles. Nanoparticles such as ZnO, which are used in pesticides, were found to have developed toxicity in soil. ZnO nanoparticles when applied in larger doses (5 g/kg of soil) can accumulate inside earthworms and cause DNA damage (Dubey & Mailapalli, 2016). Silver nanoparticles are another widely used pesticides for antimicrobial activities, however, concerns rise about silver nanoparticle toxicity on ecosystem and human health. Citrate-coated Ag nanoparticles that are used in pesticides were found to have no genotoxic, cytotoxic and phototoxic effects to humans while being used in colloidal form but were found to be toxic in powder form (Khot et al., 2012). Sodium dodecyl sulfate-modified photocatalytic TiO_2/silver nanomaterial conjugated with dimethomorph is commonly used in nanopesticide formulations (Khot et al., 2012). Silver nanoparticles when used along with surfactants such as cetylpyridinium bromide produce genotoxicity (Narayanan et al., 2013).

Nanoparticles such as clay, polymeric particles and silica capsules are used as encapsulation material for control and target delivery of many pesticides. Silica nanoparticles have also been used as insecticide as it is capable of desiccating insect cuticles. Silica nanoparticles, however, cannot be used as auxiliary as inhalation of silica nanoparticles has adverse effect on humans (Kim et al. 2015). Nanoparticles of chitosan, a polymeric material, have been explored recently for their ability for controlled release of agrochemicals. Biodegradability of chitosan makes it nontoxic, but concerns are associated with their changing absorption potential while interacting with protein or other molecules (Narayanan et al., 2013). In some cases, it is found that nanoformulation of the pesticide is less toxic than the normal formulation. A study on toxicity of chlorfenapyr nanopesticide on mice was found to be less toxic than normal formulation of chlorfenapyr (Khot et al., 2012).

2.3 Nanomaterial-Induced Toxicity

So far, we have discussed the harmful effects of nanomaterial that are of grave concern to the environment and in human health. However, at the same time, nanomaterial-based technologies have emerged as one of the promising, efficient and low-cost solutions for environmental remediation overcoming the limitations associated with existing conventional approaches (Etheridge et al., 2013; Das et al., 2015). There are two sources of nanomaterials—natural and anthropogenic. Natural sources include bacteria, viruses, volcanoes, ocean spray, dust storms, bush fires and many more, while anthropogenic sources include combustion aerosols, motor vehicle exhaust emission, coal fly ash and engineered nanomaterials (Mueller & Nowack, 2008). Nanotechnology aims to design and develop materials of nanometer scale (~1–100 nm range) that can be fabricated into advanced functionalities by varying their size and shape. Nanotechnology also has solutions for its own generated perils. Due to the small size and large surface-to-volume (S/V) ratio, they have great potential in development of highly sensitive, specific, accurate, stable and miniaturized devices that can monitor and treat various kinds of pollutants (Celebi et al., 2007; Das et al., 2015). Various nanotools including nanoparticles, nanotubes, nanospheres, nanocomposites and nanocatalysts have been employed in the control of air, water and soil pollution. This technology has also been successful in addressing the toxicity problems in various fields such as food industry, medicine, pollution control and energy. However, the hazard associated with the suspended nanoparticulates in the atmosphere cannot be ignored. As compared to natural nanomaterials, the percentage of anthropogenic nanomaterials in the atmosphere has been found to be high in the environment. Several industrial products, healthcare products and personal care products such as medicine, cosmetics and sunscreens using different forms of engineered nanomaterials are likely to enter the environment directly or indirectly during their manufacturing, use and disposal. Most widely used ENMs are metal oxide nanoparticles (Al_2O_3, MgO, ZrO_2, CeO2, TiO_2, FeO) which have industrial application. For instance, zirconium dioxide (ZrO_2) is widely used as a strong electrolyte, in structural ceramics, as catalyst in gas sensors. Titanium dioxide (TiO_2) is used as a catalyst for organic synthesis and a photocatalyst for pollutant removal, in food additives, for cosmetics, for sensors, for treatment of cancer, etc. (Westerhoff et al., 2011). In addition, mixtures of nanoparticles are also employed in the synthesis of mixed nanostructures for high performance such as in ceramic industry and rubber industry (Scheringer, 2008; Sun et al., 2014; Ding et al., 2015). Frequent use of these nanomaterials leads to "environmental toxicity" affecting the whole ecosystem (terrestrial and aquatic), which in turn affects human health via the food chain (Casals et al., 2008; Sánchez et al., 2011; Maurer-Jones et al., 2013; Exbrayat et al., 2015). Soil and water are the main sources where the leaching of nanoresidues from the industrial effluents occurs. In those ecosystems, the nanomaterials differ in their behavior undergoing aggregation, dissolution, adsorption, migration, etc. Some of the factors which influence the fate of these materials include environmental pH, salinity, hardness of water, and presence of natural organic matter (Klaine et al., 2008; Batley et al., 2013; James et al., 2014; Ma et al., 2015).

Figure 2.1 shows the various physiochemical characteristics that influence nanotoxicity. Materials manipulated at the nanoscale alter the physical and chemical

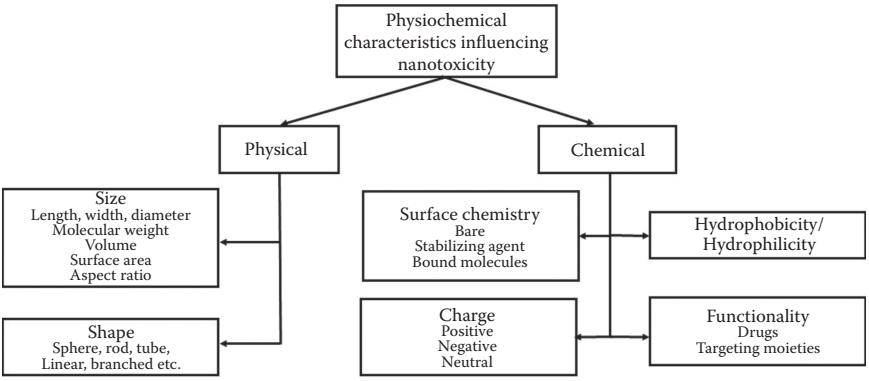


FIGURE 2.1 Physiochemical characteristics that influence nanotoxicity of materials. (Zhang, J.: Evaluation of nanotoxicity of foods and drugs: Biological properties of red elemental selenium at nano size (nano-Se) in vitro and in vivo. Zahu, S. C., & Casciano, D. A. (Eds.): *Nanotoxicity, from In Vivo and In Vitro Models to Health Risks*. pp. 97–114. 2009. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.)

properties of the material. These physical and chemical properties are the main factors behind the fate of nanomaterials to induce toxicity against biological entities. Toxicity depends on how the individual chemical properties such as surface chemistry, charge, polarity and functionality or physical properties such as size and shape interact with the biological materials.

2.3.1 Effects of Nanoparticles on Terrestrial Environment

In soil, nanomaterials occur naturally (volcanoes, glacial ice cores, organic matter, iron oxides, minerals, etc.) or are introduced to the soil matrix in engineered form (emitted from industrial, home appliances, healthcare products, etc.). Plants and microbial communities are adversely affected by the toxicity of these engineered nanomaterials. It has been observed that the engineered nanomaterial concentration is high in the soil ecosystem than in water or air (Klaine et al., 2008; Lee et al., 2010). However, there are only few studies reporting the environmental risk of engineered nanomaterials in the soil ecosystem affecting microbial communities, soil quality, soil enzymes, etc. (Simonin & Richaume, 2015). Studies reveal that engineered nannomaterials are toxic to bacteria such as *Bacillus subtilis*, *Escherichia coli* and *Vibrio fischeri*. Toxicity is induced in these bacterial species through mechanisms such as membrane disorganization, reactive oxygen species and surface coating-related photocatalytic oxidation. Studies show the toxicity accumulation on earthworms due to nanotubes (carbon nanotubes such as multiwalled carbon nanotubes and single-walled carbon nanotubes) (Peterson et al., 2008). Nanoparticles such as TiO_2, ZnO, and Al_2O_3 also show toxicity effects on earthworms. Some of these toxic effects include change in reproduction rate, DNA damage and bioaccumulation rate (Hu et al., 2010). In nematodes, the toxicity effect was mostly studied on *Caenorhabditis elegans* using various methods. Superoxide dismutase activity tests found that platinum nanoparticles induce oxidative stress in *C. elegans* through reactive oxygen species effect

(Kim et al., 2008). Nanomaterials such as silver nanoparticles, CeO_2 and TiO_2 show toxic effects in reproduction, growth and mortality of *C. elegans* (Roh et al., 2009). In another study, different nanoparticles (ZnO, Al_2O_3, and TiO_2) have been tested for their toxicity on *C. elegans* based on the particle size (bulk and nano) and were found to exhibit toxic effects by growth inhibition, particularly the reproductive power of the nematode (Wang et al., 2009). Copper oxide (CuO) copper nanoparticles could disrupt the *E. coli* membrane via K^+ leakage (Zhao et al., 2013). Further, the reduction in the soil dehydrogenase enzyme activity has been reported which was proposed to be a suitable sensor for the detection of soil quality. Other studies reported no significant toxicity on the soil enzymes (Tong et al., 2007; Hänsch & Emmerling, 2010). The effect of nanoparticles on the morphological and physiological nature of plants studied by toxicity testing is also evident from several studies (Ma et al., 2015). The level of toxicity may differ from plant species to species. Silver nanoparticles have been reported to reduce biomass as well as transpiration of *Cucurbita pepo* and germination of *Cucumis sativus* while having no effect on *Lactuca sativa*. Metal-based nanoparticles have been found to affect the chlorophyll content in many plant species as in wheat, green peas, tomatoes, etc., where it reduces the chlorophyll content while it increases in mustard, cucumber, cluster bean, etc. Studies reveal that these nanoparticles affect the cell membrane integrity and morphology of plants. Lipid peroxidation or ion leakage occurs as a result of nanoparticle exposure and also brings changes in root caps of maize, cucumber, etc. (Dimpka et al., 2012; Zhao et al., 2013; Rico et al., 2013; Pokhrel & Dubey, 2013). Few studies have reported DNA damage upon nanoparticle exposure such as dose-dependent genotoxic effects by CuO nanoparticles on terrestrial plants (transversional changes in DNA), by TiO_2, NiO, ZnO and Ag nanoparticles on plants such as onions, maize, tomato and bean (Patlolla et al., 2012; Faisal et al., 2013). Most of the studies mainly focused on nanotoxicity on individual components of the terrestrial ecosystem (plants or microorganisms), but not on soil–plant interaction. Toxicity induced by nanoparticles may not only affect the yield and quality of crops but also affect the soil microbial diversity and the ecosystem (between plants and microbes in the rhizosphere and endosphere). A decrease in the effect of ZnO nanoparticles was observed on the rhizobacterial population in a soil–plant interactive system rather than the soil systems without plants (Yeon & Kim, 2012).

2.3.2 Effect of Nanotoxicity on Aquatic Environment

The nanoparticles enter the water resources through various ways that include direct introduction, airborne deposition, groundwater transport and runoff from terrestrial resource. In aquatic systems, a large number of nanotoxicity-related studies have been carried out but studies emphasizing underlying mechanisms are scarce (Klaine et al., 2008; Wang et al., 2016). Studies report that reactive oxygen species generation is a well-studied nanotoxicity mechanism, the overproduction of which may lead to oxidative stress in the tissues of organism (Fu et al., 2014; Vale et al., 2016). It has been proposed that nanoparticles enhance the production of reactive oxygen species which in turn depends on several parameters such as the size of nanoparticles, their aggregation, solubility and coating. Studies report that the level of toxicity increases with decrease in nanoparticle size. However, a systematic correlation between particle size and toxicity levels is needed to establish which is still lacking.

Another approach to studying nanotoxicity is through the "omics" technologies, i.e., toxicogenomics, metallogenomics and proteomics (Zhang et al., 2011). Numerous studies evaluated and reported the toxicity levels of nanoparticles in freshwater organisms (Rainville et al., 2014; Kuznetsova et al., 2014). However, due to the large amount of data generated using "omics" tools and difficulty in interpretation of those data, these tools are not usually used by investigators. Majority of nanoparticles such as that of Ag, TiO_2, CuO, ZnO, fullerenes, carbon nanotubes and quantum dots (QDs) have shown adverse effects on the aquatic environment. It has been confirmed from several studies that silver nanoparticles are toxic to aquatic life, however, it is still unclear whether particulate form or ionic form of silver nanoparticles induces more toxicity (Vale et al., 2016). Particulate silver form (Ag coated with polyvinylpyrrolidone) enhances reactive oxygen species production and morphological variations in freshwater algae (Kumar et al., 2014). In zebrafish the ionic form of Ag (Ag^+) was responsible for many deformities like delaying the development of swim bladder while Ag nanoparticles showed minimum effects on the development (Powers et al., 2011). Likewise, *Cyprinus carpio*, zebrafish, and *Daphnia magna* showed changes in the morphology and gene profiles due to the effect of silver nanoparticles (Lee et al., 2012; Poynton et al., 2012). However, knowledge about the form of Ag that is responsible for biochemical changes in the snail *Lymnaea luteola* and algae *Chlorella vulgaris* is unclear (Ali et al., 2014).

TiO_2 nanoparticles have also been reported to produce toxicological effects such as increase or decrease in reactive oxygen species production and antioxidant enzyme activity (increase in chloramphenicol acetyl transferase, glutathione s-transferase, and glutathione peroxidase activity in *D. magna*; decrease in chloramphenicol acetyl transferase, glutathione s-transferase activity in *Danio rerio*; increase in superoxide dismutase activity in *C. dubia* etc.) and decrease in NaK-ATPase activity in gills and intestines of *D. magna*, *Oncorhynchus mykiss*, *Desmodesmus subspicatus*, *Artemia salina*, etc. (Kim et al., 2010; Dalai et al., 2013). Also, CuO, MgO and ZnO have been reported to cause reactive oxygen species generation, DNA damage, and antimicrobial effect on aquatic organisms such as *P. subcapitata*, *Chlamydomonas reinhardtii*, *Bacillus subtilis*, *Staphylococcus aureus*, *D. magna*, *E. coli* and *D. rerio* (Huang et al., 2005; Klaine et al., 2008; McQuillan & Shaw, 2014; Von Moos et al., 2015). In addition to the metal oxide nanoparticles, fullerenes have also been reported to be toxic to aquatic organisms such as *E. coli* (inhibiting growth due to interference in energy metabolism), *Micropterus salmoides* (largemouth bass—increase in lipid peroxidation), *D. magna* (increase in mortality rate), *Mytilus edulis* (cytotoxicity), *Crassostrea virginica* and *D. Rerio* (abnormal larval development) (Mashino et al., 1999; Oberdörster 2004, 2006).

Carbon nanotubes are another class of nanoparticles (single or multiwalled) which also affect the aquatic organisms due to their antimicrobial property. It causes membrane damage and growth inhibition activity, micronucleus and macronucleus damage, and growth inhibition in *D. magna* and *O. mykiss* by single-walled carbon nanotubes and *Stylonychia mytilus* by multiwalled carbon nanotubes (Cheng et al., 2007; James et al., 2014). Few other studies reported on the toxic effect of QDs on *Elliptio complanata* (cadmium telluride—CdTe), *C. reinhardtii* and *Pseudokirchneriella subcapitata* (CdSe/ZnS) by lipid peroxidation as well as damage to the DNA (Bouldin et al., 2008; James et al., 2014).

2.3.3 Nanotoxicity Risks within the Food Chain

The level of toxicity increases by accumulation along the food chain (from lower to higher trophic levels). Studies have been conducted to study the level of toxicity in various trophic levels of aquatic and terrestrial ecosystems.

2.3.3.1 Nanoparticle Transfer within the Food Chain of Terrestrial System

There are few studies that report on the understanding of nanoparticle transfer within the food chain of the terrestrial ecosystem in comparison to the aquatic ecosystem. Biomagnification or bioaccumulation in a terrestrial food chain depends upon parameters such as period of nanoparticle exposure and consumer growth stage. Studies report biomagnifications of gold nanoparticles from *Nicotiana tabacum* L. cv. *Xanthi* (producer level) to *Manduca sexta* (consumer level) via the food chain (Judy et al., 2010). Bioaccumulation of CeO_2 nanoparticles in zucchini (*Cucurbita pepo* L.) was found at higher levels of concentration than the bulk CeO_2, thus resulting in trophic transfer via the food chain (Hawthorne et al. 2014). A recent study also reported biomagnifications of cerium (Ce) from lower trophic levels (plants) to higher trophic levels (adult beetles followed by bugs) (Majumdar et al., 2015). Additional studies are required to gain deep insights into the biomagnification of contaminants from lower to higher trophic levels.

2.3.3.2 Nanoparticle Transfer within the Food Chain of the Aquatic System

A preliminary investigation revealed that biomagnifications of nanoparticles from *E. coli* to *Tetrahymena pyrifor* (ciliate) to *Brachionus calyciflorus* (rotifer) do not occur (Holbrook et al. 2008). Similar results were reported for TiO_2 nanoparticles and QDs from daphnia (*D. magna*) to zebrafish and from zooplankton (*Artemia franciscana* sp.) to zebrafish, respectively (Lewinski et al., 2011). Another study also reported similar observations from protozoan to microbe (Mielke et al., 2013). Further, the biomagnification of cadmium selenide (CdS) QDs at higher trophic levels (Werlin et al., 2011), as well as transfer and accumulation of TiO_2 nanoparticles from water fleas to zebrafish, dropwort roots to nematodes followed by snails, etc., through the food chain have been observed. This suggests that biomagnifications are dependent on the nature and concentration of nanoparticles and type of organisms. More studies are needed in order to observe the biomagnifications in various trophic levels in an aquatic ecosystem.

2.4 Mechanisms of Nanotoxicity

Nanomaterials are usually preferred due to the technical advantages they provide in comparison to other materials. Their unique properties also confer them more reactivity or catalytic ability ironically making them potent toxins. However, very few reports are available on the toxic effects of nanomaterials on the environment and its biotic components.

Living beings are exposed to nanomaterials through multiple modes like (a) respiratory tract by inhalation, (b) skin by contact, (c) inner body by ingestion, and (d) connective tissues such as blood by injection. Nanomaterials can pass the biological barriers easily owing to their tiny size and can be taken up into the system. Their inherent potential for toxicity can cause cellular dysfunction. The predominant mechanism observed in nanomaterial-induced toxicity is the "production of reactive oxygen species." These reactive oxygen species are produced in a cell normally and are utilized in cellular defense as well as cell signaling. However, when produced in surplus amounts, the same reactive species can cause detrimental effects to the cell. Overaccumulation of these reactive molecules leads to an imbalance of the redox state of the cell causing damage to important biological macromolecules such as proteins, lipids and DNA, thereby hindering important cellular processes such as cell signaling, motility and apoptosis. In the past decade, it has been shown through various experiments that nanoparticles incur heavy oxidative stress on the cells leading to cytotoxicity and genotoxicity (Khanna et al., 2015).

The production of reactive oxygen species by nanoparticles is mostly attributed to the charged functional groups present on the surface of the nanoparticle. For example, iron oxide nanoparticles with surface molecules poly(ethylenimine) (PEI) and PEI-poly(ethylene glycol) coupled, which are commonly used in biomedical applications, can cause higher lipid peroxidation and reactive oxygen species generation (Fu et al., 2014). In human keratinocyte cells, nano-TiO_2 particles are more photocytotoxic than the rutile form of TiO_2 proving that small size, which facilitates the penetrance of nanoparticles into any part of the body, can also act as a double-edged sword, facilitating more toxic response generation (Yin et al., 2012). Another important factor is "solubility" of the nanoparticle. Intercellular solubility plays a huge role in nanotoxicity. Copper metal nanoparticles showed that intrasoluble copper oxide nanoparticles were far more cytotoxic than insoluble and inert carbon-coated copper nanoparticles (Studer et al., 2010). Nanotoxicity which is induced by photoactivation i.e., when nanoparticles come in contact with light, is another form of nanotoxicity. A good example is the ZnO nanoparticle, which has semiconductor properties and on photoexcitation gives rise to excitons that may combine with surface-bound chemical groups such as oxygen (O_2), forming superoxide radicals/singlet oxygen radicals or like water (H_2O) forming hydroxyl radicals (Daimon & Nosaka, 2007). Other than these mechanisms, induction of inflammatory response in mucosal cells and alike as well as pH-dependent activity are also often observed as alternate ways of imparting nanotoxicity to cells by nanoparticles (Fu et al., 2014).

Due to intense research, newer mechanisms continue to be discovered that relate nanoparticles to toxicity. However, when one is developing strategies to eliminate or mitigate these toxic effects, it must be kept in mind that these mechanisms differ from one type of nanoparticle to another and from one condition to another.

Figure 2.2 shows the route of exposure of nanoparticles for inducing toxicity toward the ecosystem and human health as nanoparticles are released to air, land and water bodies by natural and anthropogenic sources. These particles accumulate in different components of the terrestrial and aquatic ecosystems including microorganisms. These particles also pass through tropic levels of food chains. Human are exposed to these particles from many different routes such as from food and medicine by ingestion, from air by inhalation, from textiles and cosmetics or medical patch or ointment by dermal contact, or from medicine by injections. Nanoparticles accumulate in the vital organs of the human body such as lungs, liver, spleen and kidney and cause health problems.

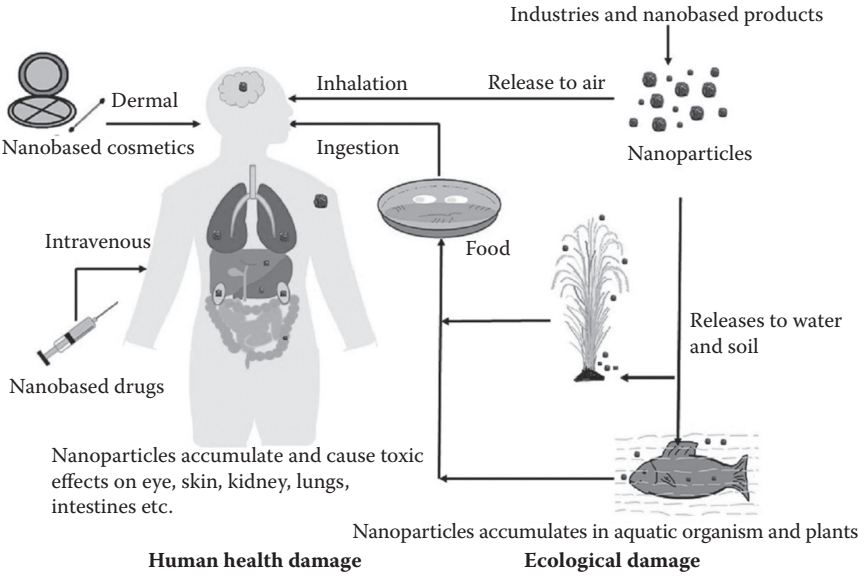


FIGURE 2.2 Route of exposure of nanoparticles for inducing toxicity toward ecosystem and human health.

2.5 Nanotoxicity Risk Assessment

The conventional risk assessment techniques of chemicals are not applicable in nanotoxicity risk assessment and hence suitable methods and regulations are needed. There are limited data and databases on nanotoxicity studies which are otherwise dominated by chemical toxicity. The nanotoxicity risk assessment techniques available at present mostly deal with risk screening for research sufficient and are not feasible for regulatory assessment. The lack of enough data on nanotoxicity and exposure is one of the major hindrances for risk assessment of engineered nanomaterials and its toxicity effects (Hristozov et al., 2012).

Risk assessment consists of a scientific approach in amalgamation with regulatory principles to describe the hazard intensity of the substance. The Organisation for Economic Co-operation and Development (OECD) defines risk assessment as the following:

> A process intended to calculate or estimate the risk to a given target organism, system or (sub) population, including the identification of attendant uncertainties, following exposure to a particular agent, taking into account the inherent characteristics of the agent of concern as well as the characteristics of the specific target system. (OECD 1994)

The risk assessment method has four steps, namely, (a) hazard identification, (b) hazard characterization, (c) exposure assessment, and (d) risk characterization. In simple words, risk assessment is to measure or analyze the intensity of harmful effect which will cause on the exposed organism or environment.

Hazard identification is the first basic step towards risk assessment for toxicity effect on the subject. It aims to determine the qualitative nature of the effect of nanotoxic contaminants. This is done by drawing conclusions of the results of the study in epidemiology, engineering or other concerned disciplines. For hazard identification, division of the nanomaterials into discrete categories is the need of the hour to speed up hazard identification and risk assessment.

Nanomaterials can be categorized based on the location of the nanoscale structure in the system. They are as follows:

- Materials that are nanostructured in bulk processing
- Materials that have a nanostructure on the surface
- Materials that contain nanostructured particles

These are further subdivided into subcategories based on physical characteristics. This narrows down the task of hazard identification on physical and chemical properties of the particles (Oberdörster et al., 2005). Extensive literature review identifies the properties such as composition, shape, structure, size, solubility, surface area and adhesion as important parameters for hazard identification (Hansen et al., 2007).

Hazard characterization basically involves relating the exposure intensity with the type of response generated. This step involves experimental studies as a need to extrapolate the results, by involving *in vivo* and *in vitro* experiments so as to substantiate the exposure response. This study involves exposing the *in vivo* and *in vitro* subjects to high and acute occupational-level exposure to low chronic environmental-level exposure. In some cases, it becomes necessary to involve genetics to study the reason for higher hazard level for any particular set of population. Dose–response analysis is the general method to analyze the intensity–effect relationship; another method is to determine the highest level of concentration of the substance that does not cause any effect.

Exposure assessment is the stage where the level of exposure to which the population, individuals or subjects receive is determined. There are various factors that may influence the exposure assessment including the climatic condition, air quality and location. The results generated in the three tasks previously mentioned are then used for risk assessment. Various computing and statistical techniques may be used for the assessment including uncertainty analysis. Conventional risk characterization of substances considered as genotoxic and carcinogenic is done by integrating the information from the hazard identification, dose–response and exposure assessments using a combination of qualitative and quantitative information and uncertainties. For risk characterization related to nanotoxicity, shape of the material and its surface charge and coating play a very important role over conventional parameters such as dose response (Tyshenko & Krewski, 2008). The decisions are made from the risk characterization step for devising risk management strategy. For new technologies with nanotechnology applications, two distinct approaches of risk management are adopted: (a) “precautionary approach” and (b) “risk-based approach.” In the precautionary approach, if the safety of the product or technology is not established to a satisfactory level, it is not allowed to introduce into the market or for wide application. The risk-based approach allows the introduction of product or technology into the market but a constant monitoring is done for its regulation in case safety is compromised (Williams et al., 2010).

Nanotoxicity of carbon-based nanoparticles or composite materials presents a challenge to the current risk assessment methodologies (Institute of Medicine, 2005). There are two important factors in risk assessment which plays a key role i.e., the chance of the event to occur and its effect or intensity (Presidential and Congressional Commission on Risk Assessment and Risk Management, 1997). Most of the countries at present do not have any proper evaluating and management technique (Tyshenko & Krewski, 2008).

Various literature reviews and studies have shown the negative consequences of nanotoxicity in human and environmental health. Databases on nanotoxicity and its effect are discrete and qualitative, and there is limited counter-control policy for nanotoxicity available (Emond & Britos, 2015). There is a lack of properly planned or designed counter management systems to deal with nanotoxicity. Risk assessment has to be entwined to risk management which is the task of reducing the risk to an acceptable level and continuous monitoring and control of the source (Zimmerman, 1986).

2.6 Conclusions

Nanotechnology and its products are playing a major role in improving many spheres of the human life. At the same time, the concern for the risk associated with it cannot be ignored; their effects on the environmental health and the population in general need to be taken care of. There is a need to focus on the study of physical and chemical properties of the nanoparticles and its impact on the environmental health.

At present, various types of novel nanotechnology and nanomaterials are being used in industries and products to enhance their desirable characteristics. The repercussions of such widely used domain need to be properly studied keeping in view its long-term future applications. Multiple studies have highlighted the adverse effects of nanotoxicity yet more studies are needed to be carried out to gain thorough insight into its long-term after-effects. The mechanism of nanotoxicity generation by industrial processes and products needs to be understood. The nanotoxicity in terms of side effects associated with drug delivery and nanomedicine needs to be further studied, as it is one of the routes where individuals are directly affected.

The mechanism and route through which nanotoxicity affects the environment and biosphere are still very less understood. A comprehensive research needs to be carried out to answer those missing links. The knowledge base on effect on terrestrial and aquatic environments still has many gaps to be covered. The biomagnification through the food chain is an important aspect which needs to be studied.

At present, there is a lack of proper infrastructure and bye-laws to tackle the problem of nanotoxicity. It is mainly due to the lack of complete understanding of the mechanism and after-effects of nanotoxicity. A global uniform regulation regarding the application, innovation, ethics and social implications of nanotechnology is needed to be put in place.

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REFERENCES

Ai, J., Biazar, E., Jafarpour, M., Montazeri, M., Majdi, A., Aminifard, S., & Rad, H. G. (2011). Nanotoxicology and nanoparticle safety in biomedical designs. *International Journal of Nanomedicine*, 6, 1117–1127.

Akhtar, M. J., Kumar, S., Murthy, R. C., Ashquin, M., Khan, M. I., Patil, G., & Ahmad, I. (2010). The primary role of iron-mediated lipid peroxidation in the differential cytotoxicity caused by two varieties of talc nanoparticles on A 549 cells and lipid peroxidation inhibitory effect exerted by ascorbic acid. *Toxicology in Vitro*, 24(4), 1139–1147.

Ali, D. (2014). Evaluation of environmental stress by comet assay on freshwater snail *Lymnea luteola* L. exposed to titanium dioxide nanoparticles. *Toxicological & Environmental Chemistry*, 96(8), 1185–1194.

Alkilany, A. M., Nagaria, P. K., Hexel, C. R., Shaw, T. J., Murphy, C. J., & Wyatt, M. D. (2009). Cellular uptake and cytotoxicity of gold nanorods: Molecular origin of cytotoxicity and surface effects. *Small*, 5(6), 701–708.

Arvizo, R., Bhattacharya, R., & Mukherjee, P. (2010). Gold nanoparticles: Opportunities and challenges in nanomedicine. *Expert Opinion on Drug Delivery*, 7(6), 753–763.

Auffan, M., Pedeutour, M., Rose, J., Masion, A., Ziarelli, F., Borschneck, D., & Bottero, J. Y. (2010). Structural degradation at the surface of a TiO_2-based nanomaterial used in cosmetics. *Environmental Science & Technology*, 44(7), 2689–2694.

Batley, G. E., Kirby, J. K., & McLaughlin, M. J. (2013). Fate and risks of nanomaterials in aquatic and terrestrial environments. *Accounts of Chemical Research*, 46(3), 854–862.

Benn, T. M. & Westerhoff, P. (2008). Nanoparticle silver released into water from commercially available sock fabrics. *Environmental Science & Technology*, 42(11), 4133–4139.

Bouldin, J. L., Ingle, T. M., Sengupta, A., Alexander, R., Hannigan, R. E., & Buchanan, R. A. (2008). Aqueous toxicity and food chain transfer of quantum dots™ in freshwater algae and *Ceriodaphnia dubia*. *Environmental Toxicology and Chemistry*, 27(9), 1958–1963.

Brakmane, G., Winslet, M., & Seifalian, A. M. (2012). Systematic review: The applications of nanotechnology in gastroenterology. *Alimentary Pharmacology & Therapeutics*, 36(3), 213–221.

Buzea, C., Pacheco, I. I., & Robbie, K. (2007). Nanomaterials and nanoparticles: Sources and toxicity. *Biointerphases*, 2(4), MR17–MR71.

Byrne, E., Mullally, G., & Sage, C. (Eds.). (2016). *Transdisciplinary Perspectives on Transitions to Sustainability*. Routledge, Abingdon, UK.

Casals, E., Vázquez-Campos, S., Bastús, N. G., & Puntes, V. (2008). Distribution and potential toxicity of engineered inorganic nanoparticles and carbon nanostructures in biological systems. *TrAC Trends in Analytical Chemistry*, 27(8), 672–683.

Celebi, O., Üzüm, Ç., Shahwan, T., & Erten, H. N. (2007). A radiotracer study of the adsorption behavior of aqueous Ba 2+ ions on nanoparticles of zero-valent iron. *Journal of Hazardous Materials*, 148(3), 761–767.

Chang, M. R., Lee, D. J., & Lai, J. Y. (2007). Nanoparticles in wastewater from a science-based industrial park—Coagulation using polyaluminum chloride. *Journal of Environmental Management*, 85(4), 1009–1014.

Chen, J. K., Ho, C. C., Chang, H., Lin, J. F., Yang, C. S., Tsai, M. H., & Lin, P. (2015). Particulate nature of inhaled zinc oxide nanoparticles determines systemic effects and mechanisms of pulmonary inflammation in mice. *Nanotoxicology*, 9(1), 43–53.

Cheng, J., Flahaut, E., & Cheng, S. H. (2007). Effect of carbon nanotubes on developing zebrafish (*Danio rerio*) embryos. *Environmental Toxicology and Chemistry*, 26(4), 708–716.

Cho, W. S., Duffin, R., Howie, S. E., Scotton, C. J., Wallace, W. A., MacNee, W., & Donaldson, K. (2011). Progressive severe lung injury by zinc oxide nanoparticles; the role of Zn^{2+} dissolution inside lysosomes. *Particle and Fibre Toxicology*, 8(1), 1.

Daimon, T. & Nosaka, Y. (2007). Formation and behavior of singlet molecular oxygen in TiO_2 photocatalysis studied by detection of near-infrared phosphorescence. *The Journal of Physical Chemistry C*, 111(11), 4420–4424.

Dalai, S., Pakrashi, S., Chandrasekaran, N., & Mukherjee, A. (2013). Acute toxicity of TiO_2 nanoparticles to Ceriodaphnia dubia under visible light and dark conditions in a freshwater system. *PloS One*, 8(4), e62970.

Das, S., Sen, B., & Debnath, N. (2015). Recent trends in nanomaterials applications in environmental monitoring and remediation. *Environmental Science and Pollution Research*, 22(23), 18333–18344.

Dimkpa, C. O., McLean, J. E., Latta, D. E., Manangón, E., Britt, D. W., Johnson, W. P., & Anderson, A. J. (2012). CuO and ZnO nanoparticles: Phytotoxicity, metal speciation, and induction of oxidative stress in sand-grown wheat. *Journal of Nanoparticle Research*, 14(9), 1–15.

Ding, L., Liu, Z., Okweesi Aggrey, M., Li, C., Chen, J., & Tong, L. (2015). Nanotoxicity: The toxicity research progress of metal and metal-containing nanoparticles. *Mini Reviews in Medicinal Chemistry*, 15(7), 529–542.

Dubey, A. & Mailapalli, D. R. (2016). Nanofertilisers, nanopesticides, nanosensors of pest and nanotoxicity in agriculture. In *Sustainable Agriculture Reviews*, pp. 307–330, Springer International Publishing, Basel.

Duncan, R. & Izzo, L. (2005). Dendrimer biocompatibility and toxicity. *Advanced Drug Delivery Reviews*, 57(15), 2215–2237.

El-Ansary, A. & Al-Daihan, S. (2009). On the toxicity of therapeutically used nanoparticles: An overview. *Journal of Toxicology*, 2009, Article ID 754810, 1–9.

Emond, C. & Britos, T. N. (2015). Human risk assessment and its application to nanotechnology: A challenge for assessors. *Journal of Physics: Conference Series*, 617(1), 012039.

Etheridge, M. L., Campbell, S. A., Erdman, A. G., Haynes, C. L., Wolf, S. M., & McCullough, J. (2013). The big picture on nanomedicine: The state of investigational and approved nanomedicine products. *Nanomedicine: Nanotechnology, Biology and Medicine*, 9(1), 1–14.

Exbrayat, J. M., Moudilou, E. N., & Lapied, E. (2015). Harmful effects of nanoparticles on animals. *Journal of Nanotechnology*, 2015, 861092.

Faisal, M., Saquib, Q., Alatar, A. A., Al-Khedhairy, A. A., Hegazy, A. K., & Musarrat, J. (2013). Phytotoxic hazards of NiO-nanoparticles in tomato: A study on mechanism of cell death. *Journal of Hazardous Materials*, 250, 318–332.

Foss Hansen, S., Larsen, B. H., Olsen, S. I., & Baun, A. (2007). Categorization framework to aid hazard identification of nanomaterials. *Nanotoxicology*, 1(3), 243–250.

Fu, P. P., Xia, Q., Hwang, H. M., Ray, P. C., & Yu, H. (2014). Mechanisms of nanotoxicity: Generation of reactive oxygen species. *Journal of Food and Drug Analysis*, 22(1), 64–75.

Fujishima, A., Zhang, X., & Tryk, D. A. (2007). Heterogeneous photocatalysis: From water photolysis to applications in environmental cleanup. *International Journal of Hydrogen Energy*, 32(14), 2664–2672.

Ge, Z. & Gao, Z. (2008). Applications of nanotechnology and nanomaterials in construction. *First International Conference on Construction in Developing Countries*, 235–240.

Geiser, M., Rothen-Rutishauser, B., Kapp, N., Schürch, S., Kreyling, W., Schulz, H., & Gehr, P. (2005). Ultrafine particles cross cellular membranes by nonphagocytic mechanisms in lungs and in cultured cells. *Environmental Health Perspectives*, 1555–1560.

Hänsch, M. & Emmerling, C. (2010). Effects of silver nanoparticles on the microbiota and enzyme activity in soil. *Journal of Plant Nutrition and Soil Science*, 173(4), 554–558.

Hawthorne, J., De la Torre Roche, R., Xing, B., Newman, L. A., Ma, X., Majumdar, S., & White, J. C. (2014). Particle-size dependent accumulation and trophic transfer of cerium oxide through a terrestrial food chain. *Environmental Science & Technology*, 48(22), 13102–13109.

Holbrook, R. D., Murphy, K. E., Morrow, J. B., & Cole, K. D. (2008). Trophic transfer of nanoparticles in a simplified invertebrate food web. *Nature Nanotechnology*, 3(6), 352–355.

Hristozov, D. R., Gottardo, S., Critto, A., & Marcomini, A. (2012). Risk assessment of engineered nanomaterials: A review of available data and approaches from a regulatory perspective. *Nanotoxicology*, 6(8), 880–898.

Hu, C. W., Li, M., Cui, Y. B., Li, D. S., Chen, J., & Yang, L. Y. (2010). Toxicological effects of TiO_2 and ZnO nanoparticles in soil on earthworm *Eisenia fetida*. *Soil Biology and Biochemistry*, 42(4), 586–591.

Huang, L., Li, D. Q., Lin, Y. J., Wei, M., Evans, D. G., & Duan, X. (2005). Controllable preparation of nano-MgO and investigation of its bactericidal properties. *Journal of Inorganic Biochemistry*, 99(5), 986–993.

Institute of Medicine. (2005). *Implications of Nanotechnology for Environmental Health Research*, Workshop Summary. Institute of Medicine, Washington, DC.

ISO (International Organization for Standardization) (2010). ISO/TS 80004-1:2010 Nanotechnologies—Vocabulary—Part 1: Core terms. ISO, Geneva.

Ivask, A., Juganson, K., Bondarenko, O., Mortimer, M., Aruoja, V., Kasemets, K., & Kahru, A. (2014). Mechanisms of toxic action of Ag, ZnO and CuO nanoparticles to selected ecotoxicological test organisms and mammalian cells in vitro: A comparative review. *Nanotoxicology*, 8(sup1), 57–71.

James J., Saxena P., Rajendran, N. (2014). Nanotoxicity of materials on marine and aquatic organisms. *International Journal of Pharmaceutical Sciences Review and Research*, 27(2), 117–124.

Jarvie, H. P., Al-Obaidi, H., King, S. M., Bowes, M. J., Lawrence, M. J., Drake, A. F., & Dobson, P. J. (2009). Fate of silica nanoparticles in simulated primary wastewater treatment. *Environmental Science & Technology*, 43(22), 8622–8628.

Judy, J. D., Unrine, J. M., & Bertsch, P. M. (2010). Evidence for biomagnification of gold nanoparticles within a terrestrial food chain. *Environmental Science & Technology*, 45(2), 776–781.

Kamat, J. P., Devasagayam, T. P. A., Priyadarsini, K. I., & Mohan, H. (2000). Reactive oxygen species mediated membrane damage induced by fullerene derivatives and its possible biological implications. *Toxicology*, 155(1), 55–61.

Kazimirova, A., Magdolenova, Z., Barancokova, M., Staruchova, M., Volkovova, K., & Dusinska, M. (2012). Genotoxicity testing of PLGA–PEO nanoparticles in TK6 cells by the comet assay and the cytokinesis-block micronucleus assay. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 748(1), 42–47.

Khanna, P., Ong, C., Bay, B. H., & Baeg, G. H. (2015). Nanotoxicity: An interplay of oxidative stress, inflammation and cell death. *Nanomaterials*, 5(3), 1163–1180.

Khot, L. R., Sankaran, S., Maja, J. M., Ehsani, R., & Schuster, E. W. (2012). Applications of nanomaterials in agricultural production and crop protection: A review. *Crop Protection*, 35, 64–70.

Kim, I. Y., Joachim, E., Choi, H., & Kim, K. (2015). Toxicity of silica nanoparticles depends on size, dose, and cell type. *Nanomedicine: Nanotechnology, Biology and Medicine*, 11(6), 1407–1416.

Kim, J. K., Shin, J. H., Lee, J. S., Hwang, J. H., Lee, J. H., Baek, J. E., & Ahn, K. (2016). 28-Day inhalation toxicity of graphene nanoplatelets in Sprague–Dawley rats. *Nanotoxicology*, 1–11.

Kim, J., Takahashi, M., Shimizu, T., Shirasawa, T., Kajita, M., Kanayama, A., & Miyamoto, Y. (2008). Effects of a potent antioxidant, platinum nanoparticle, on the lifespan of *Caenorhabditis elegans*. *Mechanisms of Ageing and Development*, 129(6), 322–331.

Kim, K. T., Klaine, S. J., Cho, J., Kim, S. H., & Kim, S. D. (2010). Oxidative stress responses of *Daphnia magna* exposed to TiO_2 nanoparticles according to size fraction. *Science of the Total Environment*, 408(10), 2268–2272.

Kim, S. Y., Jeong, S. H., Lee, E. Y., Park, Y. H., Bae, H. C., Jang, Y. S., & Son, S. W. (2011). Skin absorption potential of ZnO nanoparticles. *Toxicology and Environmental Health Sciences*, 3(4), 258–261.

Kim, Y. S., Kim, J. S., Cho, H. S., Rha, D. S., Kim, J. M., Park, J. D., & Kwon, I. H. (2008). Twenty-eight-day oral toxicity, genotoxicity, and gender-related tissue distribution of silver nanoparticles in Sprague–Dawley rats. *Inhalation Toxicology*, 20(6), 575–583.

Kittelson, D. B. (1998). Engines and nanoparticles: A review. *Journal of Aerosol Science*, 29(5), 575–588.

Klaine, S. J., Alvarez, P. J., Batley, G. E., Fernandes, T. F., Handy, R. D., Lyon, D. Y., Mahendra, S., McLaughlin, M. J., & Lead, J. R. (2008). Nanomaterials in the environment: Behavior, fate, bioavailability, and effects. *Environmental Toxicology and Chemistry*, 27(9), 1825–1851.

Köhler, A. R., Som, C., Helland, A., & Gottschalk, F. (2008). Studying the potential release of carbon nanotubes throughout the application life cycle. *Journal of Cleaner Production*, 16(8), 927–937.

Kourmpanis, B., Papadopoulos, A., Moustakas, K., Stylianou, M., Haralambous, K. J., & Loizidou, M. (2008). Preliminary study for the management of construction and demolition waste. *Waste Management & Research*, 26(3), 267–275.

Kulthong, K., Srisung, S., Boonpavanitchakul, K., Kangwansupamonkon, W., & Maniratanachote, R. (2010). Determination of silver nanoparticle release from antibacterial fabrics into artificial sweat. *Particle and Fibre Toxicology*, 7(1), 1.

Kumar, D., Kumari, J., Pakrashi, S., Dalai, S., Raichur, A. M., Sastry, T. P., & Mukherjee, A. (2014). Qualitative toxicity assessment of silver nanoparticles on the fresh water bacterial isolates and consortium at low level of exposure concentration. *Ecotoxicology and Environmental Safety*, 108, 152–160.

Kuznetsova, G. P., Larina, O. V., Petushkova, N. A., Kisrieva, Y. S., Samenkova, N. F., Trifonova, O. P. & Lisitsa, A. V. (2014). Effects of fullerene C60 on proteomic profile of *Danio rerio* fish embryos. *Bulletin of Experimental Biology and Medicine*, 156(5), 694–698.

Lam, C. W., James, J. T., McCluskey, R., Arepalli, S., & Hunter, R. L. (2006). A review of carbon nanotube toxicity and assessment of potential occupational and environmental health risks. *Critical Reviews in Toxicology*, 36(3), 189–217.

Lee, B., Duong, C. N., Cho, J., Lee, J., Kim, K., Seo, Y., Kim, P., Choi, K., & Yoon, J. (2012). Toxicity of citrate-capped silver nanoparticles in common carp (*Cyprinus carpio*). *Journal of Biomedicine and Biotechnology*, 2012, 262670.

Lee, J., Mahendra, S., & Alvarez, P. J. (2010). Nanomaterials in the construction industry: A review of their applications and environmental health and safety considerations. AC*S Nano*, 4(7), 3580–3590.

Lee, W. M., Kim, S. W., Kwak, J. I., Nam, S. H., Shin, Y. J., & An, Y. J. (2010). Research trends of ecotoxicity of nanoparticles in soil environment. *Toxicological Research*, 26(4), 253.

Lewinski, N. A., Zhu, H., Ouyang, C. R., Conner, G. P., Wagner, D. S., Colvin, V. L., & Drezek, R. A. (2011). Trophic transfer of amphiphilic polymer coated CdSe/ZnS quantum dots to *Danio rerio*. *Nanoscale*, 3(8), 3080–3083.

Liu, Y., Li, W., Lao, F., Liu, Y., Wang, L., Bai, R., Zhao, Y., & Chen, C. (2011). Intracellular dynamics of cationic and anionic polystyrene nanoparticles without direct interaction with mitotic spindle and chromosomes. *Biomaterials*, 32(32), 8291–8303.

Lowry, G. V., & Casman, E. A. (2009). Nanomaterial transport, transformation, and fate in the environment. In *Nanomaterials: Risks and Benefits* (pp. 125–137). Springer, Dordrecht.

Lu, P. J., Huang, S. C., Chen, Y. P., Chiueh, L. C., & Shih, D. Y. C. (2015). Analysis of titanium dioxide and zinc oxide nanoparticles in cosmetics. *Journal of Food and Drug Analysis*, 23(3), 587–594.

Ma, C., White, J. C., Dhankher, O. P., & Xing, B. (2015). Metal-based nanotoxicity and detoxification pathways in higher plants. *Environmental Science & Technology*, 49(12), 7109–7122.

Madureira, A. R., Nunes, S., Campos, D. A., Fernandes, J. C., Marques, C., Zuzarte, M., & Gomes, A. M. (2016). Safety profile of solid lipid nanoparticles loaded with rosmarinic acid for oral use: In vitro and animal approaches. *International Journal of Nanomedicine*, 11, 3621.

Majumdar, S., Trujillo-Reyes, J., Hernandez-Viezcas, J. A., White, J. C., Peralta-Videa, J. R., & Gardea-Torresdey, J. L. (2015). Cerium biomagnification in a terrestrial food chain: Influence of particle size and growth stage. *Environmental Science & Technology*, 50, 6782–6792.

Mashino, T., Okuda, K., Hirota, T., Hirobe, M., Nagano, T., & Mochizuki, M. (1999). Inhibition of *E. coli* growth by fullerene derivatives and inhibition mechanism. *Bioorganic & Medicinal Chemistry Letters*, 9(20), 2959–2962.

Maurer-Jones, M. A., Gunsolus, I. L., Murphy, C. J., & Haynes, C. L. (2013). Toxicity of engineered nanoparticles in the environment. *Analytical Chemistry*, 85(6), 3036–3049.

Mcquillan, J. S. & Shaw, A. M. (2014). Whole-cell *Escherichia coli*-based bio-sensor assay for dual zinc oxide nanoparticle toxicity mechanisms. *Biosensors and Bioelectronics*, 51, 274–279.

Meredith, A. N., Harper, B., & Harper, S. L. (2016). The influence of size on the toxicity of an encapsulated pesticide: A comparison of micron-and nanosized capsules. *Environment International*, 86, 68–74.

Merget, R., Bauer, T., Küpper, H., Philippou, S., Bauer, H., Breitstadt, R., & Bruening, T. (2002). Health hazards due to the inhalation of amorphous silica. *Archives of Toxicology*, 75(11–12), 625–634.

Mielke, R. E., Priester, J. H., Werlin, R. A., Gelb, J., Horst, A. M., Orias, E., & Holden, P. A. (2013). Differential growth of and nanoscale TiO_2 accumulation in *Tetrahymena thermophila* by direct feeding versus trophic transfer from *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology*, 79(18), 5616–5624.

Mueller, N. C. & Nowack, B. (2008). Exposure modeling of engineered nanoparticles in the environment. *Environmental Science & Technology*, 42(12), 4447–4453.

Müller, R. H., Petersen, R. D., Hommoss, A., & Pardeike, J. (2007). Nanostructured lipid carriers (NLC) in cosmetic dermal products. *Advanced Drug Delivery Reviews*, 59(6), 522–530.

Murphy, C. J., Gole, A. M., Stone, J. W., Sisco, P. N., Alkilany, A. M., Goldsmith, E. C., & Baxter, S. C. (2008). Gold nanoparticles in biology: Beyond toxicity to cellular imaging. *Accounts of Chemical Research*, 41(12), 1721–1730.

Narayanan, A., Sharma, P., & Moudgil, B. M. (2013). Applications of engineered particulate systems in agriculture and food industry. *KONA Powder and Particle Journal*, 30(0), 221–235.

Narayanan, K. B. & Park, H. H. (2013). Pleiotropic functions of antioxidant nanoparticles for longevity and medicine. *Advances in Colloid and Interface Science*, 201, 30–42.

Nelson, J. (2003). *The Physics of Solar Cells* (Vol. 1, pp. 1–15). Imperial College Press, London.

Nowack, B. & Bucheli, T. D. (2007). Occurrence, behavior and effects of nanoparticles in the environment. *Environmental Pollution*, 150(1), 5–22.

Oberdörster, E. (2004). Manufactured nanomaterials (fullerenes, C60) induce oxidative stress in the brain of juvenile largemouth bass. *Environmental Health Perspectives*, 1058–1062.

Oberdörster, E., Zhu, S., Blickley, T. M., McClellan-Green, P., & Haasch, M. L. (2006). Ecotoxicology of carbon-based engineered nanoparticles: Effects of fullerene (C60) on aquatic organisms. *Carbon*, 44(6), 1112–1120.

Oberdorster, G., Gelein, R. M., Ferin, J., & Weiss, B. (1995). Association of particulate air pollution and acute mortality: Involvement of ultrafine particles? *Inhalation Toxicology*, 7(1), 111–124.

Oberdörster, G., Maynard, A., Donaldson, K., Castranova, V., Fitzpatrick, J., Ausman, K., & Olin, S. (2005). Principles for characterizing the potential human health effects from exposure to nanomaterials: Elements of a screening strategy. *Particle and Fibre Toxicology*, 2(1), 1.

Oberdörster, G., Sharp, Z., Atudorei, V., Elder, A., Gelein, R., Kreyling, W., & Cox, C. (2004). Translocation of inhaled ultrafine particles to the brain. *Inhalation Toxicology*, 16(6–7), 437–445.

OECD (Organisation for Economic Cooperation and Development). (1994). *OECD Guidelines for the Testing of Chemicals*. OECD, Paris.

Ou, L., Song, B., Liang, H., Liu, J., Feng, X., Deng, B., & Shao, L. (2016). Toxicity of graphene-family nanoparticles: A general review of the origins and mechanisms. *Particle and Fibre Toxicology*, 13(1), 57.

Panyala, N. R., Peña-Méndez, E. M., & Havel, J. (2008). Silver or silver nanoparticles: A hazardous threat to the environment and human health. *Journal of Applied Biomedicine*, 6(3), 117–129.

Pardeike, J., Hommoss, A., & Müller, R. H. (2009). Lipid nanoparticles (SLN, NLC) in cosmetic and pharmaceutical dermal products. *International Journal of Pharmaceutics*, 366(1), 170–184.

Patlolla, A. K., Berry, A., May, L., & Tchounwou, P. B. (2012). Genotoxicity of silver nanoparticles in *Vicia faba*: A pilot study on the environmental monitoring of nanoparticles. *International Journal of Environmental Research and Public Health*, 9(5), 1649–1662.

Patlolla, V. R., Srikanth, M., Asmatulu, R. (2012). *Review of Various Nanomaterials and Their Major Health Issues*. International SAMPE Technical Conference, Charleston, NC.

Petersen, E. J., Huang, Q., & Weber, Jr, W. J. (2008). Bioaccumulation of radio-labeled carbon nanotubes by Eisenia foetida. *Environmental Science & Technology*, 42(8), 3090–3095.

Pokhrel, L. R. & Dubey, B. (2013). Evaluation of developmental responses of two crop plants exposed to silver and zinc oxide nanoparticles. *Science of the Total Environment*, 452, 321–332.

Powers, C. M., Badireddy, A. R., Ryde, I. T., Seidler, F. J., & Slotkin, T. A. (2011). Silver nanoparticles compromise neurodevelopment in PC12 cells: Critical contributions of silver ion, particle size, coating, and composition. *Environmental Health Perspectives*, 119(1), 37.

Powers, C. M., Slotkin, T. A., Seidler, F. J., Badireddy, A. R., & Padilla, S. (2011). Silver nanoparticles alter zebrafish development and larval behavior: Distinct roles for particle size, coating and composition. *Neurotoxicology and Teratology*, 33(6), 708–714.

Poynton, H. C., Lazorchak, J. M., Impellitteri, C. A., Blalock, B. J., Rogers, K., Allen, H. J., & Govindasmawy, S. (2012). Toxicogenomic responses of nanotoxicity in Daphnia magna exposed to silver nitrate and coated silver nanoparticles. *Environmental Science & Technology*, 46(11), 6288–6296.

Presidential and Congressional Commission on Risk Assessment and Risk Management. (1997). Framework for environmental health risk management, *Risk Assessment and Risk Management in Regulatory Decision-Making, Final Report* (Vol. 1, p. 1), DIANE Publishing, US Environmental Agency, Washington, DC.

Rainville, L. C., Carolan, D., Varela, A. C., Doyle, H., & Sheehan, D. (2014). Proteomic evaluation of citrate-coated silver nanoparticles toxicity in *Daphnia magna*. *Analyst*, 139(7), 1678–1686.

Rezić, I. (2011). Determination of engineered nanoparticles on textiles and in textile wastewaters. *TrAC: Trends in Analytical Chemistry*, 30(7), 1159–1167.

Rico, C. M., Hong, J., Morales, M. I., Zhao, L., Barrios, A. C., Zhang, J. Y., & Gardea-Torresdey, J. L. (2013). Effect of cerium oxide nanoparticles on rice: A study involving the antioxidant defense system and in vivo fluorescence imaging. *Environmental Science & Technology*, 47(11), 5635–5642.

Roh, J. Y., Sim, S. J., Yi, J., Park, K., Chung, K. H., Ryu, D. Y., & Choi, J. (2009). Ecotoxicity of silver nanoparticles on the soil nematode Caenorhabditis elegans using functional ecotoxicogenomics. *Environmental Science & Technology*, 43(10), 3933–3940.

Sánchez, A., Recillas, S., Font, X., Casals, E., González, E., & Puntes, V. (2011). Ecotoxicity of, and remediation with, engineered inorganic nanoparticles in the environment. *TrAC: Trends in Analytical Chemistry*, 30(3), 507–516.

Saraceno, R., Chiricozzi, A., Gabellini, M., & Chimenti, S. (2013). Emerging applications of nanomedicine in dermatology. *Skin Research and Technology*, 19(1), e13–e19.

Scheringer, M. (2008). Nanoecotoxicology: Environmental risks of nanomaterials. *Nature Nanotechnology*, 3(6), 322–323.

Simonin, M. & Richaume, A. (2015). Impact of engineered nanoparticles on the activity, abundance, and diversity of soil microbial communities: A review. *Environmental Science and Pollution Research*, 22(18), 13710–13723.

Som, C., Wick, P., Krug, H., & Nowack, B. (2011). Environmental and health effects of nanomaterials in nanotextiles and facade coatings. *Environment International*, 37(6), 1131–1142.

Studer, A. M., Limbach, L. K., Van Duc, L., Krumeich, F., Athanassiou, E. K., Gerber, L. C., & Stark, W. J. (2010). Nanoparticle cytotoxicity depends on intracellular solubility: Comparison of stabilized copper metal and degradable copper oxide nanoparticles. *Toxicology Letters*, 197(3), 169–174.

Sun, B., Shao, M., & Lee, S. (2016). Nanostructured silicon used for flexible and mobile electricity generation. *Advanced Materials*, 28(47), 10539–10547.

Sun, T. Y., Gottschalk, F., Hungerbühler, K., & Nowack, B. (2014). Comprehensive probabilistic modelling of environmental emissions of engineered nanomaterials. *Environmental Pollution*, 185, 69–76.

Tong, Z., Bischoff, M., Nies, L., Applegate, B., & Turco, R. F. (2007). Impact of fullerene (C60) on a soil microbial community. *Environmental Science & Technology*, 41(8), 2985–2991.

Tyshenko, M. G. & Krewski, D. (2008). A risk management framework for the regulation of nanomaterials. *International Journal of Nanotechnology*, 5(1), 143–160.

Vale, G., Mehennaoui, K., Cambier, S., Libralato, G., Jomini, S., & Domingos, R. F. (2016). Manufactured nanoparticles in the aquatic environment-biochemical responses on freshwater organisms: A critical overview. *Aquatic Toxicology*, 170, 162–174.

Von Moos, N., Maillard, L., & Slaveykova, V. I. (2015). Dynamics of sub-lethal effects of nano-CuO on the microalga *Chlamydomonas reinhardtii* during short-term exposure. *Aquatic Toxicology*, 161, 267–275.

Wang, B., Feng, W., Wang, M., Wang, T., Gu, Y., Zhu, M., & Chai, Z. (2008). Acute toxicological impact of nano- and submicroscaled zinc oxide powder on healthy adult mice. *Journal of Nanoparticle Research*, 10(2), 263–276.

Wang, H., Wick, R. L., & Xing, B. (2009). Toxicity of nanoparticulate and bulk ZnO, Al_2O_3 and TiO_2 to the nematode *Caenorhabditis elegans*. *Environmental Pollution*, 157(4), 1171–1177.

Wang, Z., Zhang, L., Zhao, J., & Xing, B. (2016). Environmental processes and toxicity of metallic nanoparticles in aquatic systems as affected by natural organic matter. *Environmental Science: Nano*, 3(2), 240–255.

Werlin, R., Priester, J. H., Mielke, R. E., Krämer, S., Jackson, S., Stoimenov, P. K., & Holden, P. A. (2011). Biomagnification of cadmium selenide quantum dots in a simple experimental microbial food chain. *Nature Nanotechnology*, 6(1), 65–71.

Westerhoff, P., Song, G., Hristovski, K., & Kiser, M. A. (2011). Occurrence and removal of titanium at full scale wastewater treatment plants: Implications for TiO_2 nanomaterials. *Journal of Environmental Monitoring*, 13(5), 1195–1203.

Williams, R. A., Kulinowski, K. M., White, R., & Louis, G. (2010). Risk characterization for nanotechnology. *Risk Analysis*, 30(11), 1671–1679.

Yeon, L. S. & Kim, S. H. (2012). Effects of soil-plant interactive system on response to exposure to ZnO nanoparticles. *Journal of Microbiology and Biotechnology*, 22(9), 1264–1270.

Yildirimer, L., Thanh, N. T., Loizidou, M., & Seifalian, A. M. (2011). Toxicology and clinical potential of nanoparticles. *Nano Today*, 6(6), 585–607.

Yin, J. J., Liu, J., Ehrenshaft, M., Roberts, J. E., Fu, P. P., Mason, R. P., & Zhao, B. (2012). Phototoxicity of nano titanium dioxides in HaCaT keratinocytes—Generation of reactive oxygen species and cell damage. *Toxicology and Applied Pharmacology*, 263(1), 81–88.

Zhang, J. (2009). Evaluation of nanotoxicity of foods and drugs: Biological properties of red elemental selenium at nano size (nano-Se) in vitro and in vivo. *Nanotoxicity, from In Vivo and In Vitro Models to Health Risks*, Zahu, S. C., & Casciano, D. A. (Eds.) (pp. 97–114). Wiley, Hoboken, NJ.

Zhang, R., Bai, Y., Zhang, B., Chen, L., & Yan, B. (2012). The potential health risk of titania nanoparticles. *Journal of Hazardous Materials*, 211, 404–413.

Zhang, Z., He, X., Zhang, H., Ma, Y., Zhang, P., Ding, Y., & Zhao, Y. (2011). Uptake and distribution of ceria nanoparticles in cucumber plants. *Metallomics*, 3(8), 816–822.

Zhao, L., Sun, Y., Hernandez-Viezcas, J. A., Servin, A. D., Hong, J., Niu, G., & Gardea-Torresdey, J. L. (2013). Influence of CeO_2 and ZnO nanoparticles on cucumber physiological markers and bioaccumulation of Ce and Zn: A life cycle study. *Journal of Agricultural and Food Chemistry*, 61(49), 11945–11951.

Zimmerman, R. (1986). The management of risk, in Covello, V. T., Menkes, J., & Munpower, J. (Eds.). *Risk Evaluation and Management* (pp. 435–460). Plenum Press, New York.

3

Nanotoxicological Evaluation in Marine Water Ecosystem: A Detailed Review

Anna Giulia Cattaneo

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3.1 Introduction

The need for an accurate definition of nature and safety of nanomaterials (nanomaterials) goes at the same pace of their growing production and economic interest. In the field of environmental sciences, the impact of engineered nanomaterials is typically two faceted (Pettitt and Lead, 2013). Table 3.1 lists recent documents issued in this field by the Organisation for Economic Co-operation and Development (OECD).

The safety of nanomaterials dispersed in the natural environment is typically two faceted. Nanomaterials either provide a brilliant solution for remediation or represent pollutants whose environmental fate and hazard are difficult to define (Heggelund et al., 2016; Vaseashta, 2009; Vunain et al., 2016; Zhang et al., 2015). In the marine ecosystem, this assessment remained neglected until recently, and its progression hits with the lack of appropriate methods of detection. The accepted workflow, reported in Figure 3.1, includes in fact highly specialized and expensive methods, not fitting the fieldwork. The only reasonable method for extensive research in natural marine water is the detection of silver nanoparticles, based on visible light photometry, as proposed by the participants to the project MARAgNANO (Lodeiro et al., 2016; MARAgNANO, 2015).

The variables interfering with the stability of nanocolloids in saltwater interplay with the manifold marine habitats, limiting the advancement of knowledge. The fate and stability of nanocolloids in seawater depend on the properties of the dispersed nanomaterials and those of water, such as salinity, temperature, and pH

TABLE 3.1

Guidelines on Nanomaterials Released from the Organisation for Economic Co-Operation and Development

Document Reference and URL*	Public Release	Content
ENV/JM/MONO(2009)21 (http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?doclanguage=en&cote=env/jm/mono(2009)21)	July 10, 2009	Preliminary review of OECD test guidelines, evaluation of applicability to nanomaterials
ENV/JM/MONO(2010)25 (http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2010)25&doclanguage=en)	May 31, 2010	Sample preparation and dosimetry of nanomaterials
ENV/JM/MONO(2012)40 (http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2012)40&doclanguage=en)	December 18, 2012	Sample preparation and dosimetry of nanomaterials
ENV/JM/MONO(2015)20 (http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2015)20&doclanguage=en)	July 3, 2015	Available methods and models for assessing nanosafety
ENV/JM/MONO(2016)2 (http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2016)2&doclanguage=en)	January 21, 2016	Physical–chemical parameters on nanoscale: methods
ENV/JM/MONO(2016)7 (http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2016)7&doclanguage=en)	February 11, 2016	Physical–chemical parameters of NM: evaluation
ENV/JM/MONO(2016)9 (http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2016)9&doclanguage=en)	February 17, 2016	Categorization of manufactured nanomaterials
ENV/JM/MONO(2016)11 (http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2016)11&doclanguage=en)	March 29, 2016 (most recent)	Ongoing projects on nanosafety, tour de table
Dossiers and endpoint on different compounds (nanomaterials)		
ENV/JM/MONO(2015)44 (http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2015)44&doclanguage=en)	December 18, 2015	Silver nanoparticles: focus on dissolution
ENV/JM/MONO(2015)7 (http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2015)7&doclanguage=en)	May 29, 2015	Gold nanoparticles
ENV/JM/MONO(2015)9 (http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2015)9&doclanguage=en)	May 29, 2015	Dendrimers
ENV/JM/MONO(2015)10 (http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2015)10&doclanguage=en)	June 2, 2015	Nanoclays
ENV/JM/MONO(2016)20 (http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2016)20&doclanguage=en)	May 30, 2016	MWCNTs
ENV/JM/MONO(2016)23 (http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2016)23&doclanguage=en)	June 8, 2016	Silica nanoparticles

(*Continued*)

TABLE 3.1 (CONTINUED)

Guidelines on Nanomaterials Released from the Organisation for Economic Co-Operation and Development

Document Reference and URL*	Public Release	Content
ENV/JM/MONO(2016)21 (http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2016)21&doclanguage=en)	July 1, 2016	C60
ENV/JM/MONO(2016)22 (http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2016)22&doclanguage=en)	July 7, 2016	SWCNTs

Note: OECD, Organization for Economic Co-operation and Development. Monographs on selected nanoparticles: titanium dioxide: http://www.oecd.org/chemicalsafety/nanosafety/titanium-dioxide-nm100-nm105-manufactured-nanomaterial.htm; oxozinc: http://www.oecd.org/chemicalsafety/nanosafety/zinc-oxide-manufactured-nanomaterial.htm; silver: http://www.oecd.org/chemicalsafety/nanosafety/silver-nanoparticles-manufactured-nanomaterial.htm (all documents accessed June 8, 2017).

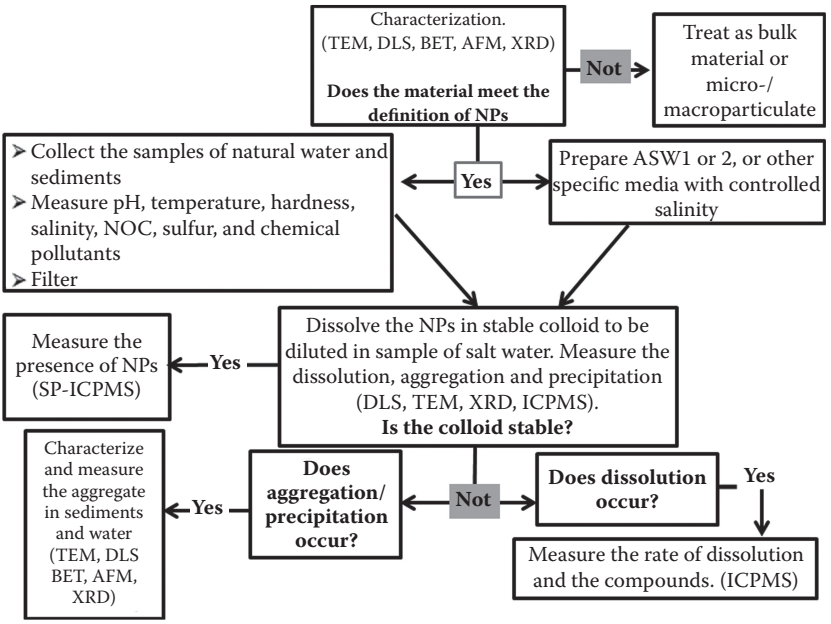


FIGURE 3.1 Decision tree for identifying and characterizing nanoparticles in water samples. Abbreviations: AFM, atomic force microscopy; BET, method of Brunauer, Emmett and Teller, based on nitrogen retention at the surface; DLS, dynamic light scattering; (SP-)ICPMS, (single particle-) inductively coupled plasma mass spectrometry; TEM, transmission electron microscopy; XRD, X-ray diffraction; ASW1 and ASW2, artificial sea water, according to ASTM E724–798. See also Tantra et al. (2015) and Heithmar (2011).

(Brunelli et al., 2013; Garner and Keller, 2014; Zhang et al., 2016). Resuspension from the bottom or bioavailability in the pelagic component of the food web could also result in partial redistribution of nanomaterials (Thain et al., 2004).

Among different marine habitats, coastal regions undergo heavy anthropic factors, especially in proximity of estuaries and harbors. Nevertheless, nanoparticles (nanoparticles) also develop in open sea from aged materials, such as plastic or beads of glass wool used as a barrier against oil spill (Andrady, 2011; Koehler et al., 2008). In Arctic regions, temperature-driven exchanges and the presence of sulfuric acid clusters should be responsible for the nucleation of suspended organic polymers into airborne nanoparticles (Karl et al., 2013). Figure 3.2 shows a scheme of factors possibly interfering with the dispersion of nanomaterials in marine ecosystems.

In this perspective, the large mass of plastic debris dispersed in the ocean acquires an even more disquieting aspect than that resulting from the published map, shown in Figure 3.3. This waste partially converts into microplastics and possibly into long-living nanoparticles of organic polymers (Andrady, 2011; Cózar et al., 2014). Their final fate, under the forces driving in the different parts of the planet, is unpredictable until affordable methods to detect environmental nanoplastics will be available. Some researchers discussed this matter in depth (Bouwmeester et al., 2015; Koelmans et al., 2015; Mattsson et al., 2015; Weis et al., 2015).

This chapter will focus on the strategy adopted to test nanotoxicity on marine organisms, which in general follows that of classical ecotoxicology, with suitable modifications. The organisms, either reared in farms or caught in natural environments, required testing at the lab facilities. Valuable results mainly depended on reproducibility and understanding of reactions to environmental changes and pollution. Tables 3.2 and 3.3 report the species, their natural habitats, and their experimental environment, such as the salinity and the type of water, natural or artificial. A further section describes the experiments in mesocosms.

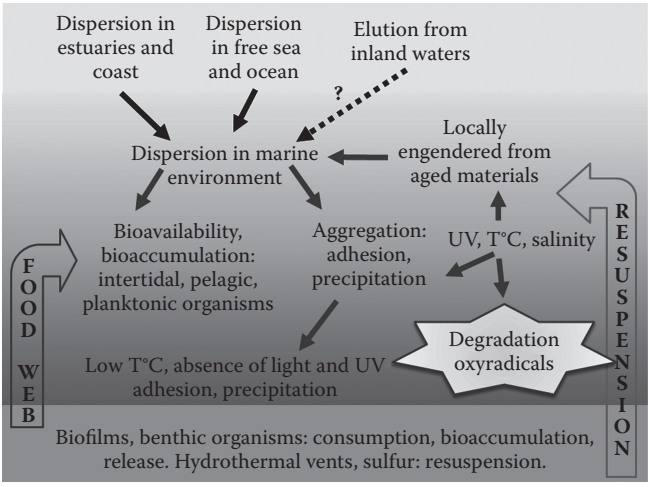


FIGURE 3.2 Scheme of the fate of dispersed nanomaterials in the marine environment (on the top, the dotted arrow indicates a poorly probable source). UV, changes of temperature, and salinity are some causes of degradation, aggregation, and precipitation. Once on the bottom, consumption, accumulation in the food chain, or resuspension could happen.

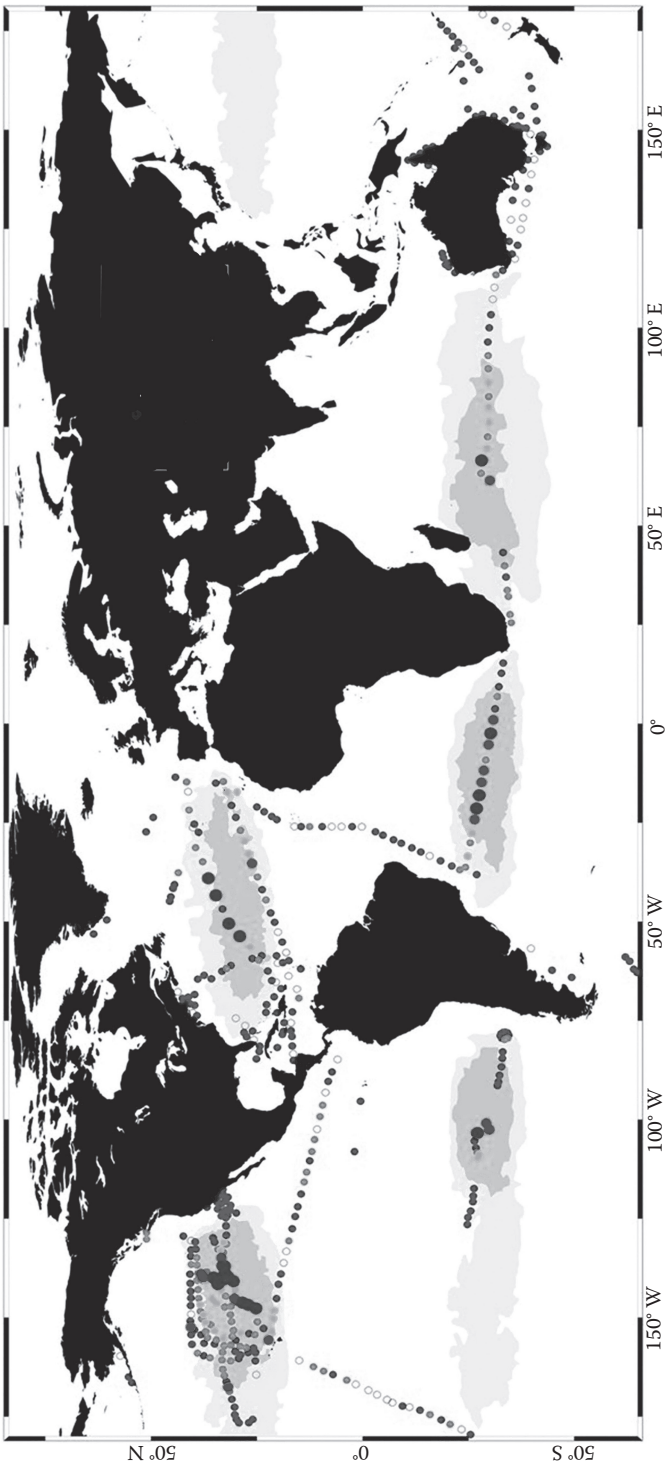


FIGURE 3.3 Concentrations of plastic debris in surface waters of the global ocean. (Reproduced from Cózar, A. et al., *PNAS USA*, 111, 10239–44, 2014. Copyright 2014 National Academy of Sciences, U.S.A. With permission.)

TABLE 3.2

Species Used in Experimental Studies: Pelagic and Benthopelagic

Species	Habitat (Adult), Experimental Environment	References or Documents
Vertebrates		
Tursiops truncatus	Pelagic[a] (cells "in vitro")	Frenzilli et al., 2014
Cyprinodon variegatus	Euryhaline, benthopelagic, nonmigratory[a,b]	**OECD 305, 2012; OECD 203, 2011; Ferry et al., 2009**
Fundulus heteroclitus	Euryhaline, benthopelagic, nonmigratory[c]	Bone et al., 2015
Oryzias latipes, *O. melastigma*	Brackish water, benthopelagic[a,d]	Kashiwada, 2006; Wu et al., 2010; Wang and Wang, 2014; Wong et al., 2010
Invertebrates		
Mytilus galloprovincialis, *M. edulis*	Marine water, intertidal, suspension feeder[a,d]	**Canesi et al., 2012; Leverett and Thain, ICES 2013; Martínez-Gómez et al., ICES 2015**
Artemia salina, *A franciscana*	Marine or saltwater, benthopelagic	**Libralato et al., 2014, 2016**
Brachionus plicatilis	Euryhaline, benthopelagic[a]	Clément et al., 2013
Platynereis dumerilii	Euryhaline, benthopelagic[a,e]	García-Alonso et al., 2014
Algae and Bacteria		
Chlorella pyrenoidosa, *Chlorella* sp.	Freshwater, pelagic, microalga[a]	**OECD 201, 2002**
Dunaliella tertiolecta, *D. salina*	Marine, halophyle, and benthopelagic[a]	**ISO 10253, 1995, revised in 2016**
Tetraselmis suecica	Marine, pelagic, microalga[a]	Aravantinou et al., 2015
Thalassiosira spp.	Euryhaline, pelagic, microalga[a,d]	Burchardt et al., 2012; Miao et al., 2009; Wong et al., 2010; Yung et al., 2015
Phaeodactylum tricornutum	Neritic, pelagic, microalga[a]	Clément et al., 2013
Vibrio fischeri	Marine, planktonic and symbiontic[a]	**ISO 11348-3, 2007**
Photobacterium phosphoreum	Marine, symbiontic[a]	Wang et al., 2014a

Note: Approved protocols or reviews (**bold**) for the species replace experimental studies.

[a] Artificial seawater (ASW; salinity: 30–36 psu) or other media.

[b] Tidal indoor mesocosm (salinity: 20%).

[c] Tidal outdoor mesocosm (salinity: 15 psu).

[d] Natural seawater (salinity: 30–38 psu) and lagoon water (32–36 psu).

TABLE 3.3

Marine Species Used in Experimental Studies: Benthic, Sediment, or Substrate-Bound

Species	Habitat (Adult)	References or Documents
Invertebrates		
Paracentrotus lividus	Marine[a,d]	**Beiras et al., ICES, 2012**; **Matranga and Corsi, 2012**
Arbacia lixula	Marine[d]	Burić et al., 2015
Sphaerechinus granularis	Marine[d]	Burić et al., 2015
Strongylocentrotus droebachiensis	Marine, circumpolar[d]	Magesky et al., 2016
Psammechinus miliaris	Marine[d]	Gallo et al., 2016; Kadar et al., 2011
Ciona intestinalis	Eurhyaline[d]	Gallo et al., 2016; Kadar et al., 2011
Americamysis bahia	Euryhaline[a]	**OECD, draft 2013**
Ampelisca abdita	Marine[a]	Wang et al., 2014b
Elasmopus rapax	Marine, water, subtidal[a]	Wong et al., 2010
Leptocheirus plumulosus	Estuarine[a]	Hanna et al., 2013
Palaemonetes pugio	Brackish, estuarine[b]	**Ferry et al., 2009**
Sarsamphiascus tenuiremis	Euryhaline[a]	**ASTM E2317-04, 2004 (active: 2012)**
Tigriopus japonicus	Euryhaline[a]	Lee et al., 2010; Wong et al., 2010
Haliotis diversicolor supertexta	Saltwater[d]	Zhu et al., 2011
Ilyanassa obsoleta	Euryhaline[b]	Ferry et al., 2009
Crassostrea gigas, C. virginica	Euryhaline[a,d]	Cole and Galloway, 2015; Ringwood et al., 2010; McCarthy et al., 2013
Scrobicularia plana	Euryhaline[c,d,e]	**Mouneyrac et al., 2014**
Mercenaria mercenaria	Euryhaline[b]	Ferry et al., 2009
Arenicola marina	Euryhaline[f]	Galloway et al., 2010
Hediste diversicolor	Euryhaline[c,d]	**Mouneyrac et al., 2014**; Cong et al., 2011
Nereis virens	Euryhaline[e]	Wang et al., 2014b
Montastraea faveolata (with Zooxanthellae)	Marine[d]	Jovanovìc and Guzmàn, 2014
Nematostella vectensis	Marine[d]	Ambrosone and Tortiglione, 2013
Plants and Algae, Photosynthetic and Bacterial Biofilms		
Fucus serratus (macroalga)	Intertidal[a]	Nielsen et al., 2008
Spartina alterniflora (plant)	Intertidal, euryhaline[b]	**Ferry et al., 2009**
Skeletonema costatum (microalga)	Marine[a]	Wong et al., 2010
Biofilm	[b]	**Ferry et al., 2009**

(*Continued*)

TABLE 3.3 (CONTINUED)

Marine Species Used in Experimental Studies: Benthic, Sediment, or Substrate-Bound

Species	Habitat (Adult)	References or Documents
Biofilm (*Alteromonas macleodii*)	Marine, planktonic[a]	Nevius et al., 2012
Biofilm (*Pelobacter propionicus*)	Estuarine[e]	Antizar-Ladislao et al., 2015
Bacterial biofilm	Estuarine[e]	Bradford et al., 2009

Note: Bold: approved protocols or reviews for the species replace experimental studies.

[a] Artificial seawater (ASW, salinity: 30–36 psu) or other media.

[b] Tidal indoor mesocosm (salinity: 20%).

[c] Tidal outdoor mesocosm (salinity: 15 psu).

[d] Natural seawater (salinity: 30–38 psu) and lagoon water (32–36 psu).

[e] Brackish water (salinity up to 30 psu).

3.2 Nanotoxicity and Marine Organisms

A wide number of marine species have been studied to assess the safety of nanomaterials. They are representative of all levels of the food web and inhabit different marine habitats, most frequently tidal. Among species reported in Tables 3.2 and 3.3, several unconventional vertebrates (mainly fish) and invertebrates emerged as model species, beside those recommended by official protocols that require adaptation to the specific needs of nanoecotoxicity.

The fish considered for nanotoxicology studies are euryhaline, benthopelagic, least-concerned, and third-level predators (http://www.fishbase.org).

The small crustacean *Artemia salina* is a recognized model organism for testing chemical toxicity. Thanks to its resistance to very high salinity, role in the food chain, and ability to swallow small particles, the brine shrimp attracted the attention of nanotoxicologists (Libralato et al., 2014, 2016; Rajabi et al., 2015). Among the emerging species, the *Mytilus* spp. provided a useful model for "in vivo" studies in suspension filter feeders and for "in vitro" studies as well. Their hemolymph and hemocytes represented an interesting start point for studies on general toxicity, membrane instability, and triggering of the innate immunity (Canesi et al., 2012; Martínez-Gómez et al., 2015). Free-spawning marine organisms, sea urchins, oyster, and cnidarians, among others, offered a convenient model to study the embryonic toxicity (Ambrosone and Tortiglione, 2013; Beiras et al., 2012; Leverett and Thain, 2013; Matranga and Corsi, 2012).

Beside animal species, photosynthetic organisms and bacterial biofilms were of great interest, for their position in the food web and, possibly, for their ability to accumulate nanoparticles.

In the next subsection, dedicated to the in vivo studies, we considered organisms able to complete a cycle of life and reproduction, including embryos and larvae, or the unicellular organisms, such as bacteria or microalgae. Observations done in cells isolated from multicellular organisms are instead the subject of the subsection dedicated to in vitro studies, with the nanotoxicity in gametes and early zygote at the end of this section.

The nanoparticles most frequently studied were the silver and titanium dioxide nanoparticles (23% and 20%, respectively). Present in many consumer products, such as sunscreens and garments, they are likely to cause pollution in saltwater. Metal and semimetal oxides, other than titanium dioxide, represented altogether the 39% of all

studies here considered; carbon based-nanoparticles, such as carbon black, fullerenes, and graphene, followed (15%). Only a few studies focused on gold nanorods. Minetto et al. (2016) deeply reviewed this matter.

3.2.1 In Vivo Toxicity Testing of Nanomaterials in Marine Ecosystem

Studies in vivo required tanks, with or without recirculation, suitable for the purposes of the experiment and the species tested. The preferred ways of exposure were with food or with different types of water. The lethal effect was more frequently observed before complete development, but sublethal toxicity developed at any age.

Species, salinity of water, and type and concentration of nanoparticles were the main factors that modulated the reaction to the exposure. The concentrations causing 50% of lethality (LC50) or of inhibition of growth (IC50) are reported in Table 3.4. The toxic effect due to ion dissolution was difficult to evaluate, because of contradictory results.

Among vertebrates, the fry of *Oncorhynchus mykiss* was sensitive to nanosilver. Lethal effects developed at lower salinity, within the range of 0.4–12%. Sublethal effects included mucous overproduction, respiratory problems, and altered swimming (Kalbassi et al., 2011). Multiple deformities and delayed development affected Japanese medaka embryos after exposure to nanosilver (Wu et al., 2010). The trophic transfer was low to *Oryzias melastigma* fed on *A. salina*, reared on sediments contaminated with nanosilver. However, the growth rate of fish was low and enzymatic activities raised (Wang and Wang, 2014). The only reaction of adult *Cyprinodon variegatus* to nanosilver was transcriptional activation in ovary and fully reversible epithelial proliferation in gills, while juveniles developed low degree of liver spongiosis and hemorrhagic trend in gills (Griffitt et al., 2012). Dose- and salinity-dependent oxidative stress, increased mucous secretion, and behavioral changes were also present in response to copper oxide nanoparticles (Ates et al., 2014). *Fundulus heteroclitus* apparently did not accumulate cadmium after feeding with pellet containing quantum dots. However, differential expression of stress-related genes and low spawning rate appeared in both sexes (Blickley et al., 2014).

While ingested nanoparticles reached the circulation or accumulated in gills, the filter feeders in the water column did not develop lethal effects. Silver nanoparticles reached the extrapallial tissue of *Mytilus edulis* through circulation (Zuykov et al., 2011). Instead, silica nanoparticles and nanofibers, as those produced by glass beads used to contain oil spill, accumulated in the gills and in the epithelial cells of the digestive tube. Lipofuscins were present in the hepatopancreas and in the digestive tube after long-term exposure (Koehler et al., 2008).

The oyster *Crassostrea virginica*, a sessile, first-level predator, reacted to the exposure to nanosilver with severe embryo toxicity and lysosomal destabilization of the hepatopancreas in adults, independently from dissolution (McCarthy et al., 2013; Ringwood et al., 2010). In *Mytilus galloprovincialis*, signs of lipoperoxidation, DNA damage, and multiple cytohistological anomalies developed after exposure to titanium dioxide nanoparticles (100% anatase). The nanoparticles mainly accumulated in gills and digestive glands, where they entered the cells and localized on the surface of villi, with vacuolization of villous cells and hemocytic infiltration of the gland (Balbi et al., 2014; Banni et al., 2016; Barmo et al., 2013; Gornati et al., 2016). Nuclear budding and segmentation occurred, signs possibly associated to the hemocytic

TABLE 3.4

Lethal Effect of Different Nanoparticles

NP	LC50/IC50	Organism	References
Silver	2–100 μg/L	Invertebrates: *Mytilus galloprovincialis*, *Paracentrotus lividus*, *Arbacia lixula*, *Sphaerechinus granularis*, *Platynereis dumerilii*, *Artemia salina* Algae: *Thalassiosira pseudonana*, *Thalassiosira weissflogii*	Arulavasu et al., 2014; Ates et al., 2013a; Burchardt et al., 2012; Burić et al., 2015; García-Alonso et al., 2014; Katsumiti et al., 2015; Kumar et al., 2012; Miao et al., 2009
Titanium dioxide (100% anatase)	3.4–267 mg/L	Invertebrates: *Artemia salina*, *Brachionus plicatilis* Algae: *Phaeodactylum tricornutum*	Callegaro et al., 2015[a]; Clément et al., 2013[b]
Oxozinc	0.85–>100 mg/L	Invertebrates: *Artemia salina*, *Tigriopus japonicus*, *Elasmopus rapax* Algae: *Tetraselmis suecica*, *Thalassiosira pseudonana*,[c] *Skeletonema costatum*, *Thalassiosira pseudo nana* Bacteria: *Photobacterium phosphoreum*	Aravantinou et al., 2015; Ates et al., 2013b; Wang et al., 2014a; Wong et al., 2010; Yung et al., 2015
Other metal and oxides	0.34–3.747 mg/L	Invertebrates: *Artemia salina*, *Paracentrotus lividus* Algae: *Dunaliella tertiolecta.* Bacteria: *Photobacterium phosphoreum*[d]	Ashtari et al., 2012, 2013c; Baumerte et al., 2013; Cornejo-Garrido et al., 2011; Falugi et al., 2012; Gambardella et al., 2014; Zamani et al., 2014; Wang et al., 2014a
ZVI, coating: polymers or iron oxide	0.9 or 9 mg/L	Algae: *Dunaliella tertiolecta*, *Thalassiosira pseudonana*, *Isochrysis galbana*	Keller et al., 2012
Carbon-based[e]	1.14–30 mg/L	Invertebrates: *Artemia salina*, *Amphiascus tenuiremis* Algae: *Dunaliella tertiolecta* Bacteria: *Vibrio fischeri*	Baumerte et al., 2013; Fatouros et al., 2011; Pretti et al., 2014; Templeton et al., 2006

Note: LC50 refers to mortality excess, IC50 to inhibition of growth. Adult vertebrate and invertebrate were usually spared, and data on lethality are mainly recorded in embryos and larvae.

[a] Anatase 80%, rutile 20%.

[b] 100% anatase.

[c] IC50 (96 hours): 2–8 mg/mL (target: photosynthetic energy yield of photosystem II).

[d] The lethality was as follows: $ZnO > Fe_3O_4 > Co_3O_4 > Fe_2O_3 > Pr_6O_{11} > Cr_2O_3 > CuO > Nd_2O_3 > NiO > WO_3$ (target: fluorescence, Wang et al., 2014).

[e] Graphene and graphite were highly toxic in algae and bacteria, not in copepods. The lethality of other nanomaterials was single-walled carbon nanotubes (SWCNTs) < carbon black < multiwalled carbon nanotubes (MWCNTs) < aged SWCNTs.

neoplasia of mussels (Bower, 2006). Enhanced antioxidative responses and metallothionein expression were reported in the same species after exposure to copper oxide nanoparticles. The nanoparticles were safer than ions; copper accumulated in the digestive gland and especially in gills (Gomes et al., 2011, 2012). Cerium and zinc oxide nanoparticles accumulated in *M. galloprovincialis*; only the first compound was present as nanoparticles in pseudofeces (Montes et al., 2012).

A cheap and robust ecotoxicity test used the larvae of *Artemia* spp. Several magnetic and metallic nanoparticles (silver, zinc, aluminum, and molybdenum oxides), nanowires based on molybdenum–sulfur–iodine, carbon black, and MWCNTs enhanced the mortality ratio in this species, while SWCNTs, lead, and other oxides (ceric, stannic, and titanium) did not. Sublethal effects occurred, such as accumulation of nanoparticles in gut and low excretion, altered swimming, overexpression of markers of stress, and altered activity of cholinesterase (Arulavasu et al., 2014; Ashtari et al., 2012; Ates et al., 2013a, 2013b, 2013c; Baumerte et al., 2013; Cornejo-Garrido et al., 2011; Fatouros et al., 2011; Gambardella et al., 2014; Kumar et al., 2012). Libralato (2014) systematically reviewed the entire subject.

The sea urchins are grazer organisms and another favorite group of species for nanotoxicology studies (Gambardella et al., 2016). The sessile, intertidal, or benthic adults generate planktonic embryos and larvae, sensitive to the teratogenic effects of nanosilver. Among the Mediterranean–Atlantic species, the most resistant is *Parancentrotus lividus*, in comparison with *Arbacia lixula* and *Sphaerechinus granularis*. The earlier stages were frailer in all organisms (Burić et al., 2015). *Strongylocentrotus droebachiensis*, a species living in colder waters, was also sensitive to the exposure. After chronic exposure, juveniles developed edema, lethargy, and immobility, and embryos interrupted morphogenesis. Mortality hindered the recovery (Magesky et al., 2016). The forced ingestion of metal oxide nanoparticles (ferrosoferric, ceric, or stannic) was lethal for the larvae of *P. Lividus*; sublethal doses induced instead oxidative stress and alterations of coelomocytes (Falugi et al., 2012).

The mud or sand bottom, the habitat of many benthic and epibenthic marine animals, could be the primary accumulation site for nanoparticles or their aggregates. Sediment tests after pollution with nanosilver gave negative results in the mysids *Ampelisca abdita* and *Americamysis bahia*. Behavioral changes, such as reduced borrowing behavior and feeding rate, and markers of oxidative stress occurred in the clam *Scrobicularia plana* and the worm *Hediste diversicolor.* Markers of oxidative stress occurred, but no lethal effects were observed. Cupric oxide nanoparticles were safe (Buffet et al., 2011, 2012, 2013a, 2013b, 2014b, 2016; Pan et al., 2012; Wang et al., 2014b). The bioavailability of silver was somewhat higher after direct exposure than feeding; that of gold increased after direct exposure (Cong et al., 2011; Pan et al., 2012). The organisms were captured at the Bay of Bourgneuf, France, and exposed to gold, silver, oxozinc, and cupric oxide nanoparticles and to cadmium selenide quantum dots (Mouneyrac et al., 2014).

Sediment pollution with nanosilver induced defective growth in the benthic worm *Alitta virens* (formerly *Nereis virens*). The pattern of dissolution and the consequent toxicity differed in nanoparticles embedded in organic matter dispersed in sediments and water (Wang et al., 2014b). Different developmental stages of *Platynereis dumerilii*, a benthic worm, were differently sensitive to exposure to nanosilver for 48 hours. IC/LC50 was lower in egg than in juvenile and adult. From the stage of trochophore, the worm was especially sensitive to developmental effects of nanoparticles and resistant

to silver ions. Coating with humic acid partially protected from toxicity the early embryos (García-Alonso et al., 2014). The size and dissolution rate of nanosilver were important factors of toxicity for the alga *Thalassiosira* spp. (Burchardt et al., 2012; Miao et al., 2009). In general, the amplified effect seen in microalgae could be a consequence of photosynthetic insufficiency, due to shading from nanoparticles adhering to the surface of the cells.

Titanium dioxide nanoparticles, safe in *Artemia franciscana*, reduced instead the growth of the diatom *Phaeodactylum tricornutum* and that of the planktonic rotifer *Brachionus plicatilis*. It was both lethal and teratogenic in embryos of *Haliotis diversicolor supertexta* (Zhu et al., 2011). The effect, when present, was dose, size, and composition dependent. Rutile–anatase was more toxic than 100% anatase (Callegaro et al., 2015; Clèment et al., 2013). The endangered coral *Orbicella faveolata* (formerly *Montastraea faveolata*) reacted with dose-dependent overexpression of HSP70, bleaching, and expulsion of the symbiont algae zooxanthellae. The coral was captured in its natural habitat, the Caribbean Sea, and reared in seawater collected at the same site. The lowest dose simulated the possible scenario of leakage from wastewater plans; the largest was instead not realistic in seawater. Large aggregates formed (>800 nm) and precipitated by flocculation, especially at the higher dose. The nanoparticles mainly accumulated in zooxanthellae (Jovanovìc and Guzmàn, 2014).

Tests with nanoparticles different from nanosilver and nano-titanium dioxide documented toxicity in pelagic invertebrates, such as *Sarsamphiascus tenuiremis*, *Tigriopus japonicus*, *Elasmopus rapax*, and *B. plicatilis* displayed adverse effects to the exposure of SWCNTs, graphene nanosheets, titanium dioxide, and oxozinc nanoparticles. Mortality developed after exposure to soft (carbon-based) and hard (metallic) nanomaterials. The animal species, nature of nanomaterials, shape, chemical purity, and/or size were factors influencing the toxicity (Clément et al., 2013; Pretti et al., 2014; Templeton et al., 2006; Wong et al., 2010). The pelagic, symbiotic bacteria *Vibrio fischeri* and *Photobacterium phosphoreum* displayed an inhibition of their bioluminescence when exposed to carbon-based nanoparticles and to metal and metal oxide nanoparticles (Pretti et al., 2014; Wang et al., 2014a).

Environmental factors and pollutants shape benthic biofilm, a complex community of bacteria and microscopic algae. Two studies evaluated the effect of nanosilver in estuarine bacterial communities from sites of great ecological importance. The study of consortia composition implemented methods such as denaturing gradient gel electrophoresis and terminal restriction fragment length polymorphism. The results obtained at Plymouth Sound, United Kingdom, a complex biosystem collecting inland waters from a highly inhabited region, did not support a clear impact of nanoparticles with the local bacterial community (Bradford et al. 2009). The second site, the Hugly estuary, India, is a tropical wetland that also includes a wide mangrove block. Anthropic activities, added to complex natural phenomena, endanger this frail but protected site. The exposure to nanosilver of samples of the benthic bacterial consortium affected the equilibrium between species, with the disappearance of key organisms, such as *Pelobacter propionicus* (Antizar-Ladislao et al., 2015). The different conclusions of the two studies pointed out the importance of local diversity in nanotoxicology. On the other hand, the biofilms intervene in determining the fate of nanoparticles, as exhaustively reviewed by Ikuma et al. in 2015.

Before concluding this subsection, we discuss experimental knowledge about the potential hazard of nanoplastics, at the present limited to the polystyrene nanoparticles.

This material did not enhance the mortality of adult medaka or the hatchability of exposed eggs, but 24 hpf embryos could not survive high concentrations (97% mortality with 30 mg/L, in medium with 5% salinity). The hatched embryo concentrated the nanoparticles in the yolk and gallbladder, not in the liver, while the most affected system in adults were the gills (Kashiwada, 2006).

Among invertebrates, *Crassostrea gigas* and *M. edulis*, fed on polystyrene nanoparticles, ingested lower amounts of isolated nanoparticles than large clusters. The excretion of microparticles was more rapid and complete than that of nanoparticles <100 nm, suggesting that aggregate and larger particles cannot reach the digestive glands of the mollusks. However, *M. edulis* accumulated the nanoparticles in tissues around the foot. The ingestion was well tolerated by the oyster, however, accumulation represented a threat to the alimentary chain (Cole and Galloway, 2015; Ward and Kach, 2009; Wegner et al., 2012). Lethal and growth inhibitory effects developed in *A. salina* and in the alga *Dunaliella tertiolecta* with surface-charged nanoparticles (Bergami et al., 2017).

The interactions between the marine bacterium *Alteromonas macleodii* and polystyrene nanoparticles demonstrated the role of biofilms in clearing the water from nanoparticles. The adhesion was larger when nanoparticles were positively charged, but it became instable at higher ionic strength (Nevius et al., 2012).

3.2.2 In Vitro Toxicity Testing of Nanomaterials in Marine Ecosystem

High standardization characterizes the toxicology studies in vitro. Cells, media, and conditions of exposure follow tiered protocols, mainly according to international guidelines. The main downside of high standardization, however, is the exclusion of media different from the environmental samples. This is especially limiting in marine ecotoxicology, where several representative cell models have reached instead a high degree of standardization. To test the toxicity of nanomaterials, the preferred cells were the phagocytes involved in mechanisms of the innate immunity, such as phagocytes of fish, hemocytes of mussel, or coelomocytes of the sea urchin. Fibroblasts and white blood cells of dolphin were also studied.

A recent document approved by the ICES revised the dominating model, developed in the hemocytes of *Mytilus* spp. (Canesi et al., 2012; Martínez-Gómez et al., 2015). In synthesis, the hemocytes were isolated from pooled samples of hemolymph, drained on ice from the posterior adductor muscle sinus, and filtrated. The main toxicological targets were the lysosomal membrane instability, the phagocytic ability, and the expression of markers of oxidative stress. Genotoxicity, apoptosis, and lethality were additional targets. The incubation in the hemolymph and the consequent formation of a corona around the particles enhanced the toxicity of inert nanoparticles of polystyrene against hemocytes, without affecting the phagocytosis rate (Canesi et al., 2016). Titanium dioxide nanoparticles localized in the cytoplasm and nucleus of hemocytes reduced the membrane stability and the phagocytosis and induced the release of lysozyme, mitochondrial damage, and signs of stress (Ciacci et al., 2012). Signs of oxidative stress, immunological activation, and lysozyme release developed after the exposure to other metal oxides and carbon-based nanoparticles, that of oxozinc being the most lethal (Canesi et al., 2008, 2010; Balbi et al., 2014; Barmo et al., 2013). The lysosome release was higher in response to titanium dioxide than to other metal-based nanoparticles, but oxozinc nanoparticles

were more toxic (Canesi et al., 2010; Ciacci et al., 2012). Reduced phagocytosis and signs of apoptosis followed the exposure to titanium dioxide nanoparticles of the hemocytes of another filter feeder bivalve, the clam *Ruditapes philippinarum* (Marisa et al., 2015).

Heavy lethality and dose-dependent genotoxicity developed in hemocytes and phagocytes of mussels and in a stabilized gonadal cell line of rainbow trout (RTG-2), after exposure to quantum dots. The lethality of quantum dots was lesser in the hemocytes of mussels than in the phagocytes of rainbow trout and mice. The human monocytes were the most sensitive cells. Silver sulfide nanoparticles were instead safe (Bruneau et al., 2015; Munari et al., 2014a, 2014b).

A different model was developed in coelomocytes of sea urchins and applied also in the worms *H. diversicolor* and *Arenicola marina*. These cells are dispersed in the coelomatic fluid with relative abundancy (10^7/mL) and is therefore easy to collect in sufficient amounts from samples drawn through a syringe. A subgroup of them is involved in phagocytosis and possibly in immune response. This model offered an interesting insight in gene expression of the sea urchins in response to metal oxide nanoparticles, especially for the set of genes responsible for the immune responses in these species (Falugi et al., 2012; Matranga and Corsi, 2012). While obviously interesting for in vitro studies, this model was implemented also in vivo, in the sea urchin. Significant DNA damage and decreased lysosomal stability were recorded after in vitro exposure of the coelomocytes of *H. diversicolor* to nanosilver and of *A. marina* to titanium dioxide nanoparticles (Cong et al., 2011; Galloway et al., 2010).

The bottlenose dolphin represents the only species of marine mammals studied in the nanoecotoxicology landscape. The fibroblasts and leukocytes, obtained from skin biopsies and blood samples, revealed significant DNA damage after exposure to titanium dioxide-uncoated particles (anatase nanoparticles or rutile microparticles). 3T3 fibroblasts, human fibroblasts, and human leukocytes, studied for comparison, showed different sensitivities (Bernardeschi et al., 2010; Frenzilli et al., 2014).

All these models offered an integrated view of the processes bound to immunity, inflammation, and phagocytosis. Further development could provide useful biomarkers to prediction of toxicity and early signal specific pollution with nanomaterials.

Studies on gametes of marine species offered a direct insight to the reproductive toxicity of nanoparticles. Most of aquatic species freely deliver their gametes in water, where the developing zygote is directly exposed to the environmental insults. All the conditions negatively affecting the viability and the fertility potential of the gametes, or the development of the zygote, are threatening the biodiversity and the safety of the food chain. Several recent works focused on the impact of nanoparticles with the fertilization of marine organisms.

Decreased fertilization potential of sperm occurred in *Ciona intestinalis*, *M. galloprovincialis*, *Paracentotus lividus*, and *Fucus serratus* exposed to nickel, zero-valent iron, and carbon black nanoparticles, respectively. Silver, zinc, and graphene did not affect this parameter. Teratogenic effects, in terms of malformed embryos and

progeny, or delayed and arrested growth, appeared in all the mentioned species and in *Nematostella vectensis*, also after exposure to nanoparticles apparently safe for the sperm. Altered expression of biomarkers, membrane disruption and lipoperoxidation, mitochondrial damage, DNA fragmentation, and inhibition of the activity of acetylcholinesterase and propionylcholinesterase at the surface of embryos represented the main sublethal effects. The dissolution did not explain the toxicity of several nanoparticles (Ambrosone and Tortiglione, 2013; Gallo et al., 2016; Gambardella et al., 2013, 2015; Manzo et al., 2013; Mesarić et al., 2015).

The teratogenicity was related not only to the type of nanomaterials, but also to the period of exposure and organism species. The embryonic toxicity of quantum dots in *N. vectensis* appeared only when gametes were exposed during the fertilization period (Ambrosone and Tortiglione, 2013). Anomalous polarization of the embryo appeared after exposure of *F. serratus* to zero-valent iron nanoparticles, but the subsequent germination and rhizoid elongation was normal (Nielsen et al., 2008). The comparison of three marine invertebrates suggested substantial interspecific differences of sperm sensitivity. The prefertilization exposure did not affect the successful fertilization rate in *Psammechinus miliaris*, but induced moderate effects in *M. galloprovincialis* and more severe effects in *Ciona intestinalis*, the most sensitive species (Kadar et al., 2011, 2013). Table 3.5 summarizes the cell lines used to set up in vitro nanotoxicity in marine organisms.

TABLE 3.5

Cell Models for In Vitro Studies on the Marine Ecotoxicity of Nanomaterials

Species	Cells	Source of Cells and Method	References
Vertebrates			
Tursiops truncatus	Fibroblasts	Fresh cutaneous biopsies treated with trypsin, cells cultured at 90% confluence	Frenzilli et al., 2014
	Leukocytes	Blood samples. Target: bioavailability of nanoparticles, DNA damage	
Oncorhynchus mykiss	Phagocytes	Head kidneys homogenized with heparin, cells collected by centrifugation. Targets: lethality, phagocytosis, lymphoblastic transformation	Bruneau et al., 2015
Oncorhynchus mykiss	RTG-2 cell line	Stabilized cell line from gonadal cell line, validated model for cytotoxicity Targets: cell proliferation, DNA damage (Comet test)	Munari et al., 2014

(*Continued*)

TABLE 3.5 (CONTINUED)

Cell Models for In Vitro Studies on the Marine Ecotoxicity of Nanomaterials

Species	Cells	Source of Cells and Method	References
Invertebrates			
Mytilus galloprovincialis	Hemocytes	Hemolymph from the posterior adductor muscle transferred on ice. Hemocytes collected by centrifugation, suspension in medium, or ASW, or hemolymph. Targets: lethality, lysosomal membrane stability, phagocytosis	Bruneau et al., 2015; Canesi et al., 2012; Martínez-Gómez et al., 2015
Ruditapes philippinarum	Hemocytes	As before. Short-term cultures of hemocytes suspended in filtered seawater. Targets: biodistribution of nanoparticles, phagocytosis	Marisa et al., 2016
Paracentrotus lividus	Coelomocytes	Coelomic fluid drawn through a syringe, separation by centrifugation	Falugi et al., 2012; Matranga and Corsi, 2012
Hediste diversicolor	Coelomocytes	Coelomic fluid drawn through a syringe, separation by centrifugation. Target: DNA damage	Cong et al., 2012
Sperm and Early Zygote			
Mytilus galloprovincialis	Sperm	Induction of gametogenesis: injection of HCl (posterior adductor muscle), heat shock (15°C > 25°C). Collection of gametes, fertilization in vitro	Kadar et al., 2011, 2013
Paracentrotus lividus	Sperm, early zygote	Induction of sperm emission with oral administration of acetylcholine, direct collection from the genital pores	Della Torre et al., 2014; Gambardella et al., 2013, 2015; Mesarić et al., 2015; Manzo et al., 2015
Nematostella vectensis	Gametes	Induction of gametogenesis: 24°C, 16 hours of light. Fertilization vitro. Count of swimming planulae (hatching: 48 hours). Morphological evaluation of embryos and larvae	Ambrosone and Tortiglione, 2013
Ciona intestinalis	Sperm	Isolation from the sperm duct. Target: plasma and mitochondrial peroxidation, pH_i, DNA damage, ultrastructural morphology	Gallo et al., 2016; Kadar et al., 2011, 2013
Fucus serratus	Gametes and zygote	Gametes, early zygote, orientation, rhizome elongation (up to 5 dpf)	Nielsen et al., 2008

3.2.3 Mesocosms: Studying Marine Ecosystems in Stabilized Environments

The mesocosms are complex systems with abiotic components and biotic communities, which reproduce natural environments in standardized conditions. These systems represent an innovative approach to the toxicology of nanomaterials dispersed in marine habitats. Until now, tests have been conducted in brackish and tidal water and in marine water with free-floating mesocosms, as shown in Table 3.6.

The free-floating mesocosms, sketched in Figure 3.4, stabilize the column of water to permit reproducible measurement of physical chemistry changes and other variables, as well as their influence on the dynamics of the plankton. The addition of sediment traps allowed the collection of benthic samples with minimal disturbance of the water column (Riebesell et al., 2013; Watts and Bigg, 2001). They could adapt to a wide range of natural habitat, from lacustrine to marine. Platforms, floating in pools filled with natural seawater, allowed to study the fate of different types of nanosilver in abiotic systems and in microscopic planktonic organisms (Toncelli et al., 2016). A simpler indoor mesocosm was included, provided with several inserted slides coated with nanoparticles. This model was especially designed to study bacteria present in biofuels that are fastidious to growth. In sunlight, zinc-based nanomaterials showed antibacterial activity (Al-Naamani et al., 2017; Sathe et al., 2016).

However, the tidal estuarine mesocosms were the preferred systems to study the impact of nanoparticles. The US National Oceanic and Atmospheric Administration validated an indoor mesocosm that recreated a tidal estuarine inlet with natural, unfiltered seawater, originally from Cherry Point Boat Landing on Wadmalaw Island, South Carolina, United States (Lauth et al., 1996; Pennington et al., 2007). This estuarine mesocosm includes a modular system that mimics tidal events with periodical submersion at three different levels. The architecture of the tanks warrantees the reproducibility between triplicated experiments. Figure 3.5 illustrates a scheme of the engine and its components, according to the pilot work of Ferry et al. (2009). The authors studied the fate of gold nanorods in a mesocosm recreated with reference sediment collected at Leadenwah Creek, Wadmalaw Island, South Carolina. Stable partitioning of gold nanoparticles between sediment (ca 3 μg/kg) and water (0.4 μg/L) occurred after 48 hours. The organisms included were, in addition to a mixed biofilm (bacteria and microalgae), plants (*Spartina alterniflora*), gastropods feeding on the biofilm (*Ilyanassa obsoleta*), a filter feeder (*Mercenaria mercenaria*), two omnivores, a grass shrimp (*Palaemonetes pugio*), and a fish (*C. variegatus*). The concentration factors of gold in different components of the mesocosm after 11 days of exposure ranged between nearly null in water to 8×10^3 μg/L in *I. obsoleta*. The plant accumulation was poor as was that of sediment; it was intermediate in clamp, shrimp, and biofilm. No signs of toxicity developed; the gold was not found in the shell of the snail and remained in the gut of the fish, and it was possible to postulate that it did not enter the blood (Ferry et al., 2009; Burns et al., 2013). This mesocosm represent a paradigm for nanoecotoxicology; other studies implemented similar implants, with "as-needed" modifications, as reported in Table 3.6.

A further study evaluated the partitioning of silver in each component of a tidal estuarine mesocosm during a 60-day exposure to different sources of silver. The only difference with the implant mentioned earlier was the presence of a sandy track for the clam and the absence of fish and worm. The team compared the exposure

TABLE 3.6

Types of Mesocosms Adopted until the Present for Studies of Environmental Nanotoxicity

Site	Features	Abiotic Components	Biotic Components	References
Cherry Point, NC, US	Indoor, three tidal levels	Water: natural sea, salinity: 20%, 370 L. Sediment: natural, mud, and sand	V: *C. variegatus*; I: *M. mercenaria*, *I. obsoleta*, *P. pugio*; P: *S. alterniflora*; Pl: algae	Ferry et al., 2009; Cleveland et al., 2012 Burns et al., 2016
Bay of Bourgneuf, France	Outdoor, two tidal levels	Water: natural seawater, salinity: 20%, >40 L. Sediment: natural, from the site	I: *H. diversicolor*, *S. plana*	Buffet et al., 2013b, 2014b
Inland, UK	Outdoor, three tidal levels	Water: modified groundwater, salinity: 15%. Sediment: silt, sand, and clay blend	V: *F. heteroclitus*; P: *E. densa*, *P diversifolius*, *L. punctatus*; zoo- and phytoplankton	Bone et al., 2015
Al-Khodh, Oman	Outdoor	Water: natural seawater 20 L, salinity: 30%	Bacteria: *N. incerta*, *P. nigrifaciens*	Al-Naamani et al., 2017; Sathe et al., 2016
Heraklion (Crete, Greece)	Outdoor, free-floating, natural seawater	Water: natural seawater, salinity: 33%, 3,000 L.	Oligothrophic water	Toncelli et al., 2016

Source: Data from Méléder, V. et al., *J Phycol*, 43, 1177–90, 2007.

Note: Water, sediment, and animals were generally sampled at the site of reference. ASW, artificial seawater; V, vertebrate; I, invertebrate; P, plant; Pl, plankton.

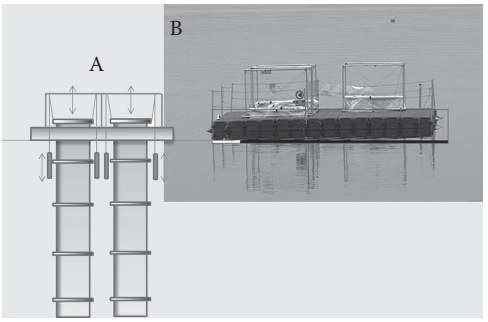


FIGURE 3.4 Schemes of free-floating mesocosms: marine (A) and lacustrine (B). The marine mesocosm includes at its bottom a sediment trap for collecting benthic samples with minimal disturbance of the water column; this is not present in the lacustrine mesocosms illustrated in (B). At the right, the aspect of a floating platform (B). (B courtesy of Professor Giuseppe Crosa, unpublished data.)

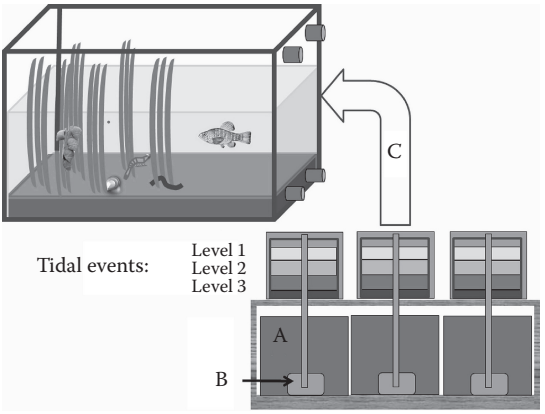


FIGURE 3.5 Scheme and aspect of the replicated modular estuarine mesocosm. The lower tanks (reservoir, A) contained natural, unfiltered tidal water. A pump (B), programmed to reproduce tides, pulled the water into the upper tank (tidal water, C). The system allowed three tidal levels (1: high, 2: medium, and 3: low). The upper tank guests a reproduction of the tidal ecosystem, with natural unfiltered water, sand and mud sediment, fish, filter and deposit feeder invertebrates, microalgae, and aquatic plants. The water recirculated through a channel. Prototypes are described in Lauth et al. (1996) and Pennington et al. (2007).

to water containing silver ions, two differently sized silver nanoparticles, and three different consumer products containing nanosilver (socks, toys, and wound dressing). The dynamics of silver in water differed with the different contaminants. Silver nanoparticles were more persistent than dissolved ions and remained in water from 6 to 12 hours, depending on size. Only the consumer products promoted a sustained release (until 30 days) and increased the silver content in sediment and in plants. As expected, the trophic transfer factor between animals and biofilm was high in detritivore organisms, such as clam, shrimp, and snail, suggesting the intake with food.

Surprisingly, the clams survived the exposure to all types of contaminants, except the smaller nanoparticles. The silver content during the exposure to consumer products reached its maximum level (15–45 μg/g) after 7 or 14 days in the exposure in the snail. In clams, the highest concentration was between 30 and 40 μg/g after 13 or 30 days, while the shrimp retained the largest amounts (44–77 μg/g) for 13 days (Cleveland et al., 2012).

Another group of workers implemented outdoor, intertidal mesocosms to study two hypobenthic species, the worm *H. diversicolor* and the clam *S. plana*, collected at the Bay of Bourgneuf, France (North-East Atlantic). Both organisms accumulated Cu, after exposure to cupric oxide bulk and nanoparticles, without changes in the mortality rate. The exposure to nanoparticles, not to the bulk compound, modified the burrowing and feeding behavior. The expression of a few biomarkers of damage and defense resulted in its alteration, and the amount of damaged DNA was comparable in both organisms and with both treatments (Buffet et al., 2013b). The same organisms accumulated silver, after exposure to silver ions or nanoparticles, without increased mortality rate or changes of feeding behavior. The borrowing behavior did not change after exposure to nanoparticles. The study revealed that several biomarkers of damage and defense increased after the exposure; only laccase and the lysozyme seemed to be exclusive for nanoparticle exposure (Buffet et al., 2014b). Embryos and larvae of *F. heteroclitus* differently reacted to the exposure to nanosilver in relation to the coating of nanoparticles and environmental conditions. The comparison of survival in mesocosms, microcosms, and conventional laboratory conditions demonstrated that a protective action of mesocosms seems dependent on sediment (Bone et al., 2015).

3.3 Conclusions

Several considerations emerge from the still fragmentary picture composed by the literature in the field of marine nanotoxicology. Standardized model organisms and mesocosms, specifically designed for nanotoxicology testing in marine environments, rapidly emerged. They gave substantial results, confirming that the toxicity of nanoparticles seems to be moderate in marine systems. However, it cannot be ignored, in the case of either sensitive reproductive stages or endangered species, as well as for rapidly growing, long-living pollutants. Major criticisms include the current restriction of the studies to the brackish, tidal environment near the coast and the lack of robust methods to conveniently detect the presence of different nanopollutants in marine waters. Future advancements should include wider exploitation of free-floating, "true" marine mesocosms and further development of methods for the fieldwork, such as those available for nanosilver (Bouwmeester et al., 2015; Heithmar, 2011; Koelmans et al., 2015; Mattsson et al., 2015).

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REFERENCES

Al-Naamani, L., S. Dobretsov, J. Dutta, and J. G. Burgess. 2017. Chitosan-zinc oxide nanocomposite coatings for the prevention of marine biofouling. *Chemosphere* 168: 408–17.

Andrady, A. L. 2011. Microplastics in the marine environment. *Mar Pollut Bull* 62: 1596–605.

Antizar-Ladislao, B., B. D. Bhattacharya, S. Ray Chaudhuri, and S. K. Sarkar. 2015. Impact of silver nanoparticles on benthic prokaryotes in heavy metal-contaminated estuarine sediments in a tropical environment. *Mar Pollut Bull* 99: 104–11.

Ambrosone, A. and C. Tortiglione. 2013. Methodological approaches for nanotoxicology using cnidarian models. *Toxicol Mech Meth* 23, no. 3: 207–16.

Aravantinou, A. F., V. Tsarpali, S. Dailiani, and I. D. Manariotis. 2015. Effect of cultivation media on the toxicity of ZnO nanoparticles to freshwater and marine microalgae. *Ecotoxicol Environ Saf* 114: 109–16.

Arulavasu, C., S. M. Jennifer, D. Prabhu, and D. Chandhirasekar. 2014. Toxicity effect of silver nanoparticles in brine shrimp *Artemia*. *Sci World J* 2014: 256919.

Ashtari, K., K. Khajeh, J. Fasihi, P. Ashtari, A. Ramazani, A., and H. Vali. 2012. Silica-encapsulated magnetic nanoparticles: Enzyme immobilization and cytotoxic study. *Int J Biol Macromol* 50, no. 4: 1063–9.

ASTM E2317-04. 2004. *Standard Guide for Conducting Renewal Microplate-Based Life-Cycle Toxicity Tests with a Marine Meiobenthic Copepod*; American Society for Testing Materials: Philadelphia, PA. (Active standard revised in 2012).

Ates, M., J. Daniels, Z. Arslan, and I. O. Farah. 2013a. Effects of aqueous suspensions of titanium dioxide nanoparticles on *Artemia salina*: Assessment of nanoparticle aggregation, accumulation, and toxicity. *Environ Monit Assess* 185: 3339–48.

Ates, M., J. Daniels, Z. Arslan, I. VO. Farah, and H. F. Rivera. 2013b. Comparative evaluation of impact of Zn and ZnO nanoparticles on brine shrimp (*Artemia salina*) larvae: Effects of particle size and solubility on toxicity. *Environ Sci Process Impacts* 15: 225–33.

Ates, M., V. Demir, Z. Arslan, J. Daniels, I. O. Farah, and C. Bogatu. 2013c. Evaluation of alpha and γ aluminum oxide nanoparticle accumulation, toxicity, and depuration in *Artemia salina* larvae. *Environ Toxicol* 30, no. 1: 109–18.

Ates, M., M. A. Dugo, V. Demir, Z. Arslan, and P. B. Tchounwou. 2014. Effect of copper oxide nanoparticles to sheephead minnow (*Cyprinodon variegatus*) at different salinities. *Dig J Nanomater Biostruct* 9, no. 1: 369–77.

Balbi, T., A. Smerilli, R. Fabbri et al. 2014. Co-exposure to n-TiO_2 and Cd^{2+} results in interactive effects on biomarker responses but not in increased toxicity in the marine bivalve *Mytilus galloprovincialis*. *Sci Total Environ* 493: 355–64.

Banni, M., S. Sforzini, T. Balbi, I. Corsi, A. Viarengo, and L. Canesi. 2016. Combined effects of n-TiO_2 and 2,3,7,8-TCDD in *Mytilus galloprovincialis* digestive gland: A transcriptomic and immunohistochemical study. *Environ Res* 145: 135–44.

Barmo, C., C. Ciacci, B. Canonico et al. 2013. In vivo effects of n-TiO_2 on digestive gland and immune function of the marine bivalve *Mytilus galloprovincialis*. *Aquat Toxicol* 132–133: 9–18.

Baumerte, A., G. Sakale, J. Zavickis et al. 2013. Comparison of effects on crustaceans: Carbon nanoparticles and molybdenum compounds nanowires. *J Phys Conf Ser* 429: 012041.

Beiras, R., I. Durán, J. Bellas, and P. Sánchez-Marín. 2012. Biological effects of contaminants: *Paracentrotus lividus* sea urchin embryo test with marine sediment elutriates. *ICES Techn. Mar Environ Sci* no. 51. http://www.ices.dk/sites/pub/Publication%20Reports/Techniques%20in%20Marine%20Environmental%20Sciences%20(TIMES)/times51/TIMES%2051%20Final%20120522.pdf.

Bergami E., S. Pugnalini, M. L. Vannuccini et al. 2017. Long-term toxicity of surface-charged polystyrene nanoplastics to marine planktonic species *Dunaliella tertiolecta* and *Artemia franciscana*. *Aquat Toxicol* 189: 159–69.

Bernardeschi M., P. Guidi, V. Scarcelli, G. Frenzilli, and M. Nigro. 2010. Genotoxic potential of TiO_2 on bottlenose dolphin leukocytes. *Anal Bioanal Chem* 396, no. 2: 619–23.

Blickley, T. M., C. W. Matson, W. N. Vreeland, D. Rittschof, R. T. Di Giulio, and P. D. McClellan-Green. 2014. Dietary CdSe/ZnS quantum dot exposure in estuarine fish: Bioavailability, oxidative stress responses, reproduction, and maternal transfer. *Aquat Toxicol* 148: 27–39.

Bone, A. J., C. W. Matson, B. P. Colman, X. Yang, J. N. Meyer, R. T., and T. Di Giulio. 2015. Silver nanoparticles toxicity to Atlantic killfish (*Fundulus eteroclitus*) and *Caenorhabditis elegans*: A comparison of mesocosm, microcosm, and conventional laboratory studies. *Environ Toxicol Chem* 34: 275–82.

Bouwmeester, H., P. C. H. Hollman, and R. J. B. Peters. 2015. Potential health impact of environmentally released micro- and nanoplastics in the human food production chain: Experiences from nanotoxicology. *Environ Sci Technol* 49, no. 15: 8932–47.

Bower, S. M. 2006. Haemocytic Neoplasia of Mussels. Synopsis of Infectious Diseases and Parasites of Commercially Exploited Shellfish at Fisheries and Oceans Canada. http://www.dfo-mpo.gc.ca/science/aah-saa/diseases-maladies/hcnmu-eng.html.

Bradford, A., R. D. Handy, J. W. Readman, A. Atfield, and M. Mühling. 2009. Impact of silver nanoparticle contamination on the genetic diversity of natural bacterial assemblages in estuarine sediments. *Environ Sci Technol* 43, no. 12: 4530–36.

Bruneau A., M. Fortier, F. Gagne et al. 2015. In vitro immunotoxicology of quantum dots and comparison with dissolved cadmium and tellurium. *Environ Toxicol* 30, no. 1: 9–25.

Brunelli, A., G. Pojana, S. Callegaro, and A. Marcomini. 2013. Agglomeration and sedimentation of titanium dioxide nanoparticles (n-TiO_2) in synthetic and real waters. *J Nanopart Res* 15: 1684.

Buffet, P. M., C. Amiard-Triquet, A. Dybowska et al. 2012. Fate of isotopically labeled zinc oxide nanoparticles in sediment and effects on two endobenthic species, the clam *Scrobicularia plana* and the ragworm *Hediste diversicolor. Ecotoxicol Environ Saf* 84: 191–8.

Buffet, P. M., O. Fossi Tankoua, J. F. Pan et al. 2011. Behavioural and biochemical responses of two marine invertebrates *Scrobicularia plana* and *Hediste diversicolor* to copper oxide nanoparticles. *Chemosphere* 84: 166–74.

Buffet, P. E., J. F. Pan, L. Poirier et al. 2013a. Biochemical and behavioural responses of the endobenthic bivalve *Scrobicularia plana* to silver nanoparticles in seawater and microalgal food. *Ecotoxicol Environ Saf* 89: 117–24.

Buffet, P. E., L. Poirier, A. Zalouk-Vergnoux et al. 2014a. Biochemical and behavioural responses of the marine polychaete *Hediste diversicolor* to cadmium sulfide quantum dots (CdS quantum dots): Waterborne and dietary exposure, *Chemosphere* 100: 63–70.

Buffet, P. M., M. Richard, F. Caupos et al. 2013b. A mesocosm study of fate and effects of CuO nanoparticles on endobenthic species (*Scrobicularia plana*, *Hediste diversicolor*). *Environ Sci Technol* 47: 1620–8.

Buffet, P. M., A., Zalouk-Vergnoux, A. Châtel et al. 2014b. A marine mesocosm study on the environmental fate of silver nanoparticles and toxicity effects on two endobenthic species: The ragworm *Hediste diversicolor* and the bivalve mollusc *Scrobicularia plana*. *Sci Total Environ* 470–471: 1151–9.

Burchardt, A. D., R. N. Carvalho, A. Valente et al. 2012. Effects of silver nanoparticles in diatom *Thalassiosira pseudonana* and cyanobacterium *Synechococcus* sp. *Environ Sci Technol* 46: 11336–44.

Burić, P., Ž. Jakšić, L. Štajner et al. 2015. Effect of silver nanoparticles on Mediterranean sea urchin embryonal development is species specific and depends on moment of first exposure. *Mar Environ Res* 111: 50–9.

Burns, J. M., P. L. Pennington, P. N. Sisco et al. 2013. Surface charge controls the fate of Au nanorods in saline estuaries. *Environ Sci Technol* 47, no. 22: 12844–51.

Callegaro, S. D. Minetto, G. Pojana et al. 2015. Effects of alginate on stability and ecotoxicity of nano-TiO_2 in artificial seawater. *Ecotoxicol Environ Saf* 117: 107–14.

Canesi, L., C. Ciacci, M. Betti et al. 2008. Immunotoxicity of carbon black nanoparticles to blue mussel hemocytes. *Environ Int* 34: 1114–19.

Canesi L., C. Ciacci, R. Fabbri et al. 2016. Interactions of cationic polystyrene nanoparticles with marine bivalve hemocytes in a physiological environment: Role of soluble hemolymph proteins. *Environ Res* 150: 73–81.

Canesi L., C. Ciacci, R. Fabbri, A. Marcomini, G. Pojana, and G. Gallo. 2012. Bivalve molluscs as a unique target group for nanoparticle toxicity. *Mar Environ Res* 76: 16–21.

Canesi, L., C. Ciacci, D. Vallotto, G. Gallo, A. Marcomini, and G. Pojana. 2010. In vitro effects of suspensions of selected nanoparticles (C60 fullerene, TiO_2, silica) on *Mytilus* hemocytes. *Aquat Toxicol* 96: 151–58.

Ciacci, C., B. Canonico, D. Bilanicovà et al. 2012. Immunomodulation by different types of N-oxides in the hemocytes of the marine bivalve *Mytilus galloprovincialis*. *PLoS One* 7, no. 5: e36937.

Clément, L., C. Hurel, and N. Marmier. 2013. Toxicity of TiO_2 nanoparticles to cladocerans, algae, rotifers and plants—Effects of size and crystalline structure. *Chemosphere* 90: 1083–90.

Cleveland D., S. E. Long, P. L. Pennington et al. 2012. Pilot estuarine mesocosm study on the environmental fate of silver nanomaterials leached from consumer products. *Sci Total Environ* 421–422: 267–72.

Cole, M. and T. S. Galloway. 2015. Ingestion of nanoplastics and microplastics by Pacific oyster larvae. *Environ Sci Technol* 49, no. 24: 14625–32.

Cong, Y., G. T. Banta, H. Selck, D. Berhanu, E. Valsami-Jones, and V. E. Forbes. 2011. Toxic effects and bioaccumulation of nano-, micron- and ionic-silver in the polychaete, *Nereis diversicolor. Aquat Toxicol* 105: 403–11.

Cornejo-Garrido, H., D. Kibanova, A. Nieto-Camacho et al. 2011. Oxidative stress, cytoxicity, and cell mortality induced by nano-sized lead in aqueous suspensions. *Chemosphere* 84, no. 10: 1329–35.

Cózar, A., F. Echevarría, J. I. González-Gordillo et al. 2014. Plastic debris in the open ocean. *Proc Natl Acad Sci USA* 111, no. 28: 10239–44.

Della Torre, C., E. Bergami, A. Salvati et al. 2014. Accumulation and embryotoxicity of polystyrene nanoparticles at early stage of development of sea urchin embryos *Paracentrotus lividus*. *Environ Sci Technol* 48, no. 20: 12302–11.

Falugi, C., M. G. Aluigi, M. C. Chiantore et al. 2012. Toxicity of metal oxide nanoparticles in immune cells of the sea urchin. *Mar Environ Res* 76: 114–21.

Fatouros, D. G., K. Power, O. Kadir et al. 2011. Stabilisation of SWNTs by alkyl-sulfate chitosan derivatives of different molecular weight: Towards the preparation of hybrids with anticoagulant properties. *Nanoscale* 3, no. 3: 1218–24.

Ferry, J. L., P. Craig, C. Hexel et al. 2009. Transfer of gold nanoparticles from the water column to the estuarine food web. *Nat Nanotechnol* 4: 441–4.

Frenzilli, G., M. Bernardeschi, P. Guidi et al. 2014. Effects of in vitro exposure to titanium dioxide on DNA integrity of bottlenose dolphin (*Tursiops truncatus*) fibroblasts and leukocytes. *Mar Environ Res* 100: 68–73.

Gallo A., R. Boni, I. Buttino, and E. Tosti. 2016. Spermiotoxicity of nickel nanoparticles in the marine invertebrate *Ciona intestinalis* (ascidians). *Nanotoxicology* 10, no. 8: 1096–104.

Galloway, T., C. Lewis, I. Dolciotti, B. D. Johnston, J. Moger, and F. Regoli. 2010. Sublethal toxicity of nano-titanium dioxide and carbon nanotubes in a sediment dwelling marine polychaete. *Environ Pollut* 158: 1748–55.

Gambardella, C., M. G. Aluigi, S. Ferrando et al. 2013. Developmental abnormalities and changes in cholinesterase activity in sea urchin embryos and larvae from sperm exposed to engineered nanoparticles. *Aquat Toxicol* 130–131: 77–85.

Gambardella, C., S. Ferrando, A. M. Gatti et al. 2016. Review: Morphofunctional and biochemical markers of stress in sea urchin life stages exposed to engineered nanoparticles. *Environ Toxicol* 31, no. 11: 1552–62.

Gambardella, C., S. Ferrando, S. Morgana et al. 2015. Exposure of *Paracentrotus lividus* male gametes to engineered nanoparticles affects skeletal biomineralization processes and larval plasticity. *Aquat Toxicol* 158: 181–91.

Gambardella, C., T. Mesarič, T. Milivojević et al. 2014. Effects of selected metal oxide nanoparticles on *Artemia salina* larvae: Evaluation of mortality and behavioural and biochemical responses. *Environ Monit Assess* 186, no. 7: 4249–59.

García-Alonso, J., N. Rodriguez-Sanchez, S. B. Misra et al. 2014. Toxicity and accumulation of silver nanoparticles during development of the marine polychaete *Platynereis dumerilii*. *Sci Total Environ* 476–477: 688–95.

Garner, K. L. and A. A. Keller. 2014. Emerging patterns for engineered nanomaterials in the environment: A review of fate and toxicity studies. *J Nanopart Res* 16: 2503.

Gomes, T., C. G. Pereira, C. Cardoso, J. P. Pinheiro, I. Cancio, and M. J. Bebianno. 2012. Accumulation and toxicity of copper oxide nanoparticles in the digestive gland of *Mytilus galloprovincialis*. *Aquat Toxicol* 118–119: 72–9.

Gomes, T., J. P. Pinheiro, I. Cancio, C. G. Pereira, C. Cardoso, and M. J. Bebianno. 2011. Effects of copper nanoparticles exposure in the mussel *Mytilus galloprovincialis*. *Environ Sci Technol* 45, no. 21: 9356–62.

Gornati, R., A., Longo, F. Rossi et al. 2016. Effects of titanium dioxide nanoparticle exposure in *Mytilus galloprovincialis* gills and digestive gland. *Nanotoxicology* 10, no. 6: 807–17.

Griffitt, R. J., N. J. Brown-Petterson, and D. A. Savin. 2012. Effects of chronic nanoparticulate silver exposure to adult and juvenile sheepshead minnows (*Cyprinodon variegatus*). *Environ Toxicol Chem* 31, no. 1: 160–67.

Hanna, S. K., R. J. Miller, D. Zhou, A. A. Keller, and H. S. Lenihan. 2013. Accumulation and toxicity of metal oxide nanoparticles in a soft-sediment estuarine amphipod. *Aquat Toxicol* 142–143: 441–6.

Heithmar, M. S. 2011. Screening Methods for Metal-Containing Nanoparticles in Water. APM 32, EPA/600/R-11/096. https://cfpub.epa.gov/si/si_public_record_report.cfm?dirEntryId=238306 (accessed June 8, 2017).

Heggelund, L., S. F. Hansen, T. F. Astrup, and A. Boldrin. 2016. Semi-quantitative analysis of solid waste flows from nano-enabled consumer products in Europe, Denmark and the United Kingdom—Abundance, distribution and management. *Waste Manag* 56: 584–92.

Ikuma, K., A. W. Decho, and B. L. Lau. 2015. When nanoparticles meet biofilms—Interactions guiding the environmental fate and accumulation of nanoparticles. *Front Microbiol* 6: 591.

ISO 10253. 2016. Water quality. Marine Algal Growth Inhibition Test with *Skeletonema costatum* and *Phaeodactylum tricornutum*. Geneva: International Organization for Standardization). http://www.iso.org/iso/home/store/catalogue_ics/catalogue_detail_ics.htm?csnumber=66657 (accessed June 8, 2017).

ISO 11348-3. 2007. *Water Quality. Determination of the Inhibitory Effect of Waters Samples on the Light Emission of* Vibrio fischeri *(Luminescent Bacteria Test). Part 3: Method Using Freeze-Dried Bacteria*; Geneva: International Organization for Standardization. http://www.iso.org/iso/catalogue_detail.htm?csnumber=40518 (accessed June 8, 2017).

Jovanovìc, B., and H. M. Guzmàn. 2014. Effects of titanium dioxide (TiO_2) nanoparticles on Carribean reef-building coral (*Monastrea faveolata*). *Environ Toxicol Chem* 33, no. 6: 1346–53.

Kadar E., O. Dyson, R. D. Handy, S. N., and Al-Subiai. 2013. Are reproduction impairments of free spawning marine invertebrates exposed to zerovalent nano-iron associated with dissolution of nanoparticles? *Nanotoxicology* 7: 135–43.

Kadar E., G. A. Tarran, A. N. Jha, and S. N. Al-Subiai. 2011. Stabilization of engineered zero-valent nanoiron with Na-acrylic copolymer enhances spermiotoxicity. *Environ Sci Technol* 45: 3245–51.

Kalbassi, M. R., H. Salari-joo, and A. Johari. 2011. Toxicity of silver nanoparticles in aquatic ecosystems: Salinity as the main cause in reducing toxicity. *Iranian Journal of Toxicology* 5, nos. 1 and 2: 436–43. http://ijt.arakmu.ac.ir/browse.php?a_id=38&sid=1&slc_lang=fa.

Karl, M., C. Leck, E. Coz, and J. Heintzenberg. 2013. Marine nanogels as a source of atmospheric nanoparticles in the high Arctic. *Geophys Res Lett* 40: 3738–43.

Kashiwada, S. 2006. Distribution of nanoparticles in the see-through medaka (*Oryzias latipes*). *Environ Health Perspect* 114: 1697–702.

Katsumiti, A., D. Gilliland, I. Arostegui, and M. P. Cajaraville. 2015. Mechanisms of toxicity of Ag nanoparticles in comparison to bulk and ionic Ag on mussel hemocytes and gill cells. *PLoS One* 10, no. 6: e0129039.

Keller, A. A., K. L. Garner, R. J. Miller, and H. S. Lenihan. 2012. Toxicity of nano-zero valent iron to freshwater and marine organisms. *PLoS One* 7, no. 8: e43983.

Koehler, A., U. Marx, K. Broeg, S. Bahns, and J. Bressling. 2008. Effects of nanoparticles in *Mytilus edulis* gills and hepatopancreas—A new threat to marine life? *Mar Environ Res* 66, no. 1: 12–4.

Koelmans, A. A., E. Besseling, and W. J. Shim. 2015. Nanoplastics in the aquatic environment: Critical review. In *Marine Anthropogenic Litter*, eds. M. Bergmann, L. Gutow, and M. Klages, 325–340. Berlin: Springer.

Kumar, P., S. Selvi, and A. L. Praba et al. 2012. Antibacterial activity and in-vitro cytotoxicity assay against brine shrimp using silver nanoparticles synthesized from *Sargassum ilicifolium*. *Dig J Nanomater Biostruct* 7, no. 4: 1447–55. http://www.chalcogen.ro/1447_Kumar.pdf.

Lauth, J. R., G. I. Scott, D. S. Cherry, and A. L. Buikema Jr. 1996. A modular estuarine mesocosm. *Environ Toxicol Chem* 15, no. 5: 630–7.

Lee, K. W., W. J. Shim, O. Y. Kwon, and J. H. Kang. 2010. Size-dependent effects of micro polystyrene particles in the marine copepod *Tigriopus japonicus*. *Environ Sci Technol* 47: 11278–83.

Leverett, D. and J. Thain. 2013. Oyster embryo-larval bioassay (Revised). *ICES Techn Mar Environ Sci*, no. 54. http://ices.dk/sites/pub/Publication%20Reports/Techniques%20in%20Marine%20Environmental%20Sciences%20(TIMES)/times54/TIMES%2054%20web.pdf (accessed June 8, 2017).

Libralato, G. 2014. The case of *Artemia* spp. in nanoecotoxicology. *Mar Environ Res* 101: 38–43.

Libralato, G., E. Prato, L. Migliore, A. M. Cicero, and L. Manfra. 2016. A review of toxicity testing protocols and endpoints with *Artemia* spp. *Ecol Indic* 69: 35–49.

Lodeiro, P., E. P. Achterberg, J. Pampín, A. Affatati, and M. S. El-Shahawi. 2016. Silver nanoparticles coated with natural polysaccharides as models to study AgNP aggregation kinetics using UV–visible spectrophotometry upon discharge in complex environments. *Sci Total Environ* 539: 7–16.

Magesky, A., C. A. Ribeiro, and E. Pelletier. 2016. Physiological effects and cellular responses of metamorphic larvae and juveniles of sea urchin exposed to ionic and nanoparticulate silver. *Aquat Toxicol* 174: 208–27.

Manzo, S., M. L. Miglietta, G. Rametta, S. Buono, and G. Di Francia. 2013. Embryotoxicity and spermiotoxicity of nanosized ZnO for Mediterranean sea urchin *Paracentrotus lividus*. *J Hazard Mater* 254–255: 1–9.

MARAgNANO. 2015. The Behaviour, Fate and Ecotoxicological Effects of Silver Nanoparticles in Estuarine and Coastal Waters—Report Summary. http://cordis.europa.eu/result/rcn/171493_en.html (accessed June 8, 2017).

Marisa I., M. G. Marin, F. Caicci, E. Franceschinis, A. Martucci, and V. Matozzo. 2015. In vitro exposure of haemocytes of the clam *Ruditapes philippinarum* to titanium dioxide (TiO_2) nanoparticles: Nanoparticle characterisation, effects on phagocytic activity and internalisation of nanoparticles into haemocytes. *Mar Environ Res* 103: 11–7.

Mattsson, K., L. A. Hansson, and T. Cedervall. 2015. Nano-plastics in the aquatic environment. *Environ Sci Process Impacts* 17, no. 10: 1712–21.

Minetto D., A. Volpi Ghirardini, and G. Libralato. 2016. Saltwater ecotoxicology of Ag, gold, CuO, TiO_2, ZnO and C60 engineered nanoparticles: An overview. *Environ Int* 92–93: 189–201.

Mouneyrac, C., P. E. Buffet, L. Poirier et al. 2014. Fate and effects of metal-based nanoparticles in two marine invertebrates, the bivalve mollusc *Scrobicularia plana* and the annelid polychaete *Hediste diversicolor*. *Environ Sci Pollut Res Int* 21: 7899–912.

Mesarić, T., K. Sepcic, D. Drobne et al. 2015. Sperm exposure to carbon-based nanomaterials causes abnormalities in early development of purple sea urchin (*Paracentrotus lividus*). *Aquat Toxicol* 163: 158–166.

Miao, A. J., K. A. Schwehr, C. Xu et al. 2009. The algal toxicity of silver engineered nanoparticles and detoxification by exopolymeric substances. *Environ Pollut* 157: 3034–41.

Martínez-Gómez, C., Bignell, J. and Lowe, D. 2015. Lysosomal membrane stability in mussels. *ICES Techniques in Marine Environmental Sciences* no. 56. http://ices.dk/sites/pub/Publication%20Reports/Techniques%20in%20Marine%20Environmental%20Sciences%20(TIMES)/times56/TIMES%2056.pdf.

Matranga, V. and I. Corsi. 2012. Toxic effects of engineered nanoparticles in the marine environment: Model organisms and molecular approaches. *Mar Environ Res* 76: 32–40.

McCarthy, M. P., D. L. Carroll, and A. H. Ringwood, A. H. 2013. Tissue specific responses of oysters, *Crassostrea virginica*, to silver nanoparticles. *Aquat Toxicol* 138–139: 123–8.

Méléder, V., Y. Rincé, L. Barillé, P. Gaudin, and P. Rosa. 2007. Spatiotemporal changes in microphytobenthos assemblages in a macrotidal flat (Bourgneuf Bay, France). *J Phycol* 43: 1177–90.

Montes, M. O., S. K. Hanna, H. S. Lenihan, and A. A. Keller. 2012. Uptake, accumulation, and biotransformation of metal oxide nanoparticles by a marine suspension-feeder. *J Hazard Mater* 225–226: 139–45.

Munari M., J. Sturve, G. Frenzilli et al. 2014a. Genotoxic effects of CdS quantum dots and Ag_2S nanoparticles in fish cell lines (RTG-2). *Mutat Res Genet Toxicol Environ Mutagen* 775–776: 89–93.

Munari M., J. Sturve, G. Frenzilli et al. 2014b. Genotoxic effects of Ag_2S and CdS nanoparticles in blue mussel (*Mytilus edulis*) haemocytes. *Chem Ecol* 30, no. 8: 719–25.

Nevius, B. A., Y. P. Chen, J. L. Ferry, A. W. Decho. 2012. Surface-functionalization effects on uptake of fluorescent polystyrene nanoparticles by model biofilms. *Ecotoxicology* 21, no. 8: 2205–13.

Nielsen, H. D., L. S. Berry, V. Stone, T. R. Burridge, T. F. Fernandes. 2008. Interactions between carbon black nanoparticles and the brown algae *Fucus serratus*: Inhibition of fertilization and zygotic development. *Nanotoxicology* 2, no. 2: 88–97.

OECD 201, draft. 2002. Guidelines for the Testing of Chemicals, Freshwater Alga and Cyanobacteria, Growth Inhibition Test. Draft revised guidelines, July 2002. http://www.oecd.org/chemicalsafety/testing/1946914.pdf (accessed June 8, 2017).

OECD 203. 2011. Guidelines for the Testing of Chemicals, Section 2. Effects on Biotic Systems. Test No. 203: Fish, Acute Toxicity Test. Adopted: July 17, 1992.

OECD 305. 2012. Guidelines for the Testing of Chemicals, Section 3. Degradation and Accumulation. Bioaccumulation in Fish: Aqueous and Dietary Exposure. Adopted: October 2, 2012.

OECD, Draft. 2013. Guidelines for Testing of Chemicals. Draft New Test Guidelines. Mysid Two-Generation Test. July 15, 2013. http://www.oecd.org/env/ehs/testing/OECD%20TG%20Mysid%202-gen_Draft%20for%20REVIEW_15%20July%202013.pdf (accessed June 8, 2017).

Pan, J. F., P. E. Buffet, L. Poirier et al. 2012. Size dependent bioaccumulation and ecotoxicity of gold nanoparticles in an endobenthic invertebrate: The Tellinid clam *Scrobicularia plana*. *Environ Pollut* 168: 37–43.

Pennington, P. L., M. E. De Lorenzo, P. B. Key, E. F. Wirth, M. H. Fulton, and G. I. Scott. 2007. The Design, Construction, Operation and Maintenance of the Replicated Modular Estuarine Mesocosm. NOAA Technical Memorandum NOS NCCOS, 62. http://aquaticcommons.org/14617/1/NOS%20NCCOS%2062.pdf (accessed June 8, 2017).

Pettitt, M. E. and J. R. Lead. 2013. Minimum physicochemical characterisation requirements for nanomaterial regulation. *Environ Int* 52: 41–50.

Pretti, C., M. Oliva, R. D. Pietro, G. Monni, G. Cevasco, F. Chiellini, C. Pomelli, and C. Chiappe. 2014. Ecotoxicity of pristine graphene to marine organisms. *Ecotoxicol Environ Saf* 101: 138–45.

Rajabi S., A. Ramazani, M. Hamidi, and T. Naji. 2015. *Artemia salina* as a model organism in toxicity assessment of nanoparticles. *DARU J Pharm Sci* 23, no. 20.

Rana, S., S. Samanta, S. Bhattacharya, K. Al-Khaledd, A. Goswami, and J. Chattopadhyay. 2015. The effect of nanoparticles on plankton dynamics: A mathematical model. *Biosystems* 127: 28–41.

Ringwood, A. H., McCarthy, M., Bates, T. C., and Carroll, D. L. 2010. The effects of silver nanoparticles on oyster embryos. *Mar Environ Res* 69: S49–S51.

Riebesell, U., J. Czerny, K. von Bröckel et al. 2013. Technical Note: A mobile sea-going mesocosm system—New opportunities for ocean change research. *Biogeosciences* 10, no. 3: 1835–47.

Sathe, P., J. Richter, M. T. Myint, S. Dobretsov, and J. Dutta. 2016. Self-decontaminating photocatalytic zinc oxide nanorod coatings for prevention of marine microfouling: A mesocosm study. *Biofouling* 32, no. 4: 383–495.

Tantra, R., D. Gohil, S. Kaliyappan, and S. Jing. 2015. Nanoparticle characterisation for ecotoxicological studies using dynamic light scattering, scanning electron microscopy and nanoparticle tracking analysis techniques. In: *ENV/JM/MONO(2015)15/ANN4, Dossier on Zinc Oxide, Annex 4. Series on the Safety of Manufactured nanomaterials*, no. 52. http://www1.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2015)15/ANN4&docLanguage=En (accessed June 8, 2017).

Templeton, R., P. L. Ferguson, K. M. Washburn, W. A. Scrivens, and Chandler, G. T. 2006. Life-cycle effects of single-walled carbon nanotubes (SWNTs) on an estuarine meiobenthic copepod. *Environ Sci Technol* 40: 7387–93.

Thain, J., Y. Allen, and S. Kirby, S. 2004. Sediment Bioassays and Their Use in Managing the Disposal of Dredged Material in the Marine Environment. Document ICES CM 2004/Z:03. http://www.ices.dk/sites/pub/CM%20Doccuments/2004/Z/Z0304.pdf (accessed June 8, 2017).

Toncelli, C., K. Mylona, M. Tsapakis, S. A. and Pergantis. 2016. Flow injection with online dilution and single particle inductively coupled plasma–mass spectrometry for monitoring silver nanoparticles in seawater and in marine microorganisms. *J Anal At Spectrom* 31: 1430–39.

Vaseashta, A. 2009. Nanomaterials for chemical–biological–physical integrity of potable water. In *Water Treatment Technologies for the Removal of High-Toxicity Pollutants*, eds. M. Václavíková, K. Vitale, G. P. Gallios and L. Ivaničová, 1–17. NATO Science for Peace and Security Series C: Environmental Security. Dordrecht: Springer.

Vunain, E., A. K. Mishra, B. B. Mamba. 2016. Dendrimers, mesoporous silicas and chitosan-based nanosorbents for the removal of heavy-metal ions: A review. *Int J Biol Macromol* 86: 570–86.

Wang, D., Y. Gao, Z. Lin, Z. Yao, and W. Zhang. 2014a. The joint effects on *P. phosphoreum* of metaloxide nanoparticles and their most likely coexisting chemicals in the environment. *Aquat Toxicol* 154: 200–6.

Wang, H., K. Y. Ho, K. G. Scheckel et al. 2014b. Toxicity, bioaccumulation, and biotransformation of silver nanoparticles in marine organisms. *Environ Sci Technol* 48: 13711–17.

Wang, J. and W. X. Wang. 2014. Low bioavailability of silver nanoparticles presents trophic toxicity to marine medaka (*Oryzias melastigma*). *Environ Sci Technol* 48, no. 14: 8152–61.

Ward, J. E., and D. J. Kach. 2009. Marine aggregates facilitate ingestion of nanoparticles by suspension-feeding bivalves. *Mar Environ Res* 68: 137–42.

Watts M. C., and G. R. Bigg. 2001. Modelling and the monitoring of mesocosm experiments: Two case studies. *J Plankton Res* 23, no. 10: 1081–93.

Weis, J., C. J. Andrews, J. E. Dyksen et al. 2015. Report of the NJDEP-Science Advisory Board. Human Health Impacts of Microplastics and Nanoplastics, http://www.state.nj.us/dep/sab/NJDEP-SAB-PHSC-final-2016.pdf (accessed June 8, 2017).

Wegner, A., E. Besseling, E. M. Foekema, P. Kamermans, and A. A. Koelmans. 2012. Effects of nanopolystyrene on the feeding behaviour of the blue mussel (*Mytilus edulis* L.). *Environ Toxicol Chem* 31: 2490–97.

Wong, S. W., P. T. Leung, A. B. Djurisić, and K. M. Leung. 2010. Toxicities of nano zinc oxide to five marine organisms: Influences of aggregate size and ion solubility. *Anal Bioanal Chem* 396, no. 2: 609–18.

Wu, Y., Q. Zhou, H. Li, W. Liu, T. Wang, and G. Jiang. 2010. Effects of silver nanoparticles on the development and histopathology biomarkers of Japanese medaka (*Oryzias latipes*) using the partial-life test. *Aquat Toxicol* 100, no. 2: 160–7.

Yung, M. M. N., S. W. Y. Wong, K. W. H. Kwok et al. 2015. Salinity-dependent toxicities of zinc oxide nanoparticles to the marine diatom *Thalassiosira pseudonana*. *Aquat Toxicol* 165: 31–40.

Zamani, H., A. Moradshahi, H. D. Jahromi, and M. H. Sheikhi. 2014. Influence of PbS nanoparticle polymer coating on their aggregation behavior and toxicity to the green algae *Dunaliella salina*. *Aquat Toxicol* 154: 176–83.

Zhang C., Z. Hu, and B. Deng. 2016. Silver nanoparticles in aquatic environments: Physiochemical behavior and antimicrobial mechanisms. *Water Res* 88: 403–27.

Zhang, Y., Y. R. Leu, R. J. Aitken, and M. Riediker. 2015. Inventory of engineered nanoparticle-containing consumer products available in the Singapore retail market and likelihood of release into the aquatic environment. *Int J Environ Res Public Health* 12, no. 8: 8717–43.

Zhu, X., J. Zhou, and Z. Cai. 2011. TiO_2 nanoparticles in the marine environment: Impact on the toxicity of tributyltin to abalone (*Haliotis diversicolor supertexta*) embryos. *Environ Sci Technol* 45: 3753–8.

Zuykov, M., E. Pelletier, and S. Demers, S. 2011. Colloidal complexed silver and silver nanoparticles in extrapallial fluid of *Mytilus edulis*. *Mar Environ Res* 71: 17–21.



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Interaction of Carbon Nanomaterials with Biological Matrices

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4.1 Introduction to Nanotechnology and Toxicology

Nanotechnology is defined as a field of science where the materials at the nanoscale were manipulated to obtain unique properties. Such materials with the improved properties deserve major opportunities in terms of their application including energy, environment, medicine, etc. However, the rapid growth in the production of nanoparticles (NPs) is equally compensated for by growing toxicity issues. This is because every unique property observed in NPs is due to their larger surface area and smaller size, which not only support their drug-loading efficacy, but also contribute toward their toxicity in vivo (Kagan, Tyurina, and Tyurin 2006; Fadeel et al. 2007). Several researchers have indicated the need to investigate the toxicity of NPs, before imposing their application in vivo. Figure 4.1 describes the dual role of carbon nanotubes (CNTs) in both biomedicine and toxicity issues. But the data available in the literature were only the hypothesis, and no complete toxicological investigations were clear, due

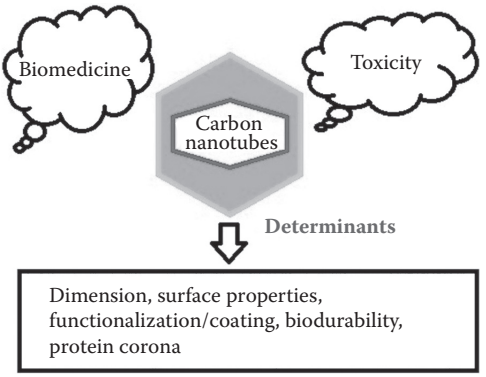


FIGURE 4.1 Dual roles of CNTs in both biomedicine and toxicology.

to the contrasting observation made by different researchers on the same particles. Few reports suggest the role of concentration-dependent toxicity, while few turn to the implication of the physicochemical properties of the NPs including size, shape, and morphology. Also, the way of the interaction of NPs with cells in vivo and the reaction-induced biological or systemic consequences attained remain to be a great question, as only few modeling works have been reported on the biological interaction of the NPs. Hence, more understanding on the interrelationship between NPs and biological molecules in vivo is needed for emphasizing the safer utilization of nanotechnology with no toxicity issue (Kagan, Tyurina, and Tyurin 2006).

In this chapter, a brief outline on the introduction to CNTs, dispersion properties, the synthesis and characterization techniques, functionalization approaches, biomedical applications, and their toxicity in terms of their interaction with biological macromolecules has been summarized. Hence, this chapter would add insight into the available knowledge on the toxicity issues of engineered NPs being produced in tones without revealing knowledge on their toxicity.

4.2 Carbon Nanotubes

The structure of CNTs was designed with the graphite sheet being rolled up into cylinders. The tube diameter ranges in nanometer dimension with the length measuring in terms of millimeters that ultimately contributes to their strange electrical transport. Certain tubes act as metals, while others act as semiconductors. Graphitic band structures were initially elucidated by Wallace (1947). Ijima in 1991 later reported diamond, graphite, and fullerene (C60) quasi-one-dimensional nanotubes to be another form of CNTs. He has made this observation during discovery of multiwalled carbon nanotubes (MWCNTs) in carbon soot being synthesized by an arc-discharge method (Iijima 1991).

MWCNTs were found to have at least two layers with an outer diameter ranging between 3 and 30 nm. Later, Ijima again discovered that the structure of single-walled carbon nanotubes (SWCNTs) (Iijima and Ichihashi 1993) is narrower than that of multiwalled tubes with a diameter range of 1–2 nm (Nugent et al. 2001; Harris 2009).

Hence, the CNTs were divided based on their graphene layers such as the single- and multiwalled CNTs. There also exist certain differences in the structural and functional properties of SW and MWCNTs.

For example, in the case of SWCNTs, there exists only one graphene sheet rolled up into a cylinder form, while in MWCNTs, many layers of sheets were arranged. Also, the synthesis of SWCNTs requires the catalyst, while that of MWCNTs does not require the catalyst to mediate the process. In SWCNTs, the bulk synthesis is difficult as the control of crystal growth and the atmospheric conditions are very difficult. In the case of MWCNTs, the bulk synthesis is very easy. The dispersion of SWCNTs leads to the formation of bundle aggregates while MWCNTs form a homogenous dispersion with no apparent bundle aggregates. The structure of MWCNTs is very complex in comparison to that of SWCNTs and hence the characterization of SWCNTs is very easy and can even be twisted for more applications (Abrahamson, Wiles, and Rhoades 1999; Meyyappan et al. 2003; Hirlekar et al. 2009).

Major applications of CNTs were mainly due to the uniqueness existing in their structure and inherent subtlety. The surface of the CNT looks like honeycomb lattices being designed as hexagonal arrays with a superior arrangement including helicity with carbon atoms. Hence, both the properties of CNTs such as local symmetry or helicity and the smaller diameter that helps in determining the size of the repeating structural unit contribute to their tremendous application in electronic devices (Ajayan, Pulickel, and Zhou 2001). Other physical properties of CNTs including the size, structure, and topology contribute to their significant mechanical and surface properties. In addition to the helical lattice structure and closed topology, topological defects in CNTs (Carroll, Redlich, and Ajayan 1997; Khare and Bose 2005) also enhance the reactivity of tube ends by opening up new possibilities for functionalization approaches with foreign substances (Ajayan 1993; Ebbesen, Ajayan, and Tanigaki 1994; Liu et al. 1998; Forró et al. 1999; Zakharova et al. 2005). Several immobilization approaches have been discovered with the wrapping of chemical and biological moieties around the CNTs and hence were considered as attractive candidates in diverse nanotechnological applications, such as fillers in polymer matrixes, molecular tanks, biosensors, and many others (Robertson and McGowan 2003). However, the lack of solubility and the precipitation of the bundles after sonication create a major limitation for their technological application (Thostenson, Ren, and Chou 2001). Also, the formation of supramolecular complexes has allowed a better processing of CNT toward the fabrication of innovative nanodevices (Lu and Chen 2005). However, concerns over their adverse and unanticipated effects on human health have also been raised. In fact, the same properties of CNTs that make the engineered nanomaterials attractive from a technological and biomedical perspective could also make these novel materials harmful to human health and the environment (Shvedova et al. 2009). Also, their interaction with biological systems in vivo including the cells, tissues, and organs and the biological macromolecules remains quite complicated due to their complexity and diverse biological role (Boncel et al. 2011).

4.2.1 Synthesis and Dispersion of Carbon Nanotubes

There are several major techniques for synthesizing CNT nanostructures. The arc-evaporation method producing good-quality nanotubes requires a current passage of about 50 A between two graphite electrodes in a helium atmosphere with metal

catalysts such as cobalt or nickel. The second method is chemical vapor deposition, where the decomposition of a carbon-containing gas occurs with the catalyst such as Fe, Co, Yt, or Ni to form CNTs. The advantage of catalytic synthesis over the arc-evaporation method remains on the ability for scaling up the production volume. Other methods commonly used are the high-pressure carbon monoxide process and the CoMoCAT process. The former method is the gas-phase homogeneous process that uses a floating catalyst approach, whereas CoMoCAT production technology is a heterogeneous process. Another method called laser ablation technology utilizes the powerful laser for vaporizing the metal (Co and Ni)-graphite target. On comparing several techniques for CNT synthesis, chemical vapor deposition remains to be the prominent one, as it gives out not only higher production volume, but also less catalyst contaminant in the synthesized structures (Awasthi, Srivastava, and Srivastava 2005; Kingston and Simard 2006).

Aggregation of CNT bundles due to their poor solubility arising out from attractive van der Waals forces limits their technological and biological applications (Vaisman, Wagner, and Marom 2006; Krause et al. 2011). To overcome such problems, several researchers have reported the utilization of organic solvents, but later these solvents were found to be incompatible with living organisms (Matarredona et al. 2003). Then, the application of surfactants that were more compatible with living organisms was performed in the fields of biochemistry and biomedical engineering (Yamamoto, Noda, and Kato 2011). So far, several surfactants (anionic, cationic, nonionic) have been used to disperse CNTs including sodium deoxycholate (Liu et al. 2010), sodium dodecylbenzenesulfonate (SDBS) (Tan et al. 2008), sodium dodecyl sulfate (Nair et al. 2008), and Tween group surfactants (Rastogi et al. 2008). However, surfactant-assisted CNT dispersion would not be done with simple mixing spontaneously and hence requires tedious methodologies in addition.

For example, sonication is a mechanical method utilized in combination with surfactants to break, disperse, or de-bundle CNT aggregates or bundles in the dispersing process (Yu et al. 2007). Studies (Matarredona et al. 2003) have reported that under optimized conditions, enhanced sonication gives better CNT structures effectively. Hence, sonication was fixed as the parameter for identifying the degree of CNT dispersion in any solvents. For example, Yang et al. have optimized the dispersion energy conditions for the dispersion of CNTs with the anionic surfactant, SDBS (Yang et al. 2013). Shi et al. have also optimized conditions for the sonication-based dispersion of SWCNTs. They have reported that with higher surfactant concentration the dispersion rate of SWCNTs increases and low-temperature sonication is required to achieve maximum dispersion. Dispersion of higher SWCNT concentrations requires longer sonication time (Liu et al. 2010).

Above all, the most advanced method for CNT dispersion is the encapsulation of molecules in the inner empty cavity of the nanotubes. That is, CNTs act as attractive carriers by providing an internal space that could be filled with molecules, NPs, or radionuclides, while the external wall could be functionalized for enhancing their biocompatibility and achieving biodegradation (Bianco, Kostarelos, and Prato 2011) or imparting novel properties for targeting applications (Fabbro et al. 2012) or imaging purposes (Hong, Gao, and Cai 2009; Liu, Yang, and Lee 2011). Such functionalized CNTs were reported to act as an effective drug delivery system to overcome some limitations such as poor solubility of drugs, rapid deactivation, unfavorable pharmacokinetics, and limited biodistribution and direct them to a target organ

(Ménard-Moyon et al. 2010; Liu et al. 2011; Vashist et al. 2011). Hence, the functionalization of CNTs with biologically active molecules helps in improving their biocompatibility and dispersibility (Karousis, Tagmatarchis, and Tasis 2010; Bianco, Kostarelos, and Prato 2011).

Functionalization approaches were classified into two categories: covalent and noncovalent. Covalent functionalization requires the oxidative formation of functional groups on the CNT surface, followed by the grafting of organic moieties on the surface (Bahr et al. 2001; Yamamoto, Noda, and Kato 2011). Noncovalent functionalization requires dispersants such as surfactants and organic solvents for promoting CNT dispersion, but leaves electronic structures and properties of the CNTs unaltered. The method of covalent functionalization remains more robust, accurate, and better controllable in comparison with the noncovalent interactions. The major disadvantage of noncovalent CNT-based conjugates is the possible dissociation in biological fluids and also creates more concerns regarding their potential hazardous effects in terms of their interaction with serum proteins during their administration in vivo. However, for certain applications the noncovalent functionalization approach is preferred as it provides gentler dispersion conditions that preserve not only the structural, optical, and electrical properties of the CNTs, but also the properties of the adsorbed molecules. But in covalent grafting, the substances on the nanotube surface dramatically change the conjugated π-electron framework of CNTs by inducing rehybridization of the sp^2-derivatized carbon atoms to sp^3-derivatized ones, which leads to the reduced intrinsic near-infrared (NIR) fluorescence and Raman scattering (Battigelli et al. 2013).

4.2.2 Characterization of Carbon Nanotubes

A large number of analytical techniques were currently available for the characterization of nanostructured materials and carbon materials. Characterization methods should be capable of deriving information about the quantitative estimate of metal content, type and amount of carbon impurities, diameter distribution and length of the nanotubes, crystalline quality, degree of bundling, presence of defects in the nanotube walls and tips, degree of functionalization of the nanotubes, and type of functional chemical group. The dispersion of CNTs in solution can be studied by UV–visible spectroscopy, atomic force microscopy, Fourier-transform infrared (FTIR) and Raman spectroscopy, thermogravimetric analysis (TGA), scanning electron microscopy (SEM), and cryogenic transmission electron microscopy (TEM) (Ci et al. 2003; Moore et al. 2003; Heller et al. 2004; Zhou et al. 2004; Liu et al. 2008; Liu, Zhang, and Gao 2009; Liao, Zheng, and Zhang 2009; Tessonnier et al. 2009).

Shi et al. have reported UV–visible spectroscopy as a major technique to evaluate the dispersion of CNTs in aqueous solution and SEM to visualize the morphology. They monitored the sonication-driven dispersion of SWCNTs in aqueous surfactant solution and reveal that the maximum concentration of dispersed SWCNTs corresponds to the maximum UV-Vis-NIR absorbance of the solution (Liu et al. 2010). FTIR studies aid in the identification of the functional group attached on the surface of the CNTs (Hussain et al. 2011). Bakieva et al. (2013) have developed and utilized IR absorption spectroscopy for the determination of SWCNTs in carbon nanomaterials. CNT Raman spectra have characteristic features with the peaks originating from different aspects of the nanotube such as the radial breathing mode (RBM), tangential mode (G-band), and disordered mode (D-band) (Graupner 2007). RBMs observed

between 100 and 300 cm^{-1} depend on the tube diameter, the higher-frequency tangential displacement G modes occur near ~1590 cm^{-1}, and the second-order G′ peaks near 2600 cm^{-1} remain much sensitive to charges that have been exchanged between nanotubes and the guest moiety. The shape and intensity of the D-mode observed at around 1290–1320 cm^{-1} correspond to the sp^3-hybridized carbon atoms. The D-mode correlates to the extent of nanotube sidewall defects and chemical sidewall functionalization (Mirershadi et al. 2009). Hence, the D-band remains as a useful diagnostic of disorder in the hexagonal framework of the tubes, whereas the relative intensity of the D- and G-bands forms an important indicator of the amount of defects introduced upon chemical treatment (Burghard 2005).

Sample impurities can be detected and identified by TEM (Martí et al. 2003). Except for metal NPs, which are recognized as dark spots in TEM images, graphitic particles look like coiled or layered globules and amorphous carbon constitutes disordered aggregates surrounding the nanotubes. TEM images in general depict the structural perfection and crystalline organization of the CNTs (Thess et al. 1996). SEM images depict the qualitative evaluation of the impurity in graphitic structures as they have been incorporated in energy-dispersive X-ray (EDX) microanalysis, also defined as energy-dispersive spectroscopy. The EDX technique estimates the quantitative assessment of the metal content in microscopic surface regions of CNTs (Itkis et al. 2005).

The Kataura plot observed from the absorption spectra of CNTs in the NIR and the visible regions shows three band features, including S11, S22, and M11 bands (Kataura, Kumazawa, and Maniwa 1999). The XRD pattern for graphite demonstrates a sharp peak at $2\theta = 26.5$, corresponding to the distance between graphite sheets (0.34 nm). This signal can be utilized to detect graphitic impurities in CNTs (García-Gutiérrez et al. 2007).

TGA is often utilized for the characterization of CNTs by heating them in an oxygen or air atmosphere at 5°C, 10°C, and 15°C/minute up to 1000°C. Combustion of carbon materials in general does not start until the temperature reaches at least 300°C. Weight losses below this temperature could be due to the evaporation of adsorbed humidity or the pyrolitic evolution of surface functional groups. Combustion of the nanotubes and carbonaceous impurities occurs at temperatures between 300°C and 600°C, and hence several band components are usually observed in the TGA derivative (Itkis, Perea, and Niyogi 2003).

4.2.3 Enzyme Immobilization and Application of Carbon Nanotubes

Practical use of enzymes has been realized in various industrial processes and is being expanded in new fields, such as fine chemical synthesis, pharmaceuticals, biosensors, and biofuel cells (Kim et al. 2009). To improve enzyme stability, enzymes have generally been studied with the enzymes immobilized on a solid support (Laurent, Haddoub, and Flitsch 2008). Recent development in nanomaterials has opened new avenues for industrial biocatalysis. In theory, nanomaterials can serve as ideal supporting materials for enzyme immobilization because they can provide the upper limits on enzyme efficiency-determining factors such as surface area/volume ratio, mass transfer resistance, and enzyme loading capacity (Asuri et al. 2006; Wang 2009).

Among the various nanostructure materials, such as NPs, nanofibers, and nanotubes, CNTs have been the focus of much research. CNTs exhibit extraordinary mechanical, electrical, and thermal properties as well as biocompatibility (Shim et al. 2002; Zhang and Henthorn 2010). SWNTs are attractive for their higher surface area for enzyme interaction, but MWNTs are desirable for their easier dispersibility and lower cost. Potential biotechnological applications of CNTs have captured the imagination of many researchers (Wang et al. 2010). However, common immobilization methods including adsorption, covalent binding, cross-linking, and encapsulation have limited applicability owing to enzyme leaching, 3D structure loss, and strong diffusion resistance (Huang et al. 2002; Karajanagi et al. 2004; Letant et al. 2004; Vidinha et al. 2006; Hong et al. 2007; Kim et al. 2007; Manyar et al. 2008). Moreover, costly enzyme purification steps are needed before immobilization. An important advantage of enzyme immobilization is the stability improvement, which expands the range of conditions suitable for enzyme function in industrial applications (Sarah, Jakki, and Edmond 2008).

In the case of CNTs, both noncovalent and covalent conjugations have been reported for the immobilization of various enzymes. Noncovalent attachment preserves the unique properties of both enzymes and CNTs, but the immobilized protein can be gradually lost during the use of the CNT–enzyme complex (Gao and Kyratzis 2008). Covalent conjugation provides durable attachment, but the enzyme structure may be more disrupted. Functionalization of CNTs with organic, polymeric, and biological molecules can provide biocompatible nanotube composites with specific groups on their surface. CNT composites can provide a basis for specific immobilization of an enzyme. No matter what method is used, the main challenge is promoting a stable attachment of enzymes while maintaining their activity and function as closely as possible to their native state. The performance of CNT–enzyme complexes is affected by a combination of the nanotube chemistry and immobilization method (Pedrosa et al. 2010). Also, the conformational changes of enzymes attached with CNTs could have a possible influence on their enzyme activity.

For example, Wang et al. have reported an efficient immobilization method based on the specific interaction between His-tagged nox and SWCNTs functionalized by Nα,Nα-bis(carboxymethyl)-L-lysine hydrate without requiring enzyme purification. This nanoscale biocatalyst has overcome the aforesaid limitations and demonstrates good loading capacity and stability while maintaining 92% maximum activity of the native enzyme. We further demonstrate that the immobilization is reversible and can retain activity for several loading cycles (Sarah, Jakki, and Edmond 2008). Few reports on enzyme immobilization strategies using CNTs as the major substrate are discussed in Table 4.1.

Sensor development using CNTs as the substrate also attracted great attention from researchers. For example, Sharghi, Ebrahimpourmoghaddam, and Doroodmand (2013) have reported the effective immobilization of copper complexes onto the activated MWCNT system. Lee et al. have prepared a new nanomaterial by grafting a layer of a sulfonated polyaniline network onto the surface of MWNT, which was effectively utilized for immobilization of an enzyme and for the fabrication of a biosensor (Lee et al. 2010). Few more reports on the enzyme immobilization strategies using CNTs as the major substrate for sensing applications are discussed in Table 4.2.

TABLE 4.1

Characteristics of CNT-Immobilized Nanostructures in Sensor Applications

Enzyme Nanocomplex	Substrate Complex	Experimental Method	Development	Effect
Beta-lactamase	MWCNTs and hematein	Layer-by-layer assembly technique	Amperometric penicillin biosensor	Enhanced sensitivity
Chitosan	Chitosan–CNT composite film	DNA hybridization detection strategy	DNA hybridization sensor	Improved electron transfer reaction of the electro active molecules
Inorganic-organic hybrid nanocomposite	Ni(II)-pyromellitic acid–bimetallic Au–Pt, CNT composite	Electrocatalytic oxidation study	Glucose electrochemical sensor	Excellent electrochemical activity toward glucose oxidation
Trypsin	Polyaniline coated nano-Fe_3O_4/CNT composite	Protein digestion	Magnetic biomaterial	Promise for protein analysis due to the high magnetic responsivity and excellent dispersability
Copper complex	Silicomolybdate and SWCNTs	Catalytic reduction	Sensor for bromate and hydrogen peroxide detection	Nanomolar detection of hydrogen peroxide and bromate
Glucose oxidase-chitosan	Gold–platinum alloy nanoparticles/MWCNTs	Electrocatalytic and reduction	Glucose biosensor	High sensitivity, a low detection limit, a wide linear range, a fast response time, and good reproducibility, stability, and selectivity
Goat anti-human IgG	Anti-human IgG/COOH–MWCNTs/Fe_3O_4	Electrochemical immunoassay	Magnetic nanocomposite	Electrochemical sensing of human tetanus IgG (hIgG) of lower concentration
Hemoglobin	Hb–MWCNTs/copper nanoparticles/polyaniline hybrid film	Covalent immobilization and electrochemical sensing	Acrylamide biosensor	The biosensor exhibited low detection with high sensitivity, fast response time, and wide linear rangeo
D-amino acid oxidase	D-amino acid oxidase/carboxylated MWCNT/copper nanoparticles/polyaniline-modified gold electrode	Covalent immobilization and electrochemical sensing	Amperometric D-amino acid biosensor	Measurement of D-amino acid level

(*Continued*)

TABLE 4.1 (CONTINUED)

Characteristics of CNT-Immobilized Nanostructures in Sensor Applications

Enzyme Nanocomplex	Substrate Complex	Experimental Method	Development	Effect
Acetylcholinesterase/ choline oxidase/ peroxidase)	CNTs, platinum nanoparticles, dendrimers, and enzymes	Electrocatalytic activity	Electrochemical biosensors	Detection of a biocontaminant inhibitor (organophosphorus pesticide) in a wide range of concentrations
Laccases	Laccase–MWCNT complex	Pirimicarb pesticide quantification	Development of a sensitive enzymatic biosensor for the determination of pirimicarb pesticide based on the immobilization of laccase on composite	Best performance, with excellent electron transfer kinetic and catalytic effects
Alcohol dehydrogenase	MWCNT–ADH–Meldola's Blue	Ethanol determination	Amperometric biosensor	The amperometric response for alcohol using this biosensor showed excellent sensitivity, operational stability and wide linear response range
Horseradish peroxidase	Core–shell organosilica@ chitosan nanospheres and MWCNTs composite	Reduction and sensor development	Amperometric hydrogen peroxide biosensor	The direct electron transfer of HRP was achieved at HRP/organosilica@ chitosan/MWNTs/GCE, which exhibited excellent electrocatalytic activity for the reduction of H_2O_2

Source: Data from Chen, B. et al., *Analytica Chimica Acta*, 674, 89–95, 2010; Wang, Q. et al., *Electrochimica Acta*, 56, 3829–3834, 2011; Gholivand, M.B., and A. Azadbakht, *Electrochimica Acta*, 76, 300–311, 2012; Wang, S. et al., *Analytica Chimica Acta*, 612,182–189, 2008; Salimi, A. et al., *Analytica Chimica Acta*, 635, 63–70, 2009; Kang, X. et al., *Analytical Biochemistry*, 369, 71–79, 2007; Zarei, H. et al., *Analytical Biochemistry*, 421, 446–453, 2012; Batra, B. et al., *Analytical Biochemistry*, 433, 210–217, 2013; Lata, S. et al., *Analytical Biochemistry*, 437, 1–9, 2013; Siriviriyanun, A. et al., *Analytical Biochemistry*, 443, 169–171, 2013; Oliveira, T. et al., *Talanta*, 106, 137–143, 2013; Santos, A.S. et al., *Electrochimica Acta*, 52, 215–220, 2006; Chen, S. et al., *Electrochimica Acta*, 54, 3039–3046, 2009.

TABLE 4.2

Characteristics of CNT-Immobilized Enzymes and Their Applications

Enzyme	Method of Immobilization	Type of CNT	Properties of the Immobilized Enzymes
Lipase from *Candida rugosa*	Simple adsorption	Raw MWCNT	Retained 85% of the initial enzyme activity
Catalase from bovine liver	Simple adsorption	Pristine SW, MW, and oxidized MWCNT	Decreased enzyme activity in the order of O-SWCNT > SWCNT > MWCNT
Papain	Physical adsorption and biomimetic silicification	Amine and carboxyl group functionalized MWCNTs	Bioconjugates retained most of the initial enzyme activity
Alkaline lipase from *Pseudomonas fluorescence*	Noncovalent immobilization	Pristine and oxidized MWCNTs	Retained enzyme activity after 10 cycles of transesterification reaction
Creatine amidinohydrolase from *Actinobacillus* sp.	Covalent immobilization with chitosan–SiO_2–MWCNT matrix	MWCNTs	Nanomatrix has higher affinity to immobilize CAH enzyme
NADH oxidase	Cloning of His-tagged NADH oxidase and immobilization	SWCNT with lysine hydrate	Good loading capacity and stability while maintaining 92% maximum activity of the native enzyme
Organophosphate hydrolase	Covalent	SWCNT and MWCNTs	Retained enzyme activity and stability
Lipase	Covalent	Amino-cyclodextrin functionalized MWCNTs	Enhanced enzyme activity

(*Continued*)

TABLE 4.2 (CONTINUED)

Characteristics of CNT-Immobilized Enzymes and Their Applications

Enzyme	Method of Immobilization	Type of CNT	Properties of the Immobilized Enzymes
Acetylcholinesterase	Click chemistry reaction	MWCNTs	Decreased enzyme activity
Yarrowia lipolytica lipase	Covalent	Magnetic-MWCNTs	Improved enzyme activity
Hydrolases	Covalent and noncovalent	Amie group functionalized graphene oxide and MWCNTs	Covalent conjugate have showed improved enzyme activity
Candida antarctica lipase B	Carbodimide chemistry	MWCNTs	Retained enzyme activity
NADH oxidase	Site-specific immobilization	Carbon nanospheres and MWCNTs	Maintained 78–87% of the native enzyme activity
Kluyveromyces lactic B galactosidase	Covalent	Glutaraldehyde-modified MWCNTs	Retained greater biocatalytic activity
C–C bond hydrolase BphD	Physical adsorption and covalent bonding	SWCNTs	Retained enzyme activity

Source: Data from Prlainović, N.Ž. et al., *Journal of Industrial and Engineering Chemistry*, 19, 279–285, 2013; Zhang, C. et al., *Talanta*, 113, 142–147, 2013; Wang, Q. et al., *Enzyme and Microbial Technology*, 49, 11–16, 2011; Boncel, S. et al., *Enzyme and Microbial Technology*, 53, 263–270, 2013; Tiwari, A., and S.R. Dhakate, *International Journal of Biological Macromolecules*, 44, 408–412, 2009; Wang, L. et al., *Journal of Biotechnology*, 150, 57–63, 2010; Pedrosa, V.A. et al., *Colloids and Surfaces B: Biointerfaces*, 77, 69–74, 2010; Li, L. et al., *Colloids and Surfaces B: Biointerfaces*, 102, 124–129, 2013; Cabral, M.F. et al., *Colloids and Surfaces B: Biointerfaces*, 111, 30–35, 2013; Tan, H. et al., *Bioresource Technology*, 115, 172–176, 2012; Pavlidis, I.V. et al., *Bioresource Technology*, 115, 164–171, 2012; Raghavendra, T. et al., *Bioresource Technology*, 140, 103–110, 2013; Wang, L. et al., *Journal of Molecular Catalysis B: Enzymatic*, 69, 120–126, 2011; Ansari, S.A. et al., *Journal of Molecular Catalysis B: Enzymatic*, 97, 258–263, 2013; Shen, E. et al., *Chinese Journal of Catalysis*, 34, 723–733, 2013.

4.2.4 Cellular Uptake and Interaction with Complement System

The cellular uptake of CNTs was mainly due to their extremely high aspect ratio. CNTs have the capacity to penetrate the cell membrane and be taken up by the cells. Once the CNTs enter the cells, they get localized inside the cell endosomes and lysosomes. CNTs individually were able to cross cellular barriers and enter into the nucleus (Smart et al. 2006; Mu et al. 2008; Liu et al. 2009). The relevant cellular internalization mechanisms for CNTs are the endocytosis–phagocytosis pathway and passive diffusion (Kam, Shi, and Dai 2005; Firme and Bandaru 2010). In the passive diffusion pathway, CNTs cross the lipid bilayer in a needle-like manner (Bianco, Kostarelos, and Prato 2005). Before applying any nanomaterials for biomedical applications, their interaction with immune cells must be understood. The immune system aids in recognizing and sorting out foreign materials including CNTs. The innate immune system specifically contains the component system as well as collectins as the major protein circulating in the bloodstream or bodily fluids and performs the immunity function. CNTs were reported to be major synthetic polymers that were recognized by innate immune systems (Salvador-Morales et al. 2006), but were found to be not susceptible to digestion inside macrophages or to cell-killing mechanisms. Their interaction with immune systems could activate certain mechanisms that ultimately lead to inflammation and tissue damage to the host (Carroll and Sim 2011). Hence, to mask the CNT aromatic structures from the immune cell attack, protein-coated CNTs were utilized. For example, DWCNTs coated with bovine serum albumin (BSA), human serum albumin, and human fibrinogen were investigated for their ability, but were not able to escape from complement activation (Rybak-Smith et al. 2011). It is mainly due to the nonsaturation of protein coverage over the surface of CNTs. Also, there exists replacement of biomolecules of the mixture to attain a specific surface of CNTs over certain periods of time. But few reports stated that the extent of the complement activation can be changed by altering the surface properties of CNTs. MWCNTs functionalized with L-alanine, poly-ε-caprolactam (Nylon 6), 1-octadecylamine, and 1,6-hexanedithiol all showed a significant decrease in classical pathway activation compared with pristine MWCNTs (Basiuk et al. 2006; Rybak-Smith and Sim 2011).

4.2.5 Biomedical Applications of Carbon Nanotubes

4.2.5.1 Drug Delivery

Carbon nanotubes possess outstanding properties and a unique physicochemical architecture that serves as an alternative platform for the delivery of various therapeutic molecules. CNTs have been proposed and actively explored as multipurpose, innovative nanocarriers for drug delivery systems. The attachment of the drug onto the surface of CNTs occurs via covalent, noncovalent, and click chemistry-based covalent approaches (Peretz and Regev 2012). But the main problem behind their drug delivery application is their recognition by the immune system of the body as foreign particles. This leads to their elution from the bloodstream. For example, opsonins as bloodstream components have the ability to bind with CNTs via hydrophobic and electrostatic interaction mechanisms. Also, the macrophages of the reticuloendothelial system recognize and excrete them out from the bloodstream within a few seconds of their entry. To overcome such issues, several researchers have tried

for the surface modification of CNTs to escape from immune cell recognition by coating with hydrophilic polymers and nonionic surfactants. Especially, polyethylene glycol (PEG) is one of the most popular CNT surface modifiers, due to its hydrophilicity, flexibility, and biocompatibility. PEG-modified CNTs may also be dispersed in relatively higher salt-concentrated aqueous solutions (Bottini, Rosato, and Bottini 2011).

4.2.5.2 Bone Tissue Regeneration and Engineering

The extracellular environment which supports cell life is composed of a hierarchy of maintenance, force, and regulatory systems which integrate from the nanoscale through to the macroscale. For this reason, strategies to recreate cell-supporting environments have been investigating the use of nanocomposite biomaterials (Newman et al. 2013). Nanoscale features have demonstrated regulatory effects over almost every facet of cell behavior including adhesion, migration, proliferation, signaling, genetic expression, and stem cell fate (Lee et al. 2011). As a consequence of these findings, many biomaterials are being designed with nanoscale details that elicit favorable cellular behavior. Such designs require that there be reliable methods to fabricate and control such details and that there be an understanding of the mechanisms by which nanoscale details influence cells. Given this, biomaterials can be optimized to best replace damaged tissues (Lin, Taylor, and Li 2004; Polizu et al. 2006). For example, the nanocomposites prepared with CNTs have major application in bone tissue engineering due to their extraordinary physical, chemical, and electrical properties. They aid in the improvement of both the mechanical properties and the biocompatibility of the composite materials in vivo (Vigolo et al. 2000). CNTs could interact well with the cell-binding proteins, thereby regulating the stem cell differentiation, and could modulate cell shape due to their cell-binding affinity (Sreekumar et al. 2004). But the major drawback of this technology is the lack of information on their cellular interaction behavior.

4.2.5.3 Carbon Nanotubes as Field Emitters and Composites

CNTs serve as very effective field emitters, allowing them to serve as electron sources for X-ray sources, with specific advantages over traditional thermionic tubes. CNT-derived X-ray sources can create X-ray pulses of any duration and frequency, gate the X-ray pulse to any source, and allow the placement of many sources in close proximity. Lee et al. have constructed a number of micro-CT systems based on CNT X-ray sources for applications in small animal imaging, specifically focused on the imaging of the heart and lungs (Lee et al. 2011). A growing interest toward the field of hybrid materials is currently observed in the biomedical world. The hybrid approach provides great fabrication versatility and encourages the creation of new materials with unique properties, depending on the nature of the incorporated functional segments. CNTs are emerging materials, which capture a great interest in the biomaterial field (Wallace 1947). It is noteworthy that the effectiveness of CNTs in hybrid materials strongly depends on the ability to disperse the nanotubes homogenously through the matrix while maintaining their integrity and ensuring the bonding between components (Iijima 1991; Iijima and Ichihashi 1993; Nugent et al. 2001). Polizu et al. engineer CNT fibers applying the particle coagulation spinning

(PCS) method, which forms macroscopic fibers with a very good alignment of nanotubes (Polizu et al. 2006).

Carbon is an important element to humans; carbon materials (such as glassy, graphite, fibers) with conventional dimensions in the micron scale have been considered for tissue implants; however, for the results of biocompatibility properties of carbon materials pertinent to tissue prosthetics, it is clear that conventional carbon formulation may not be the optimal implant material (Mortier and Engelhardt 1999). And due to their unique mechanical, physical, and chemical properties, it is expected that they will find certain applications in a range of medical techniques (Xie et al. 2000; Dresselhaus, Mildred, and Avouris 2001). Recently, high-density hydroxyapatite (HA) and CNT composites were fabricated by pressureless sintering under a complicated atmosphere: carbon monoxide and hydrogen, with a small amount of water (Curtin and Sheldon 2004). Wang et al. have developed MWCNTs/HA composites with 20%, 40%, and 60% HA which were successfully fabricated at 1200°C and 120 MPa by spark plasma sintering. The results indicated that the MWCNTs/HA composites have potential for a wide variety of bone graft applications in the future (Lin, Taylor, and Li 2004). In order to improve the biocompatibility, hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HA) was selected as binder for substituting the PCS to consolidate CNTs. HA (Ca/P ratio of 1.66) has been extensively used for maxillofacial surgery, orthopedics, and implant fabrication and is one of the most compatible biomaterials owing to its similar chemical composition and crystal structure of apatite in the human hard tissue such as bone and tooth (Wang, Ge, and Zhang 2005; White, Best, and Kinloch 2007). Ahmed et al. (2013) have developed calcium carbonate/MWCNTs/chitosan composite coatings on Ti–6Al–4V alloy for orthopedic implants. The Ti–6Al–4V and Ti–6Al–7Nb alloys are among the most commonly used implant materials, particularly for dental, orthopedic, and osteosynthesis applications (Kuroda et al. 1998; Sittig et al. 1999; Khan, Williams, and Williams 1999). As an important active material, chitosan has attracted considerable attention owing to its excellent biocompatibility, biodegradability, nontoxicity, and adsorption properties (De Campos et al. 2003; Agnihotri, Mallikarjuna, and Aminabhavi 2004; Campos et al. 2009). It has been widely used in biomedical areas, such as vehicle for drug, protein, and gene delivery and scaffold for tissue engineering (Park et al. 2010). Calcium carbonate, a natural mineral with great biocompatibility, has been widely used in industry, technology, medicine, microcapsule fabrication, and many other biorelated fields (Volodkin, Larionova, and Sukhorukov 2004). Likewise, MWCNTs are a relatively new carbon allotrope with high aspect ratio, outstanding strength, unique atomic structure, thermal conductivity, and biocompatibility (Cho et al. 2009). Making a nanostructure is suitable for biomedical coatings (Li et al. 2011). Further, the biocompatibility of CNTs in orthopedic application is also established by studies showing accelerated bone growth (in vitro) and osteoblast proliferation (Kalbacova et al. 2007), which fortifies its candidature as orthopedic biomaterial. Chew et al. (2011) have developed the reinforcement of calcium phosphate cement (CPC) with MWCNTs and BSA for injectable bone substitute applications. The new CPC composites comprise β-tricalcium phosphate (β-TCP) and dicalcium phosphate anhydrous mixed with BSA and incorporated with MWCNTs or functionalized MWCNTs (MWCNTs–OH and MWCNTs–COOH). Bioresorbable CPCs represent an interesting alternative to traditional bone graft materials. Moreover, CPC is a highly desirable material for orthopedic applications

due to its mouldability, in situ self-hardening ability, excellent osteoconductivity, adjustable resorbability rate, and bone replacement capability (Chow 2009).

4.2.5.4 Gene Delivery

Methods have recently been developed for the attachment of various biomolecules to CNTs to produce novel conjugates that are considered attractive vehicles for the delivery of drugs, vaccines, and genes (Foldvari and Bagonluri 2008). Essentially, all biological applications of CNTs depend on the large hydrophobic surface present in these nanosized materials, but require the development of an effective method for their functionalization. Many reports have shown that properly functionalized CNTs are effective nanocarriers giving efficient and safe delivery of biomolecules into different cells (Lacerda et al. 2007). Behnam et al. (2013) have performed noncovalent functionalization of SWCNTs with modified polyethyleneimine for efficient gene delivery. Few more reports on the composite strategies using CNTs as the major substrate are discussed in Table 4.3.

4.2.5.5 Biomedical Imaging

CNTs have been utilized as a multifunctional probe for biomedical imaging. The unique physical properties of CNTs, particularly SWNTs, make them extremely attractive in the area of biomedical imaging. Quasi-one-dimensional (1D) semiconducting SWNTs exhibit a narrow band gap of about 1 eV (Wilder et al. 1998), which allows fluorescence emission in the NIR regions, including the classical NIR-I region (700–900 nm) and the newly defined NIR-II region (1100–1400 nm) (Heller et al. 2005; Welsher, Sherlock, and Dai 2011; Hong et al. 2012). Utilizing the intrinsic band gap fluorescence of semiconducting SWNTs and fluorescence imaging in the NIR-II region with enhanced tissue penetration and spatial resolution has shown great promise in recent years. Raman imaging based on the resonance Raman scattering of SWNTs has also been explored by a number of groups for in vitro and in vivo imaging of biological samples. The strong absorbance of CNTs in the NIR region can be used for photoacoustic imaging, and their photoacoustic signals can be dramatically enhanced by adding organic dyes or coating with gold shells. Taking advantages of metal NP impurities attached to nanotubes, CNTs can also serve as a T2-contrast agent in magnetic resonance (MR) imaging. In addition, when labeled with radioactive isotopes, many groups have developed nuclear imaging with functionalized CNTs. Therefore, CNTs are unique imaging probes with great potential in biomedical multimodal imaging (Gong, Peng, and Liu 2013).

4.2.5.6 Photothermal Cancer Therapy

Laser-induced thermal cancer therapy can provide a minimally invasive and potentially more effective alternative compared to conventional treatment. The goal of this therapy is to supply a lethal dose of heat to the prescribed tissue volume while causing minimal damage to the surrounding normal tissue. Nanoparticles have significant potential as selective photoabsorbing agents for laser-based cancer treatment, due to their unique physical properties. Among several NPs, CNTs have been utilized as

TABLE 4.3

Hybrid Nanostructures and Their Potential Applications

Hybrid Nanostructure	Application
CNT fibers–polyvinyl alcohol (PVA) and polylactic-*co*-glycolic acid (PLGA) composite	Biocompatibility enhancement for biomedical use
CNT-derived X-ray sources	Field emitters for micro-CT scan development to small animal organs
MWCNT–HA (hydroxyapatite) composite	Bone graft application as an implant
Calcium carbonate–MWCNT–chitosan composite	Orthopedic implant
Calcium phosphate–MWCNT–BSA composite	Injectable bone substitute
SWCNT–polyethyleneimines	Gene delivery
Magnetic functionalized CNTs	Drug vehicles for cancer lymph node metastasis treatment
Polymeric nanohybrid and functionalized CNT	Drug delivery carriers for cancer therapy
Nanostructures hybrid membrane polysulfone–CNT	Hemodialysis
CNT doped poly(3,4-ethylenedioxythiophene)	Chronic neural stimulation
CNT-multilayered cellulose acetate nanofibers	Tissue engineering
Phospholipid–hyaluronan-functionalized SWCNT	Decreased toxicity and therapeutic effects
SWCNT–peptide composite	Photothermal ablation of tumor cells
Polyethylene oxide chain–CNT	Electronic biosensor
Tantalum and phosphonic acid–CNT coating on titanium surface	Conception of solid and biocompatible biomaterials
Ag–MWCNT–hydroxyapatite composite	Bioceramics with antibacterial effect
Poly(lactic-*co*-glycolic-acid) MWCNT composite	Blood-contacting application
Poly(methyl methacrylate)–MWCNT composite	Bone cement
Poly(3,4-ethylenedioxythiopene)–MWCNT composite	Improved stability of microelectrodes in neural prostheses application

Source: Data from Polizu, S. et al., *Applied Surface Science*, 252, 6750–6753, 2006; Lin, Y. et al., *Journal of Materials Chemistry*, 14, 527–541, 2004; Wang, W. et al., *Applied Surface Science*, 262, 194–199, 2012; Ahmed, R.A. et al., *Applied Surface Science*, 285, 309–316, 2013; Chew, K.-K. et al., *Journal of the Mechanical Behavior of Biomedical Materials*, 4, 331–339, 2011; Behnam, B. et al., *International Journal of Pharmaceutics*, 454, 204–215, 2013; Yang, F. et al., *European Journal of Cancer*, 47, 1873–1882, 2011; Prakash, S. et al., *Advanced Drug Delivery Reviews*, 63, 1340–1351, 2011; Nechifor, G. et al., *Desalination*, 241, 342–348, 2009; Luo, X. et al., *Biomaterials*, 32, 5551–5557, 2011; Luo, Y. et al., *Carbohydrate Polymers*, 91, 419–427, 2013; Dvash, R. et al., *Journal of Controlled Release*, 170, 295–305, 2013; Hashida, Y. et al., *Journal of Controlled Release*, 173, 59–66, 2014; Chen, R.J. et al., *Proceedings of the National Academy of Sciences*, 100, 4984–4989, 2003; Maho, A. et al., *Materials Science and Engineering: C*, 33, 2686–2697, 2013; Herkendell, K. et al., *Materials Science and Engineering: C*, 34, 455–467, 2014; Koh, L.B. et al., *Acta Biomaterialia*, 5, 3411–3422, 2009; Ormsby, R. et al., *Acta Biomaterialia*, 8, 1201–1212, 2012; Zhou, H. et al., *Acta Biomaterialia*, 9, 6439–6449, 2013.

photothermal transducers. CNTs exhibit strong optical absorption in the NIR regions (NIR I: 700–900 nm, NIR II: 1–1.4 μm). NIR optical radiation has a penetration depth of 1.6 mm into biological tissues. CNTs generate heat by light absorption and induce the thermal destruction of those cancer cells containing sufficient CNT concentrations. To avoid damage to normal tissues, targeted CNTs have been prepared by the covalent attachment of tumor-specific ligands to the CNTs. The conjugated CNTs show good stability under physiological conditions and generate a highly specific photothermal killing of the targeted cells. Internalized CNTs are more sensitive to NIR-mediated photothermal damage than cells with CNTs on their surface (Marches et al. 2009; Marches et al. 2011).

4.2.6 Toxicity of Carbon Nanotubes

Carbon nanotubes are emblematic nanomaterials, presenting unique physicochemical properties, such as mechanical, thermal, or electrical conductivity, which have led to a large number of actual applications and uses, as well as (future) developments in aerospace, automobiles, nanoelectronics, or nanomedicine. CNT are currently used in many devices (computers, aircraft airframe, and sporting goods such as tennis rackets, bicycles, golf irons) and have also emerged as efficient drug delivery carriers in the biomedical and drug delivery fields (Beg et al. 2011). Because of these actual and future applications, there is an increasing concern regarding the consequences that could result from human exposure to CNT, particularly at the respiratory level, since it represents a major route of exposure to nanomaterials. CNT in lungs can induce inflammatory response, granuloma formation, and development of pulmonary fibrosis. CNT can also modulate biological response to preexisting pathologies such as asthma and bacterial infections. The exact determinants of these effects still remain to be clearly identified, although intrinsic physicochemical characteristics of CNT such as length, catalyst residue nature and content, and dispersion state (along with aggregation potential and distribution pattern) seem to be of importance (Boczkowski and Lanone 2012). In general, there are several different mechanisms proposed on CNT toxicity in living organisms such as physical interference of CNTs, phagocyte activation, altered gene expression, and impairment of DNA. Figures 4.2 and 4.3 describe the mechanistic flow chart of the oxidative stress induced and noninduced toxicological mechanism (Ding et al. 2005; Oberdörster et al. 2005; Sayes et al. 2005; Lanone and Boczkowski 2006; Haniu et al. 2010; Holt et al. 2010; Halliwell and Gutteridge 2015).

The toxicity and biocompatibility of CNTs in general depend on several factors including CNT structure (diameter, length, and shape); production methods possibly requiring toxic metal catalysts (Co, Fe, Ni, Mo); organic materials (amorphous or microstructured, residual organic carbon), or supporting materials (alumina, magnesium oxide or silica); and CNT functionalization—covalent and noncovalent, with various moieties. Although a wide range of nanotoxicity studies have been done, the results are still controversial and await a consensus (Kaiser et al. 2011). For example, Karousis et al. have evaluated the toxicity of aryl-derivatized, water-soluble

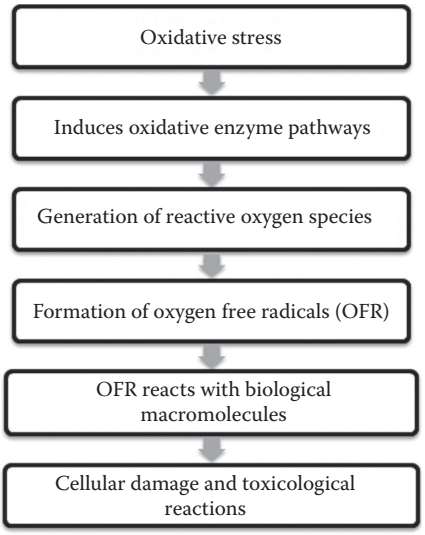


FIGURE 4.2 Oxidative stress-induced toxicity mechanism of CNTs.

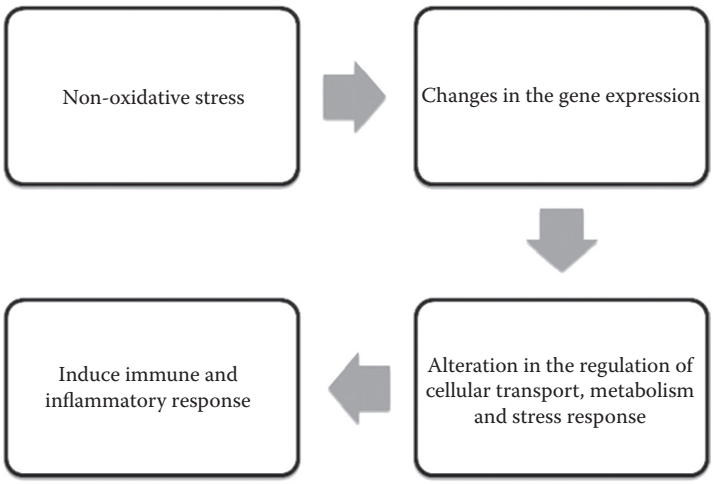


FIGURE 4.3 Nonoxidative stress-induced toxicity mechanism of CNTs.

functionalized CNTs against epithelial breast cancer-derived MCF-7 cell lines using MTT assay for testing cell viability (Mosmann 1983). They reported the higher cell viability in the samples incubated with aryl-derivatized CNTs and hence dictate the possibility of utilizing CNTs as components for biological experimentation. Also, the increasing degree of functionalization of CNTs reduced their toxicity (Sayes et al. 2006) and improved their biocompatibility (Lacerda et al. 2006). Thus, the functionalized CNTs have a decreased toxicity nature. Tong et al. have studied the influence of acid functionalization on the cardiopulmonary toxicity of single-walled

CNTs and ultrafine carbon black particles in mice. Their findings were in contrast to those previously mentioned with the result that the acid functionalization of the CNTs leads to their increased toxicity (Tong et al. 2009). Chen, Hsiao, and Chou (2013) have reported the molecular mechanism behind the toxicity of SWCNTs using *Caenorhabditis elegans* as a model animal. Amide-modified SWCNTs have been reported to cause no significant toxicity due to the amide modification (Chen, Hsiao, and Chou 2013). Mao, Kawazoe, and Chen (2013) have also studied the impact of collagen-functionalized SWCNTs against the bovine articular chondrocyte culture. They found no obvious negative cellular effects and that a high amount of SWCNTs were internalized by cells. The internalized collagen-SWCNTs were distributed in the perinuclear region and retained in the cells for more than 1 week (Mao, Kawazoe, and Chen 2013).

4.2.7 Interaction Studies with Biomolecules

Simultaneously existing concerns on the biomedical and toxicity face of CNTs have necessitated the understanding of their interaction with proteins or the biological macromolecules possessing significant physiological function in the human body. When NPs are in a physiological environment, they can selectively adsorb biomolecules, leading to the formation of a biocorona. As with any other nanomaterial, such dynamic exchanges between CNT surfaces and biofluids (i.e., proteins or lipids) have been described. This corona may lead to a modified biological "identity" of CNTs (new biological effects as well as potential modifications of the material by the biomolecules present in the corona—in terms of structure and/or functions). The formation of the biocorona is now considered as crucial in the determination of CNT toxicity (Salvador-Morales et al. 2006; Ge et al. 2011; Podila et al. 2012; Walkey and Chan 2012; Shannahan et al. 2013). For this, several spectroscopic methods including fluorescence and UV–visible spectroscopy, FTIR and Raman spectroscopy, circular dichroism, nuclear MR spectroscopy, etc., have been in use for revealing the binding-induced conformational changes in the biomolecules upon interaction with NPs. Until recently, studies on the interactions between biomolecules and nanomaterials (including CNTs) have been limited to the use of single purified proteins (chymotrypsin, fibrinogen, albumin, etc.) or of the so-called representative media. However, as CNT applications in nanomedicine are ever increasing, the blood has gained extra relevance as it is the first physiological environment that CNTs will come across after intravenous administration (Boczkowski and Lanone 2012; Kapralov et al. 2012; Cai et al. 2013). For example, Li et al. have investigated the interaction between BSA and amidated and carboxylated SWCNTs in vitro using spectroscopic methods including fluorescence quenching and synchronous studies, UV–visible absorption spectra, and circular dichroism studies. Their results have concluded the involvement of hydrophobic force in leading the binding mechanism, and the tryptophan residue remained to be the major binding site. Also, the interaction has resulted out with the influence of SWCNTs on the secondary structure of BSA (Li et al. 2013; 2014). Similarly, Ge et al. (2011) have investigated the interaction between SWCNTs and blood proteins using the spectroscopic, microscopic, and molecular dynamic simulation studies. Not only did their results suggest the interaction mechanism, but also the toxicity behaviors of CNTs were reduced upon coating with biomolecules (Ge et al. 2011). Bomboi et al. (2011) have studied the interaction between the dispersions of single-walled

and nonassociated CNTs in aqueous lysozyme solution and analyzed the stabilizing effect of both protein concentration and pH. They counteract the stabilization mechanism, with subsequent nanotube/nanotube aggregation and phase separation. Guan, Zhang, and Wang (2014) have studied the binding interaction of hydroxylated CNTs with BSA and revealed the secondary structure changes of BSA. Wang et al. have investigated the binding of hydroxylated single-walled CNTs to two hemoproteins, hemoglobin and myoglobin (Wang, Zhang, and Cao 2014). From the studies previously mentioned, it could be clear that CNTs can adsorb proteins to their surface, although this binding depends on protein-specific as well as CNT-specific characteristics (Ling, Biro, and Bally 2011).

4.3 Conclusion

Among carbon nanomaterials, CNTs, a tremendous material with extreme properties, have possessed several scopes for its industrial and biomedical sector. But their real-time application in vivo remains a great challenge due to their toxicity profile. Their interaction with biological matrices including the human immune system, cells and tissues of the organs and systems, and biological macromolecules including DNA, RNA, and proteins still raise great threats in terms of their biocompatibility. Several functionalization approaches for reducing CNT toxicity have shown promising results in vitro. Still, the same reaction cannot be expected in vivo due to the intervention from several physiological factors. Hence, a clear understanding of their interaction behavior with the living system and the environment must be stressed enough. In this chapter, we have summarized both the biomedical applications and the toxicity issues arising from CNT by revealing their interaction with different systems.

REFERENCES

Abrahamson, John, Peter G Wiles, and Brian L Rhoades. 1999. Structure of carbon fibres found on carbon arc anodes. *Carbon* 11 (37):1873–1874.

Agnihotri, Sunil A, Nadagouda N Mallikarjuna, and Tejraj M Aminabhavi. 2004. Recent advances on chitosan-based micro- and nanoparticles in drug delivery. *Journal of controlled release* 100 (1):5–28.

Ajayan, Pulickel M and Sumio Iijima. 1993. Capillarity-induced filling of carbon nanotubes. *Nature* 361 (6410):333–334.

Ajayan, Pulickel M and Otto Z Zhou. 2001. Applications of carbon nanotubes. In *Carbon nanotubes*, eds. Dresselhaus, M. S., G. Dresselhaus, and Ph. Avouris, Springer-Verlag, Berlin, pages 391–425. Springer-Verlag Berlin Heidelberg.

Ansari, Shakeel Ahmed, Rukhsana Satar, Sandesh Chibber, and Mohd Jahir Khan. 2013. Enhanced stability of Kluyveromyces lactis β galactosidase immobilized on glutaraldehyde modified multiwalled carbon nanotubes. *Journal of molecular catalysis B: Enzymatic* 97:258–263.

Asuri, Prashanth, Sandeep S Karajanagi, Hoichang Yang, Tae-Jin Yim, Ravi S Kane, and Jonathan S Dordick. 2006. Increasing protein stability through control of the nanoscale environment. *Langmuir* 22 (13):5833–5836.

Awasthi, Kalpana, Anchal Srivastava, and ON Srivastava. 2005. Synthesis of carbon nanotubes. *Journal of nanoscience and nanotechnology* 5 (10):1616–1636.

Bahr, Jeffrey L, Jiping Yang, Dmitry V Kosynkin, Michael J Bronikowski, Richard E Smalley, and James M Tour. 2001. Functionalization of carbon nanotubes by electrochemical reduction of aryl diazonium salts: A bucky paper electrode. *Journal of the American Chemical Society* 123 (27):6536–6542.

Bakieva, Yu R, GI Zvereva, MG Spirin, and AV Krestinin. 2013. IR absorption spectroscopy as a technique for measuring the content of single-walled carbon nanotubes in carbon nanomaterials. *Nanotechnologies in Russia* 8 (5–6):364–372.

Basiuk, Vladimir A, Carolina Salvador-Morales, Elena V Basiuk et al. 2006. 'Green' derivatization of carbon nanotubes with Nylon 6 and L-alanine. *Journal of materials chemistry* 16 (45):4420–4426.

Batra, Bhawna, Suman Lata, Madhu Sharma, and CS Pundir. 2013. An acrylamide biosensor based on immobilization of hemoglobin onto multiwalled carbon nanotube/copper nanoparticles/polyaniline hybrid film. *Analytical biochemistry* 433 (2):210–217.

Battigelli, Alessia, Cécilia Ménard-Moyon, Tatiana Da Ros, Maurizio Prato, and Alberto Bianco. 2013. Endowing carbon nanotubes with biological and biomedical properties by chemical modifications. *Advanced drug delivery reviews* 65 (15):1899–1920.

Beg, Sarwar, Mohammad Rizwan, Asif M Sheikh, M Saquib Hasnain, Khalid Anwer, and Kanchan Kohli. 2011. Advancement in carbon nanotubes: Basics, biomedical applications and toxicity. *Journal of pharmacy and pharmacology* 63 (2):141–163.

Behnam, Behzad, Wayne T Shier, Azadeh Hashem Nia, Khalil Abnous, and Mohammad Ramezani. 2013. Noncovalent functionalization of single-walled carbon nanotubes with modified polyethyleneimines for efficient gene delivery. *International journal of pharmaceutics* 454 (1):204–215.

Bianco, Alberto, Kostas Kostarelos, and Maurizio Prato. 2005. Applications of carbon nanotubes in drug delivery. *Current opinion in chemical biology* 9 (6):674–679.

Bianco, Alberto, Kostas Kostarelos, and Maurizio Prato. 2011. Making carbon nanotubes biocompatible and biodegradable. *Chemical communications (camb)* 47 (37):10182–10188.

Boczkowski, Jorge, and Sophie Lanone. 2012. Respiratory toxicities of nanomaterials—A focus on carbon nanotubes. *Advanced drug delivery reviews* 64 (15):1694–1699.

Bomboi, F, A Bonincontro, C La Mesa, and F Tardani. 2011. Interactions between single-walled carbon nanotubes and lysozyme. *Journal of colloid and interface science* 355 (2):342–347.

Boncel, Sławomir, Karin H Müller, Jeremy N Skepper, Krzysztof Z Walczak, and Krzysztof KK Koziol. 2011. Tunable chemistry and morphology of multi-wall carbon nanotubes as a route to non-toxic, theranostic systems. *Biomaterials* 32 (30):7677–7686.

Boncel, Sławomir, Aurelia Zniszczoł, Katarzyna Szymańska, Julita Mrowiec-Białoń, Andrzej Jarzębski, and Krzysztof Z Walczak. 2013. Alkaline lipase from *Pseudomonas fluorescens* non-covalently immobilised on pristine versus oxidised multi-wall carbon nanotubes as efficient and recyclable catalytic systems in the synthesis of Solketal esters. *Enzyme and microbial technology* 53 (4):263–270.

Bottini, Massimo, Nicola Rosato, and Nunzio Bottini. 2011. PEG-modified carbon nanotubes in biomedicine: Current status and challenges ahead. *Biomacromolecules* 12 (10):3381–3393.

Burghard, Marko. 2005. Electronic and vibrational properties of chemically modified single-wall carbon nanotubes. *Surface science reports* 58 (1):1–109.

Cabral, Murilo F, Lívia F Sgobbi, Erica M Kataoka, and Sergio AS Machado. 2013. On the behavior of acetylcholinesterase immobilized on carbon nanotubes in the presence of inhibitors. *Colloids and surfaces B: Biointerfaces* 111:30–35.

Cai, Xiaoning, Rajkumar Ramalingam, Hau San Wong et al. 2013. Characterization of carbon nanotube protein corona by using quantitative proteomics. *Nanomedicine: Nanotechnology, biology and medicine* 9 (5):583–593.

Campos, Maria GN, Neera Satsangi, Henry R Rawls, and Lucia HI Mei. 2009. Chitosan Cross-Linked Films for Drug Delivery Application. *Macromolecular symposia* 279 (1):169–174.

Carroll, DL, Ph Redlich, PM Ajayan et al. 1997. Electronic structure and localized states at carbon nanotube tips. *Physical review letters* 78 (14):2811.

Carroll, Maria V and Robert B Sim. 2011. Complement in health and disease. *Advanced drug delivery reviews* 63 (12):965–975.

Chen, Po-Hsuan, Kuang-Ming Hsiao, and Cheng-Chung Chou. 2013. Molecular characterization of toxicity mechanism of single-walled carbon nanotubes. *Biomaterials* 34 (22):5661–5669.

Chew, Kean-Khoon, Kah-Ling Low, Sharif Hussein Sharif Zein et al. 2011. Reinforcement of calcium phosphate cement with multi-walled carbon nanotubes and bovine serum albumin for injectable bone substitute applications. *Journal of the mechanical behavior of biomedical materials* 4 (3):331–339.

Cho, Johann, Katarzyna Konopka, Krzysztof Rożniatowski, Eva García-Lecina, Milo SP Shaffer, and Aldo R Boccaccini. 2009. Characterisation of carbon nanotube films deposited by electrophoretic deposition. *Carbon* 47 (1):58–67.

Chow, LC. 2009. Next generation calcium phosphate-based biomaterials. *Dental materials journal* 28 (1):1.

Ci, Lijie, Zhenping Zhou, Xiaoqin Yan et al. 2003. Resonant Raman scattering of double wall carbon nanotubes prepared by chemical vapor deposition method. *Journal of applied physics* 94 (9):5715–5719.

Curtin, William A and Brian W Sheldon. 2004. CNT-reinforced ceramics and metals. *Materials today* 7 (11):44–49.

De Campos, Angela M, Alejandro Sánchez, Ruxandra Gref, Pilar Calvo, and J Alonso. 2003. The effect of a PEG versus a chitosan coating on the interaction of drug colloidal carriers with the ocular mucosa. *European journal of pharmaceutical sciences* 20 (1):73–81.

Ding, Lianghao, Jackie Stilwell, Tingting Zhang et al. 2005. Molecular characterization of the cytotoxic mechanism of multiwall carbon nanotubes and nano-onions on human skin fibroblast. *Nano letters* 5 (12):2448–2464.

Dresselhaus, Mildred S, and Phaedon Avouris. 2001. Introduction to carbon materials research. In *Carbon nanotubes*: Springer.

Dvash, Ram, Artium Khatchatouriants, Leonardo J Solmesky et al. 2013. Structural profiling and biological performance of phospholipid–hyaluronan functionalized single-walled carbon nanotubes. *Journal of controlled release* 170 (2):295–305.

Ebbesen, TW, PM Ajayan, and K Tanigaki. 1994. Purification of nanotubes. *Nature* 367 (6463):519–519.

Fabbro, Chiara, Hanene Ali-Boucetta, Tatiana Da Ros, Kostas Kostarelos, Alberto Bianco, and Maurizio Prato. 2012. Targeting carbon nanotubes against cancer. *Chemical communications* 48 (33):3911–3926.

Fadeel, Bengt, Valerian Kagan, Harald Krug et al. 2007. There's plenty of room at the forum: Potential risks and safety assessment of engineered nanomaterials. *Nanotoxicology* 1 (2):73–84.

Firme, Constantine P and Prabhakar R Bandaru. 2010. Toxicity issues in the application of carbon nanotubes to biological systems. *Nanomedicine: Nanotechnology, biology and medicine* 6 (2):245–256.

Foldvari, Marianna and Mukasa Bagonluri. 2008. Carbon nanotubes as functional excipients for nanomedicines: II. Drug delivery and biocompatibility issues. *Nanomedicine: Nanotechnology, biology and medicine* 4 (3):183–200.

Forró, L, A Janossy, D Ugarte, and Walt A de Heer. 1999. *Conducting forms of carbon: Fullerenes, onions, nanotubes*: Elsevier Science BV: Amsterdam.

Gao, Yuan and Ilias Kyratzis. 2008. Covalent immobilization of proteins on carbon nanotubes using the cross-linker 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide—A critical assessment. *Bioconjugate chemistry* 19 (10):1945–1950.

García-Gutiérrez, Mari Cruz, Aurora Nogales Ruiz, Jaime J Hernández, Daniel R Rueda, and Tiberio A Ezquerra Sanz. 2007. X-ray scattering applied to the analysis of carbon nanotubes, polymers and nanocomposites. *Optica pura y aplicada* 40, no. 2:195–205.

Ge, Cuicui, Jiangfeng Du, Lina Zhao et al. 2011. Binding of blood proteins to carbon nanotubes reduces cytotoxicity. *Proceedings of the National Academy of Sciences* 108 (41):16968–16973.

Gholivand, Mohammad Bagher, and Azadeh Azadbakht. 2012. Fabrication of a highly sensitive glucose electrochemical sensor based on immobilization of Ni (II)–pyromellitic acid and bimetallic Au–Pt inorganic–organic hybrid nanocomposite onto carbon nanotube modified glassy carbon electrode. *Electrochimica acta* 76:300–311.

Gong, Hua, Rui Peng, and Zhuang Liu. 2013. Carbon nanotubes for biomedical imaging: The recent advances. *Advanced drug delivery reviews* 65 (15):1951–1963.

Graupner, R. 2007. Raman spectroscopy of covalently functionalized single-wall carbon nanotubes. *Journal of raman spectroscopy* 38 (6):673–683.

Guan, Yonghui, Hongmei Zhang, and Yanqing Wang. 2014. New insight into the binding interaction of hydroxylated carbon nanotubes with bovine serum albumin. *Spectrochimica acta part A: Molecular and biomolecular spectroscopy* 124:556–563.

Halliwell, Barry and John MC Gutteridge. 2015. *Free radicals in biology and medicine*: Oxford University Press, Oxford.

Haniu, Hisao, Yoshikazu Matsuda, Kenji Takeuchi, Yoong Ahm Kim, Takuya Hayashi, and Morinobu Endo. 2010. Proteomics-based safety evaluation of multi-walled carbon nanotubes. *Toxicology and applied pharmacology* 242 (3):256–262.

Harris, Peter John Frederich. 2009. *Carbon nanotube science: Synthesis, properties and applications*: Cambridge University Press, Cambridge.

Hashida, Yasuhiko, Hironori Tanaka, Shuwen Zhou et al. 2014. Photothermal ablation of tumor cells using a single-walled carbon nanotube–peptide composite. *Journal of controlled release* 173:59–66.

Heller, Daniel A, Seunghyun Baik, Thomas E Eurell, and Michael S Strano. 2005. Single-walled carbon nanotube spectroscopy in live cells: Towards long-term labels and optical sensors. *Advanced materials* 17 (23):2793–2799.

Heller, Daniel A, Rebecca M Mayrhofer, Seunghyun Baik, Yelena V Grinkova, Monica L Usrey, and Michael S Strano. 2004. Concomitant length and diameter separation of single-walled carbon nanotubes. *Journal of the American Chemical Society* 126 (44):14567–14573.

Herkendell, Katharina, Vishnu Raj Shukla, Anup Kumar Patel, and Kantesh Balani. 2014. Domination of volumetric toughening by silver nanoparticles over interfacial strengthening of carbon nanotubes in bactericidal hydroxyapatite biocomposite. *Materials science and engineering: C* 34:455–467.

Hirlekar, Rajashree, Manohar Yamagar, Harshal Garse, Mohit Vij, and Vilasrao Kadam. 2009. Carbon nanotubes and its applications: A review. *Asian journal of pharmaceutical and clinical research* 2 (4):17–27.

Holt, Brian D, Philip A Short, Andrew D Rape, Yu-li Wang, Mohammad F Islam, and Kris Noel Dahl. 2010. Carbon nanotubes reorganize actin structures in cells and ex vivo. *ACS nano* 4 (8):4872–4878.

Hong, Guosong, Jerry C Lee, Joshua T Robinson et al. 2012. Multifunctional in vivo vascular imaging using near-infrared II fluorescence. *Nature medicine* 18 (12):1841–1846.

Hong, Hao, Ting Gao, and Weibo Cai. 2009. Molecular imaging with single-walled carbon nanotubes. *Nano today* 4 (3):252–261.

Hong, Jun, Peijun Gong, Dongmei Xu, Li Dong, and Side Yao. 2007. Stabilization of α-chymotrypsin by covalent immobilization on amine-functionalized superparamagnetic nanogel. *Journal of biotechnology* 128 (3):597–605.

Huang, Weijie, Shelby Taylor, Kefu Fu et al. 2002. Attaching proteins to carbon nanotubes via diimide-activated amidation. *Nano letters* 2 (4):311–314.

Hussain, S, P Jha, A Chouksey et al. 2011. Spectroscopic investigation of modified single wall carbon nanotube (SWCNT). *Journal of modern physics* 2 (06):538.

Iijima, Sumio. 1991. Helical microtubules of graphitic carbon. *Nature* 354 (6348):56–58.

Iijima, Sumio and Toshinari Ichihashi. 1993. Single-shell carbon nanotubes of 1-nm diameter.

Itkis, ME, DE Perea, S Niyogi et al. 2003. Purity evaluation of as-prepared single-walled carbon nanotube soot by use of solution-phase near-IR spectroscopy. *Nano letters* 3 (3):309–314.

Itkis, Mikhail E, Daniel E Perea, Richard Jung, Sandip Niyogi, and Robert C Haddon. 2005. Comparison of analytical techniques for purity evaluation of single-walled carbon nanotubes. *Journal of the American Chemical Society* 127 (10):3439–3448.

Kagan, VE, YY Tyurina, VA Tyurin et al. 2006. Direct and indirect effects of single walled carbon nanotubes on RAW 264.7 macrophages: Role of iron. *Toxicology letters* 165 (1):88–100.

Kaiser, JP, M Roesslein, T Buerki-Thurnherr, and P Wick. 2011. Carbon nanotubes—Curse or blessing. *Current medicinal chemistry* 18 (14):2115–2128.

Kalbacova, M, M Kalbac, L Dunsch, and U Hempel. 2007. Influence of single-walled carbon nanotube films on metabolic activity and adherence of human osteoblasts. *Carbon* 45 (11):2266–2272.

Kam, Nadine, Wong Shi and Hongjie Dai. 2005. Carbon nanotubes as intracellular protein transporters: Generality and biological functionality. *Journal of the American Chemical Society* 127 (16):6021–6026.

Kang, Xinhuang, Zhibin Mai, Xiaoyong Zou, Peixiang Cai, and Jinyuan Mo. 2007. A novel glucose biosensor based on immobilization of glucose oxidase in chitosan on a glassy carbon electrode modified with gold–platinum alloy nanoparticles/multiwall carbon nanotubes. *Analytical biochemistry* 369 (1):71–79.

Kapralov, Alexander A, Wei Hong Feng, Andrew A Amoscato et al. 2012. Adsorption of surfactant lipids by single-walled carbon nanotubes in mouse lung upon pharyngeal aspiration. *ACS nano* 6 (5):4147–4156.

Karajanagi, Sandeep S, Alexey A Vertegel, Ravi S Kane, and Jonathan S Dordick. 2004. Structure and function of enzymes adsorbed onto single-walled carbon nanotubes. *Langmuir* 20 (26):11594–11599.

Karousis, Nikolaos, Nikos Tagmatarchis, and Dimitrios Tasis. 2010. Current progress on the chemical modification of carbon nanotubes. *Chemical reviews* 110 (9):5366–5397.

Kataura, H, Y Kumazawa, Y Maniwa et al. 1999. Optical properties of single-wall carbon nanotubes. *Synthetic metals* 103 (1):2555–2558.

Khan, MA, RL Williams, and DF Williams. 1999. The corrosion behaviour of Ti–6Al–4V, Ti–6Al–7Nb and Ti–13Nb–13Zr in protein solutions. *Biomaterials* 20 (7):631–637.

Khare, Rupesh and Suryasarathi Bose. 2005. Carbon nanotube based composites—A review. *Journal of minerals and materials characterization and engineering* 4 (01):31.

Kim, Bum Joon, Bong Keun Kang, Young Yil Bahk, Kyung Hwa Yoo, and Kook Jin Lim. 2009. Immobilization of horseradish peroxidase on multiwalled carbon nanotubes and its enzymatic stability. *Current applied physics* 9 (4):e263–e265.

Kim, Moon Il, Jungbae Kim, Jinwoo Lee et al. 2007. Crosslinked enzyme aggregates in hierarchically-ordered mesoporous silica: A simple and effective method for enzyme stabilization. *Biotechnology and bioengineering* 96 (2):210–218.

Kingston, Christopher T, and Benoit Simard. 2006. Recent advances in laser synthesis of single-walled carbon nanotubes. *Journal of nanoscience and nanotechnology* 6 (5):1225–1232.

Koh, Li Buay, Isabel Rodriguez, and Subbu S Venkatraman. 2009. A novel nanostructured poly (lactic-*co*-glycolic-acid)–multiwalled carbon nanotube composite for blood-contacting applications: Thrombogenicity studies. *Acta biomaterialia* 5 (9):3411–3422.

Krause, Beate, Tobias Villmow, Regine Boldt, Mandy Mende, Gudrun Petzold, and Petra Pötschke. 2011. Influence of dry grinding in a ball mill on the length of multiwalled carbon nanotubes and their dispersion and percolation behaviour in melt mixed polycarbonate composites. *Composites science and technology* 71 (8):1145–1153.

Kuroda, Daisuke, Mitsuo Niinomi, Masahiko Morinaga, Yosihisa Kato, and Toshiaki Yashiro. 1998. Design and mechanical properties of new β type titanium alloys for implant materials. *Materials science and engineering: A* 243 (1):244–249.

Lacerda, Lara, Alberto Bianco, Maurizio Prato, and Kostas Kostarelos. 2006. Carbon nanotubes as nanomedicines: From toxicology to pharmacology. *Advanced drug delivery reviews* 58 (14):1460–1470.

Lacerda, Lara, Simona Raffa, Maurizio Prato, Alberto Bianco, and Kostas Kostarelos. 2007. Cell-penetrating CNTs for delivery of therapeutics. *Nano today* 2 (6):38–43.

Lanone, Sophie, and Jorge Boczkowski. 2006. Biomedical applications and potential health risks of nanomaterials: Molecular mechanisms. *Current molecular medicine* 6 (6):651–663.

Laurent, Nicolas, Rose Haddoub, and Sabine L Flitsch. 2008. Enzyme catalysis on solid surfaces. *Trends in biotechnology* 26 (6):328–337.

Lee, Kwang-Pill, Shanmugasundaram Komathi, Neon Jeon Nam, and Anantha Iyengar Gopalan. 2010. Sulfonated polyaniline network grafted multi-wall carbon nanotubes for enzyme immobilization, direct electrochemistry and biosensing of glucose. *Microchemical journal* 95 (1):74–79.

Lee, Yueh Z, Laurel Burk, Ko-han Wang, Guohua Cao, Jianping Lu, and Otto Zhou. 2011. Carbon nanotube based X-ray sources: Applications in pre-clinical and medical imaging. *Nuclear instruments and methods in physics research section A: Accelerators, spectrometers, detectors and associated equipment* 648:S281–S283.

Letant, Sonia E, Bradley R Hart, Staci R Kane, Masood Z Hadi, Sharon J Shields, and John G Reynolds. 2004. Enzyme immobilization on porous silicon surfaces. *Advanced materials* 16 (8):689–693.

Li, Lili, Rui Lin, Hua He, Li Jiang, and Mengmeng Gao. 2013. Interaction of carboxylated single-walled carbon nanotubes with bovine serum albumin. *Spectrochimica acta part A: Molecular and biomolecular spectroscopy* 105:45–51.

Li, Lili, Rui Lin, Hua He, Meiling Sun, Li Jiang, and Mengmeng Gao. 2014. Interaction of amidated single-walled carbon nanotubes with protein by multiple spectroscopic methods. *Journal of luminescence* 145:125–131.

Li, Xiaoming, Xi Liu, Jin Huang, Yubo Fan, and Fu-zhai Cui. 2011. Biomedical investigation of CNT based coatings. *Surface and coatings technology* 206 (4):759–766.

Liao, Lei, Mingbo Zheng, Zhou Zhang et al. 2009. The characterization and application of p-type semiconducting mesoporous carbon nanofibers. *Carbon* 47 (7):1841–1845.

Lin, Yi, Shelby Taylor, Huaping Li et al. 2004. Advances toward bioapplications of carbon nanotubes. *Journal of materials chemistry* 14 (4):527–541.

Ling, Wai Li, Adrienn Biro, Isabelle Bally et al. 2011. Proteins of the innate immune system crystallize on carbon nanotubes but are not activated. *ACS nano* 5 (2):730–737.

Liu, Guangtong, Yuanchun Zhao, Kaihong Zheng et al. 2008. Coulomb explosion: A novel approach to separate single-walled carbon nanotubes from their bundle. *Nano letters* 9 (1):239–244.

Liu, Huaping, Ye Feng, Takeshi Tanaka, Yasuko Urabe, and Hiromichi Kataura. 2010. Diameter-selective metal/semiconductor separation of single-wall carbon nanotubes by agarose gel. *The journal of physical chemistry C* 114 (20):9270–9276.

Liu, Jie, Andrew G Rinzler, Hongjie Dai et al. 1998. Fullerene pipes. *Science* 280 (5367):1253–1256.

Liu, Zhuang, Joshua T Robinson, Scott M Tabakman, Kai Yang, and Hongjie Dai. 2011. Carbon materials for drug delivery & cancer therapy. *Materials today* 14 (7):316–323.

Liu, Zhuang, Scott Tabakman, Kevin Welsher, and Hongjie Dai. 2009. Carbon nanotubes in biology and medicine: In vitro and in vivo detection, imaging and drug delivery. *Nano research* 2 (2):85–120.

Lu, Xin and Zhongfang Chen. 2005. Curved pi-conjugation, aromaticity, and the related chemistry of small fullerenes. *Chemical reviews* 105 (10):3643–3696.

Luo, Xiliang, Cassandra L Weaver, David D Zhou, Robert Greenberg, and Xinyan T Cui. 2011. Highly stable carbon nanotube doped poly (3, 4-ethylenedioxythiophene) for chronic neural stimulation. *Biomaterials* 32 (24):5551–5557.

Luo, Yu, Shige Wang, Mingwu Shen et al. 2013. Carbon nanotube-incorporated multilayered cellulose acetate nanofibers for tissue engineering applications. *Carbohydrate polymers* 91 (1):419–427.

Maho, Anthony, Simon Detriche, Joseph Delhalle, and Zineb Mekhalif. 2013. Sol–gel synthesis of tantalum oxide and phosphonic acid-modified carbon nanotubes composite coatings on titanium surfaces. *Materials science and engineering: C* 33 (5):2686–2697.

Manyar, Haresh G, Enrica Gianotti, Yasuhiro Sakamoto, Osamu Terasaki, Salvatore Coluccia, and Simonetta Tumbiolo. 2008. Active biocatalysts based on pepsin immobilized in mesoporous SBA-15. *The journal of physical chemistry C* 112 (46):18110–18116.

Mao, Hongli, Naoki Kawazoe, and Guoping Chen. 2013. Uptake and intracellular distribution of collagen-functionalized single-walled carbon nanotubes. *Biomaterials* 34 (10):2472–2479.

Marches, Radu, Pavitra Chakravarty, Inga H Musselman et al. 2009. Specific thermal ablation of tumor cells using single-walled carbon nanotubes targeted by covalently-coupled monoclonal antibodies. *International journal of cancer* 125 (12):2970–2977.

Marches, Radu, Carole Mikoryak, Ru-Hung Wang, Paul Pantano, Rockford K Draper, and Ellen S Vitetta. 2011. The importance of cellular internalization of antibody-targeted carbon nanotubes in the photothermal ablation of breast cancer cells. *Nanotechnology* 22 (9):095101.

Martı́nez, MT, MA Callejas, AM Benito et al. 2003. Sensitivity of single wall carbon nanotubes to oxidative processing: Structural modification, intercalation and functionalisation. *Carbon* 41 (12):2247–2256.

Matarredona, Olga, Heather Rhoads, Zhongrui Li, Jeffrey H Harwell, Leandro Balzano, and Daniel E Resasco. 2003. Dispersion of single-walled carbon nanotubes in aqueous solutions of the anionic surfactant NaDDBS. *The journal of physical chemistry B* 107 (48):13357–13367.

Ménard-Moyon, Cécilia, Enrica Venturelli, Chiara Fabbro et al. 2010. The alluring potential of functionalized carbon nanotubes in drug discovery. *Expert opinion on drug discovery* 5 (7):691–707.

Meyyappan, Meyya, Lance Delzeit, Alan Cassell, and David Hash. 2003. Carbon nanotube growth by PECVD: A review. *Plasma sources science and technology* 12 (2):205.

Mirershadi, S, SZ Mortazavi, A Reyhani, N Moniri, and AJ Novinrooz. 2009. Effective condition for purification of multi-walled carbon nanotubes by nitric acid. *Synthesis and reactivity in inorganic, metal-organic, and nano-metal chemistry* 39 (4):204–208.

Moore, Valerie C, Michael S Strano, Erik H Haroz et al. 2003. Individually suspended single-walled carbon nanotubes in various surfactants. *Nano letters* 3 (10):1379–1382.

Mortier, J, and M Engelhardt. 1999. Foreign body reaction in carbon fiber prosthesis implantation in the knee joint—Case report and review of the literature. *Zeitschrift fur orthopadie und ihre grenzgebiete* 138 (5):390–394.

Mosmann, Tim. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of immunological methods* 65 (1–2):55–63.

Mu, Qingxin, Wei Liu, Yuehan Xing et al. 2008. Protein binding by functionalized multiwalled carbon nanotubes is governed by the surface chemistry of both parties and the nanotube diameter. *The journal of physical chemistry C* 112 (9):3300–3307.

Nair, Nitish, Woo-Jae Kim, Richard D Braatz, and Michael S Strano. 2008. Dynamics of surfactant-suspended single-walled carbon nanotubes in a centrifugal field. *Langmuir* 24 (5):1790–1795.

Nechifor, G, SI Voicu, AC Nechifor, and S Garea. 2009. Nanostructured hybrid membrane polysulfone-carbon nanotubes for hemodialysis. *Desalination* 241 (1):342–348.

Newman, Peter, Andrew Minett, Rutledge Ellis-Behnke, and Hala Zreiqat. 2013. Carbon nanotubes: Their potential and pitfalls for bone tissue regeneration and engineering. *Nanomedicine: Nanotechnology, biology and medicine* 9 (8):1139–1158.

Nugent, JM, KSV Santhanam, A Rubio, and PM Ajayan. 2001. Fast electron transfer kinetics on multiwalled carbon nanotube microbundle electrodes. *Nano letters* 1 (2):87–91.

Oberdörster, Günter, Andrew Maynard, Ken Donaldson et al. 2005. Principles for characterizing the potential human health effects from exposure to nanomaterials: Elements of a screening strategy. *Particle and fibre toxicology* 2 (1):1.

Oliveira, Thiago MBF, M Fátima Barroso, Simone Morais et al. 2013. Biosensor based on multi-walled carbon nanotubes paste electrode modified with laccase for pirimicarb pesticide quantification. *Talanta* 106:137–143.

Ormsby, Ross, Tony McNally, Peter O'Hare, George Burke, Christina Mitchell, and Nicholas Dunne. 2012. Fatigue and biocompatibility properties of a poly (methyl methacrylate) bone cement with multi-walled carbon nanotubes. *Acta biomaterialia* 8 (3):1201–1212.

Park, Jae Hyung, Gurusamy Saravanakumar, Kwangmeyung Kim, and Ick Chan Kwon. 2010. Targeted delivery of low molecular drugs using chitosan and its derivatives. *Advanced drug delivery reviews* 62 (1):28–41.

Pavlidis, Ioannis V, Torge Vorhaben, Theodoros Tsoufis et al. 2012. Development of effective nanobiocatalytic systems through the immobilization of hydrolases on functionalized carbon-based nanomaterials. *Bioresource technology* 115:164–171.

Pedrosa, Valber A, Sheetal Paliwal, Shankar Balasubramanian et al. 2010. Enhanced stability of enzyme organophosphate hydrolase interfaced on the carbon nanotubes. *Colloids and surfaces B: biointerfaces* 77 (1):69–74.

Peretz, Sivan and Oren Regev. 2012. Carbon nanotubes as nanocarriers in medicine. *Current Opinion in colloid & interface science* 17 (6):360–368.

Podila, R, P Vedantam, PC Ke, JM Brown, and Apparao M Rao. 2012. Evidence for charge-transfer-induced conformational changes in carbon nanostructure–protein corona. *The Journal of Physical Chemistry C* 116 (41):22098–22103.

Polizu, S, M Maugey, S Poulin, P Poulin, and L'Hocine Yahia. 2006. Nanoscale surface of carbon nanotube fibers for medical applications: Structure and chemistry revealed by TOF-SIMS analysis. *Applied surface science* 252 (19):6750–6753.

Prakash, Satya, Meenakshi Malhotra, Wei Shao, Catherine Tomaro-Duchesneau, and Sana Abbasi. 2011. Polymeric nanohybrids and functionalized carbon nanotubes as drug delivery carriers for cancer therapy. *Advanced drug delivery reviews* 63 (14):1340–1351.

Prlainović, Nevena Ž, Dejan I Bezbradica, Zorica D Knežević-Jugović et al. 2013. Adsorption of lipase from Candida rugosa on multi walled carbon nanotubes. *Journal of industrial and engineering chemistry* 19 (1):279–285.

Raghavendra, Tripti, Arpana Basak, Lalit M Manocha, Amita R Shah, and Datta Madamwar. 2013. Robust nanobioconjugates of *Candida antarctica* lipase B—Multiwalled carbon nanotubes: Characterization and application for multiple usages in non-aqueous biocatalysis. *Bioresource technology* 140:103–110.

Rastogi, Richa, Rahul Kaushal, SK Tripathi, Amit L Sharma, Inderpreet Kaur, and Lalit M Bharadwaj. 2008. Comparative study of carbon nanotube dispersion using surfactants. *Journal of colloid and interface science* 328 (2):421–428.

Robertson, Neil and Craig A McGowan. 2003. A comparison of potential molecular wires as components for molecular electronics. *Chemical society reviews* 32 (2):96–103.

Rybak-Smith, Malgorzata J, and Robert B Sim. 2011. Complement activation by carbon nanotubes. *Advanced drug delivery reviews* 63 (12):1031–1041.

Salimi, Abdollah, Aazam Korani, Rahman Hallaj, Roshan Khoshnavazi, and Hasan Hadadzadeh. 2009. Immobilization of [Cu (bpy) 2] Br 2 complex onto a glassy carbon electrode modified with α-SiMo 12 O 40 4− and single walled carbon nanotubes: Application to nanomolar detection of hydrogen peroxide and bromate. *Analytica chimica acta* 635 (1):63–70.

Salvador-Morales, Carolina, Emmanuel Flahaut, Edith Sim, Jeremy Sloan, Malcolm LH Green, and Robert B Sim. 2006. Complement activation and protein adsorption by carbon nanotubes. *Molecular immunology* 43 (3):193–201.

Santos, Antonio S, Arnaldo Cesar Pereira, Nelson Durán, and Lauro T Kubota. 2006. Amperometric biosensor for ethanol based on co-immobilization of alcohol dehydrogenase and Meldola's Blue on multi-wall carbon nanotube. *Electrochimica acta* 52 (1):215–220.

Sarah, Hudson, Cooney Jakki, and Magner Edmond. 2008. Proteins in mesoporous silicates. *Angewandte chemie international edition* 47:8582–8594.

Sayes, Christie M, Andre M Gobin, Kevin D Ausman, Joe Mendez, Jennifer L West, and Vicki L Colvin. 2005. Nano-C 60 cytotoxicity is due to lipid peroxidation. *Biomaterials* 26 (36):7587–7595.

Sayes, Christie M, Feng Liang, Jared L Hudson et al. 2006. Functionalization density dependence of single-walled carbon nanotubes cytotoxicity in vitro. *Toxicology letters* 161 (2):135–142.

Shannahan, Jonathan H, Jared M Brown, Ran Chen et al. 2013. Comparison of nanotube–protein corona composition in cell culture media. *Small* 9 (12):2171–2181.

Sharghi, Hashem, Sakineh Ebrahimpourmoghaddam, and Mohammad Mahdi Doroodmand. 2013. Facile synthesis of 5-substituted-1H-tetrazoles and 1-substituted-1H-tetrazoles catalyzed by recyclable 4′-phenyl-2,2′:6′, 2″-terpyridine copper(II) complex immobilized onto activated multi-walled carbon nanotubes. *Journal of organometallic chemistry* 738:41–48.

Shen, E, Qu Yuanyuan, Zhou Hao et al. 2013. Catalytic performance and stability of CC bond hydrolase BphD immobilized onto single-wall carbon nanotubes. *Chinese journal of catalysis* 34 (4):723–733.

Shi, Yunhua, Tawfique Hasan, Nadendla H. Babu, Felice Torrisi, Silvia Milana, Andrea C. Ferrari, and David A. Cardwell. 2012. Synthesis of YBa2Cu3O7−δ and Y2BaCuO5 nanocrystalline powders for YBCO superconductors using carbon nanotube templates. *ACS nano* 6 (6):5395–5403.

Shim, Moonsub, Nadine Wong Shi Kam, Robert J Chen, Yiming Li, and Hongjie Dai. 2002. Functionalization of carbon nanotubes for biocompatibility and biomolecular recognition. *Nano letters* 2 (4):285–288.

Shvedova, AA, ER Kisin, D Porter et al. 2009. Mechanisms of pulmonary toxicity and medical applications of carbon nanotubes: Two faces of Janus? *Pharmacology & therapeutics* 121 (2):192–204.

Siriviriyanun, Ampornphan, Toyoko Imae, and Naoki Nagatani. 2013. Electrochemical biosensors for biocontaminant detection consisting of carbon nanotubes, platinum nanoparticles, dendrimers, and enzymes. *Analytical biochemistry* 443 (2):169–171.

Sittig, C, M Textor, ND Spencer, M Wieland, and PH Vallotton. 1999. Surface characterization. *Journal of materials science: Materials in medicine* 10 (1):35–46.

Smart, SK, AI Cassady, GQ Lu, and DJ Martin. 2006. The biocompatibility of carbon nanotubes. *Carbon* 44 (6):1034–1047.

Sreekumar, Thaliyil V, Tao Liu, Byung G Min et al. 2004. Polyacrylonitrile single-walled carbon nanotube composite fibers. *Advanced materials* 16 (1):58–61.

Tan, Huishan, Wei Feng, and Peijun Ji. 2012. Lipase immobilized on magnetic multi-walled carbon nanotubes. *Bioresource technology* 115:172–176.

Tan, Xiaoli, Ming Fang, Changlun Chen, Shaoming Yu, and Xiangke Wang. 2008. Counterion effects of nickel and sodium dodecylbenzene sulfonate adsorption to multiwalled carbon nanotubes in aqueous solution. *Carbon* 46 (13):1741–1750.

Tessonnier, Jean-Philippe, Dirk Rosenthal, Thomas W Hansen et al. 2009. Analysis of the structure and chemical properties of some commercial carbon nanostructures. *Carbon* 47 (7):1779–1798.

Thess, Andreas, Roland Lee, Pavel Nikolaev, and Hongjie Dai. 1996. Crystalline ropes of metallic carbon nanotubes. *Science* 273 (5274):483.

Thostenson, Erik T, Zhifeng Ren, and Tsu-Wei Chou. 2001. Advances in the science and technology of carbon nanotubes and their composites: A review. *Composites science and technology* 61 (13):1899–1912.

Tiwari, Ashutosh, and Sanjay R Dhakate. 2009. Chitosan–SiO_2–multiwall carbon nanotubes nanocomposite: A novel matrix for the immobilization of creatine amidinohydrolase. *International journal of biological macromolecules* 44 (5):408–412.

Tong, Haiyan, John K McGee, Rajiv K Saxena, Urmila P Kodavanti, Robert B Devlin, and M Ian Gilmour. 2009. Influence of acid functionalization on the cardiopulmonary toxicity of carbon nanotubes and carbon black particles in mice. *Toxicology and applied pharmacology* 239 (3):224–232.

Vaisman, Linda, H Daniel Wagner, and Gad Marom. 2006. The role of surfactants in dispersion of carbon nanotubes. *Advances in colloid and interface science* 128:37–46.

Vashist, Sandeep Kumar, Dan Zheng, Giorgia Pastorin, Khalid Al-Rubeaan, John HT Luong, and Fwu-Shan Sheu. 2011. Delivery of drugs and biomolecules using carbon nanotubes. *Carbon* 49 (13):4077–4097.

Vidinha, Pedro, Vera Augusto, Miguel Almeida et al. 2006. Sol–gel encapsulation: An efficient and versatile immobilization technique for cutinase in non-aqueous media. *Journal of biotechnology* 121 (1):23–33.

Vigolo, Brigitte, Alain Penicaud, Claude Coulon et al. 2000. Macroscopic fibers and ribbons of oriented carbon nanotubes. *Science* 290 (5495):1331–1334.

Volodkin, Dmitry V, Natalia I Larionova, and Gleb B Sukhorukov. 2004. Protein encapsulation via porous CaCO3 microparticles templating. *Biomacromolecules* 5 (5):1962–1972.

Walkey, Carl D, and Warren CW Chan. 2012. Understanding and controlling the interaction of nanomaterials with proteins in a physiological environment. *Chemical society reviews* 41 (7):2780–2799.

Wallace, Philip Richard. 1947. The band theory of graphite. *Physical review* 71 (9):622.

Wang, Liang, Li Wei, Yuan Chen, and Rongrong Jiang. 2010. Specific and reversible immobilization of NADH oxidase on functionalized carbon nanotubes. *Journal of biotechnology* 150 (1):57–63.

Wang, Liang, Rong Xu, Yuan Chen, and Rongrong Jiang. 2011. Activity and stability comparison of immobilized NADH oxidase on multi-walled carbon nanotubes, carbon nanospheres, and single-walled carbon nanotubes. *Journal of molecular catalysis B: Enzymatic* 69 (3):120–126.

Wang, Ping. 2009. Multi-scale features in recent development of enzymic biocatalyst systems. *Applied biochemistry and biotechnology* 152 (2):343–352.

Wang, Qi, Liya Zhou, Yanjun Jiang, and Jing Gao. 2011. Improved stability of the carbon nanotubes–enzyme bioconjugates by biomimetic silicification. *Enzyme and microbial technology* 49 (1):11–16.

Wang, Qingliang, Shirong Ge, and Dekun Zhang. 2005. Nano-mechanical properties and biotribological behaviors of nanosized HA/partially-stabilized zirconia composites. *Wear* 259 (7):952–957.

Wang, Qingxiang, Juanlan Shi, Jiancong Ni et al. 2011. DNA hybridization biosensor using chitosan–carbon nanotubes composite film as an immobilization platform and [Cu(bpy)(MBZ)2(H2O)](bpy = 2,2′-bipyridine, MBZ = p-methylbenzoate) as a novel redox indicator. *Electrochimica Acta* 56 (11):3829–3834.

Wang, Sheng, Huimin Bao, Pengyuan Yang, and Gang Chen. 2008. Immobilization of trypsin in polyaniline-coated nano-Fe_3O_4/carbon nanotube composite for protein digestion. *Analytica chimica acta* 612 (2):182–189.

Wang, Wei, Yuhe Zhu, Fumio Watari et al. 2012. Carbon nanotubes/hydroxyapatite nanocomposites fabricated by spark plasma sintering for bonegraft applications. *Applied surface science* 262:194–199.

Wang, Yan-Qing, Hong-Mei Zhang, and Jian Cao. 2014. Binding of hydroxylated single-walled carbon nanotubes to two hemoproteins, hemoglobin and myoglobin. *Journal of photochemistry and photobiology B: Biology* 141:26–35.

Welsher, Kevin, Sarah P Sherlock, and Hongjie Dai. 2011. Deep-tissue anatomical imaging of mice using carbon nanotube fluorophores in the second near-infrared window. *Proceedings of the National Academy of Sciences* 108 (22):8943–8948.

White, Ashley A, Serena M Best, and Ian A Kinloch. 2007. Hydroxyapatite–carbon nanotube composites for biomedical applications: A review. *International journal of applied ceramic technology* 4 (1):1–13.

Wilder, Jeroen WG, Liesbeth C Venema, Andrew G Rinzler, Richard E Smalley, and Cees Dekker. 1998. Electronic structure of atomically resolved carbon nanotubes. *Nature* 391 (6662):59–62.

Xie, Sishen, Wenzhi Li, Zhengwei Pan, Baohe Chang, and Lianfeng Sun. 2000. Mechanical and physical properties on carbon nanotube. *Journal of physics and chemistry of solids* 61 (7):1153–1158.

Yamamoto, Tatsuhiro, Suguru Noda, and Masaru Kato. 2011. A simple and fast method to disperse long single-walled carbon nanotubes introducing few defects. *Carbon* 49 (10):3179–3183.

Yang, Feng, Chen Jin, Dong Yang et al. 2011. Magnetic functionalised carbon nanotubes as drug vehicles for cancer lymph node metastasis treatment. *European journal of cancer* 47 (12):1873–1882.

Yang, Kun, ZiLi Yi, QingFeng Jing, RenLiang Yue, Wei Jiang, and DaoHui Lin. 2013. Sonication-assisted dispersion of carbon nanotubes in aqueous solutions of the anionic surfactant SDBS: The role of sonication energy. *Chinese science bulletin* 58 (17):2082–2090.

Yu, Junrong, Nadia Grossiord, Cor E Koning, and Joachim Loos. 2007. Controlling the dispersion of multi-wall carbon nanotubes in aqueous surfactant solution. *Carbon* 45 (3):618–623.

Zakharova, Galina S, Viktor L Volkov, Victoria V Ivanovskaya, and Alexander L Ivanovskii. 2005. Nanotubes and related nanostructures of d-metal oxides: Synthesis and computer design. *Russian chemical reviews* 74 (7):587.

Zarei, Hajar, Hedayatollah Ghourchian, Khadijeh Eskandari, and Majid Zeinali. 2012. Magnetic nanocomposite of anti-human IgG/COOH–multiwalled carbon nanotubes/Fe_3O_4 as a platform for electrochemical immunoassay. *Analytical biochemistry* 421 (2):446–453.

Zhang, Chengdong, Shuiming Luo, and Wei Chen. 2013. Activity of catalase adsorbed to carbon nanotubes: Effects of carbon nanotube surface properties. *Talanta* 113:142–147.

Zhang, Pu and David B Henthorn. 2010. Synthesis of PEGylated single wall carbon nanotubes by a photoinitiated graft from polymerization. *AIChE journal* 56 (6):1610–1615.

Zhou, Haihan, Xuan Cheng, Li Rao, Tao Li, and Yanwen Y Duan. 2013. Poly (3,4-ethylene dioxythiophene)/multiwall carbon nanotube composite coatings for improving the stability of microelectrodes in neural prostheses applications. *Acta biomaterialia* 9 (5):6439–6449.

Zhou, Zhenping, Lijie Ci, Li Song et al. 2004. The intrinsic temperature effect of Raman spectra of double-walled carbon nanotubes. *Chemical physics letters* 396 (4):372–376.



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5

Interaction of Inorganic Nanoparticles with Biological Matrices

Priya Sharma, Vineet Kumar, and Praveen Guleria

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5.1 Introduction

Nanoparticles are objects that are small in size with one of their dimensions in nanometers (nm). These particles are generally 1–100 nm with properties that are not shared by nonnanoscale particles having the same chemical composition (Hansen et al., 2007). A large amount of energy is required to characterize nanoparticles that are less than 20–30 nm in size. These nanoparticles are also thermodynamically unstable (Jolivet et al., 2004). Nanoparticles have a property to interact with the cells and regulate the molecular processes essential for cell functioning (Nel et al., 2006; Thill et al., 2006; Auffan et al., 2008; Gratton et al., 2008; Jiang et al., 2008). The various properties of nanoparticles such as the rate of reactions on their surface, their adsorption capacity, and their change of redox reaction affect the fate of nanoparticles in the environment (Hoyer and Weller, 1994; Tao et al., 2008). Inorganic nanoparticles have various physical properties such as size-dependent optical, magnetic, electronic, and catalytic properties. Nanoparticles with these physical properties are also emerging as novel drug delivery systems.

The different types of inorganic nanoparticles include quantum dots, magnetic, silica, gold, silver, titanium, and zinc oxide nanoparticles (Pandeya and Dhahia, 2016).

5.1.1 Gold Nanoparticles

These nanoparticles usually range from 2–100 nm in size. Various types of gold nanoparticles have been reported: gold nanorods, gold nanoshells, gold nanocages, and gold nanospheres. Smaller-sized nanoparticles range from 20 to 50 nm. These have the most prominent application in cellular uptake. The 40–50 nm-sized gold nanoparticles have a specific cellular toxicity. Thus, these can significantly diffuse into the tumors easily and help in recovery against tumorous growth. However, the large-sized nanoparticles range from 80 to 100 nm. These nanoparticles do not diffuse easily but remain attached in and near the blood vessels (El-Sayed et al., 2006; Jadzinsky et al., 2007). However, in a study during exposure of smaller- and large-sized gold nanoparticles, 50 nm-sized particles were maximally taken by the mammalian cells and their nuclear parts (Figure 5.1). However, 14 and 100 nm-sized nanoparticles were trapped in the cytoplasmic vesicles and did not enter the nucleus (Chithrani et al., 2006). Since gold nanoparticles have various physical and chemical properties, it enhances the biocompatibility, drug loading, and drug efficiency and provides more ease of targeted delivery of drug to the site with blood flow. Further, gold nanoparticles are also noncytotoxic to the normal cells (Chithrani et al., 2010; Lan et al., 2013; Khan et al., 2014).

5.1.2 Silver Nanoparticles

Silver has always been known to be a nontoxic metal. With the advent of nanotechnology, it was found that silver can be produced at the nanoscale level. Various physical and chemical processes for the synthesis of silver nanoparticles have already been reported (Iravani et al., 2014; Gudikandula and Maringanti, 2016). These nanoparticles have been documented to possess various biomedicinal applications (Figure 5.2).

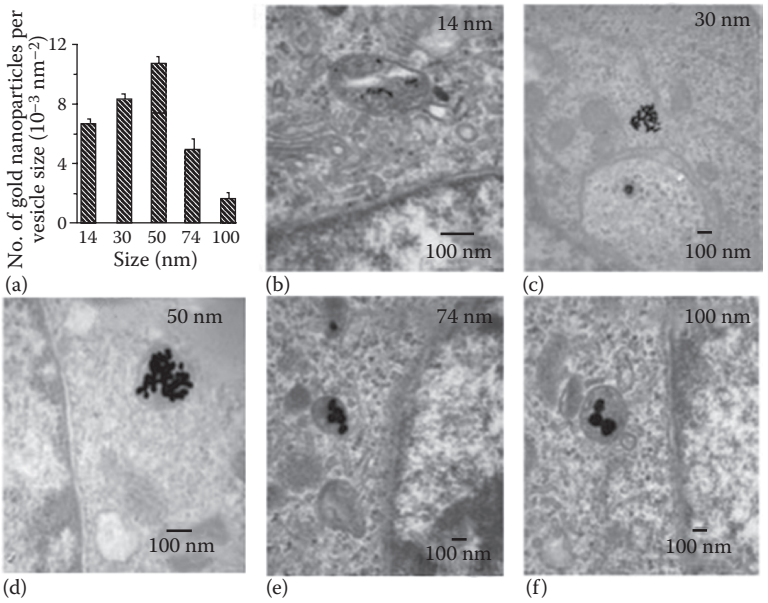


FIGURE 5.1 Measurement of gold nanoparticles present in cells supported by transmission electron microscopy. (a) The graph shows the number of gold nanoparticles present per vesicle with respect to their particle size. (b–f) TEM images representing the gold nanoparticles with sizes of 14, 30, 50, 74, and 100 nm trapped inside the vesicles of a HeLa cell. (Reprinted with permission from Chen, X. and Mao, S.S., *Chemical Reviews*, 107, 2891–2959, 2007. Copyright 2007 American Chemical Society.)

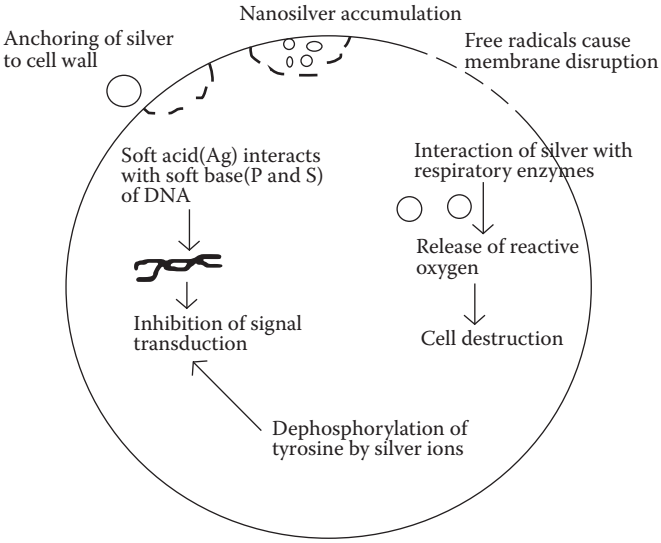


FIGURE 5.2 Various modes of interaction of silver nanoparticles with bacteria leading to their antimicrobial activity. (Adapted from Prabhu, S. and Poulose, E., *International Nano Letters*, 2, 32, 2012. With permission.)

Silver nanoparticles possess significant antibacterial, antifungal, antiviral, anti-inflammatory, and anticancer activities (Prabhu and Poulose, 2012; Ge et al., 2014). Due to these properties, silver nanoparticles are used on a large scale by medical industries. Silver nanoparticles can also be used for enhanced wound healing and as biosensors (Pulit et al., 2013; Abdalrahim, 2014; Qu et al., 2014).

5.1.3 Quantum Dots

These are small semiconductor particles ranging from 2 to 20 nm in size. These small particles exhibit different optical and electronic properties than their larger particle counterparts (Kluson et al., 2007). Quantum dots have a diameter up to 10 nm (Kral et al., 2006; Ferancova and Labuda, 2008). However, the large-sized quantum dots have a diameter varying from 10 to 12 nm. The large-sized quantum dots possess a higher wavelength that emits red or orange color. On the contrary, the small-sized quantum dots with diameter of 4–6 nm emit blue and green light due to their shorter wavelength. Quantum dots show high brightness in the excited state. These are highly resistant to photo bleaching and have simultaneous detection of multiple signals using a single excitation source (Tsutsui et al., 1993; Bera et al., 2010).

5.1.4 Silica Nanoparticles

Silica nanoparticles can be synthesized by various techniques, namely, microemulsion, sol–gel method, spray drying, template heating degradation, laser ablation, thermal annealing, thermal vaporization, hydrolysis, polycondensation hard templating, and soft templating (Yang et al., 2012; Tang and Cheng, 2013). These nanoparticles are mesoporous or nonporous in nature. These properties of silica nanoparticles are the basis behind their various biomedical applications. Mesoporous nanoparticles with 2–50 nm pore size are actively used for the delivery of active payloads that are based on their physical and chemical adsorption potential. On the other hand, encapsulation and conjugation ability of nonporous silica nanoparticles affect their targeted delivery applications (Vallet-Regi et al., 2007; Slowing et al., 2008). The major advantage of using silica nanoparticles in biomedicine is that it improves the efficiency of drug delivery system (Hergt et al., 2006).

5.1.5 Titanium Oxide Nanoparticles

Titanium dioxide nanoparticles are widely used in industries for electronics, packaging, food, and cosmetics. These nanoparticles also possess semiconducting characteristics and phytocatalytic effects and have high refractive index properties (Buso et al., 2007; Jagadale et al., 2008; Bodurov et al., 2014). But an increased level of toxicity has been reported for titanium oxide nanoparticles (Jo et al., 2016). Various methods of synthesis of titanium oxide nanoparticles along with their applications have already been described in detail (Chen and Mao, 2007).

5.2 Biological Matrices

Biological matrices are biomolecules either macromolecule or micromolecule that are present in the living organisms. The macromolecules include proteins, carbohydrates,

lipids, and nucleic acids. Simultaneously, the micromolecules are primary metabolites, secondary metabolites, and natural products. These biological matrices are mostly organic compounds (Green and Goldberger, 1967). Biological matrices can be categorized into different types: carbohydrates, proteins, nucleic acids, collagen, xenogenic extracellular matrices, fibrin, autologous extracellular matrix, and hydrogels.

5.2.1 Carbohydrates, Proteins, and Nucleic Acids

Carbohydrates are a good source of energy. They are classified into monosaccharides, oligosaccharides, and polysaccharides on the basis of number of glucose units. Monosaccharides consist of 1 sugar unit, oligosaccharides consist of 2–10 units, whereas polysaccharides consist more than 10 sugar units. Polysaccharides are unsweet and the most complex type of carbohydrates (Alberts et al., 2002). Proteins make 50% of the cellular dry weight. These are generally insoluble in water. Proteins consist of different polymers of amino acids that are further arranged in the form of polypeptide chains. They have primary, secondary, tertiary, and quaternary structures. Proteins play an important role in animals such as myosin which allows the movement by contraction of muscle. Nucleic acid is the genetic material which carries the hereditary information from parents to progeny. There are of two types: deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Their monomeric units are known as nucleotides. Nucleotides consists of acyclic nitrogenous bases such as pentose and a one to three phosphate group (adenosine triphosphate and guanosine triphosphate) which serves as source of chemical energy (Alberts et al., 2002).

5.2.2 Collagen

Collagen is the abundant protein found in mammals. Nearly 25–35% of body protein content is made up of collagen (Lullo et al., 2002). It is the main component of extracellular protein. It is found in bones as rigid tissue; in tendons and cartilage as compliant tissue, ligament, blood vessels, intervertebral discests, and skin; and as dentin in teeth (Sikorski, 2001). Collagens have various medical uses such as bone grafting, cosmetic surgeries, wound care, and tissue regeneration (Oliveira et al., 2009; Cunniffe and O'Brien, 2011).

5.2.3 Xenogeneic Extracellular Matrices

Xenogenous extracellular matrices are used for the development of bioscaffolds that are used in various tissue engineering applications (Badylak, 2004). These exogenous extracellular matrices are generally formed by decellularization and terminal sterilization of procrine organs such as small intestine or urinary bladder without any loss of function. These bioscaffolds are used in various preclinical and clinical studies of animals and humans. These find application in repairing and reconstructing of various body tissues such as musculoskeletal, cardiovascular, urogenital, and integumentary structures. The practice is to develop these bioscaffolds from the native mammalian extracellular matrix (Badylak, 2004).

5.2.4 Hydrogels

Hydrogels are biomatrices that are polymeric in nature and have the ability to swell. These are insoluble in water (Kopecek, 2002). Various hydrophilic polymers are physically and chemically cross-linked to form hydrogels (Caló and Khutoryanskiy, 2015). Hydrogels have various medical applications such as cardiac application, dental application, wound healing, and development of soft contact lenses. Further, hydrogels also act as bioscaffold for drug delivery and tissue engineering applications (Seow and Hauser, 2014; Caló and Khutoryanskiy, 2015).

5.3 Interactions of Inorganic Nanoparticles with Biological Matrices

Biomolecules share a dynamic interaction with nanoparticles on their nanoscale surfaces. Nanoparticles with advance functional properties are also being successfully synthesized to enhance their interaction potential with biological matrices (Zhang et al., 2012). Nanoparticles are engineered with various biological entities such as glucose, soluble starch, or chitosan that can link nanoparticles to biomolecules. These techniques have been implemented to immobilize DNA and proteins onto surface of the nanoparticles (Nam et al., 2003). Further, biomolecules are also used for the stabilization of nanoparticles. Cadmium sulfide (CdS) quantum dots have been reported to be stabilized by aromatic amino acids (Zhang et al., 2015). Hence, nanoparticles can be synthesized in the presence of biomolecules or can be stabilized with the help of biomolecules.

Interaction of nanoparticles with biomolecular matrices require a large number of technological integrations such as optical imaging technologies, molecular spectroscopy, and mass spectroscopy to analyze their chemical characterizations and structural changes occurring on the nano–bio interface (Nel et al., 2009). Such interaction also acts as a basis for the establishment of personalized nanomedicine and nanohealthcare (Zhang et al., 2012). Interaction of inorganic nanoparticles with various biological matrices is discussed hereafter.

5.3.1 Interaction of Gold Nanoparticles with Biological Matrices

Gold nanoparticles have various properties as plasmonic properties and geometrical properties that make them useful in fields such as analytical chemistry, catalysis, and biomedical applications and photothermal cancer therapy. One to 100 nm sized nanoparticles were mainly used in bioimaging, drug delivery, and cancer therapeutics (Bruchez et al., 1998; Huang et al., 2006; Petros and DeSimone, 2010; Liu et al., 2011; Hong et al., 2012). Gold nanoparticles have also been reported to interact with various biomatrices such as human blood, proteins, and tissues/organs as liver, lung, and spleen (Wang et al., 2015).

5.3.1.1 Interaction with Blood

Gold nanoparticles are known to possess coagulant activity. Gold nanoparticles (1.2 and 5 nanomolar, i.e., nM) having a size ranging from 12 to 85 nm have been

evaluated for their coagulation activity of blood. Nanoparticles having a size of 1.2 nM showed no effect on the blood. However, the 5 nM gold nanoparticles showed procoagulant potential. The clot strength reduced with reduction in the nanoparticle size. The nanoparticles of size 85 nm showed faster prothrombotic activity. Hence, gold nanoparticles were demonstrated to improve blood clot activity (Ajdari et al., 2017).

5.3.1.2 Interaction with Proteins

Size, shape, and curvature of gold nanoparticles play a major role in their interaction with proteins. Five, 15, and 80 nm spherical gold nanoparticles were injected in mouse serum and incubated for 24 hours. It was done to check the adsorption of proteins by nanoparticles. After incubation, absorbed and nonabsorbed proteins were separated. Centrifugation and electrophoresis were used for the same. Separated proteins were identified by matrix-assisted laser desorption ionization-time of flight mass spectrometry proteomic analyzer. Proteins absorbed on the nanoparticle surface were identified as apolipoproteins or complement C3 proteins depending on their sizes. However, with the increasing mass or size of nanoparticles, the band intensities of absorbed proteins were decreased. Hence, gold nanoparticles were reported to absorb proteins depending upon their size and shape (Schäffler et al., 2013).

Positively and negatively charged gold nanoparticles with 5–20 nm size were evaluated for their protein binding potential. Both charged nanoparticles were observed to bind with a large number of proteins. However, the neutral nanoparticles were bound to a lesser number of proteins. Hence, differentially charged nanoparticles showed different binding orientations for the proteins. Both negatively and positively charged gold nanoparticles showed highest affinity for fibrinogen protein, leading to the formation of protein corona. On the contrary, the neutral particles showed least binding to the proteins. It has also been documented that information about the protein composition is not sufficient to elucidate the biological effects of nanoparticles on proteins for the formation and composition of protein corona (Deng et al., 2013).

A density functional theory system has also been developed to study the binding of gold nanoclusters with anionic, cationic, and neutral amino acids. The anionic amino acids showed maximum tendency to interact with the nanoclusters. The major binding factor of the interaction of amino acid with nanoparticles was reported as (a) the anchoring of N–Au, O–Au, and S–Au and (b) the nonconventional N–H···Au and O–H···Au hydrogen bonds. Further, the orbital theory and atom molecule theory deciphered that partially electrostatic and partially covalent bonds were responsible for the interaction between the nanoclusters and amino acids (Pakiari and Jamshidi, 2007).

5.3.1.3 Interaction with Different Tissues/Organs

Smaller-sized gold nanoparticles have been reported to show systemic distribution in organs as compared to larger-sized nanoparticles. Rats injected with 10, 50, 100, and 250 nm gold nanoparticles have shown presence of a large number of smaller particles in various tissues/organs of rats including liver, spleen, kidney, testis, thymus, heart, lung, brain, and blood. On the contrary, the large-sized nanoparticles were detected only in blood, liver, and spleen. Hence, the biodistribution of gold nanoparticles is size dependent (Jong et al., 2008). Likewise, spherical gold nanoparticles of diameter

25 ± 8 nm have been reported to accumulate only in the liver and the rest of the organs remained unaffected. Hence, these spherical nanoparticles can find efficient applications in targeted drug delivery to liver only (Wojnicki et al., 2013).

5.3.2 Interaction of Silver Nanoparticles

Nanotechnology is growing day by day with various applications in daily life due to their increased surface–volume ratios. Nanomaterials have various chemical and physical properties that enhance their arenas of applications. Silver is known to possess antibacterial properties that make it useful for the treatment of burns and wounds and against several kinds of pathogenic bacterial infections (Cortivo et al., 2010; Sivolella et al., 2012).

5.3.2.1 Interaction with Mitochondria

Silver nanoparticles were observed to interact with mitochondria. Twenty-nanometer-sized silver nanoparticles were taken up by human dermal fibroblasts by endocytosis. TEM analysis showed that nanoparticles were absent in the cell nucleus, endoplasmic reticulum, golgi complex, and mitochondria. But they were found near the outer membrane of mitochondria. The nanoparticles were observed to release reactive oxygen (ROS) into the cytoplasm. The generation of ROS induced oxidative stress, disturbed the respiratory chain, and damaged the mitochondria (Bressan et al., 2013). Further, silver nanoparticles have also been reported to induce toxicity to the mitochondria of murine hippocampal neuronal HT22 cells by causing depolarization of the mitochondrial membrane, increased mitochondrial oxygen consumption, activation of caspase-3, and accumulation of ROS. However, the treatment of cells with sodium selenite was reported to alleviate nanoparticle-mediated toxicity (Ma et al., 2015). The toxicity induced by silver nanoparticles to mitochondria has already been discussed in detail (Maurer and Meyer, 2016).

5.3.2.2 Interaction with DNA

DNAs of different base pair compositions can be differentiated with the help of silver nanoparticles on the basis of their binding affinity and specificity. Interaction of the bacterial and mammalian DNAs with silver nanoparticles was studied by analyzing the absorption, thermal melting, isothermal calorimetry, and viscosity of the DNAs. Thermal melting showed that melting temperature (*Tm*) of DNA was decreased on interaction with nanoparticles due to the destabilization of DNA stacks. Further, the nanoparticles showed more specificity for double-stranded DNA rather than single-stranded DNA (Pramanik et al. 2016). Earlier, interaction of colloidal silver nanoparticles with DNA bases adenine, guanine, thymine, and cytosine has been documented to induce aggregation of the nanoparticles (Basu et al., 2008).

In another study, DNA–silver nanoparticle complexes have been developed and used for reactive studies against Cu^{2+}, Cl^{-}, and H_3O^{+}. The complex interactive studies showed that DNA acted like a catalyst on the surface on silver nanoparticles. Further, release of silver ions from the DNA–nanoparticle complex could be absorbed by the DNA double helix and induced cross-linkage of DNA complementary strands. These cross-links generated point mutations in the DNA and caused cell death (Bregadze

et al., 2012). Interaction of silver nanoparticles with thymus DNA has shown that nanoparticles act as liquid drops moistening the DNA surface at the point of interaction. Further, the nanoparticle–DNA complex showed DNA-dependent photoirradiation-induced conformational change in the complex (Bregadze et al., 2013).

5.3.2.3 Interaction with Proteins

Silver nanoparticles have shown spontaneous binding affinity for bovine serum albumin (BSA). The UV–visible and fluorescence spectroscopic techniques showed the formation of a ground state complex between BSA and silver nanoparticles on interaction. The silver nanoparticles possessed quenching ability and quench the intrinsic fluorescence of BSA. The ΔH, ΔG, and ΔS calculations at different temperatures showed hydrophobic and electrostatic interactions between the silver nanoparticle–BSA complexes. Hence, the interaction process/binding process between silver nanoparticles and BSA was found to be spontaneous (Roy et al., 2014).

Interaction of silver nanoparticles with light leads to surface plasmon resonance (SPR) that is defined as the collective resonance of conducting electrons. The SPR profile in the presence of several protein and varying protein concentrations was determined. With the increase in protein concentration, the SPR profiles showed a dip and rise pattern (Figure 5.3).

The consistency of SPR was dependent on the nanoparticle size and interaction level of nanoparticles with the varying protein concentration. Further, it was observed that protein usually retained its native conformation during interaction with silver nanoparticles and that the SPR phenomenon was not at all affected by the size–shape of the protein under investigation (Banerjee and Das, 2013).

The interaction of silver nanoparticles with lysozyme protein has also been documented. The interaction between lysozyme and nanoparticles has been reported to be hydrophobic and spontaneous regulated by a static quenching mechanism. The adsorption of lysozyme on the surface of silver nanoparticles was demonstrated to follow pseudo-second-order kinetic characteristics with hysteresis effect (Wang et al., 2017).

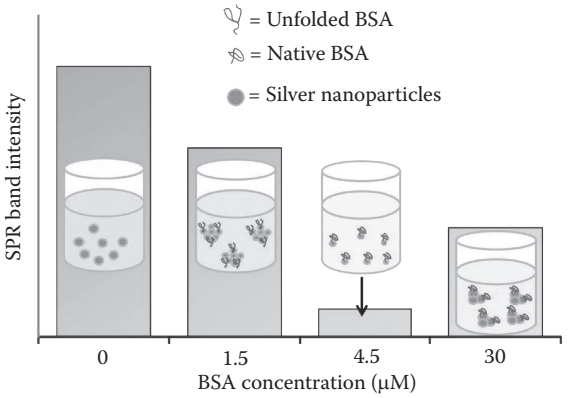


FIGURE 5.3 Graph measurements depicting the SPR band intensity of silver nanoparticles on interaction with increasing concentration of BSA protein. (Adapted from Banerjee, V. and Das, K.P., *Colloids and Surfaces B: Biointerfaces*, 111, 71–79, 2013. With permission.)

5.3.3 Interactions of Quantum Dots with Biological Matrices

5.3.3.1 Interaction with Skin

Quantum dots are known for their biomedical and electronic applications. These particles may also have the ability to penetrate skin and are useful in inhibiting the inflammatory responses of skin damage. QD621 quantum dots are generally a nail-shaped containing cadmium core with a CdS shell coated with polyethylene glycol (PEG) and have water solubility. Quantum dots were topically applied on porcine skin and human epidermal keratinocytes to assess penetration. The quantum dots were observed to penetrate the stratum corneum and were reported near the hair follicles and intercellular lipid bilayers of the membrane. Further, the penetration of quantum dots in keratinocytes reported their accumulation in the cytoplasmic vacuoles and at the periphery of the cell membranes and reduced the viability of cells (Zhang et al., 2008).

Quantum dots QD621 coated with PEG and QD565 and QD655 coated with carboxylic acid were exposed to skin to evaluate their penetration effects. QD621 has shown similar penetration responses as investigated by Zhang et al. (2008). However, the exposure of QD565 and QD655 on flexed and tape-stripped skin did not show penetration. However, these quantum dots showed penetration in the viable dermal layers in abraded skin. Hence, it was documented that penetration of quantum dots depends upon the species under investigation and anatomical complexity of skin (Monteiro-Riviere and Zhang, 2009).

5.3.3.2 Interaction with Proteins

The biological impact of quantum dots on protein conformational change was determined using tryptophan phosphorescence spectroscopy. Glutathione-coated CdS quantum dots were evaluated for interaction against model proteins having different isoelectric points and quaternary structure. The protein nanoparticles interaction was found to improve the quantum yield of nanoparticles. Tryptophan phosphorescence spectroscopy showed induction of significant conformational changes in protein structures. Further, each quantum dot was reported to interact with more than one protein molecule despite of their smaller size (Gabellieri et al., 2011). CdS quantum dots coated with mercaptoethanol, L-cysteine, and glutathione was found to interact with proteins BSA and lysozyme and change their secondary structure. Hence, the behavior of quantum dots toward proteins was dependent on the biomolecules present on their surface (Huang et al., 2011).

5.3.4 Interactions of Silica Nanoparticles

5.3.4.1 Interaction with Proteins

Silica nanoparticles contribute to the surface curvature and influence adsorbed protein structure and function. Interaction of silica nanoparticles with lysozome protein of various diameters has been reported. Four-nanometer-sized silica particles were found to form a molecular complex with lysozyme. On the contrary, interaction of 20 and 100 nm silica nanoparticles with lysozyme showed true adsorption behavior and resulted in monolayer and multilayer adsorption, respectively. The α-helix of the lysozyme was removed, and it depended on the size of nanoparticles and pH of the interaction solution (Vertegel et al., 2004).

Likewise, the interaction study of mesoporous silica nanoparticles with fetal bovine serum has also been reported. Seventy to 900 nm sized silica nanoparticles were used for this study. The smaller-sized nanoparticles having a larger external surface area showed interaction with maximum number of proteins. Most of these proteins were having low molecular weights as higher surface curvature of smaller nanoparticles could support maximum enrichment of smaller proteins. The porosity of nanoparticles further improved the deposition of low molecular weight proteins on their surface due to the size exclusion effect (Clemments et al., 2015).

5.3.4.2 Interaction with DNA

The adsorption of DNA onto mesoporous silica nanoparticles has been reported. The entrapment of DNA into mesoporous silica was regulated by the Debye length in the solution. Further, DNA was found to form direct and indirect hydrogen bonds in the pores having different diameters (Li et al., 2012). Earlier, the interaction of DNA with organically modified silica nanoparticles has been reported to be highly stable. DNase I was unable to remove or degrade the DNA present in the DNA–nanoparticle complex. The DNA was bound electrostatically to the nanoparticles because of the positively charged amino groups. Hence, these complexes were documented as efficient vehicle for DNA delivery into the cells (Roy et al., 2005).

5.4 Conclusion

The inorganic nanoparticles have tendency to interact with various biological entities. Various studies have already documented the mechanism regulating the interaction of nanoparticles with biological matrices. The formation of complexes of biological entities with nanoparticles may depend upon the size, shape, and type of nanomaterial used as well as the type, charge, and structure of biological molecule under investigation. Hence, these interactions can act as a basis for the use of nanomaterials for different medicinal applications as drug delivery and other therapeutics. However, a detailed evaluation of the tendency of several nanoparticles to bind, adsorb, or interact with various biological molecules is still midway and descriptively required.

REFERENCES

Abdalrahim, A. 2014. Preparation and characterization of silver nanoparticles. *International Journal of ChemTech Research* 6:450–459.

Ajdari, N., Vyas, C., Bogan, S. L., Lwaleed, B. A., and Cousins, B. G. 2017. Gold nanoparticle interactions in human blood: A model evaluation. *Nanomedicine: Nanotechnology, Biology and Medicine* 13:1531–1542.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Wlater, P. 2002. *Molecular Biology of the Cell* (4th ed.). New York: Garland Science. pp. 120–121. ISBN 0-8153-3218-1.

Auffan, M., Achouak, W., Rose, J., Roncato, M. A., Chanéac, C., Waite, D. T., Masion, A., Woicik, J. C., Wiesner, M. R., and Bottero, J. Y. 2008. Relation between the redox state of iron-based nanoparticles and their cytotoxicity towards *Escherichia coli*. *Environmental Science and Technology* 42:6730–6735.

Badylak, S. F. 2004. Xenogeneic extracellular matrix as a scaffold for tissue reconstruction. *Transplant Immunology* 12:367–377.

Banerjee, V. and Das, K. P. 2013. Interaction of silver nanoparticles with proteins: A characteristic protein concentration dependent profile of SPR signal. *Colloids and Surfaces B: Biointerfaces* 111:71–79.

Basu, S., Jana, S., Pande, S., and Pal, T. 2008. Interaction of DNA bases with silver nanoparticles: Assembly quantified through SPRS and SERS. *Journal of Colloid and Interface Science* 15:288–293.

Bera, D., Qian, L., Tseng, T. K., and Holloway, P. H. 2010. Quantum dots and their multimodal applications: A review. *Materials* 3:2260–345.

Bodurov, I., Yovcheva, T., and Sainov, S. 2014. PMMA films refractive index modulation via TiO2 nanoparticle inclusions and corona poling. *Colloid and Polymer Science* 292:3045–3048.

Bregadze, V. G., Melikishvili, Z. G., and Giorgadze, T. G. 2013. Photo-induced DNA-dependent conformational changes in silver nanoparticles. *Advances in Nanoparticles* 2:176–181.

Bregadze, V. G., Melikishvili, Z. G., Giorgadze, T. G., Monaselidze, J. R., Jaliashvili, Z. V., and Khuskivadze, T. B. 2012. Point Defects in Double Helix Induced by Interaction of Silver Nanoparticles with DNA. http://arxiv.org/ftp/arxiv/papers/1206/1206.4816.pdf

Bressan, E., Ferroni, L., Gardin, C., Rigo, C., Stocchero, M., Vindigni, V., Cairns, W., and Zavan, B. 2013. Silver nanoparticles and mitochondrial interaction. *International Journal of Dentistry* 2013:1–8.

Bruchez, M., Moronne, M., Gin, P., Weiss, S., and Alivisatos, A. P. 1998. Semiconductor nanocrystals as fluorescent biological labels. *Science* 281:2013–2016.

Buso, D., Pacifico, J., Martucci, A., and Mulvaney, P. 2007. Gold-nanoparticle-doped TiO_2 semiconductor thin films: Optical characterization. *Advanced Functional Materials* 17:347–354.

Caló, E. and Khutoryanskiy, V. V. 2015. Biomedical applications of hydrogels: A review of patents and commercial products. *European Polymer Journal* 65:252–267.

Chen, X. and Mao, S. S. 2007. Titanium dioxide nanomaterials: Synthesis, properties, modifications, and applications. *Chemical Reviews* 107:2891–2959.

Chithrani, B. D., Ghazani, A. A., and Chan, W. C. W. 2006. Determining the size and shape dependence of gold nanoparticle uptake into mammalian cells. *Nano Letters* 6:662–668.

Chithrani, D. B., Jelveh, S., Jalali, F., Van Prooijen, M., Allen, C., Bristow, R. G., Hill, R. P., and Jaffray, D. A. 2010. Gold nanoparticles as radiation sensitizers in cancer therapy. *Radiation Research* 173:719–728.

Clemments, A. M., Botella, P., and Landry, C. C. 2015. Protein adsorption from biofluids on silica nanoparticles: Corona analysis as a function of particle diameter and porosity. *ACS Applied Materials and Interfaces* 7:21682–21689.

Cortivo, R., Vindigni, V., Iacobellis, L., Abatangelo, G., Pinton, P., and Zavan, B. 2010. Nanoscale particle therapies for wounds and ulcers. *Nanomedicine* 5:641–656.

Cunniffe, G. and O'Brien, F. J. 2011. Collagen scaffolds for orthopedic regenerative medicine. *The Journal of the Minerals, Metals and Materials Society* 63:66–73.

Deng, Z. J., Liang, M., Toth, I., Monteiro, M., and Minchin, R. F. 2013. Plasma protein binding of positively and negatively charged polymercoated gold nanoparticles elicits different biological responses. *Nanotoxicology* 7:314–322.

El-Sayed, I. H., Huang, X., and El-Sayed, A. M. 2006. Selective laser photo-thermal therapy of epithelial carcinoma using anti-EGFR antibody conjugated gold nanoparticles. *Cancer Letters* 2:129–135.

Ferancova, A. and Labuda, J. 2008. DNA biosensors based on nanostructured materials. In: Eftekhari A. ed. *Nanostructured Materials in Electrochemistry.* Weinheim, Germany: Wiley-VCH, pp. 409–434.

Gabellieri, E., Cioni, P., Balestreri, E., and Morelli, E. 2011. Protein structural changes induced by glutathione-coated CdS quantum dots as revealed by Trp phosphorescence. *European Biophysics Journal* 40:1237–1245.

Gao, X., Cui, Y., Levenson, R. M., Chung, L. W. K., and Nie, S. 2004. *In vivo* cancer targeting and imaging with semiconductor quantum dots. *Nature Biotechnology* 22:969–976.

Gratton, S. E., Ropp, P. A., Pohlhaus, P. D., Luft, J. C., Madden, V. J., Napier, M. E., and DeSimone, J. M. 2008. The effect of particle design on cellular internalization pathways. *Proceedings of the National Academy of Sciences USA* 105:11613–11618.

Green, D. E. and Goldberger, R. 1967. *Molecular Insights into the Living Process.* New York: Academic Press.

Gudikandula, K. and Maringanti, S. C. 2016. Synthesis of silver nanoparticles by chemical and biological methods and their antimicrobial properties. *Journal of Experimental Nanoscience* 11:714–721.

Hansen, S. F., Larsen, B. H., Olsen, S. I., and Baun, A. 2007. Categorization framework to aid hazard identification of nanomaterials. *Nanotoxicology* 1:243–250.

Hergt, R., Dutz, S., Muller, R., and Zeisberger, M. 2006. Magnetic particle hyperthermia: Nanoparticle magnetism and materials development for cancer therapy. *Journal of Physics: Condensed Matter* 18:S2919-S34.

Hong, G. S., Lee, J. C., Robinson, J. T., Raaz, U., Xie, L. M., Huang, N. F., Cooke, J. P., and Dai, H. J. 2012. Multifunctional in vivo vascular imaging using near-infrared II fluorescence. *Nature Medicine* 18:1841–1846.

Hoyer, P. and Weller, H. 1994. Size-dependent redox potentials of quantized zinc oxide measured with an optically transparent thin layer electrode. *Chemical Physics Letters* 221:379–384.

Huang, D., Geng, F., Liu, Y., Wang, X., Jiao, J., and Yu, L. 2011. Biomimetic interactions of proteins with functionalized cadmium sulfide quantum dots. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 392:191–197.

Huang, X. H., El-Sayed, I. H., Qian, W., and El-Sayed, M. A. 2006. Cancer cell imaging and photothermal therapy in the near-infrared region by using gold nanorods. *Journal of the American Chemical Society* 128:2115–2120.

Iravani, S., Korbekandi, H., Mirmohammadi, S. V., and Zolfaghari, B. 2014. Synthesis of silver nanoparticles: Chemical, physical and biological methods. *Research in Pharmaceutical Sciences* 9:385–406.

Jadzinsky, P. D., Calero, G., Ackerson, C. J., Bushnell, D. A., and Kornberg, R. D. 2007. The structure of a thiol monolayer-protected gold nanoparticle at 1.1 Å resolution. *Science* 318:430–433.

Jagadale, T. C., Takale, S. P., Sonawane, R. S., Joshi, H. M., Patil, S. I., Kale, B. B., and Ogale, S. B. 2008. N-doped TiO_2 nanoparticle based visible light photocatalyst by modified peroxide sol-gel method. *The Journal of Physical Chemistry C* 112:14595–14602.

Jiang, W., Kim, B. Y. S., Rutka, J. T., and Chan, W. C. W. 2008. Nanoparticle-mediated cellular response is size-dependent. *Nature Nanotechnology* 3:145–150.

Jo, M. R., Yu, J., Kim, H. J., Song, J. H., Kim, K. M., Oh, J. M., and Choi, S. J. 2016. Titanium dioxide nanoparticle-biomolecule interactions influence oral absorption. *Nanomaterials* 6:225.

Jolivet, J. P., Froidefond, C., Pottier, A., Chanéac, C., Cassaignon, S., Tronca, E., and Euzen, P. 2004. Size tailoring of oxide nanoparticles by precipitation in aqueous medium: A semi-quantitative modelling. *Journal of Materials Chemistry* 14:3281–3288.

Jong, W. H., Hagens, W. I., Krystek, P., Burger, M. C., Sips, A. J., and Geertsma, R. E. 2008. Particle size-dependent organ distribution of gold nanoparticles after intravenous administration. *Biomaterials* 29:1912–1919.

Khan, A. K., Rashid, R., Murtaza, G., and Zahra, A. 2014. Gold nanoparticles: Synthesis and application in the drug. *Tropical Journal of Pharmaceutical Research* 13:1169–1177.

Kluson, P., Drobek, M., Bartkova, H., and Budil, I. 2007. Welcome in the nano world. *Chemické Listy* 101:262–272.

Kopecek, J. 2002. Polymer chemistry: Swell gels. *Nature* 417:388–389.

Kral, V., Sotola, J., Neuwirth, P., Kejik, Z., Zaruba, K., and Martasek, P. 2006. Nanomedicine-current status and perspectives: A big potential or just a catchword. *Chemické Listy* 100:4–9.

Lan, M. Y., Hsu, Y. B., Hsu, C. H., Ho, C. Y., Lin, J. C., and Lee, S. W. 2013. Induction of apoptosis by high-dose gold nanoparticles in nasopharyngeal carcinoma cells. *Auris Nasus Larynx* 40:563–568.

Li, X., Zhang, J., and Gu, H. 2012. Study on the adsorption mechanism of DNA with mesoporous silica nanoparticles in aqueous solution. *Langmuir* 28:2827–2834.

Liu, Z., Robinson, J. T., Tabakman, S. M., Yang, K., and Dai, H. J. 2011. Carbon materials for drug delivery & cancer therapy. *Materials Today* 14:316–323.

Lullo, G. A., Sweeney, S. M., Körkkö, J., Ala-Kokko, L., and Antonio, J. D. 2002. Mapping the ligand-binding sites and disease-associated mutations on the most abundant protein in the human, type I collagen. *The Journal of Biological Chemistry* 277:4223–4231.

Ma, W., Jing, L., Valladares, A., Mehta, S. L., Wang, Z., Li, P. A., and Bang, J. J. 2015. Silver nanoparticle exposure induced mitochondrial stress, caspase-3 activation and cell death: Amelioration by sodium selenite. *International Journal of Biological Sciences* 11:860–867.

Maurer, L. L. and Meyer, J. N. 2016. A systematic review of evidence for silver nanoparticle-induced mitochondrial toxicity. *Environmental Science: Nano* 3:311.

Monteiro-Riviere, N. A. and Zhang L. W. 2009. Assessment of quantum dot penetration into skin in different species under different mechanical actions. In: Linkov I., Steevens J. (eds.) *Nanomaterials: Risks and Benefits*. NATO Science for Peace and Security Series C: Environmental Security. Springer, Dordrecht.

Nam, J. M., Thaxton, C. S., and Mirkin, C. A. 2003. Nanoparticle-based bio-bar codes for the ultrasensitive detection of proteins. *Science* 301:1884–1886.

Nel, A. E., Mädler, L., Velegol, D., Xia, T., Hoek, E. M. V., Somasundaran, P., Klaessig, F., Castranova, V., and Thompson, M. 2009. Understanding biophysicochemical interactions at the nano-bio interface. *Nature Materials* 8:543–557.

Nel, A., Xia, T., Madler, L., and Li, N. 2006. Toxic potential of materials at the nanolevel. *Science* 311:622–627.

Oliveira, S., Ringshia, R., Legeros, R., Clark, E., Terracio, L., Teixeira, C., and Yost, M. 2009. An improved collagen scaffold for skeletal regeneration. *Journal of Biomedical Materials* 94: 371–379.

Pakiari, A. H. and Jamshidi, Z. 2007. Interaction of amino acids with gold and silver clusters. *The Journal of Physical Chemistry A* 111:4391–4396.

Petros, R. A. and DeSimone, J. M. 2010. Strategies in the design of nanoparticles for therapeutic applications. *Nature Reviews Drug Discovery* 9:615–627.

Prabhu, S. and Poulose, E. K. 2012. Silver nanoparticles: Mechanism of antimicrobial action, synthesis, medical applications, and toxicity effects. *International Nano Letters* 2:32.

Pramanik, S., Chatterjee, S., Saha, A., Devi, P. S., and Kumar, G. S. 2016. Unraveling the interaction of silver nanoparticles with mammalian and bacterial DNA. *The Journal of Physical Chemistry B* 120:5313–5324.

Pulit, J., Banach, M., Szczygłowska, R., and Bryk, M. 2013. Nanosilver against fungi, silver nanoparticles as an effective biocidal factor. *Acta Biochimica Polonica* 60:795–798.

Qu, D., Sun, W., Chen, Y., Zhou, J., and Liu, C. 2014. Synthesis and *in vitro* antineoplastic evaluation of silver nanoparticles mediated by Agrimoniae herba extract. *International Journal of Nanomedicine* 9:1871–1872.

Roy, I., Ohulchanskyy, T. Y., Bharali, D. J., Pudavar, H. E., Mistretta, R. A., Kaur, N., and Prasad, P. N. 2005. Optical tracking of organically modified silica nanoparticles as DNA carriers: A nonviral, nanomedicine approach for gene delivery. *Proceedings of the National Academy of Sciences USA* 11:279–284.

Roy, S. and Das, T. K. 2014. Spectroscopic studies of interaction between biologically synthesized silver nanoparticles and bovine serum albumin. *Journal of Nanoscience and Nanotechnology* 14:4899–4905.

Schäffler, M., Semmler-Behnke, M., Sarioglu, H., Takenaka, S., Wenk, A., Schleh, C., Hauck, S. M., Johnston, B. D., and Kreyling, W. G. 2013. Serum protein identification and quantification of the corona of 5, 15 and 80 nm gold nanoparticles. *Nanotechnology* 24:265103.

Seow, W. Y. and Hauser, C. A. E. 2014. Short to ultrashort peptide hydrogels for biomedical uses. *Materials Today* 17:381–388.

Sikorski, Z. E. 2001. *Chemical and Functional Properties of Food Proteins*. Boca Raton, FL: CRC Press. p. 242.

Sivolella, S., Stellini, E., Brunello, G., Gardin, C., Ferroni, L., Bressan, E., and Zavan, B. 2012. Silver nanoparticles in alveolar bone surgery devices and toxicity of silver nanoparticles in alveolar bone surgery devices. *Journal of Nanomaterials* 2012:1–12.

Slowing, I. I., Vivero-Escoto, J. L., Wu, C. W., and Lin, V. S. 2008. Mesoporous silica nanoparticles as controlled release drug delivery and gene transfection carriers. *Advanced Drug Delivery Reviews* 60:1278–1288.

Tang, L. and Cheng, J. 2013. Nonporous silica nanoparticles for nanomedicine application. *Nano Today* 8:290–312.

Tao, F., Grass, M. E., Zhang, Y., Butcher, D. R., Renzas, J. R., Liu, Z., Chung, J. Y., Mun, B. S., Salmeron, M., and Somorjai, G. A. 2008. Reaction-driven restructuring of Rh–Pd and Pt–Pd core-shell nanoparticles. *Science* 322:932–934.

Thill, A., Zeyons, O., Spalla, O., Chauvat, F., Rose, J., Auffan, M., and Flank, A. M. 2006 Cytotoxicity of CeO_2 nanoparticles for *Escherichia coli*: Physico-chemical insight of the cytotoxicity mechanism. *Environmental Science & Technology* 40:6151–6156.

Tsutsui, K., Hu, E. L., and Wilkinson, C. D. W. 1993. Reactive ion etched II-VI quantum dots-dependence of an etched profile on pattern geometry. *Japanese Journal of Applied Physics* 32:6233–6236.

Vallet-Regi, M., Balas, F., and Arcos, D. 2007. Mesoporous materials for drug delivery. *Angewandte Chemie International Edition* 46:7548–7558.

Vertegel, A. A., Siegel, R. W., and Dordick, J. S. 2004. Silica nanoparticle size influences the structure and enzymatic activity of adsorbed lysozyme. *Langmuir* 20:6800–6807.

Wang, G., Hou, H., Wang, S., Yan, C., and Liu, Y. 2017. Exploring the interaction of silver nanoparticles with lysozyme: Binding behaviors and kinetics. *Colloids and Surfaces B: Biointerfaces* 157:138–145.

Wang, P., Wang, X., Wang, L., Hou, X., Liu, W., and Chen, C. 2015. Interaction of gold nanoparticles with proteins and cells. *Science and Technology of Advanced Materials* 16:034610.

Wojnicki, M., Luty-Błocho, M., Bednarski, M., Dudek, M., Knutelska, J., Sapa, J., Zygmunt, M., Nowak, G., and Fitzner, K. 2013. Tissue distribution of gold nanoparticles after single intravenous administration in mice. *Pharmacological Reports* 65:1033–1038.

Yang, P., Gai, S., and Lin, J. 2012. Functionalized mesoporous silica materials for controlled drug delivery. *Chemical Society Reviews* 41:3679–3698.

Zhang, J., Guo, Y., Fang, H., Jia, W., Li, H., Yang, L., and Kui, W. 2015. Cadmium sulfide quantum dots stabilized by aromatic amino acids for visible light-induced photocatalytic degradation of organic dyes. *New Journal of Chemistry* 39:6951–6957.

Zhang, L. W., Yu, W. W., Colvin, V. L., and Monteiro-Riviere, N. A. 2008. Biological interactions of quantum dot nanoparticles in skin and in human epidermal keratinocytes. *Toxicology and Applied Pharmacology* 228:200–211.

Zhang, X. Q., Xu, X., Bertrand, N., Pridgen, E., Swami, A., and Farokhzad, O. C. 2012. Interactions of nanomaterials and biological systems: Implications to personalized nanomedicine. *Advanced Drug Delivery Reviews* 64:1363–1384.

6

Effects of Engineered Nanoparticles on Bacteria

Changjian Xie, Xiao He, and Zhiyong Zhang

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6.1 Introduction

Nanomaterials (NMs) are increasingly used in many fields, ranging from medicine, to consumer products (Kim et al., 2007; Quadros and Marr, 2011), to environmental remediation owing to their unique physicochemical characteristics, such as small size, high reactivity, and large surface area, whereas their rapid and wide applications may increase the possible release into the environment and cause potential risks to the ecosystem. Although the toxicity and biocompatibility of NMs are currently at the center of attention, its potential risks to the ecosystem remain largely unexplored. Bacteria are ubiquitous in the ecosystem. Understanding the interactions of NMs with bacteria will promote the evaluation of environmental behavior of NMs. Moreover, some traditional control strategies for infectious disease caused by bacteria, such as metal ions and antibiotics, may lead to the increasing resistance of bacteria. Recent studies have found that various NMs, such as silver (Ag) nanoparticles (NPs) (Ivask et al., 2013), vanadium pentoxide NPs (Natalio et al., 2012), Fe_3O_4 NPs (Gao et al., 2007), MoS_2 nanoflowers (NFs) (Yin et al., 2016), and graphene-based NMs (Aruguete et al., 2013; Chen et al., 2014; Wu et al., 2013), have potential for defeating the drug-resistant bacteria. Therefore, a comprehensive understanding of the interactions of NMs with

bacteria and their potential positive/adverse effects in vivo play a crucial role in further development and safe use of NMs.

This review article provides an overview of the behavior, impacts, and toxic mechanism of NMs and their derivatives to bacteria and tries to reduce the scientific "blind spots" and knowledge gaps.

6.2 Carbon Nanomaterials

6.2.1 Bacterial Toxicity of Graphene-Based Nanomaterials

Graphene, a class of two-dimensional carbon NMs, consists of a single layer of sp^2-bonded carbon atoms arranged in a hexagonal crystal structure. Due to its unique properties, it has attracted great interest as a promising NM in a rapidly expanding range of applications, including energy, water treatment, nanoelectronics, nanosensors, and bioapplications. To realize its potential, health and environmental impacts of graphene-based NMs should be thoroughly evaluated. Generally speaking, the risks of ecological exposure to graphenes are determined by their chemical behavior, environmental fate, and ecological processes.

6.2.1.1 Cytotoxicity

Graphene is a promising candidate as an antibacterial material. In 2010, graphene oxide (GO) was introduced and exploration of its antibacterial properties began by studying the interaction of Gram-negative bacteria, *Escherichia coli* (*E. coli*) *DH5a* (Hu et al., 2010). They found that GO nanosheets (after 2 hours of incubation at 37°C and at a concentration of 85 mg/L) could remarkably suppress the growth of *E. coli:* cell metabolic activity decreased to ~13%, and the colony counting showed that cell viability loss was up to 98%. Compared with GO, reduced GO (rGO) nanosheets also exhibit high antibacterial effects. The metabolic activity of *E. coli DH5a* cells was reduced to ~24% at the same conditions, and the colony-forming unit (CFU) showed that less than 10% of cells survived.

Akhavan and Ghaderi (2010) found that GO nanowalls (GONWs) reduced by hydrazine (RGNWs) exhibited more antibacterial activity as compared to unreduced GONWs. To investigate the toxicity of GONWs and RGNWs, *E. coli* and *S. aureus* were used as models for Gram-negative and Gram-positive bacteria. They found that GONWs and particularly RGNWs exhibited considerable antibacterial activities. After 1 hour of incubation, 41 ± 8% and 16 ± 3% of the *E. coli* cells survived on the surface of GONWs and RGNWs, respectively. There also ran a similar study for *S. aureus* bacteria. But GONWs and RGNWs exhibited stronger antibacterial activities against *S. aureus* than *E. coli*. In fact, after 1 hour of incubation, only 26 ± 5% and 5 ± 1% of *S. aureus* survived on the surface of GONWs and RGNWs, respectively. They also found that the concentrations of RNA in the solutions of bacteria exposed to both GONWs and RGNWs were meaningfully higher than in the control sample. To better understand the graphene-based NM antibacterial mechanisms, Liu et al. (2011) compared the antibacterial activity of four types of graphene-based NMs with *E. coli*. It was found that GO dispersion showed the highest antibacterial activity, sequentially followed by rGO, graphite (Gt), and graphite oxide (GtO), in similar

incubation condition and concentrations. No superoxide anion ($O_2^{\cdot-}$)-induced reactive oxygen species (ROS) production was detected, but all of these materials could oxidize glutathione, which serves as redox state mediator in bacteria. The lateral dimension of the GO sheets also can be related to the antibacterial activity. Liu et al. (2012) found that larger GO sheets (average area of 0.753 μm^2) showed stronger antibacterial activity (the loss of viability of 97.7% ± 1.0%) than smaller ones (average area of 0.010 μm^2), which resulted in 45.5 ± 5.5% loss (they also had different concentration- and time-dependent antibacterial activities). The effects of GO and rGO on viability of *Pseudomonas aeruginosa* (*P. aeruginosa*) were also investigated. *P. aeruginosa* population decreased in a time- and dose-dependent manner. The exposure of cells to GO and rGO could induce the production of superoxide anion. Results also showed that the cells treated with GO for 24 hours showed laddering of DNA, but use of rGO did not result in any significant fragmentation (Gurunathan et al., 2012). Later, Pham et al. (2015) investigated the antimicrobial behavior of graphene sheet surfaces using both experimental and computer simulation approaches. In this study, graphene nanofilms were fabricated to exhibit different angles of orientation and edge length in the graphene sheets. It was proven that one of the principal parameters, which contributed to the antibacterial activity of the graphene sheet films, was the density of the edges of graphene. Both experimental and theoretical evidence has demonstrated that the antibacterial activity of graphene nanosheets arises from the formation of pores in the bacterial cell wall, causing a subsequent osmotic imbalance and cell death (Pham et al., 2015). Meanwhile, GO-based nanocomposites also attracted significant interest in many different areas, one of which was antibacterial agents. Tang and colleagues fabricated GO–Ag nanocomposites with different GO to Ag NP ratios, and carefully chose two model microorganisms, *E. coli* and *S. aureus*, to investigate the interaction between materials and bacteria. Results showed that the GO–Ag nanocomposite had a highly effective antibacterial activity at very low dosages (with a minimal inhibitory concentration (MIC) of 4 mg/L against *E. coli* and 14 mg/L against *S. aureus*). Interestingly, when compared to the MICs of two commonly used antibiotics, ampicillin (with a MIC of 25 mg/L against *E. coli* and ≤1 mg/L against *S. aureus*) and kanamycin (with a MIC of 6 mg/L against *E. coli* and 6 mg/L against *S. aureus*), the MIC of GO–Ag against *S. aureus* was higher than those in two antibiotics, whereas in the case of *E. coli*, the MIC of GO–Ag dropped to the lowest among the three. Unlike GO–Ag nanocomposites, the pure GO at the same concentration had no effect on the viabilities of both *E. coli* and *S. aureus* cells (Tang et al., 2013). Recently, several GO-based nanocomposites, such as GO–TiO_2, GO–Ag, and GO–dye, were also fabricated as antibacterial products expecting synergistic efficacy. Moreover, graphene functionalized with proteins and other amines specifically wrapped around bacteria (Meyer et al., 2007; Mohanty and Berry, 2008), but did not harm them. By comparison, poly (N-vinylcarbazole) polymer-functionalized GO was more effective in preventing bacterial communities compared to untreated GO (Santos et al., 2011). However, it is too early to draw a solid conclusion about the antibacterial activity of graphene-based NMs.

6.2.1.2 Lack of Cytotoxicity

Results mentioned in the last section seem to support the idea of graphene-based NMs as a promising antibacterial material. However, recent studies also indicate that

GO may lack antibacterial properties. A study by Ruiz et al. (2011) found that GO presented neither intrinsic antibacterial activities nor cytotoxicity properties to mammalian cells (human adenocarcinoma HT-29 cell). On the contrary, when GO was added to the bacterial culture at 25 mg/L, *E. coli* cells grew even faster than the control (the cultures without GO treatment). Scanning electron microscopy (SEM) and growth inhibition zones indicated that GO did not have intrinsic antibacterial and bacteriostatic activities, which means that GO acted as a nonspecific enhancer of cellular growth. And it is easy to find that, even though GO–Ag nanocomposites could effectively inhibit *E. coli* and *P. aeruginosa* growth, but GO alone showed no significant antibacterial effects against these bacteria. Dutta et al. (2015) reported that GO was not toxic but after aging it got a surface coating of rGO and became toxic, which meant that the antibacterial activities of rGO were much higher than those of GO due to the rGO^+ intermediate reacting with a hydroxyl ion to produce GO as a coating on the surface of rGO, resulting in enhanced fluorescence and a slowdown in photoinduced ROS formation. According to Chen et al. (2014), GO nanosheets act as a membrane scaffold which can enhance and proliferate a series of gut microorganisms. Recently, Perreault et al. (2015) studied the antibacterial activity of GO as a function of its size using *E. coli* as a model microorganism. In this work, GO suspensions of average sheet area ranging from 0.01 to 0.65 μm^2 were produced and its antibacterial activity was evaluated in cell suspensions or as a model GO surface coating. As reviewed before, according to the results of this work, antibacterial activity of GO surface coating increased fourfold when the GO sheet area decreased from 0.65 to 0.01 μm^2. In contrast, GO interacted with *E. coli* in a cell entrapment mechanism in suspension assays. In this case, the antibacterial effect of GO increased with increasing sheet area, with apparent complete inactivation observed for the most area to the least area after a 3-hour exposure incubation period. However, when separated from GO sheets by mild sonication, *E. coli* cell inactivation by GO entrapment was reversible and all initially viable cells could be recovered and, rather, can be described as bacteriostatic. A recent study also found that GO significantly enhanced cell growth, biofilm formation, and biofilm development at 500 mg/L, whereas significantly inhibited cell growth and biofilm formation were discovered under rGO exposure (Guo et al., 2016).

For these controversial results, many factors may influence the antibacterial activity of graphene-based NMs, including material properties, synthetic methods, sizes, crystal structures, and surface treatments, as well as the conditions of interactions between graphene-based NMs and bacterial cells, such as incubation time, concentration, medium, and light source, which also could affect the biological behavior of cells. Detailed mechanisms and reliability of assay methods will be reviewed in the next sections to understand the interactions between graphene-based NMs and bacteria.

6.3 Bacterial Toxicity of Other Carbon Nanomaterials

Engineered carbon-based nanomaterials include graphene-based NMs, carbon nanotubes (CNTs), and fullerenes. Herein, we would describe the antibacterial effects of CNTs (single-walled nanotubes [SWNTs] and multiwalled carbon nanotubes [MWNTs]) and fullerene NMs. The major works about antibacterial effects of carbon NMs are also summarized in Table 6.1.

TABLE 6.1

Antibacterial Properties of Carbon-Based Nanomaterials

Nanomaterial	Microorganism Tested	Size	Main Findings	Reference
n-C_{60}	*B. subtilis*	Small aggregates (~2 nm) and large aggregates (97–140 nm)	MIC values ranging from 0.5 to 0.95 mg/L; membrane disruption caused by the generation of ROS	Lyon et al. (2006)
n-C_{60}	*E. coli* K12 *B. subtilis* 168	N/A	Protein oxidation, cell membrane potential, direct contact	Lyon et al. (2008)
n-C_{60}	*E. coli* *B. subtilis*	43.8 nm	Antioxidant capacity; no toxicity	Xia et al. (2010)
n-C_{60}	*E. coli* B23	N/A	Antibacterial effect; no damage to the cellular membrane structure	Aquino et al. (2010)
C_{60} derivative	*E. coli* W3310 *S. oneidensis* MR-1	C_{60}, C_{60}–OH, C_{60}–COOH, and C_{60}–NH_2; the sizes were N/A	Positively charged C_{60}–NH_2 inhibited growth of both cells; microbial central metabolism had not been effected; significant differences between control and treatments	Tang et al. (2007)
C_{60} derivative	*E. coli*	N/A	ROS inhibited the respiratory chain at a concentration of 5 μmol/L	Nakamura and Mashino (2009)
SWCNTs	*E. coli*	Tube diameters of 0.75–1.2 nm	Loss of viability was dose independent (1~50 mg/L); membrane damage by direct contact	Kang et al. (2007)
SWCNTs	*E. coli* K12	Average outer diameter 1.2 nm, lengths: 10–20 μm, specific surface area: 407 m^2/g	SWNTs in contact with cells prior to biofilm maturation inhibited their growth; EPS mitigated the toxic effects of SWNTs	Rodrigues and Elimelech (2010)
SWCNTs/ MWCNTs	*E. coli*	SWCNTs diameter of 1.2 nm and length of 17.8 nm MWCNT diameter of 17 nm and length of 91 nm	91% of *E. coli* inactivation after being exposed to a SWCNT/ MWCNT filter	Brady-Estévez et al. (2008)

(*Continued*)

TABLE 6.1 (CONTINUED)

Antibacterial Properties of Carbon-Based Nanomaterials

Nanomaterial	Microorganism Tested	Size	Main Findings	Reference
SWCNTs/ MWCNTs	*E. coli*	SWCNT diameter of 0.9 nm and length of 2 nm MWCNT diameter of 30 nm and length of 70 nm	SWCNTs 80% of *E. coli* inactivation and MWCNTs 24% of *E. coli* inactivation; membrane damage by direct contact of CNT	Kang et al. (2008)
MWCNTs	*E. coli*	Water-soluble MWCNTs, the size was N/A	No toxicity of NMs on *E. coli* colonies	Roy et al. (2012)
Gt, GO	*E. coli, S. aureus*	N/A	Cell membrane damage, the better antibacterial effects of rGO due to the better charge transfer between the bacteria and sharpened edges of rGO	Akhavan and Ghaderi (2010)
Gt, GtO, GO, rGO	*E. coli* K12	Gt, GtO, GO, rGO: DLS dispersions are 5.25, 4.42, 0.56, and 2.93 μm, respectively SEM results are 6.87 ± 3.12, 6.28 ± 2.50, 0.31 ± 0.20, and 2.75 ± 1.18 μm	GO dispersion showed the highest antibacterial effects, sequentially followed by rGO, Gt, and GtO; 92% of loss of viability at 80 mg/L and 10.5% at 5 mg/L; direct contact with graphene nanosheets disrupted cell membrane; superoxide anion-induced ROS production was not detected	Liu et al. (2011)
GO, rGO	*E. coli*	The thickness of GO sheets was ~1.1 nm, rGO was ~1 nm The thickness of GO was ~1.5 μm, rGO paper was ~4.6 μm	GO and rGO could effectively inhibit the growth of *E. coli*, but not A549 cells; GO and rGO paper can be mass-produced with low cost	Hu et al. (2010)
GO	*E. coli* *S. typhimurium,* *B. subtilis* *E. faecalis*	N/A	MICs: 1 mg/L *E. coli*, *S. typhimurium* 4 mg/L *B. subtilis*, and 8 MPT/L *E. faecalis*; lipid peroxidation involvement of ROS in antibacterial properties	Krishnamoorthy et al. (2012)

(*Continued*)

TABLE 6.1 (CONTINUED)

Antibacterial Properties of Carbon-Based Nanomaterials

Nanomaterial	Microorganism Tested	Size	Main Findings	Reference
GO	*E. coli* K12	GO-0, GO-10, GO-30, GO-50, GO-120, and GO-240 sample sizes are 0.753, 0.127, 0.065, 0.035, 0.013, and 0.010 μm^2	The antibacterial effects toward *E. coli* cells was laterally size dependent; lager GO showed stronger antibacterial effects than smaller ones and have different time- and concentration-dependent antibacterial effects; large GO easily covered cells and cells could not proliferate; cell viability loss observed in CFU test	Liu et al. (2012)
GO, rGO	*P. aeruginosa*	DLS: the size of GO was 0.525 μm and that of rGO was 3.4 μm	The exposure of cells to GO and rGO induced the production of superoxide radical anions and loss of cell viability; oxidative stress, DNA fragmentation	Gurunathan et al. (2012)
GO, rGO	*E. coli*	DLS: the size of GO was 0.479 μm and that of rGO was 2.9 μm	GO and rGO also have concentration- and time-dependent antibacterial effects; GO has higher antibacterial effects than rGO; GO produced more superoxide anions than rGO; oxidative stress DNA fragmentation	Gurunathan et al. (2013)
rGO-Ag	*E. coli* *S. aureus*	N/A	With an optimal ratio of Ag NPs to GO, the composites would be more effective and show synergistically enhanced, strong antibacterial effects at 5 mg/L; species-specific mechanisms	Tang et al. (2013)
GO	*E. coli* K12	Sonicated at high intensity for different periods: 0.65, 0.29, 0.1, and 0.01 μm^2	With the decrease in size, the antibacterial effects were enhanced; in contrast, the antibacterial effects of GO increased with sheet area, but cell inactivation by GO entrapment was reversible and all initially viable cells could be recovered when separated from GO sheets by sonication	Perreault et al. (2015)

(*Continued*)

TABLE 6.1 (CONTINUED)

Antibacterial Properties of Carbon-Based Nanomaterials

Nanomaterial	Microorganism Tested	Size	Main Findings	Reference
Gt	*P. aeruginosa* *S. aureus*	The length of edge: GT: N/A GN-R: 137.3 ± 93.9 nm GN-S: 79.7 ± 56.7 nm	The density of the edges of graphene was one of the principal parameters that contributed to the antibacterial effects of graphene nanofilms; both experimental and theoretical evidence that the antibacterial effects of material arose from the formation of pores in the cell wall, which caused a subsequent osmotic imbalance and cell death	Pham et al. (2015)
GO, rGO	*Enterobacter* sp.	N/A	rGO generates ROS under visible light and kills the cells; rGO^{+} intermediate reacted with OH– to produce GO coating on the surface of rGO, which could enhance the fluorescence and a slowdown in photoinduced ROS formation. GO is not toxic.	Dutta et al. (2015)
GO	*E. coli*	N/A	GO acted as a nonspecific enhancer of cellular growth at 25 mg/L; no biocide effect; increasing cell attachment and proliferation	Ruiz et al. (2011)

Carbon nanotubes can be classified into SWCNTs and MWCNTs by the number of graphitic-rolled sheets. Direct evidence was first provided stating that highly purified SWNTs exhibited strong antibacterial effects (Kang et al., 2007). However, with doses of SWCNTs ranging from 1 to 50 mg/L, the loss of viability of *E. coli* cells was independent of the material dose. As incubation time is extended, the inactivation rates of cells exposed to SWCNTs for 120 minutes could reach 88%. Recently, the same group found that SWCNTs were more toxic to *E. coli*, *P. aeruginosa*, *Bacillus subtilis* (*B. subtilis*), and *Staphylococcus epidermis* (*S. epidermis*) than MWCNTs and fullerenes (Kang et al., 2009). SWCNTs aggregated at a concentration of 5 mg/L and caused the inactivated 80% of *E. coli* cells; however, with the treatment of MWCNTs, cell viability remained at 76%.

Rodrigues and Elimelech (2010) studied the impact of SWCNT concentration on cell growth and biofilm formation and the toxic effects of SWCNT on mature biofilms. The results showed that SWCNTs first came into contact with *E. coli* cells prior to biofilm formation and inhibited their growth. After treatment by SWCNTs, the control was less sensitive to the presence of material. Finally, they found that soluble exopolymeric substances secreted by the *E. coli* biofilm migrated the toxic activities of SWCNTs.

Since the discovery of fullerenes in 1985, this third allotrope of carbon has become a promising compound for a large number of applications, but also has increased the risks of environmental safety. With a solubility of less than 10^{-9} mg/L, powdered C_{60} is virtually insoluble in water, and studies examining powder alone did not find any antimicrobial effects (Lyon et al., 2005). Fullerenes may become soluble following chemical modifications (Li et al., 2008). C_{60} molecules can be made to aggregate and form stable fullerene water suspensions whose properties differ from those of bulk solid C_{60}. Lyon et al. (2008) explored four of these methods and established their antibacterial effects. They found that the toxicity of C_{60} depended on the fullerene derivative and that nC_{60} showed higher antibacterial effects than did others against *B. subtilis*. Tetrahydrofuran (THF)/nC_{60} samples had the lowest MIC value of 0.09 mg/L. Afterward, Lyon and his colleagues studied the THF/nC_{60} mechanism of antimicrobial effects toward *E. coli* and found that the mechanism was not ROS-mediated damage.

As for the n-C_{60} derivatives, Tang and coworkers studied the interaction of four types of n-C_{60} (C_{60}, C_{60}–OH, C_{60}–COOH, and C_{60}–NH_2) with two model bacteria (*E. coli* W3310 and *S. oneidensis* MR-1). They found that at concentrations as low as 10 mg/L of treatment, C_{60}–NH_2 could inhibit both bacterial cell growth and reduced substrate uptake. Neutrally charged C_{60}–OH and C_{60} had mild negative effects on *S. oneidensis* MR-1; however, the negatively charged C_{60}–COOH did not affect the growth of either *E. coli* or *S. oneidensis* cells. To further investigate the effects of fullerene compounds with bacterial global metabolism, labeled [3-^{13}C] L-lactate was used to track perturbations to metabolic reaction rates in bacteria. The results showed that all fullerene-exposed cultures revealed no significant differences compared to unstressed cells, which meant that microbial central metabolism was robust to environmental stress inflicted by n-C_{60}. Both *S. oneidensis* MR-1 and *E. coli* W3110 can efficiently alleviate such stress by precipitating toxic NPs and/or cell aggregation, despite the C_{60}–NH_2 compounds causing mechanical stress on the cell wall and/or membrane. Fullerene NPs do not seem to strongly influence the central metabolism in bacteria in this work, whereas fullerene NPs with positive charges inhibited the

growth obviously. Practically, this was the first time to explore the effect by NPs on microbial metabolism (Tang et al., 2007).

However, the previous study found that the trace amount volume of THF in the n-C_{60} toxic effect study was responsible for the cytotoxicity (Isakovic et al., 2006). Xia et al. (2010) also found that the observed toxicity might be due to the THF solvent or its oxidative by-products in n-C_{60} preparations from previous studies and reported a novel method for preparing n-C_{60} without using THF as the solvent. In these experiments, no toxicity was observed for *E. coli* or *B. subtilis* at up to 342 mg/L for 16 hours of incubation time. Even though some studies had concluded that residual dissolved THF was not an influential factor in the toxicity of nC_{60} to zebrafish embryos (Zhu et al., 2007) and *Daphnia* (Lovern and Klaper, 2006), these conclusions could not be directly applied to bacteria.

6.4 Cytotoxicity Mechanisms

Few current cytotoxicity studies on graphene-based NMs reveal similarities between graphene-based NMs and other synthetic carbon NMs. The antibacterial activity of CNTs has been found to be the synergy of both "physical" and "chemical" effects, and CNTs can be regarded as rolled graphene (Geim and Novoselov, 2007).

Earlier studies have proposed that physical interactions between GO sheets and bacterial cells may be attributed to GO-induced membrane damage. Hu et al. (2010) prepared macroscopic freestanding GO and rGO papers with strong antibacterial effects. Transmission electron microscope (TEM) images revealed that antimicrobial effects were involved in cell membrane damage induced by GO, which led to leakage of the cytoplasm. Similar to the work of Hu, Akhavan and Ghaderi (2010) found that GO nanowalls could cause membrane damage of bacteria by direct contact with very sharp edges of GO nanowalls. Due to a better charge transfer ability and more sharpened edges between rGO nanowalls and bacteria, rGO nanowalls displayed stronger antimicrobial effects than GO did. The sharp, atomically thin edges of graphene sheets induce a spontaneous piercing of the membrane (Akhavan and Ghaderi, 2010).

The same as CNTs and n-C_{60} NMs, Kang et al. (2008) provided evidence that the size (diameter) of CNTs is a key factor governing their antimicrobial effects. Direct contact with CNTs, which caused the cell membrane damage, was likely the main CNT cytotoxicity mechanism. Tang et al. (2007) revealed that C_{60}–NH_2 caused higher antibacterial effects than the other C_{60} NMs, which could be explained by the strong adsorption of positively charged NPs interacting with the negative charges on the bacterial surface, hence the enhanced interaction of the NPs with the cell surface. This is also an example of cytotoxicity caused by direct contact with C_{60} NMs. A similar antibacterial mechanism of n-C_{60} was also found by Lyon and Alvarez (2008). In this work, they proposed that the mechanism required direct contact between the NPs and the bacterial cell and it was different from previously reported NM antibacterial mechanisms involving ROS generation.

Subsequently, Kang et al. (2009) elevated the concentration of NOM to reduce the attachment of bacteria on SWNT aggregates, but that did not mitigate the toxicity toward attached cells, which supported that the cytotoxic mechanism required direct contact between the NPs and the bacterial cell walls. Of late, Su et al. (2013) stated

that reactive species mainly arose through direct contact of Ag–MWCNTs with bacteria. They found that the antibacterial property of Ag–MWCNTs was not caused by silver ion dissolution, but OH_b, OHs, and H^+ play important roles in bactericidal actions.

Recently, Tu et al. (2013) found a novel mechanism for the antibacterial activities of graphene, i.e., a devastative extraction of phospholipids from *E. coli* cell membranes. During a 2.5-hour incubation process of *E. coli* with 100 mg/L GO nanosheets, roughly three stages (stages I, II, and III) of *E. coli* cell morphology were observed by TEM. In the first stage (initial morphology), *E. coli* cells were tolerant to GO for a short period of time (under low concentrations); in the second stage, the cell membranes partially lost integrity, with some bacteria showing a lower density of surface phospholipids; and in the last stage, the cell membranes were at a complete loss of membrane integrity and some were even entirely missing their cytoplasm. The results of these three distinguishable modes were confirmed by subsequent molecular dynamic simulation. First is the swing mode: the graphene nanosheet swung back and forth around the restrained atom for 10–100 for 10^{-9} s. Second is the insertion mode: the edge of the nanosheet was wrapped by cells and then the graphene nanosheet was cut into cell membranes for a few 10^{-9} s due to robust van der Waals attractions from the hydrophobic interactions and membrane lipids. Last but not the least is the extraction mode: the nanosheet drew the phospholipid molecules vigorously from the lipid bilayers onto the material's surface. These findings suggest that both graphene insertion/cutting and destructive lipid extraction lead to serious membrane stress, thus decreasing cell viability (Tu et al., 2013). However, some studies have proved that availability of the basal planes, other than sheet edges, determines the antibacterial properties of GO (áde Leon, 2015; Hui et al., 2014). In Langmuir–Blodgett films, where the sheets are totally flat on the surface and minimal cell contact with the sheet edges occurs, GO was still found to inactivate bacterial cells coming in contact with the material surface. These survey results showed that contact with the sheet edges, and thus direct piercing of the membrane by GO sheets, is not required for the antimicrobial activity of GO. Consequently, other mechanisms related to the physicochemical interactions between GO sheets and bacterial cells may play a more important role in the antibacterial effect of GO sheets (áde Leon, 2015).

Generally speaking, there have been two major paths by graphene-based NMs to mediate oxidative stress. One possible path is ROS-independent oxidative stress, where graphene-based NMs may disrupt a specific bacterial process by oxidizing or perturbing a vital cellular component or its structure without ROS production. The other is ROS-mediated oxidative stress, where graphene-based NMs could generate ROS, which induced the oxidative stress. As for the former, some researchers have used GSH oxidation to examine the possibility of its antibacterial mechanism. GSH is an antioxidant in bacteria at the concentration range from 0.1 to 10 mM, which could prevent damages to cellular components caused by oxidative stress (Fahey et al., 1978). And for the latter, as previously described, researchers measured the possibility of superoxide anion ($O_2{\cdot}^-$) production. Liu et al. (2011), based on 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl) -2H-tetrazolium-5-carboxanilide to indicate some $O_2{\cdot}^-$, which could induce ROS generated, concluded a minor role in the antimicrobial effects of graphene-based NMs. ROS typically also include hydroxyl radical (OH) and hydrogen peroxide (H_2O_2), which could harm cellular components, including DNA and proteins.

Several studies have also proposed a dominating role of oxidative stress in the antimicrobial activity of GO. Oxidative stress in bacterial cells leads to lipid peroxidation, oxidation of proteins, and damage on nucleic acid molecules and thus can ultimately result in membrane damage and cell death. In bacterial cells exposed to GO, oxidative stress was indicated by dichlorofluorescein and nitro blue tetrazolium assays, which revealed the intracellular accumulation of free radicals and superoxide anions (Chen et al., 2013; Gurunathan et al., 2012). For instance, Gurunathan et al. (2012) reported that GO and rGO showed a dose-dependent antimicrobial effect against *P. aeruginosa* cells through the generation of ROS, leading to death *of P. aeruginosa* cells, which was further confirmed through the resulting nuclear fragmentation.

Similar to previous studies, Cho et al. (2009) used a novel method to prepare a C_{60} derivative via excess ozonation in water. Ozonated treated C_{60} caused neither significant growth inhibition nor inactivation of *E. coli* cells in the absence of oxygen or light. However, it readily inactivated *E. coli* cells in the presence of both O_2 and light at a much faster rate than parent C_{60} aggregates, suggesting involvement of photochemical generation of ROS. According to TEM results, subsequent photochemical production of hydroxyl radical within the cell cytoplasm and the little surface protein released and concurrent intercellular enzyme degradation during the course of *E. coli* inactivation suggested that oxidative transformation of C_{60} can lead to a significant increase in the cytotoxicity of *E. coli* cells.

Lyon and Alvarez (2008) proposed that n-C_{60} exerted ROS-independent oxidative stress in *E. coli* and *B. subtilis* cells, with evidence of protein oxidation, which changed the cell membrane potential and interrupted cellular respiration. The results revealed that n-C_{60} interfered with electron transport proteins and membrane proteins, which might be oxidized after exposure to n-C_{60} and perturb the bacterial cell respiration functions.

Liu et al. (2011) reported the antibacterial activity of GO against *E. coli* cells. In SEM images, it was shown that GO caused irreversible damages to the cells and bacteria were found wrapped into thin GO layers. There was no significant superoxide anion detected, but the four types of materials can oxidize glutathione, which serves as redox state mediator in *E. coli* cells (glutathione is a thiol-rich tripeptide serving as one antioxidant enzyme used as indicator of the oxidative potential of NMs). Conductive rGO and Gt have higher oxidation capacities than insulating GO and GtO. Therefore, the antibacterial mechanism could be assigned to both membrane oxidative stress and the direct contact of graphene-based materials with bacterial cells, resulting in the release of intracellular material and consequent inactivation of the cells and oxidation stress. Antibacterial activity also can be related to the lateral dimension of the GO sheets, as reported by the same group (Liu et al., 2012). The possible mechanism involves the ability of larger sheets in covering cells more easily, causing the inactivation of the cell by the blockage of active sites on membranes, and cells cannot proliferate once fully covered, resulting in cell viability loss observed in the followed colony counting test. However, the opposite result was observed by Perreault et al. (2015), where the higher antibacterial activity of smaller GO sheets was attributed to oxidative mechanisms associated with the higher defect density of smaller sheets on a model GO surface coating.

Krishnamoorthy and coworkers found that antibacterial activity of graphene nanosheets against pathogenic bacteria happened via lipid peroxidation. The results also revealed that Gram-negative bacteria (*E. coli* and *S. typhimurium*) were more

sensitive because of the thinner peptidoglycan layer of Gram-negative strains (7–8 nm) as compared to Gram-positive bacterium (20–80 nm) (Krishnamoorthy et al., 2012). However, the reverse was observed by Akhavan and Ghaderi (2010), where increased resistance of *E. coli* was attributed to the Gram-negative outer membrane that is lacking in Gram-positive organisms such as *S. aureus*. Therefore, the role of the cell wall structure in minimizing graphene-based NM toxicity is unclear. These declare that antibacterial activity of graphene-based NMs is bacterial species-dependent rather than Gram-dependent.

Fang et al. (2007) investigated the effect of C_{60} on bacterial membrane phase behavior and lipid composition. The results showed that the response in membrane phase behavior and lipid composition depends on both the n-C_{60} concentration and cell wall morphology. This is the first investigation of how bacteria respond physiologically to the presence of an engineered NM.

A recent study found the GO–Ag nanocomposite to be a highly effective antibacterial agent and showed its functions as disrupting the bacterial cell wall integrity of Gram-negative *E. coli* and exhibiting a bacteriostatic effect on Gram-positive *S. aureus* by dramatically inhibiting cell division (Tang et al., 2013). To elucidate the mechanism of cell death induced by graphene-based NMs, the DNA fragmentation assay is a good indicator of cellular dysfunction. Recently, Gurunathan and coworkers used GO and rGO incubated with *E. coli* cells for a specific incubation time, and the results exhibited a remarkable effect of DNA fragmentation, which may be due to that the *E. coli* cells needed a longer incubation time exposure of graphene-based NMs to generate ROS, which then led to DNA fragmentation (Gurunathan et al., 2012).

However, some studies also have questioned the antimicrobial and bacteriostatic properties of graphene-based NMs and suggest that GO could act as a scaffold for *E. coli* bacterial attachment and play an enhancer role in bacterial proliferation and biofilm formation (Ruiz et al., 2011). Luria-Bertani (LB) contains a great deal of proteins, which could be adsorbed by GO. On the one hand, the adsorption of proteins could inhibit the antibacterial activity of GO. On the other hand, owing to the adsorption of proteins on GO, the scaffold provides sufficient nutrients that could enhance total cell growth. Compared to rGO, GO possessed higher adsorption ability of proteins that caused lower antibacterial effects (Wei et al., 2015) or even positive effects (Zou et al., 2016).

Obviously, much more careful work needs to be carried out to uncover the detailed mechanisms and controlling factors regarding the interactions between graphene-based NMs and bacteria.

6.5 Cytotoxicity of Metal-Based Nanomaterials

Researchers found that antibacterial effects of ZnO NPs were due to a reaction of the zinc oxide surface with water (Applerot et al., 2009). In this work, ZnO NPs were synthesized using ultrasonic irradiation and NP size was controlled by using different solvents in the sonication process. New findings from this study indicated that the amount of eradicated *E. coli* and *S. aureus* was strongly dependent on the particle size. Smaller NPs had more efficient antibacterial effects, which correlated with the fact that more cellular internalization was observed using the smaller NPs (data not shown).

However, few reports mentioned about the bacterial killing effect of different shapes of TiO_2 nanostructures. Wadhwa (2011) explored TiO_2 nanosphere, nanorod and nanotube, and photocatalytic antibacterial properties with respect to killing of prokaryotic Gram-negative bacterium *Agrobacterium tumefaciens* LBA4404 (*A. tumefaciens*). The TiO_2 nanotube and nanorod showed a higher antibacterial effect than spherical-shaped NPs. Tayel et al. (2011) investigated the antibacterial action of NPs and bulk ZnO particles against nine bacterial strains and found that ZnO NPs were more efficient as antibacterial agents than bulk ZnO, and Gram-positive bacteria were more sensitive to ZnO than Gram-negative ones. They also found a statistically significant concentration-dependent decrease in *E. coli* cell viability by two independent methods (CFU and flow cytometric live–dead discrimination assay), and this trend was also observed in ROS, LDH, and MDA assays. Tayel et al. (2011) demonstrated that ZnO and TiO_2 NPs induced oxidative stress leading to genotoxicity (DNA and nuclear fragmentation) in *E. coli*, but it was not found by Liu et al. (2009). In a recent study, Kim and An (2012) evaluated the effects of TiO_2 and ZnO NPs preilluminated with ultraviolet light on *B. subtilis* and *E. coli*. Results showed that ZnO NPs were more toxic when preilluminated with UVA or UVB lights than with visible light regarding both growth inhibition and CFU assays, but neither UVA nor UVB preillumination of TiO_2 NPs caused toxicity to the same bacteria. However, Dasari et al. (2013) examined four types of metal oxide NPs exposed to *E. coli*, and results showed that TiO_2 NPs had the greatest difference between the light and dark exposure groups in terms of reducing viability. Compared to the dark exposure groups, *E. coli* bacteria suffered more toxicity in the presence of light. Meanwhile, the antibacterial effects and antibiofilm activity have been focused on metal and metal oxide nanocomposites. Karunakaran et al. (2010) prepared Cu-doped TiO_2 NPs for photocatalytic disinfection of bacteria under visible light. Results showed that the composite NPs very efficiently catalyze the disinfection of *E. coli*. Gholap et al. (2013) synthesized the CdTe–TiO_2 nanocomposite as an impeder of bacterial growth and biofilm. Compared to the control, the treatment showed matrix-covered cells and continuous fluorescence, typical of biofilm formation. In experiments with *B. subtilis* and *E. coli*, the enhancement in antibacterial effect by the CdTe–TiO_2 nanocomposite was concentration- and time-dependent. However, previous work found that Cu-doped TiO_2 NPs enhanced survival of *S. oneidensis* MR-1 under UV light (Wu et al., 2011). A recent study on binary mixtures of TiO_2 and ZnO and CeO_2 NPs to *N. europaera* indicated that the mixed toxicity of CeO_2 and TiO_2 NPs was lower than its single NPs. However, in the CeO_2 and ZnO NP mixture, the binary toxicity was enhanced to exert a higher cytotoxicity than single NPs (Yu et al., 2016). Due to the high near-infrared absorption and peroxidase-like activity, polyethylene glycol-functionalized molybdenum disulfide NFs (PEG-MoS_2 NFs) provided a rapid and efficient killing outcome in vitro for *B. subtilis* and *E. coli*, when compared to the traditional strategies (Yin et al., 2016).

6.6 Cytotoxicity Mechanisms Depending on the Kind of Cell, the Medium, and the NPs

The toxic effect of metal- and metal oxide-based NPs can potentially involve at least five distinct mechanisms. Originally, NPs or their surface may interact directly with biological objects and disrupt the cell membrane or cause DNA and/or nuclear

fragmentation. Afterward, the impact by the morphology of NPs may cause a significant toxicity toward bacteria. Furthermore, toxic derivatives may be released into the media from NPs, e.g., free Zn^{2+} from ZnO NPs and Ag^{+} ions from Ag NPs. Many studies tried to elucidate the influence of water chemistry on antibacterial and bioactivity of NPs. Similarly, surface interactions with media may also produce toxic substances, e.g., chemical radicals or ROS. Last but not least, either chemically or biologically could alter NP toxicity and behavior in the environment, namely, transformation of NPs, and recent studies also focused on this field. The possible antibacterial mechanisms are illustrated in Figure 6.1. The major works about antibacterial effects of metal- and metal oxide-based NMs are also summarized in Table 6.2.

It was proposed that NMs that can physically attach to bacteria would be bactericidal if they come into contact with bacteria. Huang et al. (2008) confirmed that both Gram-positive and Gram-negative cells were damaged after ZnO NPs contacted with them, both showing membrane disorganization. They also found the cellular internalization of these NPs, and there was a huge change in the crystal structure of ZnO NPs. Later studies also showed that antibacterial activity in the liquid phase revealed that cell death occurred due to cell membrane damage (Tam et al., 2008).

Liu et al. (2009) indicated that direct contact with ZnO NPs might distort and damage bacterial cell membrane resulting in a leakage of intracellular contents and eventually the death of bacterial cells. Raman results revealed that there was no significant change in nucleic acid bands. It was shown that the reduction of the charge on the surfaces of both cells and NPs (Bae et al., 2003; Hong and Brown, 2008; Pagnout et al., 2012), which would decrease the possibility of direct contact with NPs and bacterial cell, reducing the antibacterial effect of NPs. Padmavathy and Vijayaraghavan (2016) studied the antibacterial effect of ZnO NP suspensions with various particle sizes using a standard bacterial method and found that cell wall rupture must be due to the surface activity of those ZnO NPs that were in contact with *E. coli*. The damage to the cell membrane directly led to the leakage of proteins, minerals, and genetic materials, which eventually caused cell death. Dalai et al. (2012) studied the comparative cytotoxicity of TiO_2 NPs under light and dark at low exposure concentrations, which revealed that the dominant cytotoxic mechanism under light was ROS-dependent, while that under dark the direct contact of NPs to the cell membrane caused membrane damage significantly. According to Gogniat et al. (2006), the direct contact of TiO_2 NPs with bacteria played a crucial role in bacterial photoinactivation. However, a previous study used Seine River water as a natural environmental medium to find the antibacterial effect of three types of manufactured TiO_2 NPs toward the model bacterium *E. coli*. Results showed that the presence of the anatase polymorph slightly increased the toxicity in comparison to pure rutile samples, and NPs were poorly adsorbed as aggregates onto bacterial surfaces at pH 8, which meant that toxicity existed without a direct contact and even under the aggregated state (Planchon et al., 2013).

Even though a few studies have surveyed the relationship between NP morphology and toxicity, these investigations do show evidence that morphology alters NP cytotoxicity. Pal et al. gave evidence that triangular-shaped Ag NPs displayed stronger antibacterial activity than rod and sphere Ag NPs (Pal et al., 2007). Tam et al. (2008) investigated antibacterial effects by the different concentrations of ZnO nanorods, NPs, and powder. Results showed that ZnO exhibited antibacterial effects against both *E. coli* and *Bacillus atrophaeus*, which was considerably more effective in the

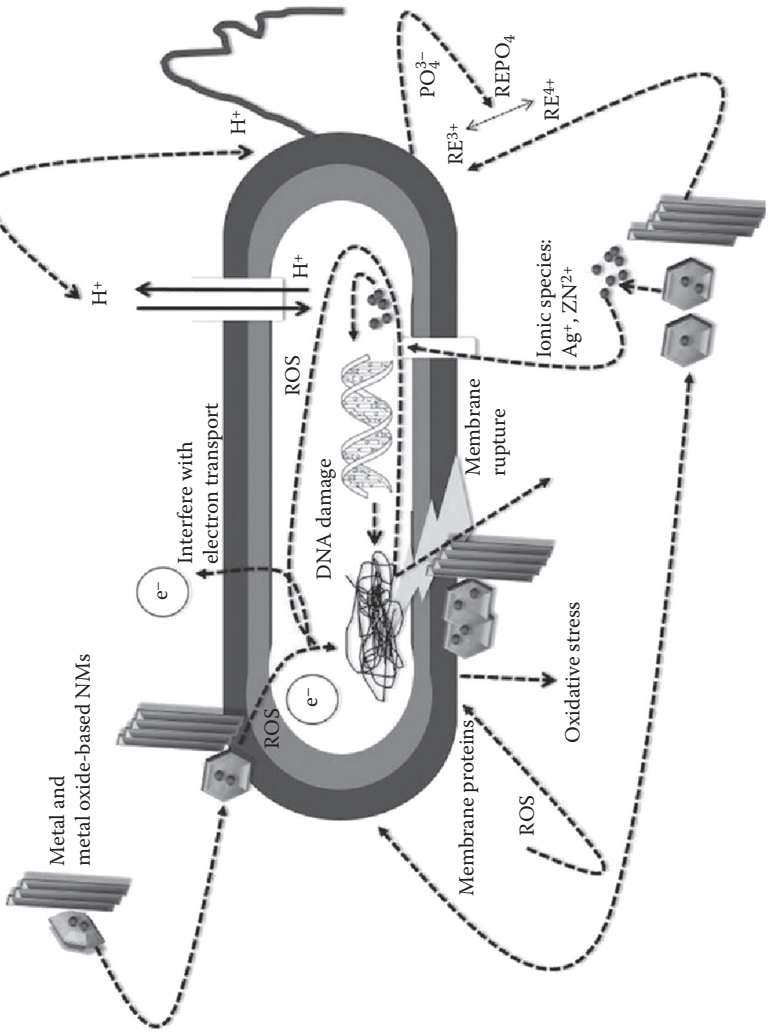


FIGURE 6.1 Mechanism of interaction of metal and metal oxide NPs with bacteria.

TABLE 6.2

Antibacterial Properties of Metal and Metal Oxide Nanomaterials

Material	Microorganism Tested	Size	Main Findings	References
ZnO	*E. coli DH 5α*	67, 820, and 44,000 nm	(48 ± 7.7) % growth inhibition at 1000 mg/L	Adams et al. (2006)
ZnO	*E. coli*	10.8 ± 2.2 nm ZnO 7.7 ± 0.9 nm ZnO–BSA 14.0 ± 0.9 nm ZnO–TOPO 11.7 ± 2.5 nm ZnO–SDS 11.7 ± 1.4 nm ZnO–Brij-76	A concentration between 10^{-2} and 3.0×10^{-3} M caused 100% inhibition of bacterial growth; *E. coli* cells after contact with DEG and ZnO were damaged show a Gram-negative triple membrane disorganization	Brayner et al. (2006)
ZnO	*S. agalactiae* *S. aureus*	Tween 20: spherical with a narrow size 150 nm SDS: irregular sheet-like organized PVA: nanorod 100 nm	Cells after contact with ZnO NPs were damaged, and the cellular internalization of these NPs; the crystal structure of ZnO NPs was changed greatly	Huang et al. (2008)
ZnO	*E. coli* *S. aureus*	Spherical 350 nm and rod shaped	The antibacterial effect toward *E. coli* increased as the particle size decreased from micro to nano size; the antibacterial action was lesser on *S. aureus* than *E. coli*	Nair et al. (2009)
ZnO	*E. coli* *S. aureus*	6.8 ± 2 nm 260 ± 40 nm 800 ± 300 nm	Small ZnO NPs produced increased levels of ROS, namely, OH·, and resulted in an increased cellular internalization of the NPs and damage of bacterial cell	Applerot et al. (2009)
ZnO	*E. coli* *S. aureus*	12, 25, 30, 88, 142, 212, and 307 nm	Smaller NPs induced higher antibacterial effects than larger ones under normal light; ROS-independent cell death	Raghupathi et al. (2011)
ZnO	*E. coli*	12 nm 45 nm 2000 nm	The bactericidal efficacy of ZnO NPs increased with decreasing particle size; cell wall ruptured; leakage of minerals, proteins, and genetic materials, causing cell death	Padmavathy and Vijayaraghavan (2016)
TiO_2	*E. coli K12* *B. subtilis*	Eight different sizes of TiO_2 NPs (5.2–120.1 nm)	Photocatalytic ROS production. TiO_2 NPs induced lipid peroxidation and cellular respiration disruption	Erdem et al. (2015b)

(*Continued*)

TABLE 6.2 (CONTINUED)

Antibacterial Properties of Metal and Metal Oxide Nanomaterials

Material	Microorganism Tested	Size	Main Findings	References
ZnO, CuO, Co_3O_4, and TiO_2	*E. coli*	ZnO 47–106 nm TiO_2 17–64 nm CuO 17–45 nm Co_3O_4 51–132 nm	LC_{50} values: ZnO < CuO < Co_3O_4 < TiO_2 under dark and ZnO < CuO < TiO_2 < Co_3O_4 under light; oxidative stress, reduced glutathione, MDA, LPO; release of metal ions	Dasari et al. (2013)
ZnO, TiO_2, SiO_2	*E. coli* *B. subtilis*	TiO_2 (66, 950, and 44 nm advertised particle size), SiO_2 (14, 930, and 60 nm advertised particle size), and ZnO (67 and 820 nm advertised particle size)	Antibacterial activity generally decreased from ZnO < TiO_2 < SiO_2; *B. subtilis* was the most susceptible; the presence of light could promote generation of ROS	Tayel et al. (2011)
ZnO, TiO_2, SiO_2, Al_2O_3	*B. subtilis* *E. coli* *P. fluorescens*	Al_2O_3 (60 nm), TiO_2 (50 nm), and ZnO (20 nm)	All NPs but TiO_2 showed higher toxicity (at 20 mg/L) than their bulk counterparts; antibacterial activity generally decreased in the ZnO< Al_2O_3 < SiO_2 order: ZnO (100% mortality for all bacteria), Al_2O_3 (57% mortality for *B. subtilis*, 36% for *E. coli*, and 70% for *P. fluorescens*); SiO_2 (40% mortality for *B. subtilis*, 58% for *E. coli*, and 70% for *P. fluorescens*)	Jiang et al. (2009)
MgO, TiO_2, Al_2O_3, CuO, CeO_2, ZnO	*S. aureus* *S. epidermidis* *S. pyogenes* *E. faecalis* *B. subtilis* *E. coli*	N/A	ZnO NPs had significantly higher antibacterial effects on *S. aureus* than five other metal oxide NPs did; size and light may be the reason for the antibacterial mechanism	Jones et al. (2008)

(*Continued*)

TABLE 6.2 (CONTINUED)

Antibacterial Properties of Metal and Metal Oxide Nanomaterials

Material	Microorganism Tested	Size	Main Findings	References
ZnO, TiO_2, CeO_2	*N. europaea*	25 nm anatase TiO_2, 200 nm anatase TiO_2, ~30 nm ZnO, ~60 nm in length and 20 nm in width CeO_2	Morphological damage; releasing of Zn^{2+} ions; the TEM and AUC results suggested that cells became heavier in the presence of CeO_2 and TiO_2 NPs	Fang et al. (2010)
Te- and Se/Te-doped anatase TiO_2 nanorods	*E. coli* *S. aureus*	Na-titanate nanotubes, with (13.8 ± 4.3) nm in diameter and (6.1 ± 1.9) mm in length; Te–TiO_2 NRs with width (106 ± 13) nm and length (824 ± 115) nm	The Te–TiO_2 NRs and Se_n/Te–TiO_2 NRs generated OH· at 2.5 and 4.5 times higher than that of commercial P25 under light, respectively	Lin et al. (2013)
CdTe–TiO_2, CdTe QDs	*E. coli* *B. subtilis*	N/A	CdTe–TiO_2~57% inhibition biofilm formation; generated ROS in cell, which caused both *E. coli* and *B. subtilis* death	Gholap et al. (2013)
Cu-doped TiO_2 NPs/ TiO_2 NPs	*M. smegmatis* *S. oneidensis* MR-1	20 nm	Not penetrated into the cell and damaged cellular structures; Cu-doped TiO_2 NPs (20 mg/L) significantly reduced the *M. smegmatis* growth rate by threefold, but did not affect *S. oneidensis* MR-1 growth; toxicity came from Cu^{2+} released by Cu-doped TiO_2 NPs; *S. oneidensis* MR-1 could tolerate a much higher concentration of Cu^{2+} or Cu-doped TiO_2 NPs due to EPS	Wu et al. (2010)
Cu-doped TiO_2 NPs	*S. oneidensis* MR-1	N/A	Compared with the control, the treatment enhanced the survival rates under UV light	Wu et al. (2011)
CeO_2 NPs	*E. coli*	7 nm	Direct contact is the lethal factor to *E. coli* and NPs; CeO_2 NP reduction (Ce^{4+} ~ Ce^{3+});	Thill et al. (2006)
CeO_2 NPs	*E. coli* *Synechocystis*	7 nm	"Direct/indirect" effects of NPs on bacteria; direct contact mechanism is suitable for *E. coli*, not *Synechocystis*	Zeyons et al. (2009)

(*Continued*)

TABLE 6.2 (CONTINUED)

Antibacterial Properties of Metal and Metal Oxide Nanomaterials

Material	Microorganism Tested	Size	Main Findings	References
CeO_2 NPs	*E. coli*	7 nm, 25 nm, and bulk counterpart (b-CeO_2)	Antibacterial activity; both NPs are more toxic than bulk CeO_2; due to direct contact with cell, NP-induced ROS was involved in the mechanism for the antibacterial effect of CeO_2	Kuang et al. (2011)
CeO_2 NPs	*E. coli*	7 nm	The antibacterial effect of CeO_2 NPs for *E. coli* were closely related to the surface charge density, and so was switchable	He et al. (2012)
La_2O_3, CeO_2, Gd_2O_3, Yb_2O_3	*E. coli* pBR 322	Not specified	Cytotoxicity; dissolution of NPs; ion-related toxicities on *E. coli*; particle-specific toxicity	He et al. (2015)

latter case (because Gram-positive bacteria have simper cell membrane structure than Gram-negative cells), and significant differences were observed in the effect of ZnO nanostructures and H_2O_2 on *B. atrophaeus*. Wadhwa (2011) explored the antibacterial effects of three different types of TiO_2 NPs (nanosphere, nanorod, and nanotube) and found TiO_2 nanotube displayed the highest antibacterial effect because of its highest surface area and surface-to-volume ratio among the studied photocatalysts. Tong et al. (2013) also found that material morphology played a key role in regulating the phototoxicity of nano-TiO_2, including nanotubes, nanorods, and nanosheets, to *E. coli* and *Aeromonas hydrophila* (*A. hydrophila*), but differ from the results of Pal et al. (2007), which showed nonspherical Ag NP was more toxic to bacteria. Results indicated that TiO_2 rod and sphere shapes were more phototoxic than their nanotube and nanosheet counterparts under simulated solar irradiation, but did not find cytotoxicity in the dark (Tong et al., 2013).

Some researchers attributed the toxicity of NPs to the dissolved toxic ions, rather than the NPs themselves; thus, the importance of the particle solubility is worth careful examination. Previous studies evaluated the contribution of dissolved metal ions released from NPs to the overall antibacterial effects, mainly on ZnO, Ag, and CdSe NPs. Dasari et al. (2013) reported that ZnO NPs and CuO NPs were toxic to *E. coli* when exposed to light due to the release of metal ions. Li et al. (2010) evaluated the influence of ZnO NP iron doping and aquatic chemistry on NP bioavailability, stability, and toxicity to *B. subtilis*, *E. coli*, and *P. putida*. The results showed that the inactivation of bacteria by ZnO NPs was most significantly influenced by the dissolution of Zn^{2+} ions. Xiu et al. (2012) demonstrated that the particle-specific antibacterial effects of Ag NPs were negligible under strictly anaerobic conditions. Results presented that the morphological properties of Ag NPs played an indirect role in releasing Ag^+, and the antibacterial effects could be adjusted by controlling Ag^+ release. Many reports also found similar results that the toxicity of Ag NPs was mainly due to Ag dissolution and the effects of free Ag^+ ions (Arnaout and Gunsch, 2012; Hachicho et al., 2014; Ma et al., 2011; Xiu et al., 2011). A recent study from our group also showed that particle cell contact could facilitate the dissolution of rare earth oxide NPs at the particle cell interface, and the additional ionic release, e.g., La^{3+}, Gd^{3+}, and Yb^{3+}, at the particle cell interface enhances the ion-related toxicity (He et al., 2015).

However, another study found that the toxicity induced by the dissolved ions was negligible. Baek and An (2011) investigated the toxicity of CuO, ZnO, NiO, and Sb_2O_3 NPs to *E. coli*, *B. subtilis*, and *S. aureus*. The release of free Zn^{2+} ions from ZnO colloidal solution was found to be negligible, and the antibacterial effect of ZnO NPs was rather dependent on the size of ZnO NPs under their experimental conditions (Raghupathi et al., 2011). According to Ivask et al. (2013), mutant *E. coli* cells were used as a model to investigate the toxicity mechanisms by various Ag NPs and the difference from Ag^+ ions. Results revealed that the toxicity of Ag NPs on bacteria was related to the physicochemical properties of Ag NPs, particularly its surface characteristics.

Indeed, water chemistry also plays very important role in NP dissolution and their antibacterial effects, which could explain the differences in published studies with so many different results. For example, it has been found that tannic acid forms stable complexes with Zn^{2+} ions and thereby reduces the free Zn^{2+} concentration in the media. Tannic acid caused less toxicity than humic, fulvic, and alginic acids (Li et al., 2010). Li et al. (2011) inspected the toxicity of ZnO NPs to *E. coli* in five commonly

used aqueous media: ultrapure water, 0.85% NaCl (normal saline), phosphate-buffered saline (PBS), minimal Davis (MD), and LB media. The toxicity of the ZnO NPs was primarily attributed to the free Zn^{2+} ions and labile zinc complexes. The presence of PO_4^{3-} in PBS (and citrate and amino acids in MD and LB media, respectively) generated a precipitation of zinc complexes, which caused a dramatic decrease in the concentration of Zn^{2+} ions and resulted in lower cytotoxicity of ZnO NPs in these media. Subsequently, Kuang et al. (2011) used normal saline and PBS to adjust the surface charge density to study the influence of surface charge on the cytotoxicity of CeO_2 NPs on *E. coli*. Results showed that the direct contact mediated by the electrostatic attraction between the positively charged CeO_2 NPs and the cell wall in normal saline would lead to outer membrane destabilization, increased ROS production, and loss of viability. Nevertheless, the inverse situation was found in PBS. With significantly reduced contacts, there was no outer membrane destabilization, there was no increased ROS production, and at the end there was no cytotoxicity found. This work as a well-designed comparison study contributes to a better understanding of the direct contact and ROS-induced oxidative stress as they apply to the antibacterial effects of NMs. Pagnout et al. (2012) investigated the role of electrostatic interactions in the toxicity of TiO_2 NPs toward *E. coli*. Results revealed that at low ionic strength, the viability of the *E. coli* cell was more severely affected at pH 5.5 than at 7.0 and 9.5; it was also shown the addition of electrolytes, e.g., NaCl, $CaCl_2$, and Na_2SO_4, which resulted in a gradual reduction of TiO_2 NP toxicity at pH 5.5, whereas an increase toxicity was found at pH 9.5, which related to the NPs' direct contact with bacteria.

Similar to previous studies, ROS play a crucial role in the antibacterial mechanisms of metal- and metal oxide-based NPs. Applerot et al. (2009) used electron spin resonance (ESR) measurements and revealed that suspension of ZnO NPs increased the levels of ROS, namely, the hydroxyl radicals (OH). This was attributed to the higher surface-to-volume ratio of the small NPs reacting with water. After antibacterial treatment, a remarkable enhancement of oxidative stress was detected. Smaller NPs may have a higher defect concentration, which could generate a larger amount of hydroxyl radicals in their aqueous suspensions, as assessed using ESR. A recent study also acknowledged that TiO_2 and ZnO NPs were photosensitive and they could generate ROS when irradiated with light (Dasari et al., 2013). On the other hand, Wu et al. (2010) found that the presence of Cu-doped TiO_2 NPs in the cultural medium could dramatically increase the survival rates of strain *S. oneidensis* MR-1. As indicated by gene expression results, the DNA repair gene recA in MR-1 was significantly induced by UV exposure, but no statistical evident was found by the influence of NPs. Plausible explanations to NP attenuation of UV stresses are as follows: firstly, TiO_2-based NPs are able to scatter and absorb UV light and therefore create a shading effect to protect MR-1 from UV radiation; secondly, which was more important against environmental stresses, Cu-doped TiO_2 NPs would coagglomerate with bacteria to create large flocks that reduced cell exposure to UV light to improve survival of cells (Wu et al., 2011). Nevertheless, due to the light-induced generation of greater quantities of ROS, the antibacterial property of the CdTe–TiO_2 nanocomposite was enhanced (Gholap et al., 2013). However, due to the short lifetime of ROS molecules, only those produced surrounding/on the bacterial surface is subsequently able to lead to phototoxicity, which causes cell death ultimately. Tong et al. (2013) provided further evidence to support these conclusions and demonstrated the essence of considering the geometry and alignment of TiO_2 NP aggregates at the bacterial surface, for the

sake of accurately estimating the probability of ROS molecule attack. Though generation of ROS was reported to be the most important reason for TiO_2 toxicity under light (Nel et al., 2009), the basic mechanism describing cytotoxicity in the dark is still not clear. Inactivation of bacterial cells by TiO_2 NPs can also occur under the absence of UV-A irradiation conditions (Adams et al., 2006; Nesic et al., 2014; Pagnout et al., 2012), indicating that mechanisms other than photocatalytic ROS were responsible for the antibacterial effect (Erdem et al., 2015a, 2015b). Under such conditions, cells also die presumably because of disturbances in the cell membrane (affecting its integrity, permeability, and osmoregulation), which in this case is due to bacterial cells' direct contact with NPs (Dalai et al., 2012; Mileyeva-Biebesheimer et al., 2010; Pagnout et al., 2012; Simon-Deckers et al., 2009). In an aerobic aqueous environment, the active electrons and h^+ generate ROS, which are strong oxidants that damage the cell wall and membranes and eventually lead to cell death. Yin et al. (2016) identified PEG-MoS_2 NFs acting as a peroxidase catalytic, which could converse H_2O_2 into •OH rapidly, and the product had higher antibacterial effects than H_2O_2 on *E. coli* and *B. subtilis* cells.

Biotransformation, which is defined as biochemical modification by living organisms, is a key factor that may modify the cytotoxicity, behavior, and fate of metal and metal oxide NPs in the environment and would cause either enhanced toxicity or detoxification. Thill et al. (2006) used CeO_2 NPs and *E. coli* as a model, and the results revealed that the adsorption of CeO_2 NPs and their reduction, which was detected by X-ray absorption near-edge structure (XANES), are associated with significant antibacterial effects. Zeyons et al. (2009) also found similar results, which showed that the direct adsorption of CeO_2 NPs on the *E. coli* cell outer membrane caused strong cytotoxicity. The probable mechanism is the oxidative power of the Ce(IV) atom, which was reduced to Ce(III) detected by XANES and ultimately caused cell death. The modification methods used by sulfidation of Ag NPs could significantly decrease their toxicity to *E. coli* growth because of the lower solubility of silver sulfide (Reinsch et al., 2012). A recent study revealed a new evidence for TiO_2 NPs leading to complete bacterial reduction under dark conditions. The results showed that the reduction of Ti^{4+} to Ti^{3+} contributed to redox interactions between TiO_2 and the *E. coli* cell wall, which provided a potential antibacterial mechanism of direct contact between bacterial cell wall and NPs (Nesic et al., 2014). The reduction of titanium from Ti(IV) to Ti(III) species which might have contributed to the redox interactions on the cell surface were confirmed using X-ray photoelectron spectroscopy (Dalai et al., 2014; Rtimi et al., 2012).

6.7 Reliability of Assay Methods

The bacterial growth or bacterial population can be measured using various methods, which are classified as direct and indirect methods. Direct methods measure the number of cells in 1 g of solid material or 1 mL of a solution. For another, the total mass of the population is directly proportional to that of cell members by indirect method measures. Similarly, the antibacterial activity of NMs can be determined by different methods that test the microbial cell live/dead after a specific incubation period of exposure. Due to the rapid development of new nano-antibacterial agents, this section will demonstrate the current methods reviewed in the literature to evaluate the

bacterial activities/viability. In addition, to evaluate the topic previously mentioned, merits and demerits of each methodology will also be reviewed.

Generally, the turbidity and counting plate are widely used to monitor the bacterial concentration/growth in a liquid suspension. The former has been extensively used to explore the antibacterial effects of NMs, i.e., GO and rGO (Guo et al., 2016; Gurunathan et al., 2012; Ruiz et al., 2011), Ag NPs (Ivask et al., 2013), CeO_2 NPs (Kuang et al., 2011), Al_2O_3 NPs (Mu et al., 2015), CuO, TiO_2, and NiO NPs (Wang et al., 2010). Using this method, the bacterial growth could be estimated by measuring the absorbance in different incubation periods of exposure in a specific wavelength (nm). But the optical density (OD) must be allowed in Lambert–Beer's law, which needs OD values from 0.2 to 0.8. If the microbial concentration was lower than 0.2 or higher than 0.8, the method should not be applied for measuring. The latter one is the most frequently used method to measure the number of bacteria (Akhavan and Ghaderi, 2010; Baek and An, 2011; Hu et al., 2010; Li et al., 2012; Liu et al., 2012; Liu et al., 2011; Pagnout et al., 2012; Perreault et al., 2015; Tang et al., 2013). However, CFU results are not always true, because sometimes the bacteria could form small agglomerates or chains. For instance, Perreault et al. (2015) revealed that due to the large GO cell aggregates ranging from 10 μm to more than 100 μm in size, these GO aggregates isolated the bacterial cells from their media and prevented their proliferation when applied on an agar medium by the CFU method. In this case, it would show incorrect CFU values and lead to false-positive antibacterial results. Due to the specificity of species, an isolated colony is not generated from a single bacterial cell but from a small number of bacterial cells. Besides, this procedure requires the use of a large number of laboratorial materials and reagents. The microbial concentration can also be quantified through the measurement of metabolic activity. This method assumes a direct correlation between the amounts of some products (organic acid or CO_2) with the number of microorganisms in a population. The measurement of dry weight is another way to quantify the microbial growth.

The MTT assay is a colorimetric method usually used to test the cellular/bacterial viability because MTT could be deoxidized by mitochondrial enzyme succinate dehydrogenase to a purple formazan, which can be related to the number of living cells. The great advantage of this method is that MTT reduction takes place only in metabolically active cells. Therefore, this assay can be extensively used to evaluate the cytotoxic effects of potential antimicrobial agents and toxic NMs against microorganisms (He et al., 2012, 2015; Kuang et al., 2011; Zimbone et al., 2015). However, if there were resting cells or cells with low metabolic activity present, the MTT values may be much reduced compared to cells in the phase of proliferation. Nevertheless, in the MTT assay, the formazan product is insoluble, and extra steps are required to dissolve the crystals. Not only can an MTT assay result be affected by the total number of cells but also succinate dehydrogenase (or other reductase) activity remained detectable in nonviable *E. coli* cells, which could result in a higher cell viability measured using the MTT assay (Tang et al., 2013). However, this method forms an insoluble formazan product, and dissolution of crystals acts as an extra step, which may cause artificial errors.

The microbial cell is considered viable if the membrane is not damaged, but if the membrane structure is corrupted, it could be regarded as a dead cell. Live/dead cell staining is a fluorescent reagent often used to test bacterial viability, allowing researchers to distinguish living from dead bacterial cells almost instantly and in

in situ analysis (Boulos et al., 1999; Chávez-Calderón et al., 2016; Kaweeteerawat et al., 2015; Li et al., 2010; Simon-Deckers et al., 2009). This method could detect planktonic or bacterial cells living in the form of biofilms, even though the population contained different bacterial strains. When these two stains are used together, it is possible to visualize dead and live cells, simultaneously. So, live bacteria will produce green fluorescence, whereas dead bacteria with damaged membranes will produce red fluorescence. The percentage of viable cells was determined through the ratio of the green to red fluorescence intensities and could be fitted by some calculation software. Otherwise, this fluorescent assay has been very useful to investigate the antiadhesion and antibiofilm property of NMs (Costerton et al., 1995; Donlan, 2002; Guo et al., 2016). If more details about the biofilm are required, confocal fluorescence microscopy provides data about the three-dimensional structure and thickness of the biofilms (Seil and Webster, 2012). However, Dumas et al. (2010) elucidated the toxicity effects to four different strains on CdTe QD exposure and found that SYTO-9 fluorescence was enhanced by red QDs, which caused an erroneous result.

To elucidate the antibacterial mechanism of cell death induced by carbon NMs and metal and metal oxide NMs, several studies have been suggested to explain the antimicrobial mode of action of these NMs, including NMs directly extracting the bacterial cell walls, the production of ROS, or oxidative stress. ROS generation can be detected using the nonfluorescent probe, such as 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). Owing to the fact that intracellular ROS (H_2O_2, $ONOO^-$, •OH, or $\mathbf{O}_2^-$) are present, DCFH-DA turns into green-fluorescent configuration (DCF) and detects its fluorescence intensity to know the level of ROS in the cells (Moyano et al., 2014). However, the intracellular redox chemistry of DCFH is complex and there are several artifacts and limitations associated with the DCF assay for intracellular H_2O_2 measurement, such as H_2O_2 which could not react with DCFH to form the fluorescent product, DCF. Many one-electron-oxidizing species can oxidize DCFH to DCF (Kalyanaraman et al., 2012). What is more, depending on some excellent features of fluorescent probes, such as high sensitivity and specificity, it can provide real-time monitoring in living cells and animals (Gutscher et al., 2008; Winterbourn 2014), which is much better than other methods for ROS detection. Nowadays, a large number of organic fluorescent probes have been applied in detecting the ROS in living cells (Albers et al., 2006; Wang et al., 2013; Yang et al., 2009). A recent study synthesized a novel fluorescent probe, MPT-Cy2, which exhibits an excellent selectivity and sensitivity toward •OH in various types of bacteria. Results also showed that MPT-Cy2 might be more useful than the nonfluorescent probe DCFH-DA for the detection of the production of •OH by different bacteria (Liu et al., 2016).

Generally, as for the physical disruption of the bacterial cell membrane interaction with graphene-based NM, atomic force microscopy (AFM) was used to survey the interactions between cells and NMs (Dorobantu et al., 2015). With different sizes of GO nanosheets, the surface roughness values of *E. coli* cells corroborated with the materials (Liu et al., 2012). Bacterial inactivation by GO was also characterized by a disruption of cell integrity, which could be demonstrated by the uptake of the membrane-impermeable PI dye in dead/live fluorescent staining, and also could be observed by SEM to find the morphological changes. Ivask et al. (2013) used TEM and AFM and found that small parts of polyvinylpyrrolidone-coating Ag NPs were attached to the *E. coli* cell surface. Many studies have also proposed that interactions between NMs and bacterial cells may be involved in NM-induced membrane damage

from the SEM and TEM results (Simon-Deckers et al., 2009; Xia et al., 2013). For nanotoxicity studies of engineered NMs, assessing the uptake of NMs and the associated cellular response becomes more and more important. Owing to the nonavailability of adequate methods for assessing cellular uptake of these NMs, however, there is a lack of information in this important area. Nowadays, flow cytometry technology can provide information not only about the viability but also about the reduction in the activity of bacteria. Dumas et al. (2010) developed this method and found that it could be applied into the different experimental conditions for several generations to detect the internalization of NMs in live bacteria. Combarros et al. (2016) employed this technique to investigate the effects of TiO_2 NP interaction with *P. putida* cells on its physiological state. By measuring parameters side scatter (SSC) and forward scatter (FSC), cell complexity and size were monitored. Owing to TiO_2 NPs which were absorbed on/in *P. putida* cells, both SSC and FSC parameters increased with higher TiO_2 NP concentration exposure.

Due to the many factors that can influence the toxic effects of NMs, such as size, surface charge, ion leakage, and light exposure, stating the antibacterial mechanisms on NM exposure is complex. Combining with the review previously described, such as OD_{600}, CFU, MTT, Dead/Live Bacterial Viability Kit, ROS detection, and some instrumental analysis methods, has revealed some positive/negative effects on the interaction between NMs and bacteria. However, this part also highlights various limitations and challenges of evaluation, characterization, and detection of nanotoxicity on bacteria. We hope that this part can provide suitable methods in some respects for the characterization and detection of the interaction between NMs and bacteria in the future nanotoxicology research.

6.8 Summary and Future Perspectives

This review summarizes recent studies on the antibacterial effects and potential mechanisms of these NMs on bacteria. It interprets mechanisms underlying the antibacterial effects of NMs including the direct contact between NMs and bacterial cells, the ability of NMs to release metal ions, to generate ROS, etc. Water chemistry and biotransformation of NMs may induce/reduce the potential bacterial toxicity. Indeed, the toxicity of NMs remains controversial as conflicting results have been found in the literature. It seems that numerous parameters are involved in the toxic effects of NMs on bacteria: factors from the environment but also the intrinsic physicochemical features of NMs, such as structure, surface defects, lateral size, C/O ratios, surface functional groups, surface charges, and particulate state. It is also very difficult to compare the antibacterial effects of NMs among various studies in that no standardized assays exist, making it really hard to draw a definite conclusion on the antibacterial effects of NMs. Even so, this review provides a well-built benchmark for researchers for assessing the potential antibacterial effects of carbon, metal, and metal oxide NPs on bacteria.

REFERENCES

Adams, L.K., Lyon, D.Y., and Alvarez, P.J. 2006. Comparative eco-toxicity of nanoscale TiO_2, SiO_2, and ZnO water suspensions. *Water research* 40, 3527–3532.

áde Leon, A. 2015. On the antibacterial mechanism of graphene oxide (GO) Langmuir–Blodgett films. *Chemical communications* 51, 2886–2889.

Akhavan, O. and Ghaderi, E. 2010. Toxicity of graphene and graphene oxide nanowalls against bacteria. *ACS nano* 4, 5731–5736.

Albers, A.E., Okreglak, V.S., and Chang, C.J. 2006. A FRET-based approach to ratiometric fluorescence detection of hydrogen peroxide. *Journal of the American Chemical Society* 128, 9640–9641.

Applerot, G., Lipovsky, A., Dror, R., Perkas, N., Nitzan, Y., Lubart, R., and Gedanken, A. 2009. Enhanced antibacterial activity of nanocrystalline ZnO due to increased ROS-mediated cell injury. *Advanced functional materials* 19, 842–852.

Aquino, A., Chan, J., Giolma, K., and Loh, M. 2010. The effect of a fullerene water suspension on the growth, cell viability, and membrane integrity of *Escherichia coli* B23. *Journal of experimental microbiology and immunology* 14, 13–20.

Arnaout, C.L. and Gunsch, C.K. 2012. Impacts of silver nanoparticle coating on the nitrification potential of *Nitrosomonas europaea*. *Environmental science and technology* 46, 5387–5395.

Aruguete, D.M., Kim, B., Hochella, M.F., Ma, Y., Cheng, Y., Hoegh, A., Liu, J., and Pruden, A. 2013. Antimicrobial nanotechnology: Its potential for the effective management of microbial drug resistance and implications for research needs in microbial nanotoxicology. *Environmental science: Processes and impacts* 15, 93–102.

Bae, H., Lee, M., Kim, W., and Rhee, C. 2003. Dispersion properties of TiO_2 nanopowder synthesized by homogeneous precipitation process at low temperatures. *Colloids and surfaces A: Physicochemical and engineering aspects* 220, 169–177.

Baek, Y.W. and An, Y.J. 2011. Microbial toxicity of metal oxide nanoparticles (CuO, NiO, ZnO, and Sb_2O_3) to *Escherichia coli*, *Bacillus subtilis*, and *Streptococcus aureus*. *Science of the total environment* 409, 1603–1608.

Boulos, L., Prevost, M., Barbeau, B., Coallier, J., and Desjardins, R. 1999. LIVE/DEAD® BacLight™: Application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. *Journal of microbiological methods* 37, 77–86.

Brady-Estévez, A.S., Kang, S., and Elimelech, M. 2008. A single-walled-carbon-nanotube filter for removal of viral and bacterial pathogens. *Small* 4, 481–484.

Brayner, R., Ferrari-Iliou, R., Brivois, N., Djediat, S., Benedetti, M.F., and Fiévet, F. 2006. Toxicological impact studies based on *Escherichia coli* bacteria in ultrafine ZnO nanoparticles colloidal medium. *Nano letters* 6, 866–870.

Chávez-Calderón, A., Paraguay-Delgado, F., Orrantia-Borunda, E., and Luna-Velasco, A. 2016. Size effect of SnO_2 nanoparticles on bacteria toxicity and their membrane damage. *Chemosphere* 165, 33–40.

Chen, H.Q., Gao, D., Wang, B., Zhao, R.F., Guan, M., Zheng, L.N., Zhou, X.Y., Chai, Z.F., and Feng, W.Y. 2014. Graphene oxide as an anaerobic membrane scaffold for the enhancement of *B. adolescentis* proliferation and antagonistic effects against pathogens *E. coli* and *S. aureus*. *Nanotechnology* 25, 165101.

Chen, J., Wang, X., and Han, H. 2013. A new function of graphene oxide emerges: Inactivating phytopathogenic bacterium *Xanthomonas oryzae* pv. Oryzae. *Journal of nanoparticle research* 15, 1–14.

Cho, M., Fortner, J.D., Hughes, J.B., and Kim, J.H. 2009. *Escherichia coli* inactivation by water-soluble, ozonated C_{60} derivative: Kinetics and mechanisms. *Environmental science and technology* 43, 7410–7415.

Combarros, R., Collado, S., and Díaz, M. 2016. Toxicity of titanium dioxide nanoparticles on *Pseudomonas putida*. *Water research* 90, 378–386.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., and Lappin-Scott, H.M. 1995. Microbial biofilms. *Annual review of microbiology* 49, 711–745.

Dalai, S., Pakrashi, S., Chakravarty, S., Hussain, S., Chandrasekaran, N., and Mukherjee, A. 2014. Studies on interfacial interactions of TiO_2 nanoparticles with bacterial cells under light and dark conditions. *Bulletin of materials science* 37, 371–381.

Dalai, S., Pakrashi, S., Kumar, R.S., Chandrasekaran, N., and Mukherjee, A. 2012. A comparative cytotoxicity study of TiO_2 nanoparticles under light and dark conditions at low exposure concentrations. *Toxicology research* 1, 116–130.

Dasari, T.P., Pathakoti, K., and Hwang, H.M. 2013. Determination of the mechanism of photoinduced toxicity of selected metal oxide nanoparticles (ZnO, CuO, Co_3O_4 and TiO_2) to *E. coli* bacteria. *Journal of environmental sciences* 25, 882–888.

Donlan, R.M. 2002. Biofilms: Microbial life on surfaces. *Emerging infectious diseases* 8, 881–890.

Dorobantu, L.S., Fallone, C., Noble, A.J., Veinot, J., Ma, G., Goss, G.G., and Burrell, R.E. 2015. Toxicity of silver nanoparticles against bacteria, yeast, and algae. *Journal of nanoparticle research* 17, 1–13.

Dumas, E., Gao, C., Suffern, D., Bradforth, S.E., Dimitrijevic, N.M., and Nadeau, J.L. 2010. Interfacial charge transfer between CdTe quantum dots and gram negative vs gram positive bacteria. *Environmental science and technology* 44, 1464–1470.

Dutta, T., Sarkar, R., Pakhira, B., Ghosh, S., Sarkar, R., Barui, A., and Sarkar, S. 2015. ROS generation by reduced graphene oxide (rGO) induced by visible light showing antibacterial activity: Comparison with graphene oxide (GO). *RSC advances* 5, 80192–80195.

Erdem, A., Metzler, D., Cha, D., and Huang, C. 2015a. Inhibition of bacteria by photocatalytic nano-TiO_2 particles in the absence of light. *International journal of environmental science and technology* 12, 2987–2996.

Erdem, A., Metzler, D., Cha, D.K., and Huang, C. 2015b. The short-term toxic effects of TiO_2 nanoparticles toward bacteria through viability, cellular respiration, and lipid peroxidation. *Environmental science and pollution research* 22, 17917–17924.

Fahey, R., Brown, W., Adams, W., and Worsham, M. 1978. Occurrence of glutathione in bacteria. *Journal of bacteriology* 133, 1126–1129.

Fang, J., Lyon, D.Y., Wiesner, M.R., Dong, J., and Alvarez, P.J. 2007. Effect of a fullerene water suspension on bacterial phospholipids and membrane phase behavior. *Environmental science and technology* 41, 2636–2642.

Fang, X., Yu, R., Li, B., Somasundaran, P., and Chandran, K. 2010. Stresses exerted by ZnO, CeO_2 and anatase TiO_2 nanoparticles on the *Nitrosomonas europaea*. *Journal of colloid and interface science* 348, 329–334.

Gao, L.Z, Zhuang, J., Nie, L., Zhang, J.B, Zhang, Y., Gu, N., Wang, T.H., Feng, J., Yang, D., and Perrett, S. 2007. Intrinsic peroxidase-like activity of ferromagnetic nanoparticles. *Nature nanotechnology* 2, 577–583.

Geim, A.K., and Novoselov, K.S. 2007. The rise of graphene. *Nature materials* 6, 183–191.

Gholap, H., Patil, R., Yadav, P., Banpurkar, A., Ogale, S., and Gade, W. 2013. CdTe–TiO_2 nanocomposite: An impeder of bacterial growth and biofilm. *Nanotechnology* 24, 195101.

Gogniat, G., Thyssen, M., Denis, M., Pulgarin, C., and Dukan, S. 2006. The bactericidal effect of TiO_2 photocatalysis involves adsorption onto catalyst and the loss of membrane integrity. *FEMS microbiology letters* 258, 18–24.

Guo, Z., Xie, C., Zhang, P., Zhang, J., Wang, G., He, X., Ma, Y., Zhao, B., and Zhang, Z. 2017. Toxicity and transformation of graphene oxide and reduced graphene oxide in bacteria biofilm. *Science of the total environment*, 580, 1300–1308.

Gurunathan, S., Han, J.W., Dayem, A.A., Eppakayala, V., and Kim, J.H. 2012. Oxidative stress-mediated antibacterial activity of graphene oxide and reduced graphene oxide in *Pseudomonas aeruginosa*. *International journal of nanomedicine* 7, 5901–5914.

Gurunathan, S., Han, J.W., Dayem, A.A., Eppakayala, V., Park, M.R., Kwon, D.N., and Kim, J.H. 2013. Antibacterial activity of dithiothreitol reduced graphene oxide. *Journal of industrial and engineering chemistry* 19, 1280–1288.

Gutscher, M., Pauleau, A., Marty, L., Brach, T., Wabnitz, G., Samstag, Y., Meyer, A., and Dick, T. 2008. Real-time imaging of the intracellular glutathione redox potential. *Nature methods* 5(6), 553–559.

Hachicho, N., Hoffmann, P., Ahlert, K., and Heipieper, H.J. 2014. Effect of silver nanoparticles and silver ions on growth and adaptive response mechanisms of *Pseudomonas putida* mt-2. *FEMS microbiology letters* 355, 71–77.

He, X., Kuang, Y.S., Li, Y.Y., Zhang, H.F., Ma, Y.H., Bai, W., Zhang, Z.Y., Wu, Z.Q., Zhao, Y.L., and Chai, Z.F. 2012. Changing exposure media can reverse the cytotoxicity of ceria nanoparticles for *Escherichia coli*. *Nanotoxicology* 6, 233–240.

He, X., Pan, Y., Zhang, J., Li, Y., Ma, Y., Zhang, P., Ding, Y., Zhang, J., Wu, Z., and Zhao, Y. 2015. Quantifying the total ionic release from nanoparticles after particle-cell contact. *Environmental pollution* 196, 194–200.

Hong, Y. and Brown, D.G. 2008. Electrostatic behavior of the charge-regulated bacterial cell surface. *Langmuir* 24, 5003–5009.

Hu, W., Peng, C., Luo, W., Lv, M., Li, X., Li, D., Huang, Q., and Fan, C. 2010. Graphene-based antibacterial paper. *ACS nano* 4, 4317–4323.

Huang, Z., Zheng, X., Yan, D., Yin, G., Liao, X., Kang, Y., Yao, Y., Huang, D., and Hao, B. 2008. Toxicological effect of ZnO nanoparticles based on bacteria. *Langmuir* 24, 4140–4144.

Hui, L., Piao, J.G., Auletta, J., Hu, K., Zhu, Y., Meyer, T., Liu, H., and Yang, L. 2014. Availability of the basal planes of graphene oxide determines whether it is antibacterial. *ACS applied materials and interfaces* 6, 13183–13190.

Isakovic, A., Markovic, Z., Nikolic, N., Todorovic-Markovic, B., Vranjes-Djuric, S., Harhaji, L., Raicevic, N., Romcevic, N., Vasiljevic-Radovic, D., and Dramicanin, M. 2006. Inactivation of nanocrystalline C_{60} cytotoxicity by γ-irradiation. *Biomaterials* 27, 5049–5058.

Ivask, A., ElBadawy, A., Kaweeteerawat, C., Boren, D., Fischer, H., Ji, Z., Chang, C.H., Liu, R., Tolaymat, T., and Telesca, D. 2013. Toxicity mechanisms in *Escherichia coli* vary for silver nanoparticles and differ from ionic silver. *ACS nano* 8, 374–386.

Jiang, W., Mashayekhi, H., and Xing, B. 2009. Bacterial toxicity comparison between nano-and micro-scaled oxide particles. *Environmental pollution* 157, 1619–1625.

Jones, N., Ray, B., Ranjit, K.T., and Manna, A.C. 2008. Antibacterial activity of ZnO nanoparticle suspensions on a broad spectrum of microorganisms. *FEMS microbiology letters* 279, 71–76.

Kalyanaraman, B., Darley-Usmar, V., Davies, K.J.A., Dennery, P.A., Forman, H.J., Grisham, M.B., Mann, G.E., Moore, K., Roberts, L.J., and Ischiropoulos, H. 2012. Measuring reactive oxygen and nitrogen species with fluorescent probes: Challenges and limitations. *Free Radical Biology and Medicine* 52(1), 1–6.

Kang, S., Herzberg, M., Rodrigues, D.F., and Elimelech, M. 2008. Antibacterial effects of carbon nanotubes: Size does matter! *Langmuir* 24, 6409–6413.

Kang, S., Mauter, M.S., and Elimelech, M. 2009. Microbial cytotoxicity of carbon-based nanomaterials: Implications for river water and wastewater effluent. *Environmental science and technology* 43, 2648–2653.

Kang, S., Pinault, M., Pfefferle, L.D., and Elimelech, M. 2007. Single-walled carbon nanotubes exhibit strong antimicrobial activity. *Langmuir* 23, 8670–8673.

Karunakaran, C., Abiramasundari, G., Gomathisankar, P., Manikandan, G., and Anandi, V. 2010. Cu-doped TiO_2 nanoparticles for photocatalytic disinfection of bacteria under visible light. *Journal of colloid and interface science* 352, 68–74.

Kaweeteerawat, C., Ivask, A., Liu, R., Zhang, H., Chang, C.H., Low-Kam, C., Fischer, H., Ji, Z., Pokhrel, S., and Cohen, Y. 2015. Toxicity of metal oxide nanoparticles in *Escherichia coli* correlates with conduction band and hydration energies. *Environmental science and technology* 49, 1105–1112.

Kim, J.S., Kuk, E., Yu, K.N., Kim, J.H., Park, S.J., Lee, H.J., Kim, S.H., Park, Y.K., Park, Y.H., and Hwang, C.Y. 2007. Antimicrobial effects of silver nanoparticles. *Nanomedicine: Nanotechnology, biology and medicine* 3, 95–101.

Kim, S.W. and An, Y.J., 2012. Effect of ZnO and TiO_2 nanoparticles preilluminated with UVA and UVB light on *Escherichia coli* and *Bacillus subtilis*. *Applied microbiology and biotechnology* 95, 243–253.

Krishnamoorthy, K., Veerapandian, M., Zhang, L.H., Yun, K., and Kim, S.J. 2012. Antibacterial efficiency of graphene nanosheets against pathogenic bacteria via lipid peroxidation. *The journal of physical chemistry C* 116, 17280–17287.

Kuang, Y., He, X., Zhang, Z., Li, Y., Zhang, H., Ma, Y., Wu, Z., and Chai, Z. 2011. Comparison study on the antibacterial activity of nano- or bulk-cerium oxide. *Journal of nanoscience and nanotechnology* 11, 4103–4108.

Li, M., Pokhrel, S., Jin, X., Mädler, L., Damoiseaux, R., and Hoek, E.M. 2010. Stability, bioavailability, and bacterial toxicity of ZnO and iron-doped ZnO nanoparticles in aquatic media. *Environmental science and technology* 45, 755–761.

Li, M., Zhu, L., and Lin, D. 2011. Toxicity of ZnO nanoparticles to *Escherichia coli*: Mechanism and the influence of medium components. *Environmental science and technology* 45, 1977–1983.

Li, Q., Mahendra, S., Lyon, D.Y., Brunet, L., Liga, M.V., Li, D., and Alvarez, P.J. 2008. Antimicrobial nanomaterials for water disinfection and microbial control: Potential applications and implications. *Water research* 42, 4591–4602.

Li, Y., Zhang, W., Niu, J., and Chen, Y. 2012. Mechanism of photogenerated reactive oxygen species and correlation with the antibacterial properties of engineered metal-oxide nanoparticles. *ACS nano* 6, 5164–5173.

Lin, Z.H., Roy, P., Shih, Z.Y., Ou, C.M., and Chang, H.T. 2013. Synthesis of anatase Se/Te-TiO_2 nanorods with dominant {100} facets: Photocatalytic and antibacterial activity induced by visible light. *ChemPlusChem* 78, 302–309.

Liu, F., Du, J., Song, D., Xu, M., and Sun, G. 2016. A sensitive fluorescent sensor for the detection of endogenous hydroxyl radicals in living cells and bacteria and direct imaging with respect to its ecotoxicity in living zebra fish. *Chemical communications* 52, 4636–4639.

Liu, S., Hu, M., Zeng, T.H., Wu, R., Jiang, R., Wei, J., Wang, L., Kong, J., and Chen, Y. 2012. Lateral dimension-dependent antibacterial activity of graphene oxide sheets. *Langmuir* 28, 12364–12372.

Liu, S., Zeng, T.H., Hofmann, M., Burcombe, E., Wei, J., Jiang, R., Kong, J., and Chen, Y. 2011. Antibacterial activity of graphite, graphite oxide, graphene oxide, and reduced graphene oxide: Membrane and oxidative stress. *ACS nano* 5, 6971–6980.

Liu, Y., He, L., Mustapha, A., Li, H., Hu, Z., and Lin, M. 2009. Antibacterial activities of zinc oxide nanoparticles against *Escherichia coli* O157: H7. *Journal of applied microbiology* 107, 1193–1201.

Lovern, S.B., and Klaper, R. 2006. Daphnia magna mortality when exposed to titanium dioxide and fullerene (C_{60}) nanoparticles. *Environmental toxicology and chemistry* 25, 1132–1137.

Lyon, D.Y., Adams, L.K., Falkner, J.C., and Alvarez, P.J. 2006. Antibacterial activity of fullerene water suspensions: Effects of preparation method and particle size. *Environmental science and technology* 40, 4360–4366.

Lyon, D.Y., and Alvarez, P.J. 2008. Fullerene water suspension (nC_{60}) exerts antibacterial effects via ROS-independent protein oxidation. *Environmental science and technology* 42, 8127–8132.

Lyon, D.Y., Brunet, L., Hinkal, G.W., Wiesner, M.R., and Alvarez, P.J. 2008. Antibacterial activity of fullerene water suspensions (nC_{60}) is not due to ROS-mediated damage. *Nano letters* 8, 1539–1543.

Lyon, D.Y., Fortner, J.D., Sayes, C.M., Colvin, V.L., and Hughes, J.B. 2005. Bacterial cell association and antimicrobial activity of a C_{60} water suspension. *Environmental toxicology and chemistry* 24, 2757–2762.

Ma, R., Levard, C., Marinakos, S.M., Cheng, Y., Liu, J., Michel, F.M., Brown Jr, G.E., and Lowry, G.V. 2011. Size-controlled dissolution of organic-coated silver nanoparticles. *Environmental science and technology* 46, 752–759.

Meyer, J.C., Geim, A.K., Katsnelson, M.I., Novoselov, K.S., Booth, T.J., and Roth, S. 2007. The structure of suspended graphene sheets. *Nature* 446, 60–63.

Mileyeva-Biebesheimer, O.N., Zaky, A., and Gruden, C.L. 2010. Assessing the impact of titanium dioxide and zinc oxide nanoparticles on bacteria using a fluorescent-based cell membrane integrity assay. *Environmental engineering science* 27, 329–335.

Mohanty, N., and Berry, V. 2008. Graphene-based single-bacterium resolution biodevice and DNA transistor: Interfacing graphene derivatives with nanoscale and microscale biocomponents. *Nano letters* 8, 4469–4476.

Moyano, A.J., Tobares, R.A., Rizzi, Y.S., Krapp, A.R., Mondotte, J.A., Bocco, J.L., Saleh, M.C., Carrillo, N., and Smania, A.M. 2014. A long-chain flavodoxin protects *Pseudomonas aeruginosa* from oxidative stress and host bacterial clearance. *PLoS genetics* 10, e1004163.

Mu, D., Mu, X., Xu, Z., Du, Z., and Chen, G. 2015. Removing *Bacillus subtilis* from fermentation broth using alumina nanoparticles. *Bioresource technology* 197, 508–511.

Nair, S., Sasidharan, A., Rani, V.D., Menon, D., Nair, S., Manzoor, K., and Raina, S. 2009. Role of size scale of ZnO nanoparticles and microparticles on toxicity toward bacteria and osteoblast cancer cells. *Journal of materials science: Materials in medicine* 20, 235–241.

Nakamura, S. and Mashino, T. 2009. Biological activities of water-soluble fullerene derivatives, *Journal of physics: Conference series* 159, 012003.

Natalio, F., André, R., Hartog, A.F., Stoll, B., Jochum, K.P., Wever, R., and Tremel, W. 2012. Vanadium pentoxide nanoparticles mimic vanadium haloperoxidases and thwart biofilm formation. *Nature nanotechnology* 7, 530–535.

Nel, A.E., Mädler, L., Velegol, D., Xia, T., Hoek, E.M., Somasundaran, P., Klaessig, F., Castranova, V., and Thompson, M. 2009. Understanding biophysicochemical interactions at the nano–bio interface. *Nature materials* 8, 543–557.

Nesic, J., Rtimi, S., Laub, D., Roglic, G.M., Pulgarin, C., and Kiwi, J. 2014. New evidence for TiO_2 uniform surfaces leading to complete bacterial reduction in the dark: Critical issues. *Colloids and surfaces B: Biointerfaces* 123, 593–599.

Padmavathy, N., and Vijayaraghavan, R. 2016. Enhanced bioactivity of ZnO nanoparticles—An antimicrobial study. *Science and technology of advanced materials* 9, 03500.

Pagnout, C., Jomini, S., Dadhwal, M., Caillet, C., Thomas, F., and Bauda, P. 2012. Role of electrostatic interactions in the toxicity of titanium dioxide nanoparticles toward *Escherichia coli*. *Colloids and surfaces B: Biointerfaces* 92, 315–321.

Pal, S., Tak, Y.K., and Song, J.M. 2007. Does the antibacterial activity of silver nanoparticles depend on the shape of the nanoparticle? A study of the gram-negative bacterium *Escherichia coli*. *Applied and environmental microbiology* 73, 1712–1720.

Perreault, F., De Faria, A.F., Nejati, S., and Elimelech, M. 2015. Antimicrobial properties of graphene oxide nanosheets: Why size matters. *ACS nano* 9, 7226–7236.

Pham, V.T., Truong, V.K., Quinn, M.D., Notley, S.M., Guo, Y., Baulin, V.A., Al Kobaisi, M., Crawford, R.J., and Ivanova, E.P. 2015. Graphene induces formation of pores that kill spherical and rod-shaped bacteria. *ACS nano* 9, 8458–8467.

Planchon, M., Ferrari, R., Guyot, F., Gélabert, A., Menguy, N., Chanéac, C., Thill, A., Benedetti, M.F., and Spalla, O. 2013. Interaction between *Escherichia coli* and TiO_2 nanoparticles in natural and artificial waters. *Colloids and surfaces B: Biointerfaces* 102, 158–164.

Quadros, M.E., and Marr, L.C. 2011. Silver nanoparticles and total aerosols emitted by nanotechnology-related consumer spray products. *Environmental science and technology* 45, 10713–10719.

Raghupathi, K.R., Koodali, R.T., and Manna, A.C. 2011. Size-dependent bacterial growth inhibition and mechanism of antibacterial activity of zinc oxide nanoparticles. *Langmuir* 27, 4020–4028.

Reinsch, B., Levard, C., Li, Z., Ma, R., Wise, A., Gregory, K., Brown Jr, G., and Lowry, G. 2012. Sulfidation of silver nanoparticles decreases *Escherichia coli* growth inhibition. *Environmental science and technology* 46, 6992–7000.

Rodrigues, D.F., and Elimelech, M., 2010. Toxic effects of single-walled carbon nanotubes in the development of *E. coli* biofilm. *Environmental science and technology* 44, 4583–4589.

Roy, M., Sonkar, S.K., Tripathi, S., Saxena, M., and Sarkar, S. 2012. Non-toxicity of water soluble multi-walled carbon nanotube on *Escherichia coli* colonies. *Journal of nanoscience and nanotechnology* 12, 1754–1759.

Rtimi, S., Baghriche, O., Pulgarin, C., Sanjines, R., and Kiwi, J. 2012. Design, testing and characterization of innovative TiN–TiO_2 surfaces inactivating bacteria under low intensity visible light. *RSC advances* 2, 8591–8595.

Ruiz, O.N., Fernando, K.S., Wang, B., Brown, N.A., Luo, P.G., McNamara, N.D., Vangsness, M., Sun, Y.P., and Bunker, C.E. 2011. Graphene oxide: A nonspecific enhancer of cellular growth. *ACS nano* 5, 8100–8107.

Santos, C.M., Tria, M.C.R., Vergara, R.A.M.V., Ahmed, F., Advincula, R.C., and Rodrigues, D.F. 2011. Antimicrobial graphene polymer (PVK-GO) nanocomposite films. *Chemical communications* 47, 8892–8894.

Seil, J.T., and Webster, T.J. 2012. Antimicrobial applications of nanotechnology: Methods and literature. *International journal nanomedicine* 7, 2767–2781.

Simon-Deckers, A., Loo, S., Mayne-L'hermite, M., Herlin-Boime, N., Menguy, N., Reynaud, C., Gouget, B., and Carrière, M. 2009. Size-, composition-and shape-dependent toxicological impact of metal oxide nanoparticles and carbon nanotubes toward bacteria. *Environmental science and technology* 43, 8423–8429.

Su, R., Jin, Y., Liu, Y., Tong, M., and Kim, H. 2013. Bactericidal activity of Ag-doped multi-walled carbon nanotubes and the effects of extracellular polymeric substances and natural organic matter. *Colloids and surfaces B: Biointerfaces* 104, 133–139.

Tam, K., Djurišić, A., Chan, C., Xi, Y., Tse, C., Leung, Y., Chan, W., Leung, F., and Au, D. 2008. Antibacterial activity of ZnO nanorods prepared by a hydrothermal method. *Thin solid films* 516, 6167–6174.

Tang, J., Chen, Q., Xu, L., Zhang, S., Feng, L., Cheng, L., Xu, H., Liu, Z., and Peng, R. 2013. Graphene oxide–silver nanocomposite as a highly effective antibacterial agent with species-specific mechanisms. *ACS applied materials and interfaces* 5, 3867–3874.

Tang, Y.J., Ashcroft, J.M., Chen, D., Min, G., Kim, C.H., Murkhejee, B., Larabell, C., Keasling, J.D., and Chen, F.F. 2007. Charge-associated effects of fullerene derivatives on microbial structural integrity and central metabolism. *Nano letters* 7, 754–760.

Tayel, A.A., El-Tras, W.F., Moussa, S., El-Baz, A.F., Mahrous, H., Salem, M.F., and Brimer, L. 2011. Antibacterial action of zinc oxide nanoparticles against foodborne pathogens. *Journal of food safety* 31, 211–218.

Thill, A., Zeyons, O., Spalla, O., Chauvat, F., Rose, J., Auffan, M., and Flank, A.M. 2006. Cytotoxicity of CeO_2 nanoparticles for *Escherichia coli*: Physico-chemical insight of the cytotoxicity mechanism. *Environmental science and technology* 40, 6151–6156.

Tong, T., Shereef, A., Wu, J., Binh, C.T.T., Kelly, J.J., Gaillard, J.F.o., and Gray, K.A. 2013. Effects of material morphology on the phototoxicity of nano-TiO_2 to bacteria. *Environmental science and technology* 47, 12486–12495.

Tu, Y., Lv, M., Xiu, P., Huynh, T., Zhang, M., Castelli, M., Liu, Z., Huang, Q., Fan, C., and Fang, H. 2013. Destructive extraction of phospholipids from *Escherichia coli* membranes by graphene nanosheets. *Nature nanotechnology* 8, 594–601.

Wadhwa, G. 2011. *Shape and size dependent bactericidal activity of photoirradiated TiO_2 nanostructures*. Thapar University, Patiala, India.

Wang, B., Li, P., Yu, F., Song, P., Sun, X., Yang, S., Lou, Z., and Han, K. 2013. A reversible fluorescence probe based on Se–BODIPY for the redox cycle between HClO oxidative stress and H_2S repair in living cells. *Chemical communications* 49, 1014–1016.

Wang, Z., Lee, Y.H., Wu, B., Horst, A., Kang, Y., Tang, Y.J., and Chen, D.R. 2010. Anti-microbial activities of aerosolized transition metal oxide nanoparticles. *Chemosphere* 80, 525–529.

Wei, X.Q., Hao, L.Y., Shao, X.R., Zhang, Q., Jia, X.Q., Zhang, Z.R., Lin, Y.F., and Peng, Q. 2015. Insight into the interaction of graphene oxide with serum proteins and the impact of the degree of reduction and concentration. *ACS applied materials and interfaces* 7, 13367–13374.

Winterbourn, C.C. 2014. The challenges of using fluorescent probes to detect and quantify specific reactive oxygen species in living cells. *Biochimica et biophysica acta (BBA)—General subjects* 1840(2), 730–738.

Wu, B., Huang, R., Sahu, M., Feng, X., Biswas, P., and Tang, Y.J. 2010. Bacterial responses to Cu-doped TiO_2 nanoparticles. *Science of the total environment* 408, 1755–1758.

Wu, B., Zhuang, W.Q., Sahu, M., Biswas, P., and Tang, Y.J. 2011. Cu-doped TiO_2 nanoparticles enhance survival of Shewanella oneidensis MR-1 under ultraviolet light (UV) exposure. *Science of the total environment* 409, 4635–4639.

Wu, M.C., Deokar, A.R., Liao, J.H., Shih, P.Y., and Ling, Y.C. 2013. Graphene-based photothermal agent for rapid and effective killing of bacteria. *ACS nano* 7, 1281–1290.

Xia, D., Ng, T.W., An, T., Li, G., Li, Y., Yip, H.Y., Zhao, H., Lu, A., and Wong, P.K. 2013. A recyclable mineral catalyst for visible-light-driven photocatalytic inactivation of bacteria: Natural magnetic sphalerite. *Environmental science and technology* 47, 11166–11173.

Xia, X.R., Monteiro-Riviere, N.A., and Riviere, J.E. 2010. Intrinsic biological property of colloidal fullerene nanoparticles (nC_{60}): Lack of lethality after high dose exposure to human epidermal and bacterial cells. *Toxicology letters* 197, 128–134.

Xiu, Z.M., Ma, J., and Alvarez, P.J. 2011. Differential effect of common ligands and molecular oxygen on antimicrobial activity of silver nanoparticles versus silver ions. *Environmental science and technology* 45, 9003–9008.

Xiu, Z.M., Zhang, Q.B., Puppala, H.L., Colvin, V.L., and Alvarez, P.J. 2012. Negligible particle-specific antibacterial activity of silver nanoparticles. *Nano letters* 12, 4271–4275.

Yang, Y.K., Cho, H.J., Lee, J., Shin, I., and Tae, J. 2009. A rhodamine–hydroxamic acid-based fluorescent probe for hypochlorous acid and its applications to biological imagings. *Organic letters* 11, 859–861.

Yin, W., Yu, J., Lv, F., Yan, L., Zheng, L.R., Gu, Z., and Zhao, Y. 2016. Functionalized nano-MoS_2 with peroxidase catalytic and near-infrared photothermal activities for safe and synergetic wound antibacterial applications. *ACS nano* 10, 11000–11011.

Yu, R., Wu, J., Liu, M., Zhu, G., Chen, L., Chang, Y., and Lu, H. 2016. Toxicity of binary mixtures of metal oxide nanoparticles to *Nitrosomonas europaea*. *Chemosphere* 153, 187–197.

Zeyons, O., Thill, A., Chauvat, F., Menguy, N., Cassier-Chauvat, C., Oréar, C., Daraspe, J., Auffan, M., Rose, J., and Spalla, O. 2009. Direct and indirect CeO_2 nanoparticles toxicity for *Escherichia coli* and *Synechocystis*. *Nanotoxicology* 3, 284–295.

Zhu, X., Zhu, L., Li, Y., Duan, Z., Chen, W., and Alvarez, P.J. 2007. Developmental toxicity in zebrafish (*Danio rerio*) embryos after exposure to manufactured nanomaterials: Buckminsterfullerene aggregates (nC_{60}) and fullerol. *Environmental toxicology and chemistry* 26, 976–979.

Zimbone, M., Buccheri, M., Cacciato, G., Sanz, R., Rappazzo, G., Boninelli, S., Reitano, R., Romano, L., Privitera, V., and Grimaldi, M. 2015. Photocatalytical and antibacterial activity of TiO_2 nanoparticles obtained by laser ablation in water. *Applied catalysis B: Environmental* 165, 487–494.

Zou, X., Zhang, L., Wang, Z., and Luo, Y. 2016. Mechanisms of the antimicrobial activities of graphene materials. *Journal of the American Chemical Society* 138, 2064–2077.

7

Comparative Risk Assessment of Copper Nanoparticles with Their Bulk Counterpart in the Indian Major Carp Labeo rohita

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7.1 Introduction

Metals are natural constituents of different ecosystems, and their distribution in the environment is governed by natural as well as anthropogenic activities (Bargagli 2000; Florea et al. 2004). Burning of fossil fuels, smelting, refining of metals, use of leaded petrol in motor vehicles and fly ash from power stations are the major sources of metal pollution in the environment (Griboff et al. 2017). The contamination of aquatic environment by metals is the consequence of industrial, agricultural, and anthropogenic activities which includes urban runoff, sewage treatment, and domestic garbage dumps as well as change in the usage of land and enhanced terrestrial runoff (Heath 1987; Rahman et al. 2012; Saha et al. 2016). Among these activities, industrial wastes constitute the major source of metal pollution in natural water (Livingstone 2001). These metals unlike other pollutants are not degraded or eliminated from the ecosystem but absorbed by living organisms and accumulated in sediments and organisms (Mendil and DoganUluozlu 2007; Rajkowska and Protasowicki 2013; Saha and Zaman 2013; Saha et al. 2016). Certain metals such as manganese, zinc, copper, iron, and nickel are essential (i.e., elements with a recognized role in biological systems) for the metabolism of aquatic organisms in low concentrations. Others, such as cadmium,

lead, and arsenic, are the most toxic nonessential metals (Canli and Atli 2003). Even essential metals may be toxic for biological activities of organisms at certain concentrations (Kucuksezgin et al. 2006).

Copper, an essential trace metal, is required for a variety of physiological and biochemical processes in many organisms and usually exists in natural waters and sediments (Lorentzen et al. 1998; Grosell et al. 2003; Shao et al. 2010). Copper acts as a cofactor for many enzymes and other proteins (Gaetke and Chow 2003; Trevisan et al. 2010). This metal is also important as a part of antioxidant enzymes (e.g., Cu-Zn SOD) (Lorentzen et al. 1998). Although copper is an essential element, it can be potentially toxic to aquatic organisms when available in excess in the water (Linder and Hazegh-Azam 1996; Fedeli et al. 2010). According to the US Environmental Protection Agency's (USEPA 2007) water quality criteria for copper, copper concentrations range from 0.2 μg/L (3.1 nmol/L) to 30 μg/L (472 nmol/L) in uncontaminated freshwaters, but may be as high as 100 μg/L (1.6μmol/L) when copper enters aquatic environments as a result of human activities. Excess amount of copper has been detected in surface water, groundwater, and seawater. There is worldwide concern about contamination of drinking water by copper. In surface water, copper is an environmental hazard and poses a threat to many aquatic organisms (USEPA 2003). Among aquatic organisms, fish are vulnerable to copper because it can induce gill injury and acute lethality. Many previous literatures report the toxicity of copper and its effect on the physiology of aquatic organisms particularly in fish (Grosell et al. 2002; Monteiro et al. 2005; Ramesh et al. 2007; Eyckmans et al. 2010; Abdel-Khalek et al. 2015; Crémazy et al. 2016).

Recently, the emergence of nanoindustries for the welfare of the humankind is considered as the next industrial revolution (Lux Research 2008; Jevgenij et al. 2013). Nanoparticles are used in many fields including drugs, wastewater treatment, cosmetics, and anticidal agents (Aitken et al. 2006; Dubey et al. 2015). However, the rapid increase of nanoproducts in the global market has raised concern about their adverse effects on living organisms and the environment (Yousefian and Payam 2012; Noureen and Jabeen 2015). Among nanoparticles, metal oxide nanoparticles are largely manufactured, and their application is found in sunscreens, paints, construction materials, coatings, catalysts, and cosmetics (Aruoja et al. 2009; Hanna et al. 2013; Tavares et al. 2014). Nowadays, nanoparticles from Au, Ag, Pt, Fe, and Cu are commonly manufactured in different sizes and shapes for their wide applications. However, these particles have gained significant attention due to their negative ecological effects and their potential toxicity (Klaine et al. 2008; Melegaria et al. 2013; Abdel-Khalek et al. 2015; Gupta et al. 2016). The bioavailability of metal nanoparticles in the aquatic environment resulted in subsequent accumulation in fish and poses a risk to human health and the environment (Shaw and Handy 2011).

Copper nanoparticles (Cu NPs) are widely used as antiviral, antibacterial, antifouling, and antifungal additives in inks, lubricants, nanocomposite coating, and heat transfer fluid for enhanced conductivity and wear resistance (Chang et al. 2005; Brownheim 2011; Ates et al. 2014; Noureen and Jabeen 2015). Due to its wide applications and also from manufacturing and remediation processes, these nanoparticles will ultimately end up in the environment. Cu NPs are insoluble in water, which may release copper ions into the surrounding water and may affect biological functions

of organisms similar to soluble copper salts such as copper sulfate (Shaw and Handy 2011; Hoseini et al. 2016). The predicted environmental concentration of Cu NPs in freshwater is 0.06 mg Cu/L, and this has raised concern on their adverse effects to aquatic organisms (Ganesh et al. 2010; Chio et al. 2012; Chen et al. 2014; Song et al. 2015). Copper nanoparticles can cause genotoxicity and oxidative DNA damage at the cellular level and induce toxicity in organisms (Nel et al. 2006; Song et al., 2012; Mu et al. 2014; Hedberg et al. 2016). Cu NPs have the ability to cross the plasma membrane, cause oxidative stress, and accumulate in the tissues such as liver and gills of fish (Gomez et al. 1998; Griffitt et al. 2007; Wang et al. 2011; Melegari et al. 2013; Wang et al. 2013).

Although the toxicity of heavy metals has been studied for many years, the link between the fate of metal nanoparticles in the environment and their toxicity is still in its infancy. Furthermore, investigations of aggregations of nanoparticles with size distributions similar to those of their bulk particles in suspension have revealed that the toxicity mechanisms of nanoparticles are more complex (Warheit et al. 2006; Zhang et al. 2008). In suspensions, nanoparticles tend to form large particles and most of the aggregates will settle out of the suspensions in a few hours, which may reduce their toxicities (Adams et al. 2006). To date, there are widespread controversies and ambiguities in relation to toxicity of nanoparticles and bulk particles to aquatic organisms. Hence, a comparative study of nanoscale and microscale materials is essential because the intrinsic characteristic of nanoparticles may be directly related to their toxicity, and comprehensive characterization of suspensions of these particles is also needed (Ribeiro et al. 2013). Furthermore, a complete understanding of the toxicity of both bulk and nanoparticle is also essential.

In most of the ecotoxicological studies, fish has been considered as an ideal test organism to examine the toxicity of pollutants (Sharma et al. 2014). In addition, fish play a significant role in assessing potential risk caused by new chemicals (Lakra and Nagpure 2009). To assess the possible disturbances in the physiology of fish in stress condition, suitable biomarkers are widely used in ecotoxicological studies (Poopal et al. 2013). In this line, biomarkers such as biochemical, enzymological, and histopathological are widely used to evaluate the toxicity of aquatic pollutants (Saravanan et al. 2012; Ramesh et al. 2014). In freshwater fish, the physiological regulations of major electrolytes (Na^+, K^+, and Cl^-) are highly sensitive to environment stressors and are commonly altered in response to pollutants including nanomaterials (McDonald and Dalxical 1989; Krishnapriya et al. 2015). The alteration of these major electrolytes is widely used as biomarkers to determine the changes that occur in the environment (Kalay 2006; Poopal et al. 2013; Abhijith et al. 2016). Similarly, the enzyme ATPase is also commonly used as a sensitive biomarker for the assessment of membrane fragility of the gills (Stagg and Shuttleworth 1982).

Recently, copper contamination has been reported in South Indian Rivers and lakes including Godavari River (Ghorade et al. 2014; Gupta et al. 2016). *Labeo rohita* is a species of the carp family and is a natural inhabitant of freshwater sections of the rivers in India. It is also a major cultivable fish species in India. Hence, the present study is aimed to compare the toxic effects of nanocopper and bulk copper using plasma electrolyte (Na^+, K^+, and Cl^-) level and Na^+/K^+-ATPase activity in the gill of a freshwater fish *L. rohita*.

7.2 Materials and Method

7.2.1 Collection of Experimental Animals

Fingerlings of *L. rohita* (average length 6–7 cm and weight 8 g) were procured from Tamil Nadu Fisheries Development Corporation Limited, Aliyar Fish Farm, Tamil Nadu, India, and acclimatized to laboratory conditions for a period of 20 days. During acclimatization, fish were fed (ad libitum) with rice bran and ground nut oil cake daily 1 hour prior to replacement of water. Dechlorinated tap water was used for the present study. Water (three-fourths) was changed regularly in order to remove the excess feed and metabolic waste, and the following water qualities were maintained during the acclimatization and experimental period: temperature: 27.0 ± 3.0°C; pH: 7.0 ± 2, dissolved oxygen: 6.9 ± 0.02 mg/L; total alkalinity: 20.0 ± 3 mg/L; salinity: 0.42 ± 0.01 ppt; total hardness: 18.2 ± 0.01 mg/L; calcium: 3.4 ± 0.6 mg/L and magnesium: 2.05 ± 0.1 mg/L.

7.2.2 Toxicant

Copper nanoparticles were purchased from Sigma-Aldrich (manufacturer information: 99.9% purity, mean particle size of <50 nm, according to TEM). A stock solution of Cu NPs was prepared fresh by dispersing 1.0 g Cu NPs L^{-1} in Millipore water, (ion-free and unbuffered) with constant stirring for 1 hour followed by sonication for about 30 minutes in a bath-type sonicator (100 W, 40 kHz). The stock was stirred for 10 minutes and sonicated for about 20 minutes prior to dosing. Likewise, $CuSO_4$ stock solution was prepared by dissolving 1.0 g Cu^{2+} L^{-1} (3.929 g $CuSO_4{\cdot}5H_2O$ in 1 L of ultrapure water). In the present study, stock solution was prepared by following the procedure of Wang et al. (2014). The stock solution of copper sulfate was prepared by dissolving 1.0 g of Cu^{2+} (3.929 g $CuSO_4{\cdot}5H_2O$) in 1 L of ultrapure water.

7.2.3 Acute Toxicity Study

Based on the predicted environmental concentration of Cu NPs (0.06 mg/L, Chio et al. 2012), three different concentrations such as 20, 50, and 100 µg/L of both Cu NPs and bulk copper were selected to determine the toxicity. To each concentration, 10 healthy fingerlings of *L. rohita* were selected from the stock tank and introduced to glass aquaria. A control group (toxicant-free) was also maintained. Four replicates were maintained for each concentration and control group. Acute toxicity study was carried out for the period of 24 hours. Feeding was withheld before 24 hours of the experiment in order to minimize the risk of toxicant absorbing to food or metabolic waste. The dead fish were removed from the glass aquaria immediately.

7.2.3.1 Collection and Preparation of Samples

Blood from control, Cu NPs-treated, and bulk copper-treated groups was collected by cardiac puncture. The collected blood sample was immediately transferred into plastic vials, which is previously rinsed with heparin. The whole blood was centrifuged at −4°C for 20 minutes at 9392 g, and the plasma was collected which was

used to estimate the electrolyte level. After the collection of blood, the fish were kept in ice in order to arrest the enzymatic activity. For the enzymological assay, the fish were washed with double-distilled water and blotted dry with absorbent paper. Then, 100 mg gill tissue was separated from control and copper-treated fish and homogenized with 1.0 mL of 0.1 M ice-cold Tris–HCl buffer (pH 7.4) using a Teflon homogenizer and centrifuged at 9392 g, at 4°C for 15 minutes. The supernatant was used for the estimation of Na^+/K^+-ATPase activity (Shiosaka et al. 1971).

7.2.3.2 Ionoregulation Studies

7.2.3.2.1 Sodium Assay

Estimation of sodium was done by the method of Maruna (1958). Twenty microliters of sample (plasma) was collected from control and toxicant-treated fish and 1000 µL of precipitating reagent were added. Similarly, 20 µL of standard reagent was added to a standard tube followed by 1000 µL of precipitating reagent. The contents were mixed well, allowed to stand at room temperature for 5 minutes, and centrifuged at 2500–3000 rpm for 5 minutes to obtain a clear supernatant. Then, 20 µL of supernatant was taken and 1000 µL of acid reagent and 100 µL of color reagent were added, and tubes were mixed well and allowed to stand at room temperature for 5 minutes. The optical density of the sample and standard was measured against distilled water using a UV spectrophotometer at 530 nm, and the readings were expressed as millimoles per liter.

7.2.3.2.2 Potassium Assay

Plasma potassium was estimated by using the method of Maruna (1958). Twenty microliters of sample (plasma) was collected from control and toxicant-treated fish and 1000 µL of potassium reagent were added. Similarly, 20 µL potassium standard reagent was added to the standard test tube followed by 1000 µL of potassium reagent. The contents in the test tubes were mixed well and allowed to stand for 5 minutes at room temperature, and the optical density was measured against the blank using a UV spectrophotometer at 630 nm. The plasma potassium level was expressed as millimoles per liter.

7.2.3.2.3 Chloride Assay

Chloride was estimated by the modified method of Tietz (1990) and Young et al. (1975). Twenty microliters of sample (plasma) was collected from control and toxicant-treated fish and 1000 µL of chloride reagent were added. Similarly, 10 µL of chloride standard reagent was taken in a test tube and 1000 µL of chloride reagent was added. The contents in the tubes were mixed well and kept for 3 minutes at room temperature. The optical density of samples was measured against the blank using a UV spectrophotometer at 505 nm, and the readings were expressed as millimoles per liter.

7.2.3.2.4 Enzymological Studies

The Na^+/K^+-ATPase activity was estimated by following the method of Shiosaka et al. (1971).

7.2.3.2.5 Statistical Analysis

Statistical analysis was made individually for each sample, and the mean value of five individual observations was taken for each parameter. The standard error for the

sample mean was calculated and is given in appropriate tables. The significance of the mean of control and Cu NPs and Cu BP-treated fish fingerlings was tested using ANOVA. The analytical data together with bar diagrams are presented on appropriate places in the text.

7.3 Results

The size of the Cu NP was <50 nm according to the manufacturer's information. In the present investigation, the mortality of fish fingerlings was noticed in 50 and 100 µg/L Cu BP-treated groups within 24 hours. However, no mortality was observed in fish exposed to all the three concentrations of Cu NP-exposed groups. In this study, plasma Na^+ level was increased significantly ($p < 0.05$) in 20 µg/L Cu NP-treated fish when compared to 20 µg/L of Cu BPs and control fish (Figure 7.1). In contrast, a significant increase in plasma sodium level was noted in 50 and 100 µg/L of Cu BP-exposed fish when compared with 50 and 100 µg/L Cu NP-exposed and control groups. A maximum percent increase of 93.34 was observed in the 100 µg/L Cu BP-exposed group after 24 hours. The plasma Na^+ level of the freshwater fish *L. rohita* exposed to different concentrations of copper nanoparticles and copper bulk particles for 24 hours was significant statistically at $F(6, 34) = 1997.790^{***}$.

Plasma K^+ level was found to be decreased significantly ($p < 0.05$) in the 20 µg/L Cu NP-treated fish when compared with the 20 µg/L Cu BP and control groups. Similarly, a significant decrease ($p < 0.05$) in plasma K^+ level was observed in 100 µg/L Cu BP-treated fish when compared to 100 µg/L Cu NP and control groups.

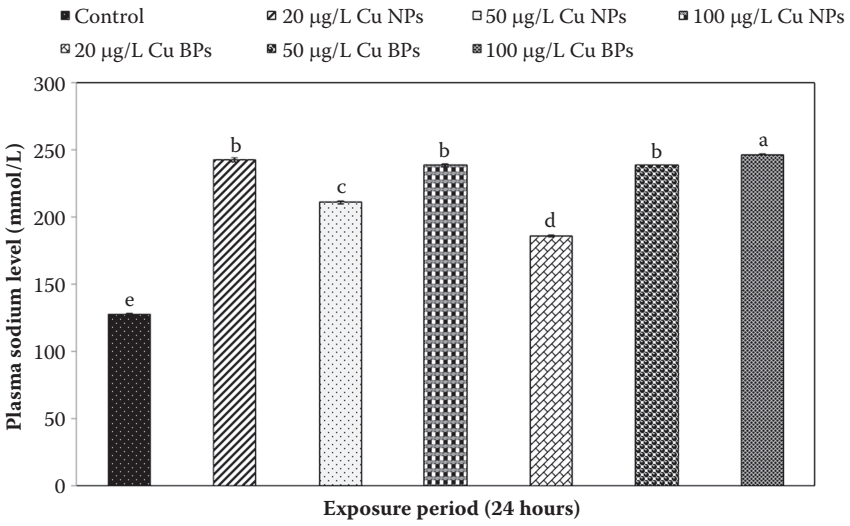


FIGURE 7.1 Changes in the plasma sodium of the freshwater fish *L. rohita* exposed to different concentrations of copper nanoparticles and copper bulk particles for 24 hours. The means in the histogram with the same letters for the compounds are not significantly different ($p < 0.05$) according to Duncan's multiple range test (DMRT).

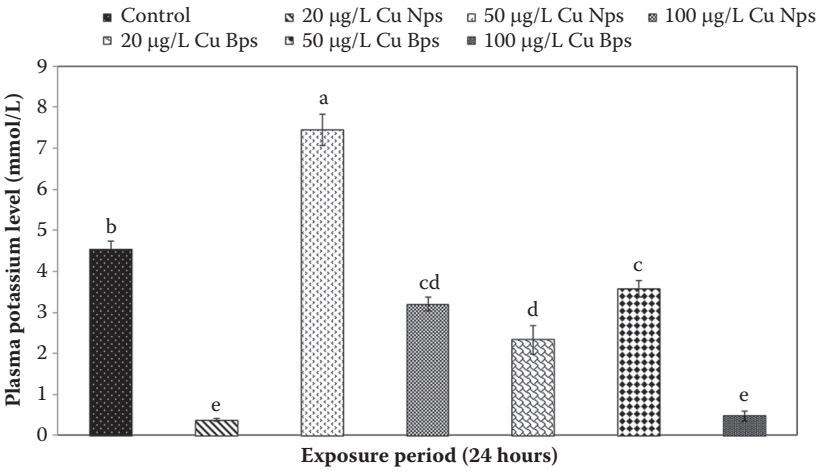


FIGURE 7.2 Changes in the plasma potassium level of the freshwater fish *L. rohita* exposed to different concentrations of copper nanoparticles and copper bulk particles for 24 hours. The means in the histogram with the same letters for the compounds are not significantly different ($p < 0.05$) according to DMRT.

A maximum percent decrease (–91.53%) of plasma potassium level was observed in 20 µg/L of Cu NP-exposed fish. In contrast to the increase previously mentioned, a significant increase ($p < 0.05$) in plasma K^+ level was observed in 50 µg/L of Cu NP-treated fish when compared to 50 µg/L Cu BP and control groups (Figure 7.2). A maximum percent increase (64.53%) in plasma potassium level was observed in fish exposed to 50 µg/L of Cu NPs. The plasma K^+ level of the freshwater fish *L. rohita* exposed to different concentrations of copper nanoparticles and copper bulk particles for 24 hours was significant statistically at F (6, 34) = 103.311***.

There was a significant increase ($p < 0.05$) in plasma chloride level in fish exposed to 20 µg/L of Cu BP-treated fish when compared with 20 µg/L of Cu NP and control groups (Figure 7.3). Similarly, a significant increase in plasma chloride level was observed in fish exposed to 50 and 100 µg/L of Cu NP-treated fish when compared to 50 and 100 µg/L of Cu BP and control groups. A percent increase of 153, 428.25, and 816.64 was observed in fish exposed to 20, 50, and 100 µg/L of Cu NP-treated fish, respectively. Similarly, a percent increase of 427.531, 158.06, and 85.67 was observed in fish exposed to 20, 50, and 100 µg/L of Cu BP-treated fish, respectively. The plasma Cl^- level of the freshwater fish *L. rohita* exposed to different concentrations of copper nanoparticles and copper bulk particles for 24 hours was significant statistically at F (6, 34) = 4722.874***. Fish exposed to Cu NPs showed a significant ($p < 0.05$) increase in gill Na^+/K^+-ATPase activity in all the three concentrations (20, 50, and 100 µg/L) when compared with Cu BP-treated fish and control groups (Figure 7.4). A maximum percent increase of 407.647 was noticed in fish exposed to 50 µg/L of Cu NPs. The plasma Na^+/K^+ ATPase activity of the freshwater fish *L. rohita* exposed to different concentrations of copper nanoparticles and copper bulk particles for 24 hours was significant statistically at F (6, 34) = 430.762***.

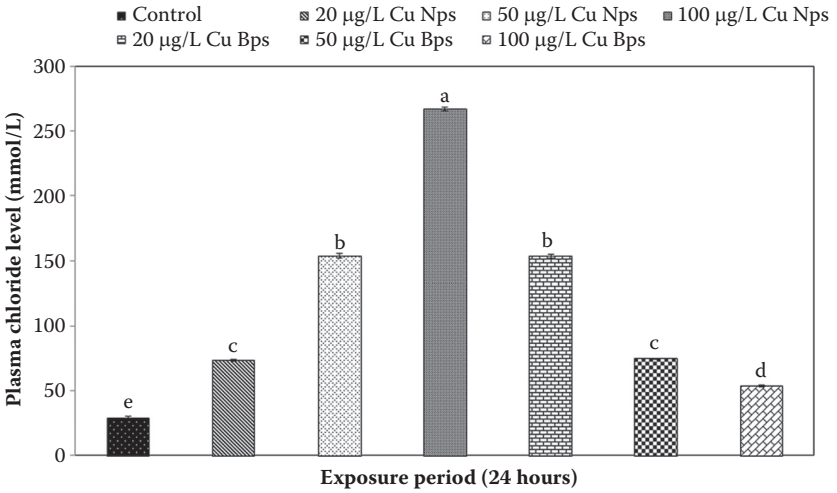


FIGURE 7.3 Changes in the plasma chloride level of the freshwater fish *L. rohita* exposed to different concentrations of copper nanoparticles and copper bulk particles for 24 hours. The means in the histogram with the same letters for the compounds are not significantly different ($p < 0.05$) according to DMRT.

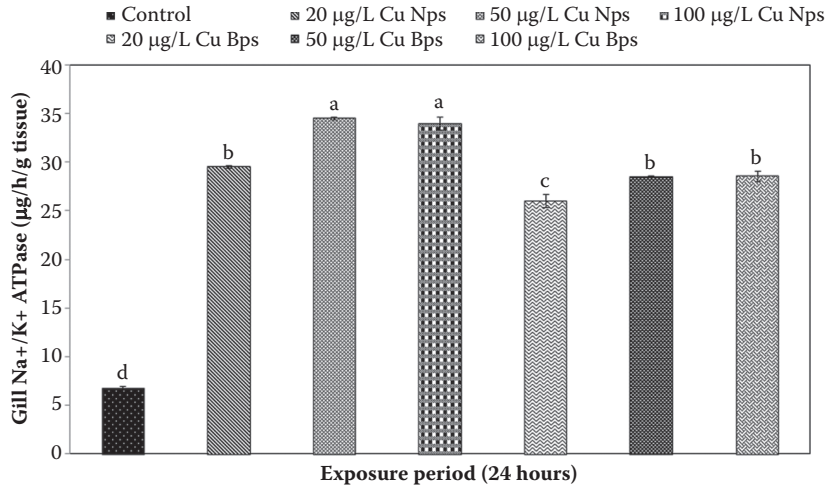


FIGURE 7.4 Changes in the gill Na^+/K^+ ATPase activity of the freshwater fish *L. rohita* exposed to different concentrations of copper nanoparticles and copper bulk particles for 24 hours. The means in the histogram with the same letters for the compounds are not significantly different ($p < 0.05$) according to DMRT.

7.4 Discussion

Maintenance of constant internal ion concentration (sodium, potassium, chloride, calcium, and magnesium) is essential for active regulation of water influx and ion efflux in aquatic organisms (Mayer et al. 1992; Logaswamy et al. 2007). Electrolytes (sodium, potassium, chloride, calcium, and magnesium) of body fluids contribute a majority of the osmotically active particles, to provide buffer systems and mechanisms for the regulations of pH (acid–base balance) (Hilmy et al. 1982). In addition, they provide proper ionic balance for normal neuromuscular irritability and tissue functions and the various metabolic events in tissues and organ system are regulated by these electrolytes (Prosser 1973; Adedeji 2010). Na^+ and Cl^- are the major cation and anion of the extracellular fluid, respectively, whereas K^+ is the major cation of the intracellular fluid (Forstner and Wittman 1983). These ions are very sensitive to environment stressors, and their measurement in blood of aquatic organisms can be used to monitor the polluted water bodies (Hemalatha et al. 2016). Furthermore, measurement of specific ion concentrations in blood of aquatic organisms can be used as sensitive biomarkers of chemical exposure and effects (Mayer et al., 1992). Generally, the concentrations of ions in blood plasma are commonly altered in response to pollutants (Larsson et al. 1985). A significant decrease in plasma electrolyte levels has been reported in many fish species exposed to metals (Van der Putte et al. 1982; Gonalez et al. 1990; Schjolden et al. 2007; Eyckmans et al. 2010). Likewise, a significant decrease in plasma electrolytes has been reported by many authors (Christensen et al. 1977; Sayer et al. 1989; Dussault et al. 2001; Pane et al. 2003; Hemalatha et al. 2016). McDonald et al. (1989) have reviewed the effects of pH and metals such as Al, Cu, Cd, Zn, Pb, Mg, Fe, and Ni on fish ionoregulation.

Metals are toxic to aquatic organisms by means of their activity on the surface of the gills, and they damage the epithelial membranes and impair the ability of the gills to regulate ions (McDonald et al. 1989; Dang et al. 2000). In the present investigation, a significant increase in plasma sodium level was observed in both bulk copper- and nanocopper-treated fish. Similar to our study, a significant increase in plasma sodium level has been reported in brook trout *Salvelinus fontinalis* exposed to methyl mercury, cadmium, and lead (Christensen et al. 1977) and in rainbow trout *Oncorhynchus mykiss* and *Platichthys flesus* exposed to copper (Stagg and Shuttleworth 1982; Handy et al. 1999). Like plasma sodium level, chloride level also increased in both bulk copper- and nanocopper-treated fish. Elevation in plasma Na^+ and Cl– level has been reported in fish exposed to silver nanoparticles, silica nanoparticles, TiO_2 nanoparticles, and single-walled carbon nanotubes (SWCNTs) (Federici et al. 2007; Smith et al. 2007; Farmen et al. 2012; Krishnapriya et al., 2015). A significant increase in plasma chloride level has also been reported in *Aphanius dispar* exposed to cadmium and mercury (Larsson et al. 1976; Hilmy et al. 1982). Pane et al. (2003) noted alterations of plasma Cl^- and Na^+ level in *O. mykiss* exposed to nickel. Fish gills play a vital role in transport of ions to maintain acid–base balance, osmotic pressure, and regulation of water influx and ion efflux (Mayer et al. 1992). Elevated levels of Na^+ and Cl^- level in fish blood may be due to the changes in the water ion equilibrium that occurred in inner and inter cells under toxic effects (Hilmy et al. 1987). Furthermore, elevated levels of sodium and chloride ions in fish may be due to toxic effects of aquatic pollutants on gill structures that are responsible for ion homeostasis (Wendelaar Bonga and Lock 1991). In the

present investigation, an increased level of plasma Na^+ and Cl^- ions in both bulk copper and nanocopper treatments might have attributed to their consequent reduction in the tissues of fish, thereby indicating the imbalances in the osmoregulation phenomenon due to copper toxicity. Loss of water from the circulation may also lead to a significant rise in electrolytes during stress condition (Fletcher 1975). Furthermore, the increase in plasma Cl^- level indicates transportation of chloride ions from other tissues into blood due to imbalances in the osmoregulation process (Suvetha et al. 2010). In general, nanoparticles enter the cell through diffusion, ion channels, transporter protein molecules, and endocytosis (Colvin et al. 2003; Nel et al. 2009; Chang et al. 2012; Krishnapriya and Ramesh 2016). Since the structure of copper ions is very similar to sodium ions, it may ultimately disturb the sodium ion homeostasis.

Similar to our study, a significant decrease in plasma K^+ level was noticed in tropical fish *Prochilodus scrofa* and in crucian carp, *Carassius carassius*, exposed to copper (Cerqueira and Fernandes, 2002; Schjolden et al. 2007). Likewise, decreased serum K^+ level has also been reported in rainbow trout, *O. mykiss*, exposed to copper, in *Salmo gairdneri* exposed to cadmium and in *Lagodon rhomboids* treated with $CC1_4$ (Giles et al. 1984; Folmar et al. 1993; Handy et al. 1999). Larsson et al. (1981) reported a pronounced decrease in potassium level during long-term exposure of flounders to acute cadmium stress. They argued that disturbed potassium regulation might be due to an impaired active reabsorption of potassium in renal tubules or renal dysfunction due to toxicant stress. Recently, a significant decrease in plasma potassium level has been reported in juvenile Atlantic salmon exposed to silver nanoparticles (Farmen et al. 2012).

Staurnes et al. (1984) reported that the ion loss may be due largely to a stimulation of passive efflux, with a small contribution from increased blockade of influx, possibly due to an inhibition of the Na^+/K^+-ATPase pump. Osmoregulatory failure may be also a reason for the decreased levels of major plasma electrolytes (Larsson et al. 1976). In the present study, the decrease in plasma potassium level might have resulted from inhibition of the Na^+/K^+-ATPase activity due to copper toxicity or structural alterations in renal tubules in the kidney of fish. However, the observed increase in plasma potassium level during Cu NP treatment might have resulted from transfer of these ions from other tissues to blood. Playle et al. (1989) reported that a significant increase of K^+ was likely due to the decreased plasma volume, which would translate into higher concentrations of this parameter.

Despite being an essential nutrient, copper at high levels can disturb fish gill ionoregulatory functions, leading to detrimental disturbance of electrolyte homeostasis (Laurén and McDonald 1985; Grosell et al. 2002; Grosell 2012). At elevated levels, it targets the gills, the main gas, and ion exchange surface in fish, interfering with respiration by increasing diffusion distance and with ion regulation by inhibiting key transport processes such as the Na^+/K^+-ATPase (Mallat 1985; Grosell et al. 2007; De Boeck et al. 2010). Waterborne copper, even at relatively low concentrations, causes mucus production, cell swelling, and epithelial lifting in the gills (Mallat 1985; Wood 2001). The enzyme Na^+/K^+-ATPase plays a central role in whole body osmoregulation (Alam and Frankel 2006). In aquatic vertebrates and invertebrates, the enzyme branchial Na^+/K^+–ATPase plays a vital role in regulation of ions and cellular water balance (Heath 1987; Canli and Stagg 1996; Grosell et al. 2003; Atli and Canli 2007). In fish gills, this enzyme is expressed abundantly in the tubular system of chloride cells and performs an integral role in the maintenance of ion balance (Flik et al. 1995; Perry 1997; Ay et al. 1999). In the present study, a significant

increase in Na^+/K^+-ATPase activity was observed in all the three concentrations of Cu NP-exposed fish.

Xenobiotics can alter the Na^+/K^+-ATPase activity by disrupting energy-producing metabolic pathways or interacting directly with the enzyme (Alam and Frankel 2006; Agrahari and Gopal 2008). Several studies demonstrated a decrease in gill Na^+/K^+-ATPase in fish exposed to metals (Watson and Beamish 1981; Lionetto et al. 2000; Atli and Canli 2007). On the other hand, a significant increase in gill Na^+/K^+-ATPase activity has been reported in American lobster *Homarus americanus* exposed to cadmium and in salmon *Salmo salar* and trout, *O. mykiss*, exposed to zinc (Lorz and McPherson 1976; Tucker 1979; Watson and Beamish 1980). Grosell et al. (2004) reported an elevation of Na^+/K^+-ATPase activity in the intestine of *Opsanus beta* exposed to copper. Likewise, an elevation in the Na^+/K^+-ATPase activity has been reported in rainbow trout (*O. mykiss*) exposed to SWCNT, in *L. rohita* treated with silica nanoparticle, and in *Carassius auratus* exposed to metal oxide nanoparticles (Smith et al. 2007; Jun et al. 2013; Krishnapriya et al. 2015).

According to Hogstrand et al. (1999), the increase in gill Na^+/K^+-ATPase could be explained as a compensation for a dysfunctional regulation of chloride and sodium levels. A slight increase in Na^+/K^+-ATPase activity in arsenic-treated *Dreissena polymorpha* may be due to a process to restore electrolyte losses (Bouskill et al. 2006). In the present study, also the significant increase in gill Na^+/K^+-ATPase activity during both bulk copper and nanocopper toxicity might have resulted from a compensation for a dysfunctional regulation of chloride and sodium levels. Gupta (1987) reported that inhibition or stimulation in enzyme activity was found to be dose and duration dependent. Gill Na^+/K^+-ATPase activity appeared to be more sensitive to the chronic waterborne copper exposure in *O. mykiss* compared to intestinal tissue (Kamunde and Wood 2003). This may be due to tissue-specific differences in Na^+/K^+-ATPase enzyme kinetics and the induction of metallothionein with greater sequestration of Cu in the intestine than in the gill.

Metallic copper is toxic to fish, induces gill injury and osmoregulatory disturbances, alters cell morphology, and causes oxidative stress (Grosell et al. 2002; Handy 2003; Ramesh et al. 2007; Eyckmans et al. 2011; Sovová et al. 2014; Abdel-Khalek et al. 2015). Likewise, nanocopper ions which are released from Cu NPs can easily enter the cells through sodium ion exchange and gills in fish. As a result, it disturbs the ion homeostasis and alters the Na^+/K^+-ATPase activity (Shaw and Handy 2011; Kong et al. 2013; Song et al. 2015; Hedayati et al. 2016). Cu NPs disrupt ionoregulatory homeostasis causing efflux of electrolytes from the blood by the gill epithelium into the water and can lead to mortality in fish (Handy 2003; Gupta et al. 2016). The toxicity of the nanoparticle is mainly increased by the size, shape, solubility, charge, zeta potential, dissolution, and aggregation (Keller et al. 2010; Chang et al. 2012; Park et al. 2014; Noureen and Jabeen 2015). However, physicochemical characteristics of water, such as pH, ionic strength, and hardness, may persuade the aggregation and toxicity of NPs (Hoecke et al. 2011; McLaughlin and Bonzongo 2012; Park et al. 2014).

7.5 Conclusion

Cu NPs and bulk copper could cause adverse effects on the nontargeting organism *L. rohita*, and the alterations of electrolytes and Na^+/K^+-ATPase activity can be used as a potential biomarker in analyzing the health of aquatic organisms and the

ecosystem. However, to confirm the toxicity of nanoparticles over the bulk metal, chronic toxicity studies are needed. Further research is needed in physiochemical characteristics of nanoparticles and its level of toxicity in the aquatic organisms.

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REFERENCES

Abdel-Khalek, A. A., M. A. M. Kadry, S. R. Badran et al., 2015. Comparative toxicity of copper oxide bulk and nanoparticles in Nile Tilapia; *Oreochromis niloticus*: Biochemical and oxidative stress. *Journal of Basic and Applied Zoology* 72:43–57.

Abhijith, B. D., M. Ramesh, and R. K. Poopal, 2016. Responses of metabolic and antioxidant enzymatic activities in gill, liver and plasma of *Catla catla* during methyl parathion exposure. *Journal of Basic and Applied Zoology* 77:31–40.

Adams, L., D. Lyon, and P. Alvarez, 2006. Comparative eco-toxicity of nanoscale TiO_2, SiO_2, and ZnO in water suspensions. *Water Research* 40:3527–32.

Adedeji, O. B., 2010. Acute effect of diazinon on blood plasma biochemistry in the African catfish (*Clarias gariepinus*). *Journal of Clinical Medicine and Research* 2(1):001–006.

Agrahari, S. and K. Gopal, 2008. Inhibition of Na^+/K^+-ATPase in different tissues of freshwater fish *Channa punctatus* (Bloch) exposed to monocrotophos. *Pesticide Biochemistry and Physiology* 92:57–60.

Aitken, R., M. Chaudhry, A. Boxall et al., 2006. Manufacture and use of nanomaterials: Current status in the UK and global trends. *Occupational Medicine* 56:300–306.

Alam, M. and T. Frankel, 2006. Gill ATPase of silver perch, *Bidyanus bidyanus*, and golden perch, *Macquaria ambigua*: Effects of environmental salt and ammonia. *Aquaculture* 251:118–133.

Aruoja, V., H. C. Dubourguier, K. Kasemets et al., 2009. Toxicity of nanoparticles of CuO, ZnO and TiO_2 to microalgae *Pseudokirchneriella subcapitata*. *Science of the Total Environment* 407:1461–1468.

Ates, M., M. A. Dugo, V. Demir et al., 2014. Effect of copper oxide nanoparticles to sheepshead minnow (*Cyprinodon variegatus*) at different salinities. *Digest of Journal of Nanomaterials and Biostructures* 9:1,369–377.

Atli, G. and M. Canli, 2007. Enzymatic responses to metal exposures in a freshwater fish *Oreochromis niloticus*. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology* 145:282–287.

Ay, O., M. Kalay, L. Tamer, and M. Canli, 1999. Copper and lead accumulation in tissues of a freshwater fish Tilapia zillii and its effects on the branchial Na^+/K^+-ATPase activity. *Bulletin of Environmental Contamination and Toxicology* 62:160–168.

Bargagli, R., 2000. Trace metals in Antarctica related to climate change and increasing human impact. *Review of Environmental Contamination and Toxicology* 166:129–173.

Bouskill, N. J., R. D. Handy, T. E. Ford et al., 2016. Differentiating copper and arsenic toxicity using biochemical biomarkers in *Asellus aquaticus* and *Dreissena polymorpha*. *Ecotoxicology and Environmental Safety* 65(3):342–349.

Brownheim, S. V., 2011. Characterization and *in vitro* toxicity of copper nanoparticles (Cu-NPs) in murine neuroblastoma (N2A) cells. Master's thesis, Department of Systems and Engineering Management, Graduate School of Engineering and Management, Air Force Institute of Technology, Air University, Wright-Patterson Air Force Base, OH.

Canli, M. and G. Atli, 2003. The relationships between heavy metal (Cd, Cr, Cu, Fe, Pb and Zn) levels and the size of six Mediterranean fish species. *Environmental Pollution* 121:129–136.

Canli, M. and R. M. Stagg, 1996. The effects of in vivo exposure to cadmium, copper and zinc on the activities of gill ATPases in the Norway lobster, *Nephrops norvegicus*. *Archives of Environmental Contamination and Toxicology* 31:494–501.

Cerquiera, C. C. C. and M. N. Fernandes, 2002. Gill tissue recovery after copper exposure and blood parameter responses in the tropical fish *Prochilodus scrofa*. *Ecotoxicology and Environmental Safety* 52:83–91.

Chang, H., C. Jwo, C. Lo et al., 2005. Effect of copper oxide nanoparticles to sheepshead minnow (*Cyprinodon variegatus*) at different salinities. *Reviews on Advanced Materials Science* 10:128.

Chang, Y. N., M. Zhang, L. Xia et al., 2012. The toxic effects and mechanisms of CuO and ZnO nanoparticles. *Materials (Basel)* 5(12):2850–2871.

Chen, H., Y. Chen, X. Zheng et al., 2014. How does the entering of copper nanoparticles into biological wastewater treatment system affect sludge treatment for VFA production. *Water Research* 63:125–134.

Chio, C. P., W. Y. Chen, W. C. Chou et al., 2012. Assessing the potential risks to zebra fish posed by environmentally relevant copper and silver nanoparticles. *Science of the Total Environment* 420:111–118.

Christensen, G. M., E. Hund, and J. Fiandt, 1977. The effect of methyl mercury chloride, cadmium chloride, and lead nitrate on six biochemical factors of the brook trout, *Salvelinus fontinalis*. *Toxicology and Applied Pharmacology* 42:523–530.

Colvin, R. A., C. P. Fontaine, M. Laskowski et al., 2003. Zn^{2+} transporters and Zn^{2+} homeostasis in neurons. *European Journal of Pharmacology* 479(1–3):171–185.

Crémazy, A., C. M. Wood, D. S. Smith et al., 2016. Investigating copper toxicity in the tropical fish cardinal tetra (*Paracheirodon axelrodi*) in natural Amazonian waters: Measurements, modeling, and reality. *Aquatic Toxicology* 180:353–363.

Dang, Z., R. A. C. Lock, G. Flick et al., 2000. Na^+/K^+–ATPase immunoreactivity in branchial chloride cells of *Oreochromis mossambicus* exposed to cooper. *Journal of Experimental Biology* 151:517–428.

De Boeck, G., R. Smolders, R. Blust, 2010. Copper toxicity in gibel carp *Carassius auratus gibelio*: Importance of sodium and glycogen. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology* 152(3):332–337.

Dubey, A., M. Goswami, K. Yadav et al., 2015. Oxidative stress and nano-toxicity induced by TiO_2 and ZnO on WAG cell line. *Plos One* 10(5):e0127493.

Dussault, E. B., R. C. Playle, D. G. Dixon et al., 2001. Effects of sublethal, acidic aluminum exposure on blood ions and metabolites, cardiac output, heart rate, and stroke volume of rainbow trout, *Oncorhynchus mykiss*. *Fish Physiology and Biochemistry* 25(4):347–357.

Eyckmans, M., C. Tudorache, V. M. Darras et al., 2010. Hormonal and ion regulatory response in three freshwater fish species following waterborne copper exposure. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology* 152(3):270–278.

Eyckmans, M., N. Celis, N. Horemans et al., 2011. Exposure to waterborne copper reveals differences in oxidative stress response in three freshwater fish species. *Aquatic Toxicology* 103:112–120.

Farmen, E., H. N. Mikkelsen, O. Evensen et al., 2012. Acute and sublethal effects in juvenile Atlantic salmon exposed to low 1 g/L concentrations of Ag nanoparticles. *Aquatic Toxicology* 108:78–84.

Fedeli, D., M. Carloni, G. Falcioni, 2010. Oxidative damage in trout erythrocyte in response to "in vitro" copper exposure. *Marine Environmental Research* 69(3):172–177.

Federici, G., B. J. Shaw, R. D. Handy, 2007. Toxicity of titanium dioxide nanoparticles to rainbow trout (*Oncorhynchus mykiss*): Gill injury, oxidative stress, and other physiological effects. *Aquatic Toxicology* 84(4):415–430.

Flik, G., P. M. Verbost, S. E. Wendelaar Bonga, 1995. Calcium transport processes in fishes. In *Fish Physiology*, eds. Hoar, W. S., D. J. Randall and A. P. Farrell, vol. 14. Academic Press, San Diego, CA, pp. 317–342.

Fletcher, G. L., 1975. The effects of capture stress and storage of whole blood on the red blood cells, plasma proteins, glucose and electrolytes of the winter flounder, *Pseudopleuronectes americanus*. *Canadian Journal of Zoology* 53:197–206.

Florea, A. M., E. Dopp, G. Obe et al., 2004. Genotoxicity of organometallic species. In *Organic Metal and Metalloid Species in the Environment: Analysis, Distribution, Processes and Toxicological Evaluation*, eds. Hirner, A. V. and H. Emons. Springer-Verlag, Heidelberg, pp. 205–219.

Folmar, L. C., S. Bonomelli, T. Moody et al., 1993. The effect of short term exposure to three chemicals on the blood chemistry of the pinfish, *Lagodon romboides*. *Archives of Environmental Contamination and Toxicology* 24:83–86.

Forstner, N. and G. T. W. Wittman, 1983. Heavy metals in aquatic organisms. In *Metal Pollution in the Aquatic Environment*. 2nd ed., Springer-Verlag, Berlin, Germany, pp. 271–323.

Gaetke, L. M., and C. K. Chow, 2003. Copper toxicity, oxidative stress, and antioxidant nutrients. *Toxicology* 189:147–163.

Ganesh, R., J. Smeraldi, T. Hosseini et al., 2010. Evaluation of nanocopper removal and toxicity in municipal wastewaters. *Environmental Science and Technology* 44:7808–7813.

Ghorade, I. B., S. V. Lamture, S. S. Patil, 2014. Assessment of heavy metal content in Godavari river water. *International Journal of Research in Applied, Natural and Social Sciences* 2:23–26.

Giles, M. A., H. S. Majewski, B. Hobden, 1984. Osmoregulatory and hematological responses of rainbow trout (*Salmo gairdneri*) to extended environmental acidification. *Canadian Journal of Fisheries and Aquatic Sciences* 41:1686–1694.

Gomez, J., A. C. Martínez, A. Gonzalez et al., 1998. Dual role of Ras and rho proteins: At the cutting edge of life and death. *Immunology and Cell Biology* 76: 125–134.

Gonzalez, R. J., R. S. Grippo, W. A. Dunson, 1990. The disruption of sodium balance in brook char, *Salvelinus fontinalis* (Mitchell), by manganese and iron. *Journal of Fish Biology* 37:765–774.

Griboff, J., D. A. Wunderlin, M. V. Monferran, 2017. Metals, As and Se determination by inductively coupled plasma-mass spectrometry (ICP-MS) in edible fish collected from three eutrophic reservoirs. Their consumption represents a risk for human health? *Microchemical Journal* 130:236–244.

Griffitt, R. J., R. Weil, K. A. Hyndman et al., 2007. Exposure to copper nanoparticles causes gill injury and acute lethality in zebrafish (*Danio rerio*). *Environmental Science and Technology* 41:8178–8186.

Grosell, M., M. D. McDonald, P. J. Walsh et al., 2004. Effects of prolonged copper exposure in the marine gulf toadfish (*Opsanus beta*). II. Drinking rate, copper accumulation and Na^+/K^+-ATPase activity in osmoregulatory tissues. *Aquatic Toxicology* 68:263–275.

Grosell, M., C. Nielsen, and A. Bianchini, 2002. Sodium turnover rate determines sensitivity to acute copper and silver exposure in freshwater animals. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology* 133:287–303.

Grosell, M., C. M. Wood, and P. J. Walsh, 2003. Copper homeostasis and toxicity in the elasmobranch *Raja erinacea* and the teleost *Myoxocephalus octodecemspinosus* during exposure to elevated water-borne copper. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology* 135:179–190.

Grosell, M., J. Blanchard, K. V. Brix et al., 2007. Physiology is pivotal for interactions between salinity and acute copper toxicity to fish and invertebrates. *Aquatic Toxicology* 84:162–172.

Grosell, M., 2012. Copper. In *Fish Physiology: Homeostasis and Toxicology of Essential Metals* eds. Wood, C. M., A. P. Farrell, C. J. Brauner. vol. 31A. Elsevier, London, pp. 53–133.

Gupta, S., 1987. Physiological stress induced by vegetable oil factory effluent in *Channa punctatus* (Bloch): Measurement of hepatic dehydrogenases. *Bulletin of Environmental Contamination and Toxicology* 39:417–424.

Gupta Y. R., D. Sellegounder, M. Kannan et al., 2016. Effect of copper nanoparticles exposure in the physiology of the common carp (*Cyprinus carpio*): Biochemical, histological and proteomic approaches. *Aquaculture and Fisheries* 1:15–23.

Handy, R. D., D. W. Sims, A. Giles et al., 1999. Metabolic trade-off between locomotion and detoxification for maintenance of blood chemistry and growth parameters by rainbow trout (*Oncorhynchus mykiss*) during chronic dietary exposure to copper. *Aquatic Toxicology* 47:23–41.

Handy, R. D., 2003. Chronic effects of copper exposure versus endocrine toxicity: Two sides of the same toxicological process? *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology* 135:25–38.

Hanna, S. K., R. J. Miller, D. Zhou et al., 2013. Accumulation and toxicity of metal oxide nanoparticles in a soft-sediment estuarine amphipod. *Aquatic Toxicology* 142–143:441–446.

Heath, A. G., 1987. *Water Pollution and Fish Physiology*, CRC Press, Boca Raton, FL, p. 245.

Hedayati, A., S. M. Hoseini, S. H. Hoseinifar, 2016. Response of plasma copper, ceruloplasmin, iron and ions in carp, *Cyprinus carpio* to waterborne copper ion and nanoparticle exposure. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology* 179:87–93.

Hedberg, J., H. L. Karlsson, Y. Hedberg et al., 2016. The importance of extracellular speciation and corrosion of copper nanoparticles on lung cell membrane integrity. *Colloids and Surface B: Biointerfaces* 14:291–300.

Hemalatha, D., A. Muthukumar, B. Rangasamy, B. Nataraj, M. Ramesh et al., 2016. Impact of sublethal concentration of a fungicide propiconazole on certain health biomarkers of Indian major carp *Labeo rohita*. *Biocatalysis and Agricultural Biotechnology* 8:321–327.

Hilmy, A. M., M. B. Shabana, M. M. Saied, 1982. Ionic regulation of the blood in the Syprinodont, *Apanius dispar* (Rupp.), under the effect of experimental mercury pollution. *Water Air Soil Pollution* 18:467–473.

Hilmy, A. M., N. A. El-Domiaty, K. Weshana, 1987. Acute and chronic toxicity of nitrite to *Clarias lazera*. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology* 86:247–253.

Hoecke, K. V., K. A. C. De Schamphelaere, P. V. D. Meeren et al., 2011. Aggregation and ecotoxicity of CeO_2 nanoparticles in synthetic and natural waters with variable pH, organic matter concentration and ionic strength. *Environmental Pollution* 159:970–976.

Hogstrand, C., E. A. Ferguson, F. Galvez et al., 1999. Physiology of acute silver toxicity in the starry founder (*Platichthys stellatus*) in seawater. *Journal of Comparative Physiology B*, 169:461–73.

Hoseini, S. M., A. Hedayati, A. T. Mirghaed et al., 2016. Toxic effects of copper sulfate and copper nanoparticles on minerals, enzymes, thyroid hormones and protein fractions of plasma and histopathology in common carp *Cyprinus carpio*. *Experimental and Toxicologic Pathology* 68(9):493–503.

Jevgenij, A. K., S. K. Ružena, Z. Dagmar et al., 2013. Acute toxicity of 31 different nanoparticles to zebrafish (*Danio rerio*) tested in adulthood and in early life stages—Comparative study. *Interdisciplinary Toxicology* 6(2):67–73.

Jun, X., Z. H. Zhou, L. U. G. Hua, 2013. Effects of selected metal oxide nanoparticles on multiple biomarkers in *Carassius auratus*. *Biomedical and Environmental Sciences* 26(9):742–749.

Kalay, M., 2006. The effect of cadmium on the levels of Na^+, K^+, Ca^{++} and Mg^{++} in serum of *Tilapia nilotica L. Ekoloji* 15:1–7.

Kamunde, C. N. and C. M. Wood 2003. The influence of ration size on copper homeostasis during sublethal dietary copper exposure in juvenile rainbow trout *Oncorhynchus mykiss*. *Aquatic Toxicology* 62:235–54.

Keller, A. A., H. Wang, D. Zhou et al., 2010. Stability and aggregation of metal oxide nanoparticles in natural aqueous matrices. *Environmental Science and Technol*ogy 44(6):1962–1967.

Klaine, S. J., P. J. J. Alvarez, G. E. Batley et al., 2008. Nanomaterials in the environment: Behavior, fate, bioavailability, and effects. *Environmental Toxicology and Chem*istry 27:1825–1851.

Kong, X., H. Jiang, S. Wang et al., 2013. Effects of copper exposure on the hatching status and antioxidant defense at different developmental stages of embryos and larvae of goldfish *Carassius auratus*. *Chemosphere* 92:1458–1464.

Krishnapriya, K., and M. Ramesh, 2016. Copper and copper nanoparticles induced hematological changes in a freshwater fish *Labeo rohita*—A comparative study: Copper and copper nanoparticle toxicity to fish. Chapter 15, IGI Global, Hershey, PA, pp. 352–375.

Krishnapriya, K., M. Ramesh, M. Saravanan et al., 2015. Ecological risk assessment of silicon dioxide nanoparticles in a fresh-water fish *Labeo rohita*: Hematology, ionoregulation and gill Na^+/K^+ATPase activity. *Ecotoxicology and Environmental Safety* 120:295–302.

Kucuksezgin, F., A. Kontas, O. Altay et al., 2006. Assessment of marine pollution in Izmir Bay. Nutrient, heavy metal ant total hydrocarbon concentrations. *Environmental International* 32(1):41–51.

Lakra, W. S. and N. S. Nagpure, 2009. Genotoxicological studies in fishes: A review. *Indian Journal of Animal Sciences* 79:93–98.

Larsson, A., C. Haux, M. L. Sjobeck, 1985. Fish physiology and metal pollution: Results and experiences from laboratory and field studies. *Ecotoxicology and Environmental Safety* 9:250–281.

Larsson, A., B. E. Bengtsson, C. Haux, 1981. Disturbed ion balance in flounder, Platichthys flesus L. exposed to sublethal levels of cadmium. *Aquatic Toxicology* 1(1):19–35.

Larsson, A., B. Bengtsson, and O. Svanberg, 1976. Some haematological and biochemical effects of cadmium in fish. In *Effects of Pollutants on Aquatic Organisms*, ed. Lockwood, A:35–45. Soc. Exper. Biol., Seminar Ser. no. 11, Cambridge University Press, Cambridge.

Laurén, D. J. and D. G. McDonald, 1985. Effects of copper on branchial ionoregulation in the rainbow trout, *Salmo gairdneri* Richardson. *Journal of Comparative Physiology B* 155:635–644.

Linder, M. C., and M. Hazegh-Azam, 1996. Copper biochemistry and molecular biology. *American Journal of Clinical Nutrition* 63:797–811.

Lionetto, M. G., M. E. Giordano, S. Vilella et al., 2000. Inhibition of cell enzymatic activities by cadmium. *Aquatic Toxicology* 48:561–571.

Livingstone, D. R., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. *Marine Pollution Bulletin* 42:656–666.

Logaswamy, S., G. Radha, S. Subhashini et al., 2007. Alterations in the levels of ions in blood and liver of freshwater fish, *Cyprinus carpio* var. *communis* exposed to dimethoate. *Environmental Monitoring and Assessment* 131:439–444.

Lorentzen, M., A. Maage, K. Julshamn, 1998. Supplementing copper to a fish meal basal diet fed to Atlantic salmon parr affects liver copper and selenium concentrations. *Aquaculture Nutrition* 4:67–72.

Lorz, H. W. and B. P. McPherson, 1976. Effects of copper or zinc in fresh water on the adaptation to sea water and ATPase activity, and the effects of copper on migratory disposition of Coho salmon (*Oncorhynchus kisutch*). *Journal of Fisheries Research Board Canada* 33:2023–2030.

Lux Research, 2008. Nanomaterials State of the Market Q3 2008: Stealth Success, Broad Impact. Report. https://portal.luxresearchinc.com/research/document_excerpt/3735.

Mallatt, J., 1985. Fish gill structural changes induced by toxicants and other irritants: A statistical review. *Canadian Journal of Fisheries and Aquatic Sciences* 42:630–648.

Maruna, R. F. L., 1958. Quantitative estimation of sodium (Na) and potassium (K) in human serum by colorimetric method. *Clinical Chemistry Acta* 2:581–585.

Mayer, F. L., D. J. Versteeg, M. J. Mckee et al., 1992. Physiological and nonspecific biomarkers. In: *Biomarkers: Biochemical, Physiological, and Histological Markers of Anthropogenic Stress*. eds. Huggett, R. J., R. A. Kimerle, P. M. Mehrle Jr et al. SETAC Special Publication Series, Lewis Publishers, Boca Raton, FL, pp. 5–85.

McDonald, D. G. and T. R. K. Dalxical, 1989. The combined effects of pH and trace metals on fish ion regulation. In *Acid toxicity and aquatic animals*. eds. Morris, R., E. Taylor, D. J. A. Brown et al., Cambridge University Press, Cambridge, pp. 221–242.

McLaughlin, J., J. C. J. Bonzongo, 2012. Effects of natural water chemistry on nanosilver behavior and toxicity to *Ceriodaphnia dubia* and *Pseudokirchneriella subcapitata*. *Environmental Toxicology and Chemistry* 31:168–175.

Melegari, S. P., F. Perreault, R. H. Costa et al., 2013. Evaluation of toxicity and oxidative stress induced by copper oxide nanoparticles in the green alga *Chlamydomonas reinhardtii*. *Aquatic Toxicology* 143:431–440.

Mendil, D. and O. Dogan Uluozlu, 2007. Determination of metal levels in sediment of five fish species from lakes in Tokat, Turkey. *Food Chemistry* 101:739–745.

Monteiro, S. M., J. M. Manceva, A. F. Fernandes et al., 2005. Copper induced alterations of biochemical parameters in the gill and plasma of *Oreochromis niloticus*. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology* 141:375–383.

Mu, Q., G. Jiang, L. Chen et al., 2014. Chemical basis of interactions between engineered nanoparticles and biological systems, *Chemical Reviews* 114:7740–7781.

Nel, A., T. Xia, L. Madler et al., 2006. Toxic potential of materials at the nanolevel. *Science* 311:622–662.

Nel, A. E., L. Madler, D. Velegol et al., 2009. Understanding biophysicochemical interactions at the nano-bio interface. *Nature Materials* 8(7):543–557.

Noureen, A. and F. Jabeen, 2015. The toxicity, ways of exposure and effects of Cu nanoparticles and Cu bulk salts on different organisms. *International Journal of Biology* 6:2,147–156.

Pane, E. F., J. G. Richards, C. M. Wood, 2003. The acute waterborne nickel toxicity in the rainbow trout (*Oncorhynchus mykiss*) occurs by a respiratory rather than ionoregulatory mechanism. *Aquatic Toxicology* 63:65–82.

Park, J., S. Kim, J. Yoo et al., 2014. Effect of salinity on acute copper and zinc toxicity to *Tigriopus japonicus*: The difference between metal ions and nanoparticles. *Marine Pollution Bulletin* 85:526–531.

Perry, S. F., 1997. The chloride cell: Structure and function in the gills of freshwater fishes. *Annual Review of Physiology* 59:325–347.

Playle, R. C., G. G. Goss, C. M., Wood et al., 1989. Physiological disturbances in rainbow trout (*Salmo gairdneri*) during acid and aluminum exposures in soft water of two calcium concentrations. *Canadian Journal of Zoology* 67:314–324.

Poopal, R. K., M. Ramesh, B. Dinesh, 2013. Short-term mercury exposure on Na^+/K^+-ATPase activity and ionoregulation in gill and brain of an Indian majorcarp, *Cirrhinus mrigala*. *Journal of Trace Element in Medicine Biology* 27:70–75.

Prosser, C. L., 1973. *Comparative Animal Physiology*. 3rd ed. vol. 99. W. B. Saunders, Philadelphia, PA, pp. 181–186.

Rahman, M. S., A. H. Molla, N. Saha et al., 2012. Study on heavy metals levels and its risk assessment in some edible fishes from Bangshi River, Savar, Dhaka. Bangladesh. *Food Chemistry* 134(4):1847–1854.

Rajkowska, M. and M. Protasowicki, 2013. Distribution of metals (Fe, Mn, Zn, Cu) in fish tissues in two lakes of different trophy in Northwestern Poland. *Environmental Monitoring and Assessment* 185:3493–3502.

Ramesh M., S. Senthil kumaran, C. Kavitha et al., 2007. Primary stress responses of common carp, *Cyprinus carpio*, exposed to copper toxicity. *Acta Ichthyologica et Piscatoria* 37(2):81–85.

Ramesh, M., M. Sankaran, V. Veera-Gowtham et al., 2014. Hematological, biochemical and enzymological responses in an Indian major carp *Labeo rohita* induced by sublethal concentration of waterborne selenite exposure. *Chemico Biological Interactions* 207:67–73.

Ribeiro, J., K. DaBoit, D. Flores et al., 2013. Extensive FE-SEM/EDS, HR-TEM/EDS and ToF-SIMS studies of micron- to nano-particles in anthracite fly ash. *Science of the Total Environment* 452–453:98–107.

Saha, N. and M. R., Zaman., 2013. Evaluation of possible health risks of heavy metals by consumption of foodstuffs available in the central market of Rajshahi City Bangladesh. *Environmental Monitoring and Assessment* 185(5):3867–3878.

Saha, N., M. Z. I. Mollah, M. F. Alam et al., 2016. Seasonal investigation of heavy metals in marine fishes captured from the Bay of Bengal and the implications for human health risk assessment. *Food Control* 70:110–118.

Saravanan, M., K. Usha Devi, A. Malarvizhi et al., 2012. Effects of Ibuprofen on haematological, biochemical and enzymological parameters of blood in an Indian major carp, *Cirrhinus mrigala*. *Environmental Toxicology and Pharmacology* 34:14–22.

Sayer, M. D. J., J. P. Reader, R. Morris, 1989. The effect of calcium concentration on the toxicity of copper, lead and zinc to yolk-sac fry of brown trout, *Salmo trutta* (L.), in soft, acid water. *Journal of Fish Biology* 35(3):323–332.

Schjolden, J., J. Sorensen, G. E. Nilsson et al., 2007. The toxicity of copper to crucian carp (*Carassius carassius*) in soft water. *Science of the Total Environment* 384:239–251.

Shao, X., W. Liu, W. Xu et al., 2010. Effects of dietary copper sources and levels on performance, copper status, plasma antioxidant activities and relative copper bioavailability in *Carassius auratus gibelio*. *Aquaculture* 308(1–2):60–65.

Sharma, Y. C., B. Singh, D. Madhu et al., 2014. Fast synthesis of high quality biodiesel from 'Waste Fish Oil' by single step trans esterification. *Biofuel Research Journal* 3:78–80.

Shaw, B. J. and R. D. Handy, 2011. Physiological effects of nanoparticles on fish: A comparison of nanometals versus metal ions. *Environmental International* 37(6):1083–1097.

Shiosaka, T., H. Okuda, and S. Fungi, 1971. Mechanisms of phosphorylation of thymidine by the culture filtrate of *Clostridium perfringens* and rat liver extract. *Biochemistry and Biophysics Acta* 246:171–183.

Smith, C. J., B. J. Shaw, and R. D. Handy, 2007. Toxicity of single walled carbon nanotubes on rainbow trout, (*Oncorhynchus mykiss*): Respiratory toxicity, organ pathologies and other physiological effects. *Aquatic Toxicology* 82(2):94–109.

Song, L., M. G. Vijver, W. J. G. Peijnenburg, M. et al., 2015. A comparative analysis on the in vivo toxicity of copper nanoparticles in three species of freshwater fish. *Chemosphere* 139:181–189.

Sovová, T., D. Boyle, K. A. Sloman et al., 2014. Impaired behavioural response to alarm substance in rainbow trout exposed to copper nanoparticles. *Aquatic Toxicol*ogy 152:195–204.

Stagg, R. and T., Shuttleworth, 1982. The accumulation of copper in *Ptatichthys flesus* L. and its effects on plasma electrolyte concentrations. *Journal of Fish Biology* 21:491–500.

Staurnes, M., T. Sigholt, and O. B. Reite, 1984. Reduced carbonic anhydrase and $Na^{+/}$ K^{+}-ATPase activity in gills of salmonids exposed to aluminium containing acid water. *Experientia* 40:226–227.

Suvetha, L., M. Ramesh, and M. Saravanan, 2010. Influence of cypermethrin toxicity on ionic regulation and gill Na^{+}/K^{+}-ATPase activity of a freshwater teleost fish *Cyprinus carpio*. *Environmental Toxicology and Pharmacology* 29:44–49.

Tavares, K. P., Á. CaloTooliveira, D. S. ViCenTini et al., 2014. Acute toxicity of copper and chromium oxide nanoparticles to *Daphnia similis*. *Ecotoxicology and Environmental Contamination* 9(1):43–50.

Tietz, N. W., 1990. *Clinical Guide to Laboratory Test*, 2nd ed. W. B. Saunders, Philadelphia, PA, p. 118.

Trevisan, R., D. F. Mello, A. S. Fisher et al., 2010. Selenium in water enhances antioxidant defenses and protects against copper-induced DNA damage in the blue mussel *Mytilus edulis*. *Aquatic Toxicology* 101(1):64–71.

Tucker, R. K., 1979. Effects of in vivo cadmium exposure on ATPase in gill of the lobster, *Homarus americanus*. *Bulletin of Environmental Contamination and Toxicology* 23:33–35.

USEPA, 2003. 2003 draft update of ambient water quality criteria for copper. US Environmental Protection Agency, Office of Water, Office of Science and Technology, Washington, DC.

USEPA, 2007. Aquatic life ambient freshwater quality criteria—Copper 2007 revision. EPA-822-R-07-001. http://www.epa.gov/waterscience/criteria/copper/index.htm.

Van Der Putte, I., M. B. H. M. Laurier, G. J. M. Van Eijk, 1982. Respiration and osmoregulation in rainbow trout (*Salmo gairdneri*) exposed to hexavalent chromium at different pH values. *Aquatic Toxicology* 2:99–112.

Wendelaar Bonga, S., and R. Lock, 1991. Toxicants and osmoregulation in fish. *Netherland Journal of Zoology* 42:2–3.

Wang, T., X. Long, Y. Cheng et al., 2014. The potential toxicity of copper nanoparticles and copper sulphate on juvenile *Epinephelus coioides*. *Aquatic Toxicology* 152:96–104.

Wang, Z., J. Li, J. Zhao et al., 2011. Toxicity and internalization of CuO nanoparticles to prokaryotic alga *Microcystis aeruginosa* as affected by dissolved organic matter. *Environmental Science and Technology* 45:6032–6040.

Wang, Z., A. von-dem-Bussche, P. K. Kabadi et al., 2013. Biological and environmental transformations of copper-based nanomaterials *ACS Nano* 7:8715–8727.

Warheit, D., T. Webb, C. Sayes et al., 2006. Pulmonary instillation studies with nanoscale TiO_2 rods and dots in rats: Toxicity is not dependent upon particle size and surface area. *Toxicological Sciences* 91:227–236.

Watson, T. A., and F. W. H. Beamish, 1980. Effects of zinc on branchial ATPase activity in vivo in rainbow trout, *Salmo gairdneri*. *Comparative Biochemistry and Physiology* 66C:77–82.

Watson, T. A., and W. H. Beamish, 1981. The effects of zinc on branchial adenosine triphosphatase enzymes in vitro from rainbow trout, *Salmo gairdneri*. *Comparative Biochemistry and Physiology* 68C:167–173.

Wood, C. M., 2001. Toxic responses of the gill. In *Target Organ Toxicity in Marine and Freshwater Teleosts*, eds. Schlenck, D. and W. H Benson. vol. I. Taylor & Francis, London, pp. 1–89.

Young, D. S., L. C. Pestaner, V. Gibberman, 1975. Effects of drugs on clinical laboratory tests. *Clinical Chemistry* 21(5):1–432.

Yousefian, M. and B. Payam, 2012. Effects of nanochemical particles on some histological parameters of fish (Review). *Advances in Environmental Biology* 6(3):1209–1215.

Zhang, Y., Y. Chen, P. Westerhoff et al., 2008. Stability of commercial metal oxide nanoparticles in water. *Water Research* 42:2204–2212.

8

Toxic Effects of Nanomaterials to Plants and Beneficial Soil Bacteria

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8.1 Introduction

Nanotechnology is presently being explored a lot because of its research and application benefits. Nanoparticles (NPs) have preferred applications in various biomedical and bioscientific areas. There are different types of human-made, plant- and microbe-synthesized nanoparticles. Various human-made nanomanufacturing technologies have been developed and are being used in various industrial areas (Zhao and Castranova 2011). At present, humans have developed a range of NPs. In contrary to the benefits, so far, no information is elaborated on systematic investigation that is raising concerns about the toxic effect of nanomaterials to plants and beneficial soil bacteria (Bernhardt et al. 2010).

The microbial environment is an important part of the ecosystem. Important among these are agriculture and associated microbial species. The interaction of microbes with plants is necessary for the overall growth and productivity of plants. In recent times, the extent of nanoparticle exposure to plants and microbes has increased because of the increase in NP production and in the use of engineered NPs in different products (Pan and Xing 2010). There are reports which highlights the effects of silver, copper, nanoceria, ZnO, TiO, etc., NPs on plant sand microbes (Lee et al. 2008; Choi et al. 2008; Kumari et al. 2009; López-Moreno et al. 2010a; Ge et al. 2011; Yin et al. 2011; Ghosh et al. 2010; Ge et al. 2012; El-Temsah and Joner 2012; Sahu et al. 2012). Further, it expresses concern with emphasis on ecology for assessing nanoparticles in relation to nanowastes, and toxicity to agricultural/forest crops and its soil microbial

environment. Hence, the effect of NPs on microbial populations and the interaction of plants and microbes should necessarily be assessed.

It has been considered that both plants and microbes emerged in the presence of various natural nanomaterials. However, the impact of engineered NPs on plants and microbes is still not evaluated. Therefore, the present chapter discusses the impact of NPs on plants and microbes with detailed discussion on plant–mirobial interactions. NPs have found wide applications in various areas. As a result, various biological and chemical technologies for the synthesis of NPs are being explored. The application of NPs means the exposure of living organisms to the various types of NPs. Further, the release of nanowaste after the application of NPs is also an issue of prime concern. Darban et al. (2011) have reviewed the environmental and health hazards of unrestricted release of NPs.

There is urgent need to study hazards of nanotechnology along with its potential applications. Long-term biotic and abiotic impacts of nanotechnology should be accessed before being applied on a large scale. The United States' National Nanotechnology Initiative and the Royal Society and the United Kingdom's Royal Academy of Engineering have potentially addressed the uncertainties of NP toxicity and risks. It has been documented that precise information about the exposure level of consumers to NPs is not present. Since, it has not been documented that how many marketed products are based on nanotechnology and what is the consumption rate of such products. Consumers should be aware about health benefits as well as hazards of nanomaterial-containing products. Complete information regarding product composition and effects must be available on label. Moreover, numerous engineered nanomaterials have been developed; however, there effect on human, plant, and microbe health and on the environment are not yet accessed. Various reports explained the ill effects of nanoproducts such as carbon nanotubes (CNTs) and asbestos (Vejerano et al. 2015). Asbestos has been used in various parts of world for decades, because of its thermal and mechanical properties as thermal insulator. It is made up of thin, long, fibrous crystals of silicate nanomaterial and may produce health hazards due to release of microscopic fibrils after abrasion. Now, it has been banned due to its fatal properties. Hence, short- as well as long-term studies to assess the nanotoxicity post-exposure are highly recommended.

8.2 Involvement of Plants and Microbes in Nanoparticle Synthesis

Chemical syntheses of NPs have been widely employed. However, huge toxic effects are associated with the chemical NP synthesis. Hence, the focus of the present section is on the green synthesis of NPs using plants and microbes.

8.2.1 Phytosynthesis of NPs

There are few reports regarding the production of NPs by using plants and plant extracts (Bar et al. 2009; Ahmad et al. 2010; Mallikarjuna et al. 2011). However, extensive research has been carried out on the microbial production of NP in the past. Therefore, the phytoproduction of NPs is an unrevealed area of research.

Moreover, phytoproduction is advantageous compared to microbial production, because of the absence of pathogenicity. Hence, in comparison to various chemical and physical methods of NP synthesis plant-mediated NP synthesis has been considered safer and eco-friendly. Various plant-mediated syntheses have been reported to date. Pantidos and Horsfall (2014) have discussed in detail the approaches adopted for the synthesis of silver and gold NPs. It has been reported that the phytochemicals derived from plants act as surfactant that maintains the stability of synthesized NPs. It has further been stated that plant extract can act as potential precursor for the synthesis of NPs in a nonhazardous manner. Various reports have documented the synthesis of NPs from plants. Some of them are discussed as follows. Dried root and stem sections of *Ocimum sanctum* have been employed for the rapid synthesis of silver NPs. Further, it was found that the phytochemicals of *O. sanctum* were providing robustness to the NPs against agglomerations (Ahmad et al. 2010). The weed *Chenopodium album* has also been reported to support the synthesis of silver as well as gold NPs (Dwivedi and Gopal 2010). The silver NPs synthesized from *Artemisia nilagirica* (Asteraceae) have been reported to possess antimicrobial properties (Vijayakumar et al. 2013).

A much broad range of NP size has been observed from bacteria and fungi. However, the homogenous production of NPs has been reported from various plants. Bar et al. (2009) illustrated fairly homogenous (10–20 nm) AgNP production from Jatropha curcas extract. Krishnaraj's group has also reported the homogenous production of AgNPs (20–30 nm) from *Acalypha indica* leaf extracts (Krishnaraj et al. 2010). There is another report of the reduction of Ag and the production of AgNP of size 3–20 nm by *O. sanctum* (Mallikarjuna et al. 2011).

In contrast to other studies, Kasthuri et al. (2008) showed that only phyllanthin extracted from the plant *Phyllanthus amarus* has the ability to produce AgNPs and AuNPs instead of whole plants or plant extracts. The shape of NPs is based upon the concentration of phyllanthin; i.e., higher concentrations of phyllanthin gave rise to greater levels of spherical NPs, and low concentrations resulted in the slow formation of triangular and hexagonal AuNPs. These facts were confirmed by ultraviolet-visible and transmission electron microscopy analysis (Kasthuri et al. 2008). The production of NPs has also been reported from the extract of medicinal plants. Extracts of the Neem plant and *Azadirachta indica* rapidly produced AuNPs and AgNPs within 2 hours (Shankar et al. 2004).

8.2.2 Microbial Synthesis of Nanoparticles

The microbial synthesis of NPs involves the interaction of nanotechnology with microbial biotechnology. Various reports have already discussed the synthesis of NPs using microbes or microbial extracts. The biological reduction of aqueous silver ions by extracellular components of *Streptomyces hygroscopicus* has been reported for the greener synthesis of technologically important silver NPs. These NPs significantly inhibited the growth of medically important pathogenic gram-positive bacteria (*Bacillus subtilis* and *Enterococcus faecalis*), gram-negative bacteria (*Escherichia coli* and *Salmonella typhimurium*) and yeast, *Candida albicans* (Sadhasivam et al. 2010). The eco-friendly synthesis of cadmium sulfide NPs using *Enterococcus* sp has been reported. The synthesized NPs showed antimicrobial activity against *Vibrio* sp.,

Serratia nematodiphila, *Planomicrobium* sp., *E. coli*, and *Klebsiella planticola* (Sadhasivam et al. 2010).

The reason of sustainable life on earth is regular interaction between biological entities and inorganic material. Recently, there are a number of reports regarding inorganic material production from biological entities (Ma et al. 2005; Kasthuri et al. 2008; Park et al. 2011; Ramanathan et al. 2013). Intracellular and extracellular microbial productions of nanoparticles are current topic of research. Various reports have already discussed the synthesis of NPs using microbes such as metallic NPs including gold, silver, alloy and other metal NPs, oxide NPs, and sulfide NPs (Ma et al. 2005; Kasthuri et al. 2008; Park et al. 2011; Ramanathan et al. 2013; Schlüter et al. 2014) (Table 8.1).

The synthesis of NPs by microorganism follows different mechanism: (i) entrapment of metal ions on the surface or inside of the microbial cells and followed by the reduction of trapped metal ions to NPs in the presence of enzymes (Kalishwaralal et al. 2008); (ii) NPs can be formed due to genetic and proteomic responses of metallophilic microorganism toward toxic environments (Mergeay et al. 2003; Reith et al. 2007); (iii) the synthesis of metallic NPs using microbes, which consists of both

TABLE 8.1

Microbial Production of Nanoparticles

Type of NPs		Microorganism	References
Metallic	Gold	*Fusarium oxysporum*, *Thermomonospora*, *Rhodococcus sp*, *Verticillium*, *Pseudomonas aeruginosa*	Ma et al. 2005; Kasthuri et al. 2008; Park et al. 2011; Ramanathan et al. 2013; Narayanan and Sakthivel 2010
	Silver	Bacillus licheniformis *Klebsiella pneumonia*, *Lactobacillus sp.*, *Enterococcus faecium*, *Lactococcus garvieae, Escherichia coli*, *Enterobacter cloacae*	Schlüter et al. 2014; Kalimuthu et al. 2008; Shahverdi et al. 2007
	Copper	Bacillus sphaericus JG-A12	Das et al. 2014
	Alloy	*Fusarium oxysporum*, *Fusarium semitectum*	Senapati et al. 2005; Sawle et al. 2008
	Lead	*Desulfovibrio desulfuricans*, *Escherichia coli*	Cai et al. 2009; Deplanche et al. 2010
	Platinum	Escherichia coli, Neurospora crassa	Deplanche et al. 2010; Sanghi and Verma 2009
Oxide NPs	Magnetic	Fe_3O_4: *Shewanella oneidensis*, yeast cells, *Shewanella oneidensis* MR–1	Perez-Gonzalez et al. 2010; Zhou et al. 2009; Bose et al. 2009
	Nonmagnetic Oxide	TiO_2: *Lactobacillus* sp., *Fusarium oxysporum,* ZrO_2: *Fusarium oxysporum*; $BaTiO_3$: *Lactobacillus* sp.	Bansal et al. 2004; Bansal et al. 2005; Jha et al. 2009; Jha and Prasad 2010
	Sulfide	CdS: *Fusarium oxysporum*, AsS: *Shewanella* sp., ZnS: *Rhodobacter sphaeroides*	Raveendran et al. 2003; Bai et al. 2006; Rai et al. 2009

passive and active mechanisms (Perez-Gonzalez et al. 2010); and (iv) NPs can be formed through the production of organic polymers, which can impact nucleation by favoring (or inhibiting) the stabilization of mineral (Benzerara et al. 2010).

8.3 Effect of Nanoparticles on Plant Growth-Promoting Bacteria

Plant growth-promoting rhizobacteria (PGPR) are free-living, root-colonizing, soil-borne bacteria exerting phytostimulatory actions, when applied to seeds or crops by a combination of physiological attributes. Soil microorganisms play a very important role in maintaining soil health, ecosystem functions, and crop productivity. The soil microbial world is the largest unexplored reservoir of microorganism on the Earth. In recent decades, it is an important frontier in biology under intensive investigations, since they perform numerous functions for the biosphere that include nutrient cycling and plant growth promotion. The PGPR are known to participate in many important ecosystem processes, such as the biological control of plant pathogens, nutrient cycling, and/or seedling growth. PGPR help plant growth by a combination of physiological attributes such as asymbiotic N_2 fixation and phytohormones production, namely, indole-3-acetic acid, cytokinin, gibberellins, solubilizing insoluble mineral phosphate, and siderophore production (Guleria et al. 2014).

The introduction of NPs into the environment might have significant impacts as they may be extremely resistant to degradation and have the potential to accumulate in bodies of water or in soil. However, NPs can act on living cells at the nanolevel resulting in biologically desirable effects. The research field of PGPR and their interactions with plants is highly promising for possible applications to contribute to eco-friendly sustainable agriculture and environmental biotechnology. Furthermore, NPs are introduced into the soil as a result of a number of human activities, including deliberate releases via soil and water remediation technologies; potential agricultural uses (e.g., fertilizers); and unintentional releases via air, water, and sewage sludge applied to the land. However, ecotoxicological properties and the risks of these NPs have not yet been fully characterized. Many NPs have already been reported to have antimicrobial properties and thus directly affect microorganisms. Microbial toxicity has been reported for titanium dioxide and fullerene NPs. However, how nanomaterials affect living organisms remains unknown, although reactive oxygen species generation and oxidative stress are proposed to explain the toxicity of inhaled NPs.

Engineered NP products should be tested before being released in the environment. They can affect macro- as well as microorganisms. The adverse effect of NPs on plant growth-promoting (PGP) bacteria can influence soil fertility, plant health, and production. Only a few reports have been published until now about aftereffects of engineered nanoproducts on PGPR. Although, there are studies on the effects of metallic NPs such as fullerens, gold, silver, aluminum on PGPR (Mishra and Kumar 2009). Different crops plants have been sprayed with NPs in order to study the effect of NPs on rhizospheric bacteria. After a few days of spraying, rhizosphere soil sampling and isolation of rhizospheric bacteria have been performed (Mehta et al. 2016). Silver NPs were applied through foliar spray to *Brassica*. After 20 and 40 days of *Brassica* growth, rhizospheric soil sampling has been carried out. Soil samples were analyzed by cultural and molecular techniques. Analysis results evidenced that there

was change in soil bacterial diversity before and after of spraying (Mehta et al. 2016). Furthermore, bactericidal effects of silver NPs have been studied even at very low concentration. Soni and Bondi (2004) reported cell death of *E. coli* due to silver NP exposure. It may be due to increased accumulation of silver NPs in the cell wall, leading to cell wall damage, increased cell permeability, and ultimately death of the cell. On the basis of various studies, it is evidenced that the bactericidal effect of silver NPs may be due to the following reasons: damage to cell wall and membrane; increased cell permeability; destruction of nutrient transport enzymes; inhibition of bacterial DNA replication by nanosilver (Zeng et al. 2006). The toxic effect of silver NPs on microorganism may be due the large surface area of NPs compared to its volume. Nanosilver particles of size 1–10 nm have the ability to decrease the infectivity of human immunodeficiency virus-1 because these particles have the ability to inhibit the host cell-binding capacity of the virus (Doshi et al. 2008). Aluminum NPs are highly toxic and reactive against seed germination and plant root growth. There are mainly two types of aluminum NPs, those with aluminum oxide coating and those with carboxylate ligand coating, Alex and LAlex, respectively. These are being tested to reveal the impact of aluminum NPs on environmental and soil microorganisms (Doshi et al. 2008). However, there are no studies so far on the toxicity effect of Al NPs on PGP bacteria. On the other hand, NPs of zinc, magnesium, and copper are toxic against microorganisms (Adams et al. 2006; Saliba et al. 2006). These engineered NPs have been exploited as food preservatives such as NPs of zinc oxide (ZnO) and magnesium oxide (Saliba et al. 2006). The acute toxicity of ZnO NPs has been observed on *E. coli* (Brayner et al. 2006). The toxicity of ZnO-, TiO_2-, and SiO_2-engineered NPs has been reported against *B. subtilis* and *E.coli* bacteria in water suspensions containing citrate and PO_4 (Adams et al. 2006).

8.4 Effect of Nanoparticles on Plant Health

After the development and use of beneficial nanoproducts, there is regular threat on human health and wildlife due to the release of by-products. Numerous studies have been carried out in order to study adverse effects and toxicity level of industrially produced NPs. The following section is about the release of NPs in the environment and their effects on plant health. The topic mainly focused on is the effects of commonly used NPs: CNT and metal oxide and metallic NPs.

Studies on the toxicity of metal oxide NPs on plants are limited. The toxic effect of metal oxide NPs including cerium dioxide, iron oxide, titanium dioxide, zinc oxide, and silicon dioxide on a few plant species has been recently studied. TiO_2 NPs has the ability to reduce the hydraulic conductivity of primary roots, transpiration, and leaf growth of maize (Asli and Neumann 2009). However, titanium dioxide NPs along with silicon dioxide NPs has been reported to increase nitrate reductase activity and water and fertilizer uptake in soybean (Lu et al. 2002). Similarly, in spinach, there is increase in photosynthesis, chlorophyll formation, and plant dry weight due to exposure of TiO_2 NPs (Zheng et al. 2005; Hong et al. 2005). Just like TiO_2 NPs, Fe_3O_4 NPs have been found to affect soybean.

Similarly, iron oxide NPs induced various levels of responses in different plants. In soybean, iron oxide NPs induced the increase in pod and leaf dry weight

(Sheykhbaglou et al. 2010). Further, increase in root elongation due to iron dissolution, inducing higher antioxidative enzyme activity, was induced by iron oxide NPs in pumpkin. Oxidative stress was induced due to the adsorption of NPs on the root surface or absorbed by the roots which disturb the metabolic activities in roots and ultimately leading to local instability of the cell wall and/or membrane (Wang et al. 2010).

Zinc oxide NPs inhibited the root elongation in soybean (López-Moreno et al. 2010a). While seed germination was observed in corn, tomato, cucumber, and alfalfa on zinc oxide NPs exposure (Lin and Xing 2007; López-Moreno et al. 2010b). Multiwalled CNTs accelerated seed germination and vegetative mass in tomato (Khodakovskaya et al. 2009). However, some studies reported no effect of multiwalled CNTs on seed germination and root in rape, wheat, radish, lettuce, corn, and cucumber (Lin and Xing 2007; Wild and Jones 2009). Exposure of multiwalled CNTs is also reported to delay the flowering in rice (Lin et al. 2009), while single-walled CNTs adversely affected root elongation in tomato (Canas et al. 2008). Thus, the same NPs can induce different types and levels of toxicity response in plants.

8.5 Processing of Nanoparticles Containing Products after Use

So, before the disposal of NPs, the following points need to be considered: (i) disposal and recycling procedures must be developed on the basis of different nanomaterial type; (ii) before developing disposal procedure for particular nanowaste, proper understanding of nanowaste properties is very important; (iii) already existing legislation and current knowledge should be taken into account while developing procedures for nanowaste handling; (iv) recycling of nanowaste; (v) neutralization of chemically reactive nanomaterials; and (vi) the nanowaste should be completely deactivated at the time of disposal.

REFERENCES

Adams, L.K., Lyon, D.Y., and Alvarez, P.J.J. 2006. Comparative eco-toxicity of nanoscale TiO2, SiO2, and ZnO water suspensions. *Water Research* 40: 3527–3532.

Ahmad, N., Sharma, S., Alam, K., Singh, V.N., Shamsi, S.F., Mehta, B.R., and Fatma, A. 2010. Rapid synthesis of silver nanoparticles using dried medicinal plant of basil. *Colloids and Surfaces B: Biointerfaces* 81: 81–86.

Asli, S. and Neumann, M. 2009. Colloidal suspensions of clay or titanium dioxide nanoparticles can inhibit leaf growth and transpiration via physical effects on root water transport. *Plant, Cell & Environment* 32: 577–584.

Bai, H.J., Zhang, Z.M., and Gong, J. 2006. Biological synthesis of semiconductor zinc sulfide nanoparticles by immobilized *Rhodobacter sphaeroides*. *Biotechnology Letters* 28: 1135–1139.

Bansal, V., Rautaray, D., Ahmad, A., Sastry, M. 2004. Biosynthesis of zirconia nanoparticles using the fungus *Fusarium oxysporum*. *Journal of Materials Chemistry*, 14: 3303–3305.

Bansal, V., Rautaray, D., Bharde A. et al. 2005. Fungus-mediated biosynthesis of silica and titania particles. *Journal of Materials Chemistry* 15: 2583–2589.

Bar, H., Bhui, D.K., Sahoo, G.P. et al. 2009. Green synthesis of silver nanoparticles using latex of *Jatropha curcas*. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 339: 134–139.

Benzerara, K., Miot, J., Morin, G., Ona-Nguema, G., Skouri-Panet, F., and Férard, C. 2010. Significance, mechanisms and environmental implications of microbial biomineralization.*Comptes Rendus Geoscience* 343: 160–167.

Bernhardt, E.S., Colman, B.P., Hochella, M.F. Jr. et al. 2010. An ecological perspective on nanomaterial impacts in the environment. *Journal of Environmental Quality* 39: 1954–1965.

Bose, S., Hochella, M.F., Gorby, Y.A. et al. 2009. Bioreduction of hematite nanoparticles by the dissimilatory iron reducing bacterium *Shewanella oneidensis* MR-1. *Geochimica et Cosmochimica Acta* 73: 962–976.

Brayner, R., Ferrari-lliou, R., Brivois, N., Djediat, S., Benedetti, M.F., and Fievet, F. 2006. Toxicological impact studies based on escherichia coli bacteria in ultrafine ZnO nanoparticles colloidal medium. *Nano Letters* 6: 866–870.

Cai, J., Kimura, S., Wada, M., and Kuga, S. 2009. Nanoporous cellulose as metal nanoparticles support. *Biomacromolecules* 10: 87–94.

Canas, J.E., Long, M., Nations, S., Vadan, R., Dai, L., Luo, M., Ambikapathi, R., Lee, E.H., and Olszyk, D. 2008. Effects of functionalized and nonfunctionalized single-walled carbon-nanotubes on root elongation of select crop species. *Nanomaterial Environment* 27: 1922–1931.

Choi, O., Deng, K.K., Kim, N.J. et al. 2008. The inhibitory effects of silver nanoparticles, silver ions, and silver chloride colloids on microbial growth. *Water Research* 42: 3066–3074.

Darban, A.K., Kiani Y., Forotan, A., Rahnamaee, B., Partani, S., Khodadadi, A., and Yong, R.N. 2011. *A Review of Nanowastes Environmental and Health Risk Assessment*. International Conference on Chemical, Biological and Environment Sciences (ICCEBS 2011), Bangkok.

Das, V., Thomas, R., Varghese, R. et al. 2014. Extracellular synthesis of silver nanoparticles by the Bacillus strain CS 11 isolated from industrialized area. 3 *Biotech* 4: 121–126.

Deplanche, K., Caldelari, I., Mikheenko, I.P., Sargent, F., and Macaskie, L.E. 2010. Involvement of hydrogenases in the formation of highly catalytic Pd(0) nanoparticles by bioreduction of Pd(II) using *Escherichia coli* mutant strains. *Microbiology* 156: 2630–2640.

Doshi, R., Braida, W., Christodoulatos, C., Wazne, M., and O'Connor, G. 2008. Nano-aluminum: Transport through sand columns and environmental effects on plants and soil communities. *Environmental Research* 106: 296–303.

Dwivedi, A.D. and Gopal, K. 2010. Biosynthesis of silver and gold nanoparticles using Chenopodium album leaf extract. *Colloids and Surfaces: A Physicochemical and Engineering Aspects* 369: 27–33.

El-Temsah, Y.S. and Joner, E.J. 2012. Impact of Fe and Ag nanoparticles on seed germination and differences in bioavailability during exposure in aqueous suspension and soil. *Environmental Toxicology* 27: 42–49.

Ge, Y., Schimel, J.P., and Holden, P.A. 2011. Evidence for negative effects of TiO_2 and ZnO nanoparticles on soil bacterial communities. *Environmental Science and Technology* 45: 1659–1664.

Ge, Y., Schimel, J.P., and Holden, P.A. 2012. Identification of soil bacteria susceptible to TiO_2 and ZnO nanoparticles. *Applied Environmental Microbiology* 78: 6749–6758.

Ghosh, M., Bandyopadhyay, M., and Mukherjee, A. 2010. Genotoxicity of titanium dioxide (TiO2) nanoparticles at two trophic levels: Plant and human lymphocytes. *Chemosphere* 81: 1253–1262.

Guleria, S., Walia, A., Chauhan, A., and Shirkot, C.K. 2014. Genotypic and phenotypic diversity analysis of alkalophilic proteolytic *Bacillus* sp. associated with rhizosphere of apple trees in trans Himalayan region of Himachal Pradesh. *Proceedings of National Academy of Sciences, India Section B: Biological Sciences* 86: 331–341.

Hong, F., Zhou, J., Liu, C., Yang, F., Wu, C., Zheng, L., and Yang, P. 2005. Effect of nano-TiO_2 on photochemical reaction of chloroplasts of spinach. *Biological Trace Element Research* 105: 269–279.

Jha, A.K. and Prasad, K. 2010. Ferroelectric $BaTiO_3$ nanoparticles: Biosynthesis and characterization. *Colloids and Surfaces B* 75: 330–334.

Jha, A.K., Prasad, K., and Kulkarni, A.R. 2009. Synthesis of TiO_2 nanoparticles using microorganisms. *Colloids and Surfaces B* 71: 226–229.

Kalimuthu, K., Suresh Babu, R., Venkataraman, D., Bilal, M., and Gurunathan, S. 2008. Biosynthesis of silver nanocrystals by *Bacillus licheniformis*. *Colloids and Surfaces B: Biointerfaces* 65: 150–153.

Kalishwaralal, K., Deepak, V., Ramkumarpandian, S., Nellaiah, H., and Sangiliyandi, G. 2008. Extracellular biosynthesis of silver nanoparticles by the culture supernatant of *Bacillus licheniformis*. *Materials Letters* 62: 4411–4413.

Kasthuri, J., Kathiravan, K., and Rajendiran, N. 2008. Phyllanthin-assisted biosynthesis of silver and gold nanoparticles: A novel biological approach. *Journal of Nanoparticle Research* 11: 1075–1085.

Kasthuri, J., Kathiravan, K., and Rajendiran, N. 2008. Phyllanthin-assisted biosynthesis of silver and gold nanoparticles: A novel biological approach. *Journal of Nanoparticle Research* 11: 1075–1085.

Khodakovskaya, M., Dervishi, E., Mahmood, M., Xu, Y., Li, Z., Watanabe, F., Biris, A.S. 2009. Carbon nanotubes are able to penetrate plant seed coat and dramatically affect seed germination and plant growth. *ACS Nano* 3: 3221–3227.

Krishnaraj, C., Jagan, E.G., Rajasekar, S., Selvakumar, P., Kalaichelvan, P.T., and Mohan, N. 2010. Synthesis of silver nanoparticles using Acalypha indica leaf extracts and its antibacterial activity against water borne pathogens. *Colloids Surf B Biointerfaces* 76:50–60.

Kumari, M., Mukherjee, A., and Chadrasekaran, N. 2009. Genotoxicity of silver nanoparticle in Allium cepa. *Science of Total Environment* 407: 5243–5246.

Lee, W.M., An, Y.J., Yoon, H., and Kweon, H.S. 2008. Toxicity and bioavailability of copper nanoparticles to the terrestrial plants mung bean (*Phaseolus radiatus*) and wheat (*Triticum aestivum*): Plant agar test for water-insoluble nanoparticles. *Environmental Toxicology and Chemistry* 27: 1915–1921.

Lin, D. and Xing, B. 2007. Phytotoxicity of nanoparticles: Inhibition of seed germination and root growth. *Environmental Pollution* 50: 243–250.

Lin, C., Fugetsu, B., Su, Y., and Watari, F. 2009. Studies on toxicity of multi-walled carbon nanotubes on *Arabidopsis* T87 suspension cells. *Journal of Hazardous Materials* 170: 578–583.

López-Moreno, M.L., De La Rosa, G., Hernandez-Viezcas, J.A., Castillo-Michel, H., Botez, C.E., Peralta-Videa, J.R., and Gardea-Torresdey, J.L. 2010a. Evidence of the differential biotransformation and genotoxicity of ZnO and CeO_2 nanoparticles on soybean (*Glycine max*) plants. *Environmental Science and Technology* 44: 7315–7320.

López-Moreno, M.L., De La Rosa, G., Hernandez-Viezcas, J.A., Peralta-Videa, J.R., and Gardea-Torresdey, J.L. 2010b. X-ray absorption spectroscopy (XAS) corroboration of the uptake and storage of CeO_2 nanoparticles and assessment of their differential toxicity in four edible plant species. *Journal of Agricultural and Food Chemistry* 58: 3689–3693.

Lu, C.M., Zhang, C.Y., Wen, J.Q., Wu, G.R., and Tao, M.X. 2002. Research of the effect of nanometer materials on germination and growth enhancement of *Glycine max* and its mechanism. *Soybean Science* 21: 168–172.

Ma, Y., Li, N., Yang, C., and Yang, X. 2005. One-step synthesis of amino-dextran protected gold and silver nanoparticles and its application in biosensors. *Analytical and Bioanalytical Chemistry* 382: 1044–1048.

Mallikarjuna, K., Narasimha, G., Dillip, G.R. et al. 2011. Green synthesis of silver nanoparticles using *Ocimum* leaf extract and their characterization. *Digest Journal of Nanomaterials and Biostructures* 6: 181–186.

Mehta, P.C.M., Srivastava, R., Arora, S., and Sharma, A.K. 2016. Impact assessment of silver nanoparticles on plant growth and soil bacterial diversity. *3 Biotech* 6: 254.

Mergeay, M., Monchy, S., Vallaeys, T. et al. 2003. *Ralstonia metallidurans*, a bacterium specifically adapted to toxic metals: Towards a catalogue of metal-responsive genes. *FEMS Microbiology Reviews* 27: 385–410.

Mishra, V.K. and Kumar, A. 2009. Impact of metal nanoparticles on the plant growth promoting rhizobacteria. *Digest Journal of Nanomaterials and Biostructures* 4: 587–592.

Narayanan, K.B. and Sakthivel, N. 2010. Biological synthesis of metal nanoparticles by microbes. *Advances in Colloid* and *Interface Science* 156: 1–13.

Pan, B. and Xing, B. 2010. Manufactured nanoparticles and their sorption of organic chemicals. *Advances in Agronomy* 108: 137–181.

Pantidos, N., Horsfall, L.E. 2014. Biological synthesis of metallic nanoparticles by bacteria, fungi and plants. *Journal of Nanomedicine and Nanotechnology* 5: 233.

Park, Y., Hong, Y.N., Weyers, A., Kim, Y.S., and Linhardt, R.J. 2011. Polysaccharides and phytochemicals: A natural reservoir for the green synthesis of gold and silver nanoparticles. *IET Nanobiotechnology* 5(3): 69–78.

Perez-Gonzalez, T., Jimenez-Lopez, C., Neal A.L. et al. 2010. Magnetite biomineralization induced by *Shewanella oneidensis*. *Geochimica et Cosmochimica Acta* 74: 967–979.

Rai, M., Yadav, A., and Gade, A. 2009. Silver nanoparticles as a new generation of antimicrobials. *Biotechnology Advances* 27: 76–83.

Ramanathan, R., Field, M.R., O'Mullane, A.P. et al. 2013. Aqueous phase synthesis of copper nanoparticles: A link between heavy metal resistance and nanoparticle synthesis ability in bacterial systems. *Nanoscale* 5: 2300–2306.

Raveendran, P., Fu, J., and Wallen, S.L. 2003. Completely "green" synthesis and stabilization of metal nanoparticles. *Journal of the American Chemical Society* 125: 13940–13941.

Reith, F., Lengke, M.F., Falconer, D., Craw, D., Southam, G. 2007. The geomicrobiology of gold. *The ISME Journal* 1: 567–584.

Sadhasivam, S., Shanmugam, P., and Yun K. 2010. Biosynthesis of silver nanoparticles by *Streptomyces hygroscopicus* and antimicrobial activity against medically important pathogenic microorganisms. *Colloids and Surfaces B: Biointerfaces* 81: 358–362.

Sahu, N., Soni, D., Chandrashekhar, B. et al. 2012. Synthesis and characterization of silver nanoparticles using *Cynodon dactylon* leaves and assessment of their antibacterial activity. *Bioprocess and Biosystems Engineering* 36: 999–1004.

Saliba, A.M., Nishi, R., Raymond, B. et al. 2006. Implications of oxidative stress in the cytotoxicity of Pseudomonas aeruginosa ExoU. *Microbes and Infection* 8: 450–459.

Sanghi, R. and Verma, P. 2009. Biomimetic synthesis and characterisation of protein capped silver nanoparticles. *Bioresource Technology* 100: 501–504.

Sawle, B.D., Salimath, B., Deshpande, R., Bedre, M.D., Prabhakar, B.K., and Venkataraman, A. 2008. Biosynthesis and stabilization of Au and Au–Ag alloy nanoparticles by fungus, *Fusarium semitectum*. *Science and Technology of Advanced Materials* 9: 1–6.

Schlüter, M., Hentzel, T., Suarez, C. et al. 2014. Synthesis of novel palladium nanocatalysts by microorganisms from heavy-metal influenced high-alpine sites for dehalogenation of polychlorinated dioxins. *Chemosphere* 117: 462–470.

Senapati, S., Ahmad, A., Khan, M.I., Sastry, M., and Kumar, R. 2005. Extracellular biosynthesis of bimetallic Au-Ag alloy nanoparticles. *Small* 1: 517–520.

Shahverdi, A.R., Minaeian, S., Shahverdi, H.R., Jamalifar, H., and Nohi, A.A. 2007. Rapid synthesis of silver nanoparticles using culture supernatants of Enterobacteria: A novel biological approach. *Process Biochemistry* 42: 919–923.

Shankar, S.S., Rai, A., Ahmad, A., and Sastry, M. 2004. Rapid synthesis of Au, Ag, and bimetallic Au core-Ag shell nanoparticles using Neem (*Azadirachta indica*) leaf broth. *Journal of Colloid and Interface Sciences* 275: 496–502.

Sheykhbaglou, R., Sedghi, M., Shishevan, M.T., and Sharifi, R.S. 2010. Effects of nano-iron oxide particles on agronomic traits of soybean. *Notulae Scientia Biologicae* 2: 112–113.

Soni, S.B. and Bondi, J. 2004. Impact of metal nanoparticles on the plant growth promoting Rhizobacteria. *Journal of Colloid and Interface Science* 275: 1770–1782.

Vejerano, E.P., Ma, Y., Holder, A.L., Pruden, A., Elankumarana, S., and Marr, L.C. 2015. Toxicity of particulate matter from incineration of nanowaste. *Environmental Science: Nano* 2: 143–154.

Vijayakumar, M., Priya, K., Nancy, F.T., Noorlidah, A., and Ahmed, A.B.A. 2013. Biosynthesis, characterization and antibacterial effect of plant-mediated silver nanoparticles using *Artemisia nilagirica*. *Industrial Crops and Products* 41: 235–240.

Wang, H., Kou, X., Pei, Z., Xiao, J.Q., Shan, X., and Xing, B. 2010. Physiological effects of magnetite (Fe_3O_4) nanoparticles on perennial ryegrass (*Lolium perenne* L.) and pumpkin (*Cucurbita mixta*) plants. *Nanotoxicology.*

Wild, E. and Jones, K.C.2009. Novel method for the direct visualization of in vivo nanomaterials and chemical interactions in plants. *Environmental Science and Technology* 43: 5290–5294.

Yin, L., Cheng, Y., Espinasse, B. et al. 2011. More than the ions: The effects of silver nanoparticles on *Lolium multiflorum*. *Environmental Science and Technology* 45: 2360–2367.

Zeng, Q., Liao, B., Zhang, L., Zhou, X., and Tang, H. 2006. Short-term alleviation of aluminum phytotoxicity by urea application in acidic soils from south China. *Chemosphere* 63: 860–868.

Zhao, J. and Castranova, V. 2011. Toxicology of nanomaterials used in nanomedicine. *Journal of Toxicology and Environmental Health B Critical Review* 14: 593–632.

Zheng, L., Hong, F., Lu, S., and Liu, C. 2005. Effect of nano-TiO_2 on strength of naturally aged seeds and growth of spinach. *Biological Trace Element Research* 104: 83–91.

Zhou, W., He, W., Zhong, S. et al. 2009. Biosynthesis and magnetic properties of mesoporous Fe_3O_4 composites. *Journal of Magnetism and Magnetic Materials* 321(8): 1025–1028.



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Nanotoxicity of Silver Nanoparticles: From Environmental Spill to Effects on Organisms

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9.1 Introduction

Nanotechnologies are a remarkable source of modern innovation leading to the production of cutting-edge products with high potential for new processes and goods. From a recent survey, we know that 622 companies from 32 countries manufacture 1814 products claiming the presence of engineered nanomaterials or engineered nanoparticles in their goods (Vance et al. 2015). Silver nanoparticles (AgNPs) are the most popularly advertised nanomaterial with 438 products (Vance et al. 2015). Each year, thousands of tons of engineered nanomaterials are produced. These engineered nanomaterials are very small objects having at least one dimension between 1 and 100 nm (ISO 2008). These tiny structures can be embedded in solid products to form nanostructured materials or surface-bound particles. However, in most of consumer products, they are present as minuscule suspended particles in a variety of fluids or dispersed in powders (Vance et al. 2015). During the life cycle of engineered nanomaterials, which includes production, use, and disposal (Caballero-Guzman and Nowack 2016; Som et al. 2010), it is estimated that 10 of the major engineered nanomaterials, SiO_2, CeO_2, carbon nanotubes, nanoclays, Al_2O_3, Cu, Fe, ZnO, TiO_2, and Ag, could release about 190,000, 50,000, 8,000 and and 69,000 metric t per year to landfill, soil, air, and water, respectively (Keller and Lazareva 2014). Even if important uncertainties exist on the production and real mass flows of nanoproducts released in the environment and the difficulty to measure their concentrations in environmental matrices (Keller and Lazareva 2014; Piccinno et al. 2012; Vance et al. 2015; Holden et al. 2014), proofs of the leaching of engineered nanomaterials have been published. For instance, it is well known that nanoparticle liberation can occur from textiles or coating or from solid nanocomposites products (Benn and Westerhoff 2008; Froggett et al. 2014; Kaegi et al. 2010; Mitrano et al. 2015). What would happen if important spills of engineered nanomaterials occur in wastewater, river, lake, or estuary? What is the fate and toxic action of those engineered nanomaterials dropped in the environment? Today, our knowledge concerning their environmental risks and their nanotoxicity is limited.

As shown in Figure 9.1, throughout their life cycle, engineered nanomaterials undergo variations in their particle size distributions from production to disposal with a general increase in size explained by various processes discussed in the following (Caballero-Guzman and Nowack 2016). This is because the production of engineered nanomaterials is processed under monitored parameters exerting control over the small size of native engineered nanomaterials. However, the inclusion of engineered nanomaterials into products and their use, weathering and aging under variable conditions result in the aggregation or transformation at the time of disposal. Engineered nanomaterials can be released from nanomaterials during their entire life cycle, but should be more important at the use phase (Caballero-Guzman and Nowack 2016; Gottschalk and Nowack 2011). After their release, engineered nanomaterials can be processed in recycling systems, incinerators and wastewater treatment plants or delivered to environmental compartments, landfill, air, soil, and water (Caballero-Guzman and Nowack 2016). At this moment, exposure and risk begin to be a reality for bacteria, microalgae, zooplankton, fish, and ultimately human beings as shown in Figure 9.1. Throughout their journey, engineered nanomaterials can experience numerous changes in their physicochemical speciation and environmental exposure to organisms.

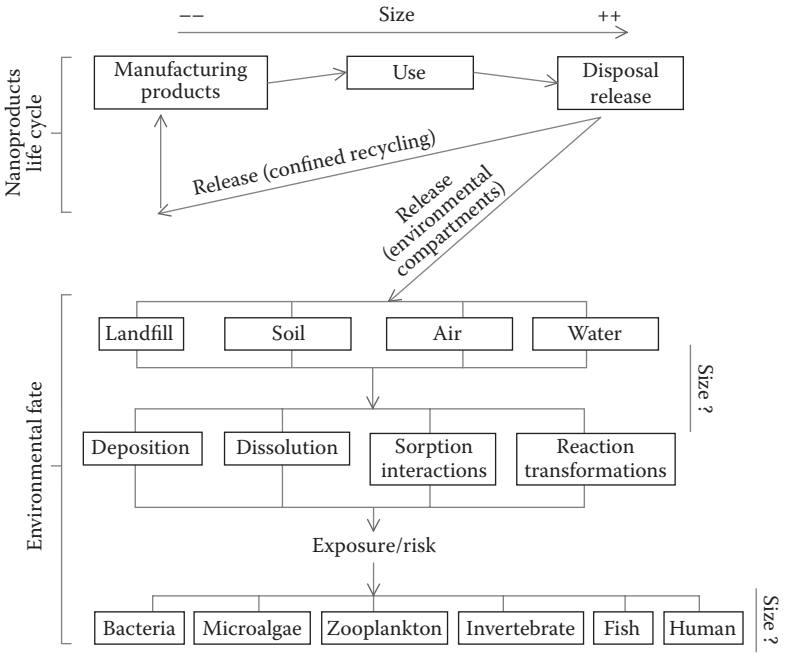


FIGURE 9.1 Nanomaterial life cycle, environmental fate, and exposure/risk of organisms to native nanomaterials or by-products.

It is impossible to describe all biophysicochemical interactions affecting engineered nanomaterials in complex aqueous media or near biological interfaces, but Nel et al. (2009) described a useful conceptual framework to guide us in understanding what happens at the nanobiointerface and in providing clues to mechanisms of nanomaterial toxicity. Following Nel et al. (2009), the nanobiointerface is composed of three dynamically interacting components: (i) the nanoparticle surface, the characteristics of which are determined by its physicochemical composition; (ii) the solid–liquid interface and the changes that occur when the particle interacts with components in the surrounding medium; and (iii) the contact zone of the solid–liquid interface with biological substrates. The main parameters affecting the nanobiointeractions are related to the nature of engineered nanomaterials, the composition of the suspending media, the solid–liquid interface present in the environmental compartments, and the interactions occurring at the nanobiointerface as shown in Table 9.1.

At the production step, the chemical composition, size, surface coating, geometry, crystallinity, porosity, roughness, hydrophobicity, or hydrophilicity are fixed. The suspending media where engineered nanomaterials are dispersed determine the pH, ionic strength, zeta potential, aggregation, state of dispersion and stability, dissolution characteristics, and the presence of large organic molecules (such as humic substances, proteins, polysaccharides) or detergents (Nel et al. 2009). The particle attributes foster the interactions with the medium by (i) promoting the adsorption of ions, the sorption of proteins, natural organic matter (NOM) or detergents; (ii) double

TABLE 9.1

Main Biophysicochemical Parameters Affecting Nanomaterials Speciation and the Interface between Nanomaterials, Suspending Media, and Biological Systems

Nanoparticle Speciation
Size, size distribution, shape, surface area
Surface charge, energy, roughness and porosity
Chemical properties (element, valence, functional groups/coating)
Crystallinity and defect
Hydrophobicity and hydrophilicity
Solubility
Suspending Media
Water molecules
Acids and bases
Salts and multivalent ions
Natural organic matter (NOM such as humic substances, proteins, and lipids)
Oxygen, sulfur compounds
Surfactants
Polymers
Polyelectrolytes
Solid–Liquid Interface
Surface hydration and dehydration
Surface reconstruction and release of free energy
Ion adsorption and charge neutralization
Electrical double-layer formation, zeta potential, isoelectric point
Sorption organic matter, steric molecules, or toxins
Electrostatic, steric, and electrosteric interactions
Aggregation, dispersion, and dissolution
Hydrophilic and hydrophobic interactions
Nanobiointerface
Membrane interaction: specific and nonspecific forces
Receptor–ligand binding interactions
Membrane wrapping: resistive and promotive forces
Biomolecule interactions (lipids, proteins, DNA) leading to structural and functional effects
Free energy transfer to biomolecules
Oxidant injury to biomolecules
Mitochondrial and lysosomal damage, decrease ATP

Source: Modified from Nel, A. E. et al., *Nature Materials*, 8, 543–557, 2009.

layer formation; (iii) dissolution; or (iv) minimizing free surface energy by surface restructuring (Khan et al. 2011; Nel et al. 2009). These effects can results from forces arising from attractive van der Waals (VDW) and (generally) repulsive electrostatic double-layer interactions, plus short-range forces arising from charge, steric, depletion, and solvent interactions (Nel et al. 2009). Nel et al. (2009) suggested that hydrodynamic, electrodynamic, electrostatic, solvent, steric, and polymer bridging

interactions are the main forces controlling the interfacial interactions between nanomaterials and biological systems.

In this review, we focused our interest to silver nanoparticles (AgNPs), the most widespread used nanomaterial (Vance et al. 2015). AgNPs have important applications in food packaging materials, odor-resistant textiles, paints, cosmetics, medical devices, and water disinfectants, because of their potential bactericidal effects (SCENIHR 2014). Multiple uses of AgNPs make inevitable their release and transport in wastewaters and their migration toward rivers and coastal waters explaining why they are listed as aquatic emergent contaminants (SCENIHR 2014; USEPA 2009). Little is yet known about the fate of AgNPs in natural waters and their potential environmental impacts and toxicity on aquatic organisms from bacterial communities to vertebrates. This review aims to provide a critical assessment of the current understanding of the fate of AgNPs in natural waters and to link this behavior to toxic effects observed in some prokaryotes and eukaryotes organisms. Our survey is describing the behavior of AgNPs in aqueous media and then discusses the toxic effects reported for bacteria, microalgae, zooplankton, invertebrates, and fish. A special attention is given to marine organisms when information was available.

9.2 Physical and Chemical Behavior and Fate of Silver Nanoparticles in Aquatic Systems

As mentioned earlier, some critical physical and chemical transformations of AgNPs occur in aquatic environments (Figure 9.2). Two important processes are commonly observed: (i) single particle or individual behavior such as oxidation and sulfidation and (ii) multiple particles or group behavior as autoaggregation or heteroaggregation. Multiple sorption processes or interactions between nanomaterials and NOM are located at the

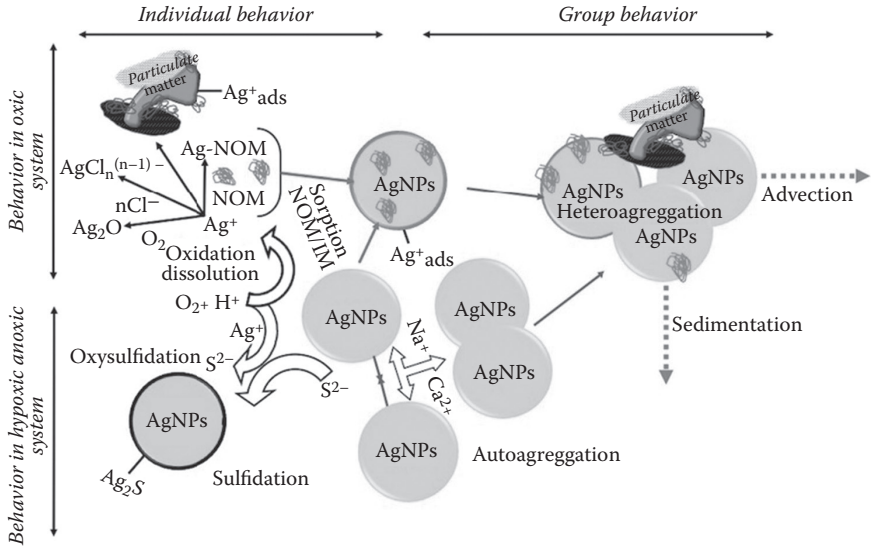


FIGURE 9.2 Complex behavior of silver nanoparticles in aquatic systems.

confluence of single and group behavior. This NOM can be formed by dissolved small molecules or large geomolecules generated *in situ* or by particulate detritus released by biological activities. The way nanoparticles behave in the environment is controlled by their own properties (size, geometry, coating, hydrophobicity, etc.) and by the characteristics of the receiving environment, i.e., redox conditions (oxic or hypoxic/anoxic), ionic strength, pH, NOM, and suspended particulate matter (SPM) (SCENIHR 2014).

9.2.1 Single Nanoparticle and Individual Behavior

9.2.1.1 Oxidation–Dissolution

The presence of dissolved oxygen can promote surface oxidation of AgNP (Liu and Hurt 2010; Zhang et al. 2016a), according to

$$\mathrm{AgNP} + \tfrac{1}{4}\mathrm{O_{2(aq)}} + \mathrm{H^+} \leftrightarrow \mathrm{Ag^+_{(aq)}} + \tfrac{1}{2}\mathrm{H_2O} \tag{9.1}$$

This oxidation–dissolution process is directly influenced by the pH of the solution, being higher under acidic conditions (Peretyazhko 2014; Zhang et al. 2016a). Intermediate reaction with the formation of H_2O_2 can enhance the dissolution (Zhang et al. 2016a). The dissolution of silver nanoparticles is also affected by the size of AgNPs (Dobias and Bernier-Latmani 2013; Lowry et al. 2012; Peretyazhko 2014; Zhang et al. 2016a). This can be explained by the higher reactivity of small AgNPs due to their higher surface/volume ratio. However, many studies have shown that this process is generally slow under natural conditions. Authors reported 2–3% AgNP dissolution after 14 days and 30% after 4 months (Dobias and Bernier-Latmani 2013; Li et al. 2012; Zhang et al. 2016a).

Some studies (Dobias and Bernier-Latmani 2013; Li et al. 2010b) mentioned strong variations in silver ions levels during the first minutes of experiments where a quick increase was followed by a strong decrease. Sotiriou et al. (2012) hypothesized desorption or dissolution of silver oxide layers from AgNP surface. This layer could result from hydrolysis or oxidation of AgNP surface (Elimelech et al. 1998; Zhang et al. 2016a). The desorption of chemisorbed silver at the surface of AgNPs (Dobias and Bernier-Latmani 2013; Li et al. 2012; Sotiriou et al. 2012) was affected by the presence of sodium–halogen salts (Espinoza et al. 2012). For instance, this process is not observed at NaCl concentrations below 27 mM, but enhanced at higher concentrations (Espinoza et al. 2012).

Silver ions released from AgNPs can interact with inorganic or organic compounds present in aqueous media. In an oxic system, silver ions interact with chlorides, carbonates, or sulfates (Levard et al. 2012). Due to the concentration of these anions in natural waters, the formation of silver chlorocomplexes $\left(\mathrm{AgCl_n^{(n-1)-}}\right)$ is thermodynamically favored (Levard et al. 2012), and these complexes ($\mathrm{AgCl^-}$, $\mathrm{AgCl_2^-}$, etc.) are strongly influenced by the Cl/Ag ratio (Levard et al. 2013). These formed salts can be sorbed around AgNPs modifying their coating or can precipitate to form new silver nanomaterials ($K_{\mathrm{spAgCl}} = 1.77 \times 10^{-10}$ [Levard et al. 2012]). Moreover, silver ions can also interact with NOM to form organic silver nanomaterials (see also Section 9.2.2.2 for AgNP interactions with NOM) (Adegboyega et al. 2013; Akaighe et al. 2012; Akaighe et al. 2011) or sorb on SPM as clay (Zhou et al. 2012).

9.2.1.2 Sulfidation

Sulfidation of AgNPs is a process mainly observed in hypoxic and anoxic systems where levels of sulfur ions (S^{2-}) are high (Levard et al. 2012). This transformation mostly occurs in wastewater treatment plants (Kaegi et al. 2011; Levard et al. 2012), some S-rich soils (Colman et al. 2013), and sediments (Dale et al. 2013; Lowry et al. 2012). Sulfur ions can interact with AgNPs by two pathways. In the first one, the oxysulfidation, S^{2-} interacts with silver ions in the presence of oxygen and sulfur, under hypoxic conditions (Liu et al. 2011). Due to the low solubility of Ag_2S (K_{sp} = 5.92×10^{-51} [Levard et al. 2012]), this salt can precipitate to form new silver nanomaterials and/or sorb around AgNPs (Levard et al. 2011; Liu et al. 2011). The second process could be a direct sulfidation, inducing the formation of a new Ag_2S coating over the surface of AgNPs (Baalousha et al. 2015; Liu et al. 2011). This process is known to preferentially occur in anoxic environments with high sulfur levels (Liu et al. 2011). These two processes are strongly influenced by the original coating of AgNPs (Baalousha et al. 2015; Liu et al. 2011), and depending on this coating, the sulfidation can be limited and some properties of native AgNPs can be preserved, as reported for citrate-coated AgNPs (Baalousha et al. 2015).

9.2.2 Multiple Particles or Group Behavior

9.2.2.1 Aggregation

Aggregation is defined as a process where dispersed particles tend to interact together to form an assemblage of particles named aggregates (McNaught and Wilkinson 2014). In aqueous solution, AgNPs move randomly due to the Brownian motion (Elimelech et al. 1998; Petosa et al. 2010) and can collide and interact together to form larger particles or aggregates. Depending on various physicochemical parameters of the medium, these interactions can be efficient or not.

Interactions between free-moving particles in liquid have been modeled by Derjaguin, Landau, Verwey, and Overbeek (DLVO) (Derjaguin and Landau 1941; Petosa et al. 2010; Verwey and Overbeek 1948; Zhang et al. 2016a). The DLVO theory indicates that the attractive and repulsive forces, respectively, VDW forces and electrical double layer repulsion, are controlling the aggregative behavior. The stability of AgNPs in suspension is observed when the repulsive forces are stronger than attractive ones, as seen in nanopure water for citrate–AgNPs (Millour et al. 2015). The addition of dissolved salts to citrate–AgNP solution induced a destabilization of the steady state of the suspension (Millour et al. 2015; Baalousha et al. 2013; El Badawy et al. 2010). When the ionic strength increases, the thickness of electrical double layer decreases and favors the close encounter of AgNPs (Millour et al. 2015; Petosa et al. 2010). In these conditions, the repulsive forces decrease and tend to be weaker than attractive forces. The presence of dissolved salts also modulates surface charges of AgNPs (Millour et al. 2015; Petosa et al. 2010) as measured with the zeta potentials. The surface charges are screened by counterions (Millour et al. 2015; Petosa et al. 2010), as cations tend to interact with negatively charged AgNPs, whereas anions are attracted by positive AgNP surface. The pH of the medium can also influence the stability of AgNPs through surface charges or isoelectric points (Petosa et al. 2010) by adding H^+ or OH^- ions. The collision between two AgNPs resulting in aggregation is not observed to be awfully efficient when the ion concentration is too low to diminish

electrical double-layer repulsion (slow or reaction limited aggregation), but could be fully efficient when ions are sufficiently concentrated to impact electrical double-layer repulsion (fast or diffusion limited aggregation) (Millour et al. 2015; Petosa et al. 2010). The transition between these two steps is determined by the critical coagulation concentration (CCC) (Millour et al. 2015; Petosa et al. 2010). Moreover, the valence of counterions strongly influences the CCC (Petosa et al. 2010). The CCC decreases with increasing valence. The CCC determined for AgNPs in water are generally higher than 50 mM in the presence of monovalent salts and smaller than 60 mM for divalent salts depending on the organic coating of AgNPs (Baalousha et al. 2013; Chen and Zhang 2012; Huynh and Chen 2011).

Using DLVO model, we can assume that the CCC is correlated to zeta potentials, as observed for citrate–AgNPs, polyethyleneimine–AgNPs, or bare AgNPs (El Badawy et al. 2010; Philippe and Schaumann 2014). However, this is not the case with AgNPs coated with polymers as polyvinylpyrrolidone. With this coating, AgNPs have low zeta potentials, but the CCC is observed at higher ion concentrations (Chen and Zhang 2012; El Badawy et al. 2010; Huynh and Chen 2011). NOM sorption can also modulate the aggregation and CCC values (Baalousha et al. 2013; Chen and Zhang 2012; Huynh and Chen 2011; Millour et al. 2015). The effect of NOM is to modify the forces involved to reach the aggregation equilibrium. In this case, it becomes important to use the extended DLVO theory to describe aggregative behavior, because additional interactions due to polymers or NOM are taken in account (Grasso et al. 2002; Petosa et al. 2010), including hydrogen bonding, hydrophobic interaction, hydration pressure, noncharge transfer Lewis acid–base interactions, and steric interactions. These complex interactions and effects of medium are fully described in an exhaustive review by Grasso et al. (2002).

Multiple interactions of AgNPs with other particles or compounds (Table 9.1 and Figure 9.2) are observed with NOM as humic substances (Chen and Zhang 2012; Huynh and Chen 2011, Millour et al. 2015; Wang et al. 2016), clay minerals (Liu et al. 2015; Zhou et al. 2012), exopolysaccharides, and extracellular polymeric substances (Khan et al. 2011; Lau et al. 2013), as well as proteins and other biological compounds (Nel et al. 2009; Philippe and Schaumann 2014). Depending on particles interacting together, two modes of aggregation are observed. Autoaggregation describes the aggregation of many identical particles, whereas heteroaggregation occurs between particles of different nature (Yu and Borkovec 2002). In natural aquatic environment, the heteroaggregation of AgNPs with other particles or colloids is the primary and most important process, because AgNP concentrations are between nanograms per liter to a few micrograms per liter (Coll et al. 2015; Gottschalk et al. 2013), whereas natural particles, colloids, or dissolved NOM are at milligrams per liter level (Klaine et al. 2008; Tremblay and Gagné 2009). Moreover, interactions between AgNPs and colloids are modulated by physicochemical conditions of aquatic environments. In natural waters, with a complex mixture of ions, the aggregation of AgNPs or other particles are driven by additive or synergistic effects of each salt and controlled by the dominant salt (Baalousha et al. 2013). For negatively charged AgNPs, one can assume that divalent cations drive the aggregation in freshwater, whereas in seawater, monovalent cations are activating the process. This is caused by the average concentrations of major cations in world rivers: Na^+ (0.23 mM), Ca^{2+} (0.33 mM), Mg^{2+} (0.15 mM), whereas concentrations are Na^+ (468 mM), Ca^{2+} (10.2 mM), and Mg^{2+} (53.2 mM) in seawater (Bianchi 2007). For anions, the concentrations are HCO_3^- at 0.86 mM, Cl^- at

0.16 mM, and SO_4^{2-} at 0.069 mM in freshwater and 2.38, 545, and 28.2 mM in seawater. The toxicity mechanisms of AgNPs and their environmental fate can be different depending on the size of aggregates (Millour et al. 2015; Nel et al. 2009; Zhu et al. 2012). Small aggregates can directly interact with cellular surface and be internalized, while large aggregates cannot directly penetrate the cell membrane. Moreover, large aggregates can rapidly settle, whereas small ones can stay a long time in suspension increasing possible interactions with NOM, detritus, or living cells or even be transported far from their discharge point in the environment.

9.2.2.2 Interaction or Sorption with Natural Organic Matter

Sorption is a process where a substance (sorbate) interacts with a solid (sorbent) by adsorption, absorption, and desorption (McNaught and Wilkinson 2014; Gagné et al. 2011). Adsorption occurs when sorbate interacts just with the surface of the sorbent, whereas absorption appears when sorbate is internalized by sorbent. Desorption is the process where the sorbate separates from the sorbent. Sorption is an exothermic and spontaneous process (Somorjai 1994). This process implies some possible interactions with nanomaterials: (i) physical interactions as VDW and hydrophobic interactions; (ii) electrostatic interactions as charge transfer complex, ligand exchange, ionic bridging, and hydrogen bound; and (iii) chemical interactions as covalent bound (Gagné et al. 2011; Philippe and Schaumann 2014; Wang et al. 2016). As described earlier, the surface of AgNPs can be a sorbent for salts and/or organic matter inducing their transformation. Moreover, transformed AgNPs can also interact with heterogeneous surfaces such as cell membranes. Depending on the surface involved in the interaction, the fate of AgNPs can differ.

NOM is ubiquitous in pristine natural waters, wastewaters, or industrial waters or inside biological cells. Humic substances are particularly present in freshwater with more than 50% of NOM composition (Thurman 1985; Tremblay and Gagné 2009), but polysaccharides, lipids, proteins, and other organic compounds are also present (Philippe and Schaumann 2014) as well as in living cells. Interactions between NOM and AgNPs in aqueous environment are mostly studied with humic substances and more specifically with humic acids (Chen and Zhang 2012; Doiron et al. 2014; Huynh and Chen 2011; Kim et al. 2013; Philippe and Schaumann 2014; Wang et al. 2016). However, to increase the knowledge of NOM–AgNPs interactions, it is important to describe effects with fulvic acids. In aquatic systems, the presence of fulvic acids is more important than humic acids (the fulvic acid/humic acid ratio is between 2 and 10 [Tremblay and Gagné 2009]). Moreover, proteins, lipids, and polysaccharides are more representative of biological systems and should receive more attention. Considering the composition of NOM, the interactions of AgNPs with NOM in natural waters or into cells exposed to nanomaterials could be different due to the nature and the relative proportion of the organic matter found in each medium. It is important to note that bare nanomaterials do not exist under biological conditions due to protein adsorption, which, at any time, provides an organic coating (Soenen et al. 2015). Thus, it is clear that the toxicity of nanomaterials to bacteria, algae, invertebrates, and fish is modulated by the presence of NOM (Grillo et al. 2015; Petersen et al. 2014; Wang et al. 2016). The presence of inorganic and some organic surfaces, as SPM, can decrease the bioavailability of AgNPs, although organic surfaces, such as biofilms, could favor interactions of AgNPs with organisms and induce toxicity.

9.3 Effects of AgNPs on Aquatic Organisms

9.3.1 Nanoparticles and Prokaryotes

Heterotrophic prokaryotes constitute a key component in the functioning of aquatic microbial food web and serve integral environmental functions (Azam and Malfatti 2007; Pomeroy et al. 2007). Through its capacity to degrade organic matter and regenerate nutrients, this biological compartment plays an essential role in the transfer of matter and energy to higher trophic levels (Azam et al. 1983). Thereby, the potential impact of bactericidal AgNPs on aquatic heterotrophic bacteria might strongly affect their capacity to provide ecosystem services listed earlier. In addition, due to their high growth rates, their short generation times (Del Giorgio and Cole 1998), and the vast diversity of their metabolic activities, bacterial communities constitute very interesting biological models to assess, in real time, the toxicological effects of AgNPs in aquatic ecosystems (Ghiglione et al. 2016). Many studies have described the effects of AgNPs on model microorganisms such as bacterial pathogens of the genera *Vibrio*, *Escherichia*, and *Pseudomonas* (Dibrov et al. 2002; Holt and Bard 2005; Xu et al. 2004; Dorobantu et al. 2015). Some results are now available on their potential effects on complex bacterial communities, in terms of diversity and abundance, in different environmental compartments (soil, sediment, and water) but they are generally reporting conflicting results (Maurer-Jones et al. 2013). As an example, a 30-day experiment conducted on the bacterial diversity of estuarine sediments did not demonstrate a correlation between the presence of AgNPs and change in the bacterial community (Bradford et al. 2009). However, other researchers (Choi et al. 2008; Choi and Hu 2008) came to the conclusion that the presence of AgNPs reduced the natural nitrifying by about 90%, which means a drastic change in the bacterial community. Similarly, Fabrega et al. (2011b) have shown that the presence of AgNPs influenced the bacterial community within marine biofilms by reducing the development and succession of normal bacterial biofilm within 4 days of growth. The modification of the taxonomic diversity of natural bacterial communities was also demonstrated in estuarine waters at low and environmentally relevant AgNP concentrations (5 and 50 μg L^{-1}) (Doiron et al. 2012). These contrasting results demonstrate that additional knowledge of nanoparticle physicochemistry, as well as biotic and abiotic factors affecting AgNP behavior over time (see Section 9.2), is needed to fully understand the mode of action of AgNPs on prokaryotic cells inhabiting aquatic environments (Fabrega et al. 2011a).

9.3.1.1 Potential Antimicrobial Mechanisms of AgNPs

As AgNPs are expected to affect bacteria by many different ways simultaneously, the development of a specific resistance against AgNPs is quite impossible (Silver et al. 2006; Rai et al. 2012). Given the worldwide emergence of multidrug-resistant bacteria and the need to synthesize new antimicrobials, the use of AgNPs as antimicrobial agent has become of great interest in many applications ranging from antiodor products to food packaging (Rai et al. 2012; SCENIHR 2014). In the last decade, many studies have reported efficient bactericidal activities of AgNPs against bacterial models, human, and phytopathogens as well as multidrug-resistant pathogens (Marambio-Jones and Hoek 2010; Lara et al. 2010; Mishra and Singh 2015). Bacterial

models used in nanotoxicity studies greatly varied and have included common research species such as *Escherichia coli*, *Bacillus subtilis*, *Vibrio splendidus*, and *Pseudomonas aeruginosa* along with bacteria that play key roles within environmental compartments, such as *Nitrosomonas europaea*, a nitrifying bacteria with a key role in wastewater treatment (Dorobantu et al. 2015; Yang et al. 2013; Doiron et al. 2014; Maurer-Jones et al. 2013). Interestingly, AgNPs not only are active against free cells, but have also demonstrated a great efficiency against bacterial biofilms (Abdel Rahim and Ali Mohamed 2015; Flores et al. 2013; Radzig et al. 2013). Moreover, the combined use of AgNPs and classical antimicrobial compounds tends to enhance their bactericidal potential in a synergistic way (Abdel Rahim and Ali Mohamed 2015; Lara et al. 2010; Shahverdi et al. 2007; Deng et al. 2016).

Despite the increasing use of AgNPs as an antimicrobial agent, the scientific community still debates about the nature of the nanosilver antimicrobial mechanisms. Although silver ion (Ag^+) released by AgNP dissolution close to membranes or within bacterial cells is likely the predominant antimicrobial mechanism of AgNPs (Martínez-Gutierrez et al. 2012; Zhang et al. 2016a; Xiu et al. 2012), the debate on the existence of a "particle-specific" effect of nanosilver is still open (Marambio-Jones and Hoek 2010; Zhou et al. 2015; Zhang et al. 2016a). Antimicrobial properties of AgNPs are negatively correlated with the particle size and shape; small size (<10 nm) AgNPs being the particles most likely to be taken up by microorganisms and affecting cellular viability through their rapid dissolution in silver ions within cells (Choi et al. 2008; Choi and Hu 2008; Morones et al. 2005; Shrivastava et al. 2007; Sondi and Salopek-Sondi 2004; Kumari et al. 2016). Generally speaking, nanosilver exposure can seriously damage cellular structures (such as cell membranes and cell walls) and cellular components (nucleic acids, lipids, and proteins) and inhibit critical cellular processes (adenylpyrophosphate [ATP]-associated metabolism, DNA replication, transcriptional activities) (Prabhu and Poulose 2012; Yang et al. 2013; Marambio-Jones and Hoek 2010; Morones et al. 2005; Zhang et al. 2016a; Zhou et al. 2015). Some recent studies reported that the contribution of particulate (ion-free) nanosilver and reactive oxygen species (ROS) generation (Choi et al. 2008) to the overall toxicity of nanosilver must not be neglected and need to be carefully explored (Zhang et al. 2016a; Fabrega et al. 2009). Many studies concluded that silver ion release is plausibly the major antimicrobial mechanism of nanosilver. Nevertheless, others suggested that silver ion release from AgNP dissolution might not be the exclusive toxicity factor of nanosilver. These authors reported direct and indirect antimicrobial activities of particulate nanosilver as well as antimicrobial effects related to the generation of ROS under aerobic conditions (Zhang et al. 2016a). The main AgNP-related damages are presented in Table 9.2.

Cell membranes might be the main biological target of nanosilver. Many studies reported interactions of AgNPs with bacterial membranes leading to an accumulation of nanoparticles on the surface of bacterial cells, a local release of silver ions generating membrane damages (pits formation), and finally an increased membrane permeability by membrane protein denaturation (Flores et al. 2013; Krishnaraj et al. 2010; Li et al. 2010a; Lok et al. 2006, 2007; Morones et al. 2005; Raffi et al. 2008; Sondi and Salopek-Sondi 2004). Membrane alterations allow the penetration of small AgNPs within the cells and the release of lipopolysaccharidesand cellular components in the extracellular medium, resulting at least to microbial cell lysis (Krishnaraj et al. 2010; Lara et al. 2010; Morones et al. 2005; Sondi and Salopek-Sondi 2004). The interaction

TABLE 9.2

Main AgNP-Related Damages on Bacterial Cells

Membrane or Wall Damage
Accumulation of AgNPs on cell membranes
Membrane proteins denaturation by AgNPs binding on sulfur-containing proteins
Increased membrane permeability allowing penetration of AgNPs inside the cells
Membrane destabilization or disruption
Cell lysis
Inhibition of wall synthesis
Alteration of Cellular Components
Penetration and dissolution of AgNPs inside cells generating local concentration of silver ions
Enzymes denaturation
Inhibition of proteins and nucleic acids synthesis
Interaction with nucleic acids (mutations)
Physiological Damages
Catalytic generation of ROS inside the cell
Inhibition of respiratory process
Inhibition or decrease of ATP-mediated metabolism
Inhibition of DNA replication
Inhibition of transcriptional activity
Inhibition of transduction by phosphotyrosine and tyrosine phosphorylation modulation
Dissipation of the proton motive force
Reduction of growth rate
Increase of the lag phase
Enhanced phosphate efflux

Note: For review, see Zhang et al. (2016a) and Zhou et al. (2015).

of AgNPs with cell membranes is essentially due to their binding on sulfur-containing proteins or enzymes that interfere with their proper functions (Zhou et al. 2015). In addition, positively charged nanoparticles might alter the state and organization of lipids and phospholipids, generating serious damages to the structure, function, and permeability of cellular membranes (Zhang et al. 2016a).

9.3.1.2 Effects of AgNPs on Complex Natural Bacterial Communities

Although the antimicrobial capabilities of AgNPs have been widely reported on bacterial monocultures, their impacts on ecologically important microbial communities are still not well understood. Monoculture bacteria nanotoxicity studies revealed the mechanisms of nanoparticle-induced toxicity (e.g., ROS production, dissolution of AgNPs to toxic ions within cells, membrane damages) and the significance of nanoparticle physical properties such as particle and aggregate sizes and surface modifications in determining toxicity. Natural isolate studies, on the other hand, involve complex mixtures of bacterial species and have generally focused on understanding toxicity in more complex and ecologically relevant environments, often at the bacterial community level (Maurer-Jones et al. 2013).

AgNP toxicity was reported to depend on concentration, size, shape, and coating type of the particles (Kumari et al. 2016). In natural environments, AgNP

antimicrobial mechanisms reported on monocultures might also be modulated by biotic (e.g., AgNP bioaccumulation) and abiotic (e.g., salinity, presence of NOM, aggregation) factors that might modify shape and size of nanoparticles, as previously described in Section 9.2.

Presently, an increasing number of studies focused on the effects of AgNPs on various microbial communities in different environments (from activated sludge to estuarine water) (Alito and Gunsch 2014; Yang et al. 2014;, Samarajeewa et al. 2017, Zhang et al. 2016a; Blakelock et al. 2016; Echavarri-Bravo et al. 2015; Dorobantu et al. 2015; Antizar-Ladislao et al. 2015). Unfortunately, results are conflicting and difficult to compare due to the high variety of nanoparticle types, sizes, and coatings and the wide range of concentrations used from few micrograms per liter to more than 1 g L^{-1} (Zhang et al. 2016a). The concentration of AgNPs in the environment is one of the main drivers of antimicrobial effects on bacterial communities, and some studies revealed a clear dose–response effect (Calabrese 2005). In monocultures, this dose–response effect resulted in the observation of an hormesis response at low AgNP concentrations (less than 20 µg L^{-1}) (Doiron et al. 2014) and in the inhibition of bacterial growth (i.e., increase of the lag phase or total growth inhibition) at higher AgNPs (Irwin et al. 2010; Martínez-Castañón et al. 2008; Morones et al. 2005).

Most of the AgNPs released from consumer products enter sewer systems and wastewater treatment plants. As a consequence, wastewater and activated sludge microbial communities are particularly exposed to nanosilver (Kim et al. 2011; Shon et al. 2007; Sun et al. 2013). The investigation of the bacterial toxicity of AgNPs in wastewater is critical for risk assessment since autotrophic nitrifying organisms allowing water depuration are among the most sensitive prokaryotes to AgNPs (Choi et al. 2008; Choi and Hu 2008). The potential perturbations of nanosilver to microbial mediated nitrogen cycling might be important to consider in many environments since nitrifiers could be highly affected by the presence of AgNPs (Langdon et al. 2014; Masrahi et al. 2014) in terms of enzymatic activities (Colman et al. 2013), nitrification activities (Jeong et al. 2014), and microbial taxonomic and functional diversity (Samarajeewa et al. 2017; Shah et al. 2014, Yang et al. 2014).

Only a limited number of studies have been conducted to evaluate the impacts of AgNPs on complex microbial communities after AgNPs are released by wastewater effluents to aquatic ecosystems (Bradford et al. 2009; Echavarri-Bravo et al. 2015). In 2012, Das et al. demonstrated that AgNPs rapidly, but temporarily, inhibited natural bacterioplankton production in freshwater. Similarly, Colman et al. (2012) observed a decrease in the microbial respiration of pelagic communities, but found no impact on benthic communities. However, Blakelock et al. (2016) did not report any toxic effects of AgNPs on bacterioplankton abundance, biomass, production, or chlorophyll a content in a boreal lake. These recent results contradict the observations of Pradhan et al. (2011), who have demonstrated that the presence of AgNPs induced a decrease in microbial biomass and in community richness in freshwater streams. Only scarce data are available concerning the effects of AgNPs on estuarine or marine microbial communities, and they reported highly conflicting results. In 2009, Bradford et al. did not observe any effects of AgNPs on the taxonomic diversity of microbial communities inhabiting estuarine sediments, whereas Antizar-Ladislao et al. (2015) recently reported an increase in total bacterial abundance characterized by a richness modification and the loss of key species such as *Pelobacter propionicus* within the bacterial community. Similar observations were reported by Doiron et al. (2012) in estuarine

waters where the addition of low concentrations of AgNPs (5–50 μg L^{-1}) induced a rapid, but temporary, reduction of total bacteria abundance and a significant reduction of planktonic bacteria richness. Echavarri-Bravo et al. (2015) have also reported short-term modifications of the functional diversity of impacted estuarine benthic bacterial communities followed by a recovery after 120 hours at 1 mg L^{-1} of AgNPs.

Two different hypotheses have been proposed to explain the rapid recovery of the bacterial community in fresh and estuarine waters: a selection of silver resistant strains within the community and/or AgNP pressure alleviation on microbial communities due to aggregation, sedimentation, or environmental modifications of nanoparticles structure along time (Das et al. 2012; Doiron et al. 2014; Doiron et al. 2012; Fabrega et al. 2009). Even if the mechanisms involved in the reduction of AgNP toxicity along time are presently not well understood, it was proposed that natural organic matter compounds, such as humic substances, could sorb on the surface of AgNPs (Fabrega et al. 2009; Doiron et al. 2014), lessen the dissolution into silver ions, and, consequently, reduce the overall toxicity of AgNPs to bacterial cells. As a result, the inconsistency of the results on AgNP toxicity to environmental microbial communities may be related to their interactions with different environmental components (e.g., NOM and salts; see Section 9.2 for detailed information). In addition, impacts of AgNPs on complex microbial communities could be modulated not only by the structure of AgNP aggregates but also by the spatial distribution of microorganisms (free cells versus attached cells or biofilms) (Sheng and Liu 2011).

9.3.2 Effects of AgNPs on Phytoplankton

9.3.2.1 Effects and Mechanisms of AgNPs on Phytoplankton

Although AgNPs are expected to have minor effects on human health and only affect prokaryotes, there are increasing evidences that AgNPs can be a threat to eukaryotic aquatic organisms. Indeed, it was postulated that silver is the second most toxic metal for aquatic organisms, after mercury (Moreno-Garrido et al. 2015). In this regard, a great interest has been given to the effects and mechanisms of AgNPs on eukaryotic organisms.

9.3.2.1.1 Interactions of AgNPs with Microalgae

Phytoplankton assemblages play a major role in the aquatic food web, as these microscopic plants are the main source of energy for many aquatic herbivores. Although toxic concentrations of AgNPs are generally higher for phytoplankton than bacteria, many studies have shown impacts of AgNPs on microalgae leading to phenomena such as growth inhibition (Das et al. 2012; Moreno-Garrido et al. 2015; Navarro et al. 2008), reduction of algal viability (He et al. 2012; Huang et al. 2016a), and morphological malformations (He et al. 2012; Moreno-Garrido et al. 2015).

The toxic effects of AgNPs on microalgae cell membranes are quite similar to those described on bacterial membranes (see Section 9.3.1.1). First, AgNPs may partly dissolve in the media, thus releasing silver ions that could diffuse through the algal surface and penetrate the cells, thus affecting vital enzymes (Miao et al. 2009). Second, AgNPs within the diffusion layer of microalgae or directly attached to the cell surface may dissolve, providing additional silver ions directly to the surface of algae (Dash et al. 2012; Zouzelka et al. 2016). Third, as cell pores of microalgae are in

the range of 5–20 nm (Bisalputra and Weier 1963), AgNPs smaller than 20 nm could penetrate through cellular membrane pores (Zouzelka et al. 2016). However, larger AgNPs and aggregates can destabilize the cell membrane to form new pores increasing the cellular permeability (Zouzelka et al. 2016). This would facilitate AgNP entry inside the cytoplasm, where they can exert their toxic effects. Moreover, the entry of AgNPs inside algal cells through ion channels or endocytosis has been suggested by Zhang et al. (2016b). All these mechanisms are nonmutually exclusive, and they can simultaneously take place providing synergistic effects (Burchardt et al. 2012).

9.3.2.1.2 Toxic Effects of AgNPs on Microalgae

Many studies have highlighted direct interactions (sorption) of AgNPs with the outer shell of microalgal cells (Huang et al. 2016a; Oukarroum et al. 2012a). The formation of aggregates on cell membranes may inhibit cell growth (Navarro et al. 2008) and alter the cellular acquisition of essential nutrients by clogging to the walls (Bundschuh et al. 2016). Moreover, such sorption of AgNPs onto the cell surface may reduce the photosynthesis due to shading effects of AgNPs (Huang et al. 2016a). Further, Moreno-Garrido et al. (2015) suggested that the adsorption of nanoparticles on and in microalgae could increase their cellular weight and remove the microalgae from the photic zone, thus affecting the photosynthesis.

After penetrating inside the cell cytoplasm, AgNPs or released silver ions may induce various toxic effects. They can interact with thiol- and phosphate-containing proteins and enzymes, thus altering their activity and function (Mitrano et al. 2015; Zouzelka et al. 2016), disturbing the management of cellular phosphate, causing the collapse of the proton pump and denaturation of ribosomes (Moreno-Garrido et al. 2015), inhibiting transcriptomic processes of the mRNA, penetrating the nucleus through the nuclear pores to induce DNA damage, and inhibiting its repair process (Dash et al. 2012; Zhang et al. 2016b). Furthermore, the increase in ROS production by microalgae is one of the most important responses to AgNPs and/or Ag^+ ion exposure and has been described in numerous studies (He et al. 2012; Huang et al. 2016a). Qian et al. (2016) observed that *Chlorella vulgaris* is able to efficiently detoxify AgNP-induced ROS via the induction of antioxidant enzymes, thus reducing the toxicity of AgNPs. Nevertheless, other studies observed that high levels of intracellular ROS may impair cell viability (Carlson et al. 2008; Oukarroum et al. 2013), inducing lipid peroxidation, increasing cell membrane permeability and DNA and RNA damages, and activating apoptotic pathways (He et al. 2012).

9.3.2.1.3 Effects of AgNPs on Mitochondria and Chloroplasts

Contrary to bacteria, eukaryotic cells possess specific organelles such as mitochondria and chloroplasts. As both these organelles are considered as ancient bacteria, AgNPs are expected to interact with them and inhibit their functions. Engineered nanoparticles can induce mitochondrial damages via a number of processes (Leonardo et al. 2016; Stensberg et al. 2014). Maurer and Meyer (2016) categorized four main modes of mitochondrial toxicity. The first one was the induction of ROS, which is associated with the impairment of mitochondrial function that could reduce cell viability after exposure to AgNPs (Carlson et al. 2008). Moreover, mitochondria as well as chloroplast membranes contain polyunsaturated lipids which are especially prone to oxidant attack by ROS (von Moos and Slaveykova 2014). The second mode of action was the loss of mitochondrial inner membrane potential. The depolarization is the most

frequently studied mitochondrial outcome as a result of AgNP exposure (Maurer and Meyer 2016). Third, AgNP exposure may reduce the mitochondrial activity. The fourth suggested mode was the inhibition of the electron transport chain. The alteration of mitochondria could lead to a loss of membrane potential, inhibition of enzymes involved in oxidative phosphorylation, induction of more ROS, and induction of apoptotic pathways among others (Maurer and Meyer 2016).

Chloroplasts are also affected by AgNPs. Indeed, the reduction of chlorophyll a and carotenoids and inhibition of the photosynthesis are commonly observed in microalgae exposed to AgNPs (Zhang et al. 2016c; Zou et al. 2016). Besides the shading effect of AgNPs, other mechanisms have been described to explain the reduction of the photosynthesis. The reduction in chlorophyll a content and direct alteration of the chloroplast by the inhibition of the photosystem II electron transport and induction of photosystem I photosynthetic membrane damage by AgNPs or silver ions inside the cell have been shown to play a major role in photosynthesis inhibition (Huang et al. 2016a; Navarro et al. 2008).

9.3.2.1.4 Silver Detoxification Process of Microalgae

While some authors suggested that AgNP toxicity is only due to the release of silver ion (Moreno-Garrido et al. 2015), other postulated that the toxicity is due to combination of both AgNPs and silver ions (Bundschuh et al. 2016). Interestingly, it has been demonstrated that microalgae are able to reduce toxic metals and produce engineered nanoparticles based on the dissolved ions (Moreno-Garrido et al. 2015). Leonardo et al. (2016) suggested that the detoxification of silver in green alga *Coccomyxa actinabiotis* may be due to interaction with cytosolic S bearing compounds that reduce the toxicity of Ag and transport it to the chloroplast where they are reorganized into small, stable nanoparticles that apparently do not interact with other cellular components.

In an aquatic environment, the presence of NOM is known to reduce AgNP toxicity (Huang et al. 2016b). The sorption of NOM on algal cells and AgNP surface could promote repulsive charges due to increased negative surface, by NOM a negatively polyelectrolyte in natural water (Philippe and Schaumann 2014; Thurman 1985), preventing their direct interaction or uptake of AgNPs (Li et al. 2016). The excretion of exopolysaccharides and NOM by phytoplanktonic biofilms could potentially reduce AgNP toxicity by the complexation of silver ions (González et al. 2016; Li et al. 2016) or by coating and stabilization of engineered nanoparticles (Liu and Hurt 2010; Gao et al. 2009). Li et al. (2016) found that amide, carboxyl, and phosphorus functional groups of extracellular polymeric substances were involved in the binding and removal of AgNPs and/or dissolved silver. Such processes have been found to be accelerated under light conditions and may affect speciation and transformation of silver in natural waters (Zhang et al. 2016d). This was also suggested by Schiavo et al. (2017) as they proposed that the release of exopolymeric substances could also play a role in higher AgNP tolerance. The reduction of silver and the formation of AgNP ions outside and/or inside cells can explain why microalgae are less sensitive to AgNPs than other organisms such as bacteria or zooplanktons.

9.3.2.2 Varying Sensitivity to AgNPs Depending on the Species

Not all microalga species elicit the same sensitivity to AgNPs; thus, their effects may vary from one species to another (Kwak et al. 2016; Das et al. 2014). Kwak et al.

(2016) have observed that the 72-hour EC_{50} values of the two phytoplanktonic algae *Chlamydomonas reinhardtii* and *Chlorococcum infusionum* were 13.53 and 0.68 mg L^{-1}, respectively. Angel et al. (2013), showed that the toxicity of citrate–AgNPs was much higher against freshwater alga *Pseudokirchneriella subcapitata* (72-hour EC_{50} of 3.0 μg L^{-1}) than against marine diatom *Phaeodactylum tricornutum* (72-hour EC_{50} of 2380 μg L^{-1}). Planktonic cyanobacteria such as *Microcystis aeruginosa* appeared much more sensitive to AgNPs than eukaryotic phytoplankton like *C. vulgaris* (Park et al. 2010; Chen et al. 2016). *Dunaliella tertiolecta* has been shown to produce much more ROS than *C. vulgaris* when exposed to AgNPs (Oukarroum et al. 2012b), which could explain the difference in sensitivity. The composition of the cell membranes and cell walls could at least partially explain the difference in sensitivity to AgNPs (Piccapietra et al. 2012; Zouzelka et al. 2016) as microalgae cell walls are thick and rigid, which may induce a better resistance to the entry of AgNPs inside the cells. In this regard, Piccapietra et al. (2012) suggested that algal cell walls and membranes could affect the bioavailability of AgNPs by limiting their uptake. In accordance to these results, Schiavo et al. (2017) found that *P. tricornutum* are less sensitive to AgNPs than *Tetraselmis suecica*. They attributed this difference in sensitivity, at least in part to the presence of a resistant silicified cell wall in the diatom species.

In the preceding section, we described main toxic effects, modes of action, and detoxification process of AgNPs in microalgae. We also pointed out that AgNPs can sorb onto cell surface as well as penetrate inside the cell cytoplasm. As microalgae play a major role in the food chain, it is likely that AgNPs-contaminated microalgae may contaminate other organisms due to trophic transfer.

9.3.3 Effects of AgNPs on Zooplankton

Zooplankton are heterotrophic planktonic organisms playing an important role in the marine and freshwater food web as they feed on microalgae and are a resource for consumers on higher trophic levels. Due to their role in the food web, interactions of zooplankton with environmental contaminants are highly important. In this regard, a great interest has been given on the effects of AgNPs on zooplankton, small crustaceans found in the sea and freshwater. Different studies reported on the impacts of AgNPs on zooplankton and observed that the main toxicity end points concerning AgNPs on these organisms are mostly increased mortality (Arulvasu et al. 2014; Chae and An 2016), reduced reproduction (Arulvasu et al. 2014; Sakka et al. 2016), and swimming alteration (Chae and An 2016; Cupi et al. 2015).

Based on the high sensitivity of *Daphnia magna* toward AgNPs, Asghari et al. (2012) suggested, according to the Globally Harmonized System of classification and labeling of chemicals (UNECE 2013), that AgNPs should be classified as "category acute 1" to aquatic organisms such as *D. magna* as their 48-hour EC_{50} is less than 1 mg L^{-1}.

9.3.3.1 Physical Interactions of AgNPs with Zooplankton

AgNPs have been found to accumulate on the carapace and filtering appendages (Asghari et al. 2012; Shanthi et al. 2016) and inside the gut of crustaceans (Asghari et al. 2012; Vijayakumar et al. 2016). Authors observed that the attachment of engineered nanoparticles to the carapace of zooplankton may cause a reduction or even an inhibition of molting. The exposure of *D. magna* to AgNPs induced a production

of bubbles under the carapace and pigmentation of the brood chamber, which was not observed for soluble $AgNO_3$ exposure (Asghari et al. 2012; Shanthi et al. 2016). The presence of AgNPs on filtering appendages and inside the gut indicated that one of the major interactions of AgNPs with zooplankton is taught to be through ingestion. Indeed, *D. magna* is known to uptake AgNPs mainly via filtering appendages, while the transport of silver from the surrounding media to its body includes diffusion at the cell membrane, endocytosis at the cell surface, and adhesion to the gut internal wall (Bhatt and Tripathi 2011). It has been suggested that AgNP uptake in *D. magna* is mostly through ingestion (Kalman et al. 2015). Recently, Sakka et al. (2016) demonstrated that AgNP size may impact their uptake through filtration as only particles in the range of 300–500 nm are actively filtered by the *Daphnia* and may thus have higher ingestion than smaller engineered nanoparticles (Kwon et al. 2014). In contrast, Ribeiro et al. (2014) hypothesized that the attachment of AgNPs on filtrating appendages may decrease the filtering activity and feeding rate.

As previously described, AgNP exposure can induce a reduction of swimming ability, which was attributed to three nonexclusive major mechanisms. The first one is the binding of AgNPs to the carapace and filtering appendages of zooplankton (Asghari et al. 2012). The second one is massive ingestion of AgNPs that may alter their floating ability, thus leading to its sinking. The third hypothesis is that as the disruption of energy production has been observed (Cupi et al. 2015; Stensberg et al. 2014), reduced energy content may induce reduced swimming ability (Cupi et al. 2015).

9.3.3.2 Toxic Effects of AgNPs to Zooplankton

Investigating AgNP toxicity, Stensberg et al. (2014) observed the alteration of the proton efflux of mitochondria and mitochondrial membrane damages induced by AgNPs, while Cupi et al. (2015) also observed the alteration of Na^+/K^+ ATPase (NKA) activity. Studies on the effects of AgNPs on zooplankton demonstrated heart rate reduction, disturbance in energy metabolism, as well as oxidative stress (Li et al. 2015; Vijayakumar et al. 2016). Furthermore, elevated lactate levels were observed in all AgNP treatments, while it was not the case for ionic Ag treated groups, thus providing evidence that AgNPs may enhance anaerobic metabolism, probably caused by increased movement and perturbed breathing. The reduction of lysine, arginine, and glucose levels was also observed in both AgNP and $AgNO_3$ treatments. Such a reduction of amino acids was also observed for Cu^{2+} toxicity and was suggested to be a response to the induction of defense and repair mechanisms, such as the synthesis of stress proteins and DNA repair enzymes (Smolders et al. 2005). Other alterations due to AgNP exposure have been demonstrated such as altered morphology of the eyes (Becaro et al. 2015), formation of curved shell spine, developmental arrest in neonates, rupture of the carapace (Shanthi et al. 2016), translocation across the intestinal epithelium (Mattsson et al. 2016), destruction of digestive organs (Chae and An 2016), production of ROS, enzymatic activity (Ulm et al. 2015), DNA damages, induction of apoptosis (Arulvasu et al. 2014), and induction of gene expression. Such AgNP effects have already been described in Sections 9.3.1 and 9.3.2 and are mostly applicable to all kinds of eukaryotic cells. Moreover, AgNPs also inhibited multixenobiotic resistance transporter activity in *D. magna* (Georgantzopoulou et al. 2016). This inhibition reduced the excretion of toxicants out of *Daphnia* cells; thus,

AgNPs could also affect the survival of this crustacean by increasing the effects of other pollutants and synergistically acting with the contaminants. Nevertheless, zooplankton was shown to be able to, at least partially, eliminate bioaccumulated silver. As already observed for other species, the elimination of silver was biphasic with a first fast elimination through feces and a second slower diffuse excretion not clearly identified (Kalman et al. 2015; Khan et al. 2015). Further, Ribeiro et al. (2017) postulated that bigger engineered nanoparticles have a much faster clearance than smaller engineered nanoparticles. Although acute and chronic experiments on the exposure of AgNPs to zooplankton have been reported, only few research teams worked on impacts of AgNPs on successive generations of zooplanktonic organisms. Völker et al. (2013) found that AgNPs could negatively affect different *Daphnia* sp. survival and reproduction, as well as their intrinsic rates of population increase on multiple generations. They showed some evidences of the bioaccumulation of AgNPs along the different generations and observed potential induction of tolerance to AgNPs through generations potentially due to the induction of metallothionein-like proteins.

As seen for other aquatic species discussed in this chapter, it is still under debate whether AgNP-induced toxicity to zooplanktonic species directly results from AgNPs or is driven by the released silver ions. While some authors suggested that the toxicity was due to the silver ions, Poynton et al. (2012) analyzed the gene expression profiles of *Daphnia* exposed to silver ions or to AgNPs and highlighted that the major biological processes disrupted by AgNPs included protein metabolism and signal transduction, while $AgNO_3$ mainly caused a downregulation of developmental processes, particularly in the sensory development. So, it could be an addition of effects from both chemical forms.

9.3.3.3 Influence of Environmental Conditions on AgNP Toxicity to Zooplankton

Examining the effects of environmental conditions, Macken et al. (2012) showed that salinity may increase the toxicity of certain types of AgNPs and suggested that the increase in chloride ions or other inorganic ligands within the media may change the behavior and bioavailability of the AgNPs to planktonic crustaceans. As described in Section 9.3.2.2, the presence of natural organic matter could reduce the toxicity of AgNPs to zooplankton (Cupi et al. 2016). Blinova et al. (2013) supported this hypothesis by demonstrating that the toxicity of AgNPs was decreased in natural water compared to artificial water. They attributed this difference to the interactions of AgNPs with organic/inorganic matter present in natural waters that may modify silver speciation and bioavailability/toxicity, but did not clearly identify chemical reactions that could be involved. This finding was confirmed by Cupi et al. (2015), who observed a reduction of AgNP toxicity against *D. magna* in presence of NOM. Indeed, in their experiment, *D. magna* had alteration of swimming ability when exposed to AgNPs only; however, when exposed to AgNPs and natural organic matter, no swimming alteration was observed. AgNPs have also been found to modify the toxicity of other toxicants. Bioaccumulation and toxicity of cadmium on *D. magna* increased in the presence of AgNPs, while Cu bioaccumulation decreased in the presence of AgNPs (Kim et al. 2016). In this regard, Lopes et al. (2016) found that binary mixture of AgNPs and ZnONPs showed a synergistic toxicity pattern on the immobilization and

feeding inhibition of *D. magna*; thus, they suggested that AgNPs together with other chemicals could induce synergistic effects, although mechanisms have not been suggested. Moreover, it has been highlighted that most laboratory studies were using continuous exposure to AgNPs, which is not likely to be environmental conditions. Sørensen et al. (2016) performed a 3-hour pulse exposure of 10–50 μg L^{-1} AgNPs to *D. magna* and observed that after 21 days, no significant mortality, body length, nor any effect on molting were observed.

9.3.4 Behavior and Impact of AgNPs on Eukaryotic Macroorganisms

9.3.4.1 Acute Toxic and Sublethal Effects of AgNPs on Young and Mature Marine Invertebrates

Marine invertebrates have been largely used as biological models to unravel the main sublethal effects caused by AgNPs on marine fauna (Table 9.3). Among all published studies, 57% had the adult stage exposed to nude or coated silver nanoparticles. Gametes and/or early stages of development (embryos, larvae, and juveniles) of oysters, sea urchins, polychaetes, copepods, coral, branchiopods, jellyfish, barnacles, gastropods, and bivalves accounted for 46% of the studied models. Interestingly, 65% of all the reported experiments have been using AgNPs with a large range of sizes from 20 to 100 nm. Moreover, two-thirds of the studies described toxic effects of organic coated AgNPs. As shown in Sections 9.2 and 9.3.2, it is well known that the size of AgNPs is important to their cytotoxicity, but this point is not fully recognized by authors. For instance, smaller AgNPs (<20 nm) induced more oxidative stress, DNA damage, and lethality in mammalian models than larger ones when compared at higher doses (Kim and Ryu 2013). Although small nanoparticles have the potential to be strongly harmful to cells, little is known about their toxicity mechanisms with invertebrates in both adult and early stages.

Another key stone to understand AgNP effects on marine organisms is how their physicochemical behavior (see Section 9.2) may influence toxicity during short- or long-term experiments. Even though AgNP behavior in seawater has been described (Levard et al. 2012), there is much more to be understood concerning their agglomeration and dissolution inside the multicellular organisms. As an example, poly(allylamine)-coated AgNPs tend to form small aggregates in seawater due to high ionic strength, but the presence of amine groups along the polymer chains prevent the formation of large aggregates that could tend to precipitate (Magesky et al. 2016). AgNPs might be able to go through coelomic circulation as small aggregates and/or single particles or even as larger aggregates that may dissociate inside the coelomic fluid (Magesky et al. 2016).

Behavioral responses such as altered swimming, lethargy, immobility, and burrowing impairments have been reported as the first signs of nanosilver intoxication in zooplankton (see Section 9.3.3), crustaceans, sea urchins, jellyfish, and polychaetes (Bertrand et al. 2016; Gambardella et al. 2015a; Magesky et al. 2016). Considered as quick assessment end points, lethargy and disrupted swimming can also indicate different early stages of sea urchin development being more or less affected by AgNPs and dissolved silver contamination (Magesky and Pelletier 2015). However, main physiological effects observed in embryos and larvae exposed to AgNPs greatly reflect the impairment of fate and activity of mesenchyme cells at different steps

TABLE 9.3

Summary of Studies on the Effects of AgNPs on Marine Invertebrates

Biological Models	AgNP Size in Ultrapure Water (nm)	Physiological Effects Observed	References
Oyster (*Crassostrea virginica*)	15 ± 6	Embryonic development impaired at low concentrations; high metallothionein mRNA levels in both embryos and adult oysters; lysosomal integrity affected in hepatopancreas tissues	Ringwood et al. (2010)
Polychaeta (*Nereis diversicolor*)	30 ± 5	Internalization of AgNPs by gut epithelium; association of nano-Ag with inorganic granules, organelles, and heat denatured proteins	García-Alonso et al. (2011)
Polychaeta (*Nereis diversicolor*)	<100	Highest genotoxicity effects compared to dissolved silver and micron-silver (2–3.5 μm)	Cong et al. (2011)
Mussel (*Mytilus edulis*)	20–35	Accumulation of >60% of Ag in soft tissues in nano-Ag and Ag^+ treatments with maximum concentrations located in the digestive organ; low bulk activity of Ag in the extrapallial fluid	Zuykov et al. (2011)
Estuarine snail (*Peringia ulvae*)	16.5 ± 4.5	Fast elimination of Ag from AgNPs in the first days of depuration compared to dissolved silver	Khan et al. (2012)
Oyster (*Crassostrea virginica*)	26 ± 1.2	Reduction of phagocytosis by 1.64-fold in exposed hemolymph	Abbott Chalew et al. (2012)
Copepod (*Tisbe battagliai*)	56.9 ± 7.8	Mortality increased in a concentration-dependent manner for AgNPs	Macken et al. (2012)
Sea urchin (*Paracentrotus lividus*)	5–35	Agglomerated AgNPs found in larvae tissue; Ag oxidized species found with S and O/N ligands in larvae; loss of calcite	Piticharoenphun et al. (2012)
Brine shrimp (*Artemia salina*), sea urchin (*Paracentrotus lividus*), barnacle (*Amphibalanus amphitrite*)	1–10	Mortality (0.1 g L^{-1}), abnormal development (0.001 g L^{-1}), skeletal abnormality (0.001 g L^{-1}) for sea urchins; high mortality of barnacle nauplii for all tested solutions; larvae of brine shrimp less sensitive to nano-Ag	Falugi et al. (2012)
Mussel (*Mytilus galloprovincialis*)	<100	Alteration of proteins associated to stress response, cytoskeleton, and cell structure (actin and α-tubulin)	Gomes et al. (2013b)
Mussel (*Mytilus galloprovincialis*)	<100	DNA damage in hemolymph cells	Gomes et al. (2013a)

(*Continued*)

TABLE 9.3 (CONTINUED)

Summary of Studies on the Effects of AgNPs on Marine Invertebrates

Biological Models	AgNP Size in Ultrapure Water (nm)	Physiological Effects Observed	References
Oyster (*Crassostrea virginica*)	20–30	Total protein levels increased in hepatopancreas tissues pointing to tissue damaging and metabolic impairment	McCarthy et al. (2013)
Sea urchin (*Paracentrotus lividus*)	1–10	Developmental anomalies, larval enzymatic activity (AchE and PrChE) affected; potential neurotoxic damage	Gambardella et al. (2013)
Sea urchin (*Paracentrotus lividus*)	5–35	Development arrest before gastrula stage; strong morphological changes on skeletal parts	Šiller et al. (2013)
Scallop (*Chlamys islandica*)	10–20; 70–80	Strong accumulation (3800 $mL.g^{-1}$) of AgNPs in short time followed by efficient depuration process	Al-Sid-Cheikh et al. (2013)
Polychaeta (*Laeonereis acuta*)	33; 205	Symbiotic bacteria reduced; posterior region of the worms with lower antioxidant capacity	Marques et al. (2013)
Bivalve (*Scrobicularia plana*)	40–45	Response of CAT, glutathione S-transferase, SOD; feeding behavior impaired	Buffet et al. (2013)
Gastropod (*Littorina littorea*)	45 ± 33	Presence of silver in the head, gills, and foot; little measurable Ag accumulation in the kidney or stomach when introduced by Ag nanoform	Li et al. (2013)
Polychaeta *(P. dumerilii*)	16.5 (citrate-AgNPs), 13.1 (humic acid–AgNPs)	Mortality, abnormal development and high uptake rate particularly for humic acid–AgNPs	García-Alonso et al. (2014)
Coral (*Acropora japonica*)	57.2 ± 3.6	Larval deformation and metamorphosis failure under 50 μg L^{-1} exposures; high mortality of gametes, larvae and polyps with 500 μg L^{-1},growth impairment of polyps	Suwa et al. (2014)
Mussel (*Mytilus galloprovincialis*)	41.68 ± 9.62	Oxidative stress; antioxidant enzymes activation; lipid peroxidation higher in gills; metallothionein seem to be the main detoxification mechanism of nano-Ag in the gills	Gomes et al. (2014)

(*Continued*)

TABLE 9.3 (CONTINUED)

Summary of Studies on the Effects of AgNPs on Marine Invertebrates

Biological Models	AgNP Size in Ultrapure Water (nm)	Physiological Effects Observed	References
Amphipod (*Ampelisca abdita*), mysid (*Americamysis bahia*) and polychaete (*Nereis virens*)	30	No toxicity for amphipods and mysids at concentrations up to 75 mg Kg^{-1} dw; worms exposed to citrate–AgNPs had a similar distribution among Ag metal, AgCl, and Ag_2S; whereas those exposed to polyvinylpyrrolidone-AgNPs contained mostly Ag metal and AgCl	Wang et al. (2014)
Polychaete (*Nereis (Hediste) diversicolor*)	20–80	Lysosomal membrane permeability and DNA damage of *Nereis* coelomocytes increased in a concentration-dependent manner, AgNPs were more toxic than Ag^+	Cong et al. (2014)
Polychaeta (*Hediste diversicolor*) and bivalve (*Scrobicularia plana*)	40–45	DNA damages in the digestive gland of *S. plana*, and higher presence of phenoloxidase and lysozyme activities in both species	Buffet et al. (2014)
Brine shrimp (*Artemia salina*)	30–40	With increasing concentrations of nano-Ag, the mortality rate, aggregation in gut region, apoptosis, and DNA damage increased in nauplii; *Artemia* cysts hatching decreased	Arulvasu et al. (2014)
Sea urchins (*Arbacia lixula, Paracentrotus lividus* and *Sphaerechinus granularis*)	59.67 ± 3.06	Larval deformities and developmental arrest	Burić et al. (2015)
Polychaeta (*Nereis diversicolor*)	63 ± 27; 202 ± 56 mm (bulk)	Significant increases in total glutathione concentrations, SOD depletion; inhibition of CAT	Cozzari et al. (2015)
Barnacle (*Balanus amphitrite*), gasteropod (*Crepidula onyx*) and polychaete (*Hydroides elegans*)	30–50	Retardation in growth and development; reduction of larval settlement rate; internalization of nano-Ag by vacuoles of epithelial cell in the digestive tract of *C. onyx*	Chan and Chiu (2015)
Crustaceans (*Amphibalanus amphitrite* and *Artemia salina*), jellyfish (*Aurelia aurita*), and sea urchin (*Paracentrotus lividus*)	990	Mortality, cnidaria immobility and frequency of pulsations alteration, crustacean swimming speed alteration and sea urchin sperm motility change	Gambardella et al. (2015a)

(*Continued*)

TABLE 9.3 (CONTINUED)

Summary of Studies on the Effects of AgNPs on Marine Invertebrates

Biological Models	AgNP Size in Ultrapure Water (nm)	Physiological Effects Observed	References
Sea urchin (*Paracentrotus lividus*)	1–10	Altered fibronectin suggesting a role in alteration of skeletogenic cells migration; different degree of anomalies related to Golgi apparatus modification; AgNPs interfered with biomineralization	Gambardella et al. (2015b)
Mussel (*Mytilus galloprovincialis*)	<10; 20, 80 and 2 μm (bulk)	Higher sensitivity of gill cells compared to hemocytes, ROS production, CAT activity, DNA damage in both cells; activation of lysosomal AcP activity, disruption of actin cytoskeleton, stimulation of phagocytosis in hemocytes; increase of multixenobiotic resistance transport activity and inhibition of NKA in gill cells	Katsumiti et al. (2015)
Mussel (*Mytilus galloprovincialis*)	42 ± 10	Up-regulation of cytochrome CYP4y1 and cathepsin; downregulation of CAT, glutathione transferase, caspase, HSP70, and elongation factor	Bebianno et al. (2015)
Gastropod (*Potamopyrgus antipodarum*) and polychaeta (*Capitella teleta*, *Capitella* sp. S)	10–15	Low Ag accumulation; no apparent toxicity to snails; survival and growth affected (worms)	Ramskov et al. (2015)
Sea urchin (*Strongylocentrotus droebachiensis*)	15 ± 5	Developmental deformities in different embryonic stages, vacuolization of blastocoelar cells, lack of skeleton, abnormal clusters of PMCs	Magesky and Pelletier (2015)
Sea urchin (*Strongylocentrotus droebachiensis*)	15 ± 5	Failure of metamorphosis completion, high larval mortality during recovery period; lethargy, edema, and immobility in juveniles; cellular immune reaction in both larvae and juveniles	Magesky et al. (2016)
Bivalve (*Scrobicularia plana*)	2–10 ± 5	Burrowing impairments; significant bioaccumulation of Ag in cytosolic fraction of the soft tissues of the digestive gland; reduced antioxidative capacities and impairment of metabolic activity	Bertrand et al. (2016)
Sea urchin (*Strongylocentrotus droebachiensis*)	14 ± 6	Increasing levels of HSP60 and HSP70 expressions, coelomic contamination, and cell immune response	Magesky et al. (2017)

during the development. Activated in primary mesenchyme cells (PMCs) entering the blastocoel from sea urchin blastula, acetylcholinesterase (AchE) regulates the balance between cell proliferation and cell death and cell adhesion and migration (Drews 1975). Additionally, AchE and propionylcholinesterase (PrChE) enzymatic activities can be differently affected by very small AgNPs (1–10 nm) in developing embryos of *Paracentrotus lividus* (Gambardella et al. 2013). While AchE activity was impaired by AgNPs at several concentrations (1.0^{-4} to 1.0 mg L^{-1}), PrChE levels were generally enhanced by them at similar concentrations. AchE activity regulates neurotransmission and some other crucial biological functions in larvae, whereas PrChE plays a role in the nervous and neuromuscular systems (Falugi and Aluigi 2012; Talesa et al. 1993). Because both enzymes have very specific roles during sea urchin development, the onset of developmental anomalies corresponds to the change in AchE and PrChE expressions. Taking into account potential neurotoxic damages caused by small AgNPs (1–10 nm) in embryos (Gambardella et al. 2013), morphological studies focusing in neurogenesis in embryonic stages, prisma and early pluteus larvae might reveal how AgNPs could interfere with neurotransmitters expression, neurons organization, and swimming behavior.

Crucial events in the early life of sea urchins such as skeleton mineralization, secondary mesenchyma cells differentiation, PMCs, and red spherulocytes migrations could be disrupted by AgNPs in different species (Burić et al. 2015; Falugi et al. 2012; Gambardella et al. 2015a; Magesky and Pelletier 2015). Sublethal effects caused by citrate–AgNPs (5–35 nm) on developing embryos of *Paracentrotus lividus* are stronger and cannot be correlated only with release of ions (0.030 mg L^{-1} between 30 and 40 hours) at 18–19°C, while in *Strongylocentrotus droebachiensis*, the nanotoxicity effects arose from early blastula to midgastrula stage probably as a consequence of fast release of free Ag ions (~37.5% between 48–96 hours) at 5°C (Magesky and Pelletier 2015; Šiller et al. 2013). Comparatively, apoptosis and mortality of branchiopod *Artemia salina* nauplii emerged after 24/48 hours of short exposures to citrate–AgNPs (30–40 nm) in a concentration-dependent manner (Arulvasu et al. 2014). Polychaete *Platynereis dumerilii* exposed to AgNPs endured malformations and mortality during early development (García-Alonso et al. 2014; Suwa et al. 2014). Humic acid-capped AgNPs were more toxic in terms of mortality and abnormality rate than citrate–AgNPs (16.5 nm) and dissolved silver for all life stages of *P. dumerilii*, except for fertilized eggs. Humic acid–AgNPs also showed the highest uptake rate (about 20 μg g^{-1}) in adult worms compared to citrate–AgNPs and dissolved silver (5 μg g^{-1}) (García-Alonso et al. 2014). Negative impacts on fertilization, larval survival, larval metamorphosis, and primary polyp growth of the lecithotrophic species *Acropora japonica* emerged after exposure to silver nanocolloids (57.2 ± 3.6 nm) at concentration higher than 50 μg L^{-1} (Suwa et al. 2014). According to the authors, some degree of AgNP ionization in addition to concentration and uptake of particles were probably the main factors leading to nanotoxicity. More information about mechanisms of toxicity of AgNPs in lecithotrophic species as well as their defensive mechanisms against silver is still needed.

There is a growing body of evidence indicating that metamorphosis of both planktotrophic and lecithotrophic species can be particularly affected by nanocontamination. With total silver concentration relatively high over a 24-hour exposure (nominal concentration of 50 μg L^{-1}), only 14% of larvae of *A. japonica* went through metamorphic process (Suwa et al. 2014). Because coral species appear to be sensitive to

metal contamination (Reichelt-Brushett and Harrison 2005), low concentrations of trace metals such as silver might trigger strong toxicity effects. In addition, polyvinylpyrrolidone-coated AgNPs, oleic acid-coated AgNPs (OAg-NPs) (30–50 nm), and dissolved silver reduced the larval growth rate of gastropod *Crepidula onyx* as well as the settlement rate in barnacle *Balanus amphitrite* and *C. onyx* by a similar extent and a concentration-dependent manner (Chan and Chiu 2015). Both organic-coated AgNPs were even more disruptive to growth and larval settlement rate of polychaete *Hydroides elegans* when compared to dissolved silver, which is pointing toward the effects of the nanoparticle itself. As another example, fed metamorphic echinoplutei of *S. droebachiensis* chronically exposed to poly(allylamine)-AgNPs (15 ± 5 nm, 100 μg L^{-1}) did not undergo metamorphosis and showed strong mortality rate during recovery period (Magesky et al. 2016). Conversely, Ag^+-treated larvae experienced high mortality and a slow and constant morphogenic process throughout juvenile stage during exposure period. With large nanoaggregates (130–400 nm) flowing through larval blastocoel, some internally distributed AgNPs could not only harm tissular integrity, but also act as a source of Ag ions. Recognized as foreign bodies inside the coelom, AgNPs triggered cellular immune response evolving phagocytic amoebocyte, spherulocytes, and petaloid phagocytes (Magesky et al. 2016). Even though AgNPs internalized by sea urchin larvae might be oxidized and complexed with S and O/N ligands (Piticharoenphun et al. 2012), the metabolic cost allowed to nanodetoxification during metamorphosis is likely to be very high and difficult to overcome over time.

Molluscs and polychaetes mostly in adult stage have been extensively used as biological models to study AgNP effects, exploring silver biodistribution and bioaccumulation, and defensive mechanisms against silver (Abbott Chalew et al. 2012; Al-Sid-Cheikh et al. 2013; Bebianno et al. 2015; Buffet et al. 2013, 2014; Cozzari et al. 2015; Gomes et al. 2013a, 2013b, 2014; Katsumiti et al. 2015; Khan et al. 2012; Li et al. 2013;, Marques et al. 2013; Ramskov et al. 2015). After poly(allylamine)–AgNPs (20–35 nm) exposure, bivalve *Mytilus edulis* can accumulate more than 60% of Ag (both nanoforms and ionic forms) in their soft tissues with maximum concentrations located in the digestive organ and between 0.07% and 7% of total Ag in the extrapallial fluid (Zuykov et al. 2011). In *Mytilus galloprovincialis* exposed to AgNPs (42 ± 10 nm), metallothionein expression as well as the antioxidative enzymatic system represented by superoxide dismutase (SOD), CAT, and glutathione peroxidase were activated against lipid peroxidation along a 15-day exposure (Gomes et al. 2014). Interestingly, the antioxidant enzymatic activity remained either unchanged or decreased with time exposure in the digestive organ, suggesting differential and less pronounced responses by these defensive mechanisms compared to gills. Comparatively, the digestive organ of oyster (*Crassostrea virginica*) contaminated by citrate–AgNPs (15 ± 6 nm and 20–30 nm) showed not only lysosomal impairment, tissue damage, and metabolic disruption, but also high metallothionein messenger RNA (mRNA) levels (McCarthy et al. 2013; Ringwood et al. 2010).

Increasing salinity associated with polyvinylpyrrolidone–AgNPs exposure, Macken et al. (2012) observed high mortality rate in young copepods *Tisbe battagliai*. Notwithstanding, working with polyoxyethylene glycerol trioleate-stabilized AgNPs (PGT-AgNPs) and Tween 20-stabilized AgNPs (2–10 ± 5 nm), Bertrand et al. (2016) showed that Ag^+ might be a predominant source of toxicity at different salinities (15 and 30 psu) in clam *Scrobicularia plana*. More importantly, the dissolution

rate of AgNPs appeared faster under lower salinity, ~81.7% (15 psu) compared to ~68.1% (30 psu) of Ag^+. In this study, an increased amount of Ag was accumulated as cytosolic fraction (as soluble Ag and AgNPs) of the digestive organ with no measured change of metallothionein levels. In addition, the decreasing salinity condition seemed to enhance oxidative mechanisms, apoptosis, and energetic reserve depletion. According to Berthet et al. (1992), silver sulphide-loaded amoebocytes can strongly increase in the vesiculous tissue and in the gills of *Crassostrea gigas* chronically exposed to dissolved silver in brackish water. Silver might be trapped within insoluble fractions as relatively nontoxic Ag_2S in basement membranes for extrusion.

Similar mechanisms have also been described for polychaetes chronically exposed to different coated AgNPs. As an example, *Nereis diversicolor* exposed to sediment amended with citrate–AgNPs (30 ± 5 nm) internalized nano-Ag in gut epithelium over 10 days (García-Alonso et al. 2011). Strikingly, Ag accumulated from citrate–AgNPs was strongly associated with metal-rich granules, organelles, and heat-sensitive proteins while dissolved silver was basically localized to the metallothionein fraction. *N. diversicolor* is tolerant of heavy metals and able to control its body metal concentration using different mechanisms: (1) physiological detoxification pathway with formation of metal-containing extracellular granules, (2) formation of mineralized lysosomes, (3) excretion of metals via coelomocytes, and (4) synthesis and turnover of metal-binding proteins (Cong et al. 2011). Yet, 1-month exposure to polyvinylpyrrolidone–AgNPs (20–80 nm) caused lysosomal membrane permeability and DNA damage in *N. diversicolor* coelomocytes (Cong et al. 2014). It is interesting to note that AgNP surface-capping agents might also influence distinct pathways of accumulation, biotransformation, and depuration in polychaetes. For instance, *Nereis virens* exposed to citrate–AgNPs (30 nm) had a relatively similar distribution of Ag metal, AgCl, and Ag_2S, whereas those exposed to polyvinylpyrrolidone–AgNPs (30 nm) and dissolved silver mostly accumulated Ag metal and AgCl (Wang et al. 2014). These findings indicated that different distributions of Ag species might be due to the chemical transformation of AgNPs, *in vivo* bioreduction or the preferential uptake or excretion of particular Ag chemical forms.

9.3.4.2 Toxicity to Fish Early Developmental Stages and Adults

9.3.4.2.1 Early Toxicity Assays and Observations

The toxic effects of AgNPs on fish species attracted the attention of a large number of aquatic toxicologists, and early works demonstrated that early life stages were, as expected, much more sensitive than later stages and adults when exposed to dissolved and nanoparticulate silver (Asharani et al. 2008; Bilberg et al. 2010, 2011; Choi et al. 2010; Cowart et al. 2011; Farkas et al. 2010; Wu et al. 2010; Lee et al. 2007). Most of these studies involved embryos at different stages, larvae and juveniles. Lee et al. (2007) mentioned that individual AgNPs were observed inside embryos at each developmental stage, and the toxicity of Ag nanoparticles and types of abnormalities of zebrafish highly depended on the dose of Ag nanoparticles. Rates of passive diffusion and accumulation of nanoparticles in embryos are likely responsible for the dose-dependent abnormalities. Malformation such as edema, spinal, and fin fold abnormalities were observed as well as heart malformation and eye defects (Lee et al. 2007). Results suggested that while ingestion was common, gills were the main

sites of AgNP uptake. AgNPs were recognized as a source of toxic Ag ions, while nanoparticles themselves partially contributed to toxicity to fish (Kwok et al. 2012). It was suggested by Wu et al. (2010) that most of the abnormalities detected in juvenile and larval medaka (*Oryzias latipes*) were related to homeostasis malfunctions. Additionally, authors found that the most severe abnormalities were associated with yolk edema. As edema are known to be caused by disturbance in osmoregulation (Kiener et al. 2008), their observation was in accordance with the postulate suggesting that many of the deformities observed in fish after exposure to a toxic compound were related to the disrupted circulatory system and osmoregulation (Peters et al. 2007). Severe circulatory abnormalities were associated with the occurrence of pericardial edema and tubular heart (Wu and Zhou 2012). Polyvinylpyrrolidone–AgNPs have been shown to induce sluggish circulation, hemostasis, hemocytes overfilling in blood vessels, hemorrhages in different parts of the larval body, and global basophilia, indicating once again that AgNPs can lead to the modification of the circulatory system (Griffitt et al. 2012; Wu et al. 2010; Wu and Zhou 2013).

A number of early studies highlighted the fact that high concentrations of AgNPs tend to interact and bind with fish gills inducing major undesired effects (Bilberg et al. 2010; Kwok et al. 2012; Griffitt et al. 2012, 2013; Wu and Zhou 2013). Among them, Kwok et al. (2012) demonstrated that polyvinylpyrrolidone– and citrate–AgNP distributions on the gills of medaka embryos were ubiquitous, while smaller amounts were present in other organs such as the liver and brain. Based on their results, they concluded that although ingestion was common, the main uptake site for AgNPs in medaka remained the gills. A significant thickening of the epithelial gill tissues was observed in sheepshead minnows *Cyprinodon vagiegatus* (Griffitt et al. 2012). Wu and Zhou (2013) showed that in medaka at high concentrations of AgNPs (0.5 mg L^{-1}), lesions to the branchia became severe and the desquamation of lamellar epithelium and disruption of cartilaginous rod were prominent. Moreover, Wu and Zhou (2013) suggested that as gills are surrounded by aqueous media and mucus, AgNPs may be trapped and retained in the mucus, thus preventing their uptake. According to this hypothesis, their results showed that the exposure of medaka to polyvinylpyrrolidone–AgNPs led to increased hyperplasia and mucus production, which in turn could affect the uptake of AgNPs by the gills. Bilberg et al. (2010) observed the impairment of the tolerance to hypoxia in Eurasian perch *Perca fluviatilis* exposed to AgNPs and proposed that AgNPs may, by interacting with the gills, reduce their diffusion conductance, thus leading to hypoxia during low water oxygen tension.

Although AgNPs can be ingested by adult fish and found in their guts, the other organ after the gills accumulating most of silver was the liver (Griffitt et al. 2012; Choi et al. 2010; Wu and Zhou 2013). Liver abnormalities included spongiosis, cyst, disruption of hepatic cord and apoptotic changes, hepatocyte enlargement, loosened liver parenchyma, disorganization of hepatocytes, focal necrosis, and focal lymphocytic infiltration were reported. Choi et al. (2010) attributed the observed alterations to the presence of silver ions released by AgNPs in liver. However, the real contribution from AgNPs and dissolved silver was not clear and is still subject to controversy as already mentioned for other aquatic organisms. When paying attention to bioaccumulation only, fish treated with AgNPs had significantly higher silver burden in tissue than both control and soluble silver-treated fish, which could indicate a specific AgNP toxicity and propable AgNP bioaccumulation (Farkas et al. 2010). An *in vitro* study on hepatocytes of rainbow trout demonstrated that citrate–AgNPs caused significant

reduction in membrane integrity and metabolic activity in a concentration-dependent manner. At high AgNP concentrations, a significant reduction of mitochondrial activity happened (Farkas et al. 2010).

At the intracellular level, Wise et al. (2010) found that citrate–AgNPs could induce chromosomal aberration such as chromatid lesion or chromatid exchanges, aneuploidy, and damages in metaphase leading to cell cycle arrest. Chromatin condensation, pyknosis, and DNA damages, probably due to double strand break, were also observed (Choi et al. 2010). The presence of agglomerated AgNPs in cytoplasmic location such as the vicinity of the plasma membrane and the nuclear membrane was also demonstrated (Choi et al. 2010). Furthermore, the expression of a variety of genes and proteins were modified by exposure to AgNPs (Choi et al. 2010). The expression of metallothionein 2 (MT2), a protein involved in metal detoxification (Hogstrand et al. 1996), has been found in zebrafish after exposure to AgNPs (Choi et al. 2010).

Dose-dependent decrease in antioxidant enzymes in liver of medaka were reported as well as depletion in glutathione in the liver, gill, and brain and increased lipid peroxidation in their liver and gills (Wu and Zhou 2012, 2013). Authors suggested that the oxidative stress induced by AgNP could be associated with a large number of histological changes in the fish. The observed decrease in catalase (CAT) and SOD activities suggested an excessive consumption of these antioxidant enzymes. The reduction of SOD combined with limited catalysis activity could lead to a failure in the catalysis of oxyradicals, thus indicating that the antioxidant defenses in medaka liver were depressed and that a potential enhancement of ROS was produced. Griffitt et al. (2013) found that the expression of a variety of zebrafish genes was affected by exposure to AgNPs. As reported in Section 9.3.3, many genes were related to DNA repair, cellular restructuring, and developmental processes. Their results tend to indicate a direct effect of AgNPs more than an effect of released silver ions. These observations were consistent with the hypothesis that metallic nanoparticles, including AgNPs, could cause toxicity through enhanced production of ROS, which will in turn induce oxidative DNA damages, resulting in the upregulation of genes involved in DNA repairing processes. Finally, it should be mentioned that Farkas et al. (2010) did not observe a reduction of AgNPs cytotoxicity on the hepatocytes in the presence of dissolved organic carbon (DOC). Their results showed that neither size changes nor presence of DOC appeared to influence the AgNP toxicity to a significant degree, suggesting that AgNP might be toxic even under ecologically relevant exposure. Nevertheless, Kim et al. (2013) observed that a reduction in AgNP toxicity was related to the role of coating of humic acid to the surface of AgNPs and bridging between the nanoparticles. The role of AgNP coating and DOC is still unclear and subject to recent research efforts.

Possible impacts of AgNP on fish reproductive organs have also been highlighted as it has been shown that they could alter the expression of genes in *Cyprinodon vagiegatus* gonads (Griffitt et al. 2012) as well as modulate the expression of a variety of genes responsible for the steroidogenesis in marine medaka *Oryzias melastigma* primary ovarian cell culture (Degger et al. 2015). Authors evaluated the effects of two differently coated AgNP particles: oleic acid–*n*Ag and polyvinylpyrrolidone–AgNPs on fish ovarian tissues, using $AgNO_3$ as a positive control. They observed that the greatly enhanced silver toxicity using polyvinylpyrrolidone–AgNPs may be due to difference in aggregation and suggested, for the first time, that AgNPs can affect specific genes regulating steroidogenesis, involving AgNP as a potential endocrine disruptor.

9.3.4.2.2 Recent Progress in AgNP Toxicity Mechanisms in Fish

Recently published papers on effects of silver nanoparticles have been selected and summarized in Table 9.4. The first striking point is the broad use of zebrafish as a model to study AgNP toxicity, first for practical reasons (small size, low cost, diverse adaptability, short breeding cycle, high fecundity, and transparent embryos) and second for its high sensitivity to a large range of toxicants and possible application of transgenic technology (Dai et al. 2014). Otherwise, nine other species have been used (mostly early-life stages) which provide a large diversity in species used and ensure a better understanding of variable effects between species. The second point to be mentioned is the use of more realistic concentrations by a number of authors (Clark et al. 2016; Oprsal et al. 2015; Murray et al. 2016; Bruneau et al. 2016; Hawkins et al. 2015; Garcia-Reyero et al. 2015) in an attempt to derive conclusions that could be applied to field risk assessment.

Molecular mechanisms for AgNP toxicity were reviewed by McShan et al. (2014) and more specifically examined in zebrafish embryos by van Aerle et al. (2013) and in Japanese medaka by Kwok et al. (2016). The main mechanism generally accepted is that AgNPs generate ionic silver (Ag^+) causing oxidative stress through the generation of ROS and cause damage to cellular components including DNA damage, activation of antioxidant enzymes, depletion of antioxidant molecules, disabling of proteins, and damage to the cell membrane (McShan et al. 2014). Using zebrafish embryos as a model organism, van Aerle et al. (2013) examined the genotoxicity of very small AgNPs (10 nm) in comparison with large micrometer-sized Ag particles (0.6–1.6 μm) and soluble Ag^+. Their results were in accordance with accepted mechanisms as "most transcriptomic alterations caused by AgNPs and its ionic and bulk counterparts were common across all treatments, supporting the hypothesis that the toxicity of AgNPs is principally associated with the toxicity of Ag^+ at the surface of the particles or dissolved in the water" (van Aerle et al. 2013). However, they found some gene changes unique to each treatment, first not only attributing to the proportion of biologically available Ag^+ in the different treatments, but also suggesting that there may be some particle-specific effects. As a consequence of these findings, the understanding of the bioavailability of dissolved Ag^+ originating from AgNPs should be prioritized in risk assessment studies.

Recently, Kwok et al. (2016) examined the toxicity of AgNPs with different coatings and sizes using medaka larvae as a biological model in order to understand how coating could affect AgNP toxicity in vertebrates. Authors found that toxicity was independent of particle size, but related to the size of aggregates in test medium, as also observed by Oprsal et al. (2015) and Osborne et al. (2015). They also confirmed that toxicity was heavily influenced by the coating material most probably due to difference in aggregation following the coating chemistry. Interestingly, AgNP exposure did not cause a significant response in three out of four oxidative marker genes examined, a result not in accordance with accepted mechanism described earlier. Rather, Kwok et al. (2016) observed that AgNPs cause disruption of Na regulation as often observed with Ag ions with upregulation of NKA, leading to a decrease in whole body Na levels. The toxicity of AgNPs did not correlate with dissolved Ag concentration in the test medium and may instead be related to the localized release of Ag^+ on the surface of fish.

AgNP effects are highly variable following fish species, development stages, and, among all, exposure protocols and Ag concentrations. Dissolution and aggregation

TABLE 9.4

Selection of Recently Published Papers on the Effects of AgNPs on Some Fish Species

Species	Exposure and Main Observed Effects	References
Acipenser baerii	Larval Siberian sturgeon exposed to AgNPs for acute and 21-day exposure. 96-hour LC50 was 1.41 ± 0.24 mg L^{-1}. AgNPs negatively influenced survival, body length and mass and morphology and physiology of the epidermis, gills, and liver of Siberian sturgeon larvae. Pathological changes are irregular structure and pyknotic nuclei of epidermis, aplasia and/or fusion of lamellae, telangiectasis, epithelial necrosis and lifting of the gills, dilation of sinusoidal space, overfilled blood vessels, and pyknotic nuclei of the liver.	Ostaszewska et al. (2016)
Acipenser persicus; Acipenser stellatus	Sturgeon fertilized eggs and embryos exposed to AgNPs (16.6 nm) at concentrations from 0.025 to 8 mg L^{-1}. 96-hour LC50 values in both species varied between 0.163 and 0.158 mg L^{-1}. AgNPs induced a concentration-dependent toxicity in both species during early life stages.	Banan et al. (2016)
Carassius auratus	Goldfish exposed to AgNPs (5 nm) from 0.1 to 5 mg L^{-1} over 14-day period. Same pathologies for AgNPs and $AgNO_3$, including hyperplasia and lamellar fusion of the gills, and hemosiderosis and pyknotic nuclei of the liver.	Abarghoei et al. (2016)
Coregonus palaea	Whitefish embryos at late-eyed stage exposed to low concentrations (0.5 or 100 μg L^{-1}) of differently sized AgNPs. The induced hatching did not vary with nanoparticle size and was stronger in the silver nitrate group. Results suggest that the evolutionary potential for adaptation to these types of pollutants may be low.	Clark et al. (2016)

(*Continued*)

TABLE 9.4 (CONTINUED)

Selection of Recently Published Papers on the Effects of AgNPs on Some Fish Species

Species	Exposure and Main Observed Effects	References
Cyprinus carpio	Embryos of common carp exposed repeatedly for 6 hours to AgNPs (5–50 μM) either under conditions varying short-time exposure, concentration, and maximum agglomerate size of 200 or 400 nm. Importance of AgNP agglomerates in nanosilver adverse effects. Agglomerates locally enhanced toxicity particularly with larger 400 nm ones.	Oprsal et al. (2015)
Danio rerio	Embryos exposed to AgNPs (4 and 10 nm) from 0.5 to 23 mg L^{-1}. AgNPs could affect the neural development of zebrafish embryos, and the toxicity of AgNPs may be partially attributed to the comparatively higher uptake in the head area. Potential neurotoxicity of AgNPs and could be extended to other aquatic organisms.	Xin et al. (2015)
	Adult zebrafish: estimated 96 h LC50 value 16.76 mg L^{-1}. Long-term exposure (14 days) indicate that AgNPs could inhibit the activities of NKA and AChE, thus interfering with the proper ionoregulation and neuroregulation.	Katuli et al. (2014)
	Zebrafish exposed to citrate–AgNPs (20 and 110 nm) for 4 hours, 4 days, or 4 days plus a 7-day depuration period. Both particle types retained in the intestines even after depuration. Striking size-dependent differences in the ultrastructural features and histopathology in the target organs in response to the particulates.	Osborne et al. (2015)
	Embryos exposed to AgNPs (10 and 40 nm) at a concentration range from 0.2 to 1 mg L^{-1}. Specification of hematopoietic progenitor cells and differentiation of erythroids detected at different developmental stages. Erythrogenesis found to be inhibited in developmental stage-specific and cell-specific manners. AgNPs affected erythrogenesis mostly by their particles other than their releasing ions.	Cui et al. (2016)
	Embryos exposed to small (9 nm) and larger (30 nm) AgNPs at concentrations ranging from 0 to 20 mg L^{-1}. Smaller AgNPs induce increased mortality rates and decreased hatchability rates than the larger particles in a dose-dependent manner. Striking discrepancies in the phenotypic defects that were induced by AgNPs and $AgNO_3$.	Mosselhy et al. (2016)

(*Continued*)

TABLE 9.4 (CONTINUED)

Selection of Recently Published Papers on the Effects of AgNPs on Some Fish Species

Species	Exposure and Main Observed Effects	References
Oncorhynchus mykiss	Fish exposed to environmentally relevant (average 0.28 μg L^{-1}) and higher (average 47.60 μg L^{-1}) AgNP concentrations. Significant increase in blood plasma cortisol for both treatments and Ag detected in fish muscle tissue. Low AgNP concentrations did not affect growth or condition under the experimental conditions investigated.	Murray et al. (2016)
	Juvenile rainbow trout exposed to commercial AgNPs (22 nm) at 40 μg L^{-1} in diluted municipal treated wastewater. Induced morphological modifications without visible nanoparticle bioaccumulation. AgNPs in wastewater were bioavailable to fish despite of their formation of aggregates.	Bruneau et al. (2016)
Oryzias latipes	Medaka larvae exposed to a total of 10 AgNPs of different coating and diameters. The aggregation behavior and toxicity of various AgNPs were dependent on coating materials. 96-hour LC50 values correlated with AgNP aggregate sizes. Gene expression patterns suggested toxicity related to disruption of sodium regulation and not to oxidative stress.	Kwok et al. (2016)
Pimephales romelas	Fathead minnows exposed to $AgNO_3$ (1.3 or 3.7 μg L^{-1} as Ag^+), citrate–AgNPs (15 or 39 μg L^{-1}), and polyvinylpyrrolidone–AgNPs (11 or 50 μg L^{-1}). Results indicated that both $AgNO_3$ and AgNP created similar disruptions in gill structure and ionic regulation, possibly due to the ionic Ag portion of each treatment.	Hawkins et al. (2015)
	Fathead minnows 96-hour exposure to 20 nm polyvinylpyrrolidone–AgNPs (50.3 μg L^{-1}) or citrate–AgNPs (56.0 μg L^{-1}) or $AgNO_3$ (3.81 μg/L). All silver treatments affected mucus production in fish gills and lead to common and unique transcriptional changes in expressed genes.	Garcia-Reyero et al. (2015)

chemistry of coated and uncoated AgNPs within exposure media are of prime importance, but this fact is no yet fully recognized by authors, which leads to more confusion and conflicting results.

9.4 Conclusion

Many conflicting results are presented in the scientific literature about the toxicity of AgNPs or other nanomaterials. To partially explain or reconcile these results, we must consider that experimental protocols rarely use the same nanomaterials and concentrations and systematically differ in the exposure time. However, a conceptual framework is needed to help explain the results. Silver nanomaterials are interesting probes to sketch the myriad behaviors of engineered nanomaterials delivered in complex aqueous systems. They are also very interesting compounds as a chemical model to unravel the spectrum of adverse biological outcomes caused to organisms by an exposition to engineered nanomaterials. In this review, we examined the behavior of AgNPs when introduced into waters with different chemical compositions where microorganisms such as bacteria and microalgae and macroorganisms such as zooplankton, invertebrates, and fish are living and reproducing.

To understand biological effects induced by the exposure to nanomaterials such as AgNPs, environmental chemists and toxicologists have to consider critical information about physicochemical properties of the engineered AgNPs used in laboratory exposure or released in the environment, such as chemical composition, size, surface coating, geometry, crystallinity, porosity, roughness, hydrophobicity, or hydrophilicity. We also need detailed data on the composition of the aqueous media, including pH, ionic strength, zeta potential, oxygen, sulfide, dissolved ions, buffers, and presence of organic molecules such as humic substances, proteins, polysaccharides, and detergents. These pool of data are essential to determine the stability of native engineered nanomaterials and their inevitable transformation in complex aqueous media. At the end, engineered nanomaterials can coexist under a more or less broad particle size distribution. All tiny particles and aggregates present in experimental media and natural receiving waters are susceptible to produce a wide range of cellular responses depending of the particle size, duration of contact, and metal concentration achieved in the organisms. Many important damages could result to cells at the membrane and/or wall level. Moreover, alteration on cellular components and modification to physiological process such as production of ROS can occur in bacteria, microalgae, and higher organisms. Sometimes, first signs of nanosilver intoxication can be detected in crustaceans, sea urchins, jellyfish, and polychaetes when altered swimming, lethargy, immobility, and burrowing impairments are observed. Alterations can likewise reach gametes and/or early stages of development (embryos, larvae, and juvenile). About fish, gills could be the target of choice to concentrate engineered nanomaterials, but other organs such as liver or brain show levels of engineered nanomaterials. The transport of AgNPs to internal tissues elicits oxidative stress and alteration of normal physiological functions. Because AgNPs or other engineered nanomaterials show a large range of biological injuries to aquatic organisms, it is mandatory to continue to develop a conceptual framework to better understand and assess the exposure and risk from using nanomaterials in upcoming decades.

REFERENCES

Abarghoei, S., A. Hedayati, R. Ghorbani, H. K. Miandareh, and T. Bagheri. 2016. Histopathological effects of waterborne silver nanoparticles and silver salt on the gills and liver of goldfish *Carassius auratus*. *International Journal of Environmental Science and Technology* 13:1753–1760.

Abbott Chalew, T. E., J. F. Galloway, and T. K. Graczyk. 2012. Pilot study on effects of nanoparticle exposure on *Crassostrea virginica* hemocyte phagocytosis. *Marine Pollution Bulletin* 64 (10):2251–2253.

Abdel Rahim, K. A. A. and A. M. Ali Mohamed. 2015. Bactericidal and antibiotic synergistic effect of nanosilver against methicillin-resistant *Staphylococcus aureus*. *Jundishapur Journal of Microbiology* 8 (11):e25867.

Adegboyega, N. F., V. K. Sharma, K. Siskova et al. 2013. Interactions of aqueous Ag^+ with fulvic acids: Mechanisms of silver nanoparticle formation and investigation of stability. *Environmental Science & Technology* 47 (2):757–764.

Akaighe, N., S. W. Depner, S. Banerjee, V. K. Sharma, and M. Sohn. 2012. The effects of monovalent and divalent cations on the stability of silver nanoparticles formed from direct reduction of silver ions by Suwannee River humic acid/natural organic matter. *Science of the Total Environment* 441:277–289.

Akaighe, N., R. I. Maccuspie, D. A. Navarro et al. 2011. Humic acid-induced silver nanoparticle formation under environmentally relevant conditions. *Environmental Science & Technology* 45 (9):3895–3901.

Al-Sid-Cheikh, M., C. Rouleau, and E. Pelletier. 2013. Tissue distribution and kinetics of dissolved and nanoparticulate silver in Iceland scallop (*Chlamys islandica*). *Marine Environmental Research* 86:21–28.

Alito, C. L. and C. K. Gunsch. 2014. Assessing the effects of silver nanoparticles on biological nutrient removal in bench-scale activated sludge sequencing batch reactors. *Environmental Science & Technology* 48 (2):970–976.

Angel, B. M., G. E. Batley, C. V. Jarolimek, and N. J. Rogers. 2013. The impact of size on the fate and toxicity of nanoparticulate silver in aquatic systems. *Chemosphere* 93 (2):359–365.

Antizar-Ladislao, B., B. D. Bhattacharya, S. Ray Chaudhuri, and S. K. Sarkar. 2015. Impact of silver nanoparticles on benthic prokaryotes in heavy metal-contaminated estuarine sediments in a tropical environment. *Marine Pollution Bulletin* 99 (1–2): 104–111.

Arulvasu, C., S. M. Jennifer, D. Prabhu, and D. Chandhirasekar. 2014. Toxicity effect of silver nanoparticles in brine shrimp *Artemia*. *The Scientific World Journal* 2014:256919.

Asghari, S., S. A. Johari, J. H. Lee et al. 2012. Toxicity of various silver nanoparticles compared to silver ions in *Daphnia magna*. *Journal of Nanobiotechnology* 10 (14).

Asharani, P. V., Y. L. Wu, Z. Gong, and S. Valiyaveettil. 2008. Toxicity of silver nanoparticles in zebrafish models. *Nanotechnology* 19 (25):255102.

Azam, F., T. Fenchel, J. G. Field et al. 1983. The ecological role of water column microbes in the sea. *Marine Ecology Progress Series* 10:257–263.

Azam, F. and F. Malfatti. 2007. Microbial structuring of marine ecosystems. *Nature Reviews: Microbiology* 5 (10):782–791.

Baalousha, M., K. P. Arkill, I. Romer, R. E. Palmer, and J. R. Lead. 2015. Transformations of citrate and Tween coated silver nanoparticles reacted with Na_2S. *Science of the Total Environment* 502:344–353.

Baalousha, M., Y. Nur, I. Römer, M. Tejamaya, and J. R. Lead. 2013. Effect of monovalent and divalent cations, anions and fulvic acid on aggregation of citrate-coated silver nanoparticles. *Science of the Total Environment* 454–455:119–131.

Banan, A., M. R. K. M Shahi, M. Bahmani, and M. A. Y. Sadati. 2016. Toxicity assessment of silver nanoparticles in Persian sturgeon (*Acipenser persicus*) and starry sturgeon (*Acipenser stellatus*) during early life stages. *Environmental Science and Pollution Research* 23 (10):10139–10144.

Bebianno, M. J., M. Gonzalez-Rey, T. Gomes et al. 2015. Is gene transcription in mussel gills altered after exposure to Ag nanoparticles? *Environmental Science and Pollution Research* 22 (22):17425–17433.

Becaro, A. A., C. M. Jonsson, F. C. Puti et al. 2015. Toxicity of PVA-stabilized silver nanoparticles to algae and microcrustaceans. *Environmental Nanotechnology, Monitoring & Management* 3:22–29.

Benn, T. M. and P. Westerhoff. 2008. Nanoparticle silver released into water from commercially available sock fabrics. *Environmental Science & Technology* 42 (11):4133–4139.

Berthet, B., J. C. Amiard, C. Amiard-Triquet, M. Martoja, and A. Y. Jeantet. 1992. Bioaccumulation, toxicity and physico-chemical speciation of silver in bivalve molluscs: Ecotoxicological and health consequences. *Science of the Total Environment* 125:97–122.

Bertrand, C., A. Zalouk-Vergnoux, L. Giambérini et al. 2016. The influence of salinity on the fate and behavior of silver standardized nanomaterial and toxicity effects in the estuarine bivalve *Scrobicularia plana*. *Environmental Toxicology and Chemistry* 35 (10):2550–2561.

Bhatt, I. and B. N. Tripathi. 2011. Interaction of engineered nanoparticles with various components of the environment and possible strategies for their risk assessment. *Chemosphere* 82 (3):308–317.

Bianchi, T. S. 2007. *Biogeochemistry of Estuaries*. New York: Oxford University Press.

Bilberg, K., K. B. Døving, K. Beedholm, and E. Baatrup. 2011. Silver nanoparticles disrupt olfaction in Crucian carp (*Carassius carassius*) and Eurasian perch (*Perca fluviatilis*). *Aquatic Toxicology* 104 (1):145–152.

Bilberg, K., H. Malte, T. Wang, and E. Baatrup. 2010. Silver nanoparticles and silver nitrate cause respiratory stress in Eurasian perch (*Perca fluviatilis*). *Aquatic Toxicology* 96 (2):159–165.

Bisalputra, T. and T. E. Weier. 1963. The cell wall of *Scenedesmus quadricauda*. *American Journal of Botany* 50 (10):1011–1019.

Blakelock, G. C., M. A. Xenopoulos, B. C. Norman, J. L. Vincent, and P. C. Frost. 2016. Effects of silver nanoparticles on bacterioplankton in a boreal lake. *Freshwater Biology* 61 (12):2211–2220.

Blinova, I., J. Niskanen, P. Kajankari et al. 2013. Toxicity of two types of silver nanoparticles to aquatic crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. *Environmental Science and Pollution Research* 20 (5):3456–3463.

Bradford, A., R. D. Handy, J. W. Readman, A. Atfield, and M. Mu¨hling. 2009. Impact of silver nanoparticle contamination on the genetic diversity of natural bacterial assemblages in estuarine sediments. *Environmental Science & Technology* 43 (12):4530–4536.

Bruneau, A., P. Turcotte, M. Pilote, F. Gagné, and C. Gagnon. 2016. Fate of silver nanoparticles in wastewater and immunotoxic effects on rainbow trout. *Aquatic Toxicology* 174:70–81.

Buffet, P. E., J. F. Pan, L. Poirier et al. 2013. Biochemical and behavioural responses of the endobenthic bivalve *Scrobicularia plana* to silver nanoparticles in seawater and microalgal food. *Ecotoxicology and Environmental Safety* 89:117–124.

Buffet, P. E., A. Zalouk-Vergnoux, A. Châtel et al. 2014. A marine mesocosm study on the environmental fate of silver nanoparticles and toxicity effects on two endobenthic species: The ragworm *Hediste diversicolor* and the bivalve mollusc *Scrobicularia plana*. *Science of the Total Environment* 470–471:1151–1159.

Bundschuh, M., F. Seitz, R. R. Rosenfeldt, and R. Schulz. 2016. Effects of nanoparticles in fresh waters: Risks, mechanisms and interactions. *Freshwater Biology* 61 (12):2185–2196.

Burchardt, A. D., R. N. Carvalho, A. Valente et al. 2012. Effects of silver nanoparticles in diatom *Thalassiosira pseudonana* and *Cyanobacterium Synechococcus* sp. *Environmental Science & Technology* 46 (20):11336–11344.

Burić, P., Ž. Jakšić, L. Štajner et al. 2015. Effect of silver nanoparticles on Mediterranean sea urchin embryonal development is species specific and depends on moment of first exposure. *Marine Environmental Research* 111:50–59.

Caballero-Guzman, A. and B. Nowack. 2016. A critical review of engineered nanomaterial release data: Are current data useful for material flow modeling? *Environmental Pollution* 213:502–517.

Calabrese, E. J. 2005. Paradigm lost, paradigm found: The re-emergence of hormesis as a fundamental dose response model in the toxicological sciences. *Environmental Pollution* 138 (3):378–411.

Carlson, C., S. M. Hussain, A. M. Schrand et al. 2008. Unique cellular interaction of silver nanoparticles: Size-dependent generation of reactive oxygen species. *Journal of Physical Chemistry B* 112 (43):13608–13619.

Chae, Y. and Y. J. An. 2016. Toxicity and transfer of polyvinylpyrrolidone-coated silver nanowires in an aquatic food chain consisting of algae, water fleas, and zebrafish. *Aquatic Toxicology* 173:94–104.

Chan, C. Y. S. and J. M. Y. Chiu. 2015. Chronic effects of coated silver nanoparticles on marine invertebrate larvae: A proof of concept study. *PLOS ONE* 10 (7):e0132457.

Chen, S. F. and H. Zhang. 2012. Aggregation kinetics of nanosilver in different water conditions. *Advances in Natural Sciences: Nanoscience and Nanotechnology* 3 (3):035006.

Chen, S., Y. Jin, M. Lavoie et al. 2016. A new extracellular von Willebrand A domain-containing protein is involved in silver uptake in *Microcystis aeruginosa* exposed to silver nanoparticles. *Applied Microbiology and Biotechnology* 100 (20):8955–8963.

Choi, J. E., S. Kim, J. H. Ahn et al. 2010. Induction of oxidative stress and apoptosis by silver nanoparticles in the liver of adult zebrafish. *Aquatic Toxicology* 100 (2):151–159.

Choi, O., K. K. Deng, N. J. Kim et al. 2008. The inhibitory effects of silver nanoparticles, silver ions, and silver chloride colloids on microbial growth. *Water Research* 42 (12):3066–3074.

Choi, O. and Z. Hu. 2008. Size dependent and reactive oxygen species related nanosilver toxicity to nitrifying bacteria. *Environmental Science & Technology* 42 (12):4583–4588.

Clark, E. S., M. Pompini, A. Uppal, and C. Wedekind. 2016. Genetic correlations and little genetic variance for reaction norms may limit potential for adaptation to pollution by ionic and nanoparticulate silver in a whitefish (*Salmonidae*). *Ecology and Evolution* 6 (9):2751–2762.

Coll, C., D. Notter, F. Gottschalk et al. 2015. Probabilistic environmental risk assessment of five nanomaterials (nano-TiO_2, nano-Ag, nano-ZnO, CNT, and fullerenes). *Nanotoxicology* 5390 (2009):436–444.

Colman, B. P., C. L. Arnaout, S. Anciaux et al. 2013. Low concentrations of silver nanoparticles in biosolids cause adverse ecosystem responses under realistic field scenario. *PLOS ONE* 8 (2):e57189.

Colman, B. P., S. Y. Wang, M. Auffan, M. R. Wiesner, and E. S. Bernhardt. 2012. Antimicrobial effects of commercial silver nanoparticles are attenuated in natural streamwater and sediment. *Ecotoxicology* 21 (7):1867–1877.

Cong, Y., G. T. Banta, H. Selck et al. 2011. Toxic effects and bioaccumulation of nano-, micron- and ionic-Ag in the polychaete, *Nereis diversicolor. Aquatic Toxicology* 105 (3–4):403–411.

Cong, Y., G. T. Banta, H. Selck et al. 2014. Toxicity and bioaccumulation of sediment-associated silver nanoparticles in the estuarine polychaete, *Nereis* (*Hediste*) *diversicolor. Aquatic Toxicology* 156:106–115.

Cowart, D. A., S. M. Guida, S. I. Shah, and A. G. Marsh. 2011. Effects of Ag nanoparticles on survival and oxygen consumption of zebra fish embryos, *Danio rerio. Journal of Environmental Science and Health, Part A* 46 (10):1122–1128.

Cozzari, M., A. C. Elia, N. Pacini et al. 2015. Bioaccumulation and oxidative stress responses measured in the estuarine ragworm (*Nereis diversicolor*) exposed to dissolved, nano- and bulk-sized silver. *Environmental Pollution* 198:32–40.

Cui, B., L. Ren, Q. H. Xu et al. 2016. Silver nanoparticles inhibited erythrogenesis during zebrafish embryogenesis. *Aquatic Toxicology.*

Cupi, D., N. B. Hartmann, and A. Baun. 2015. The influence of natural organic matter and aging on suspension stability in guideline toxicity testing of silver, zinc oxide, and titanium dioxide nanoparticles with *Daphnia magna*. *Environmental Toxicology and Chemistry* 34 (3):497–506.

Cupi, D., N. B. Hartmann, and A. Baun. 2016. Influence of pH and media composition on suspension stability of silver, zinc oxide, and titanium dioxide nanoparticles and immobilization of *Daphnia magna* under guideline testing conditions. *Ecotoxicology and Environmental Safety* 127:144–152.

Dai, Y. J., Y. F. Jia, N. Chen et al. 2014. Zebrafish as a model system to study toxicology. *Environmental Toxicology and Chemistry* 33 (1):11–17.

Dale, A. L., G. V. Lowry, and E. A. Casman. 2013. Modeling nanosilver transformations in freshwater sediments. *Environmental Science & Technology* 47 (22):12920–12928.

Das, P., C. D. Metcalfe, and M. A. Xenopoulos. 2014. Interactive effects of silver nanoparticles and phosphorus on phytoplankton growth in natural waters. *Environmental Science & Technology* 48 (8):4573–4580.

Das, P., M. A. Xenopoulos, C. J. Williams, M. E. Hoque, and C. D. Metcalfe. 2012. Effects of silver nanoparticles on bacterial activity in natural waters. *Environmental Toxicology and Chemistry* 31 (1):122–130.

Dash, A., A. P. Singh, B. R. Chaudhary, S. K. Singh, and D. Dash. 2012. Effect of silver nanoparticles on growth of eukaryotic green algae. *Nano-Micro Letters* 4 (3):158–165.

Degger, N., A. C. Tse, and R. S. Wu. 2015. Silver nanoparticles disrupt regulation of steroidogenesis in fish ovarian cells. *Aquatic Toxicology* 169:143–151.

Del Giorgio, P. A. and J. J. Cole. 1998. Bacterial growth efficiency in natural aquatic systems. *Annual Review of Ecology and Systematics* 29:503–541.

Deng, H., D. McShan, Y. Zhang et al. 2016. Mechanistic study of the synergistic antibacterial activity of combined silver nanoparticles and common antibiotics. *Environmental Science & Technology* 50 (16):8840–8848.

Derjaguin, B. V. and L. Landau. 1941. Theory of the stability of strongly charged lyophobic sols and of the adhesion of strongly charged particles in solutions of electrolytes. *Acta Physica Chimica URSS* 14:633–662.

Dibrov, P., J. Dzioba, K. K. Gosink, and C. C. Häse. 2002. Chemiosmotic mechanism of antimicrobial activity of Ag^+ in *Vibrio cholerae*. *Antimicrobial Agents and Chemotherapy* 46 (8):2668–2670.

Dobias, J. and R. Bernier-Latmani. 2013. Silver release from silver nanoparticles in natural waters. *Environmental Science & Technology* 47 (9):4140–4146.

Doiron, K., M. Millour, J. P. Gagné, and K. Lemarchand. 2014. Combined effects of silver nanoparticles and humic and fulvic acids on *Vibrio splendidus* growth. *Journal of Xenobiotics* 4:4893 (2):59–61.

Doiron, K., E. Pelletier, and K. Lemarchand. 2012. Impact of polymer-coated silver nanoparticles on marine microbial communities: A microcosm study. *Aquatic Toxicology* 124–125:22–27.

Dorobantu, L. S., C. Fallone, A. J. Noble et al. 2015. Toxicity of silver nanoparticles against bacteria, yeast, and algae. *Journal of Nanoparticle Research* 17 (4):172.

Drews, U. 1975. Cholinesterase in embryonic development. *Progress in Histochemistry and Cytochemistry* 7 (3):1–52.

Echavarri-Bravo, V., L. Paterson, T. J. Aspray et al. 2015. Shifts in the metabolic function of a benthic estuarine microbial community following a single pulse exposure to silver nanoparticles. *Environmental Pollution* 201:91–99.

El Badawy, A. M., T. P. Luxton, R. G. Silva et al. 2010. Impact of environmental conditions (pH, ionic strength, and electrolyte type) on the surface charge and aggregation of silver nanoparticles suspensions. *Environmental Science & Technology* 44 (4):1260–1266.

Elimelech, M., J. Gregory, X. Jia, and R. A. Williams. 1998. *Particle Deposition & Aggregation measurement, Modelling and Simulation*. Oxford, UK: Butterworth–Heinemann.

Espinoza, M. G., M. L. Hinks, A. M. Mendoza, D. P. Pullman, and K. I. Peterson. 2012. Kinetics of halide-induced decomposition and aggregation of silver nanoparticles. *Journal of Physical Chemistry C* 116 (14):8305–8313.

Fabrega, J., S. R. Fawcett, J. C. Renshaw, and J. R. Lead. 2009. Silver nanoparticle impact on bacterial growth: Effect of pH, concentration, and organic matter. *Environmental Science & Technology* 43 (19):7285–7290.

Fabrega, J., S. N. Luoma, C. R. Tyler, T. S. Galloway, and J. R. Lead. 2011a. Silver nanoparticles: Behaviour and effects in the aquatic environment. *Environment International* 37 (2):517–531.

Fabrega, J., R. Zhang, J. C. Renshaw, W. T. Liu, and J. R. Lead. 2011b. Impact of silver nanoparticles on natural marine biofilm bacteria. *Chemosphere* 85 (6):961–966.

Falugi, C. and M. G. Aluigi. 2012. Early appearance and possible functions of non-neuromuscular cholinesterase activities. *Frontiers in Molecular Neuroscience* 5 (54): 1–12.

Falugi, C., M. G. Aluigi, M. C. Chiantore et al. 2012. Toxicity of metal oxide nanoparticles in immune cells of the sea urchin. *Marine Environmental Research* 76:114–121.

Farkas, J., P. Christian, J. A. Urrea et al. 2010. Effects of silver and gold nanoparticles on rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Aquatic Toxicology* 96 (1):44–52.

Flores, C. Y., A. G. Miñán, C. A. Grillo et al. 2013. Citrate-capped silver nanoparticles showing good bactericidal effect against both planktonic and sessile bacteria and a low cytotoxicity to osteoblastic cells. *ACS Applied Materials & Interfaces* 5 (8):3149–3159.

Froggett, S. J., S. F. Clancy, D. R. Boverhof, and R. A. Canady. 2014. A review and perspective of existing research on the release of nanomaterials from solid nanocomposites. *Particle and Fibre Toxicology* 11 (1):17.

Gagné, J. P., B. Gouteux, Y. D. Soubaneh, and J. R. Brindle. 2011. Sorption of pesticides on natural geosorbents. In *Pesticides—Formulations, Effects, Fate*, edited by M. Stoytcheva, 785–802. Rijeka: Intech.

Gambardella, C., M. G. Aluigi, S. Ferrando et al. 2013. Developmental abnormalities and changes in cholinesterase activity in sea urchin embryos and larvae from sperm exposed to engineered nanoparticles. *Aquatic Toxicology* 130–131:77–85.

Gambardella, C., E. Costa, V. Piazza et al. 2015a. Effect of silver nanoparticles on marine organisms belonging to different trophic levels. *Marine Environmental Research* 111:41–49.

Gambardella, C., S. Ferrando, S. Morgana et al. 2015b. Exposure of *Paracentrotus lividus* male gametes to engineered nanoparticles affects skeletal bio-mineralization processes and larval plasticity. *Aquatic Toxicology* 158:181–191.

Gao, J., S. Youn, A. Hovsepyan et al. 2009. Dispersion and toxicity of selected manufactured nanomaterials in natural river water samples: Effects of water chemical composition. *Environmental Science & Technology* 43 (9):3322–3328.

García-Alonso, J., F. R. Khan, S. K. Misra et al. 2011. Cellular internalization of silver nanoparticles in gut epithelia of the estuarine polychaete *Nereis diversicolor. Enviromental Science & Technology* 45 (10):4630–4636.

García-Alonso, J., N. Rodriguez-Sanchez, S. K. Misra et al. 2014. Toxicity and accumulation of silver nanoparticles during development of the marine polychaete *Platynereis dumerilii*. *Science of the Total Environment* 476–477:688–695.

Garcia-Reyero, N., C. Thornton, A. D. Hawkins et al. 2015. Assessing the exposure to nanosilver and silver nitrate on fathead minnow gill gene expression and mucus production. *Environmental Nanotechnology, Monitoring & Management* 4:58–66.

Georgantzopoulou, A., S. Cambier, T. Serchi et al. 2016. Inhibition of multixenobiotic resistance transporters (MXR) by silver nanoparticles and ions in vitro and in *Daphnia magna*. *Science of the Total Environment* 569–570:681–689.

Ghiglione, J. F., F. Martin-Laurent, and S. Pesce. 2016. Microbial ecotoxicology: An emerging discipline facing contemporary environmental threats. *Environmental Science and Pollution Research* 23 (5):3981–3983.

Gomes, T., O. Araújo, R. Pereira et al. 2013a. Genotoxicity of copper oxide and silver nanoparticles in the mussel *Mytilus galloprovincialis*. *Marine Environmental Research* 84:51–59.

Gomes, T., C. G. Pereira, C. Cardoso, and M. J. Bebianno. 2013b. Differential protein expression in mussels *Mytilus galloprovincialis* exposed to nano and ionic Ag. *Aquatic Toxicology* 136–137:79–90.

Gomes, T., C. G. Pereira, C. Cardoso et al. 2014. Effects of silver nanoparticles exposure in the mussel *Mytilus galloprovincialis*. *Marine Environmental Research* 101:208–214.

González, A. G., L. Fernández-Rojo, J. Leflaive, O. S. Pokrovsky, and J. L. Rols. 2016. Response of three biofilm-forming benthic microorganisms to Ag nanoparticles and Ag^{+}: The diatom *Nitzschia palea*, the green alga *Uronema confervicolum* and the cyanobacteria *Leptolyngbya* sp. *Environmental Science and Pollution Research* 23 (21):22136–22150.

Gottschalk, F. and B. Nowack. 2011. The release of engineered nanomaterials to the environment. *Journal of Environmental Monitoring* 13 (5):1145–1155.

Gottschalk, F., T. Sun, and B. Nowack. 2013. Environmental concentrations of engineered nanomaterials: Review of modeling and analytical studies. *Environmental Pollution* 181:287–300.

Grasso, D., K. Subramaniam, M. Butkus, K. Strevett, and J. Bergendahl. 2002. A review of non-DLVO interactions in environmental colloidal systems. *Reviews in Environmental Science and Biotechnology* 1 (1):17–38.

Griffitt, R. J., N. J. Brown-Peterson, D. A. Savin et al. 2012. Effects of chronic nanoparticulate silver exposure to adult and juvenile sheepshead minnows (*Cyprinodon variegatus*). *Environmental Toxicology and Chemistry* 31 (1):160–167.

Griffitt, R. J., C. M. Lavelle, A. S. Kane, N. D. Denslow, and D. S. Barber. 2013. Chronic nanoparticulate silver exposure results in tissue accumulation and transcriptomic changes in zebrafish. *Aquatic Toxicology* 130–131:192–200.

Grillo, R., A. H. Rosa, and L. F. Fraceto. 2015. Engineered nanoparticles and organic matter: A review of the state-of-the-art. *Chemosphere* 119:608–619.

Hawkins, A. D., C. Thornton, A. J. Kennedy et al. 2015. Gill histopathologies following exposure to nanosilver or silver nitrate. *Journal of Toxicology and Environmental Health, Part A* 78 (5):301–315.

He, D., J. J. Dorantes-Aranda, and T. D. Waite. 2012. Silver nanoparticle-algae interactions: Oxidative dissolution, reactive oxygen species generation and synergistic toxic effects. *Environmental Science & Technology* 46 (16):8731–8738.

Hogstrand, C., F. Galvez, and C. M. Wood. 1996. Toxicity, silver accumulation and metallothionein induction in freshwater rainbow trout during exposure to different silver salts. *Environmental Toxicology and Chemistry* 15 (7):1102–1108.

Holden, P. A., F. Klaessig, R. F. Turco et al. 2014. Evaluation of exposure concentrations used in assessing manufactured nanomaterial environmental hazards: Are they relevant? *Environmental Science & Technology* 48 (18):10541–10551.

Holt, K. B., and A. J. Bard. 2005. Interaction of silver(I) ions with the respiratory chain of *Escherichia coli*: An electrochemical and scanning electrochemical microscopy study of the antimicrobial mechanism of micromolar Ag^+. *Biochemistry* 44 (39):13214–13223.

Huang, J., J. Cheng, and J. Yi. 2016a. Impact of silver nanoparticles on marine diatom *Skeletonema costatum*. *Journal of Applied Toxicology* 36 (10):1343–1354.

Huang, T., M. Sui, X. Yan, X. Zhang, and Z. Yuan. 2016b. Anti-algae efficacy of silver nanoparticles to *Microcystis aeruginosa*: Influence of NOM, divalent cations, and pH. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 509:492–503.

Huynh, K. A. and K. L. Chen. 2011. Aggregation kinetics of citrate and polyvinylpyrrolidone coated silver nanoparticles in monovalent and divalent electrolyte solutions. *Environmental Science & Technology* 45 (13):5564–5571.

ISO (International Organization for Standardization). 2008. Technical Specification ISO/TS 27687:2008(E): Nanotechnologies—Terminology and Definitions for Nano-objects—Nanoparticle, Nanofibre and Nanoplate. Geneva: ISO.

Irwin, P., J. Martin, L. H. Nguyen et al. 2010. Antimicrobial activity of spherical silver nanoparticles prepared using a biocompatible macromolecular capping agent: Evidence for induction of a greatly prolonged bacterial lag phase. *Journal of Nanobiotechnology* 8 (1):34.

Jeong, E., W. T. Im, D. H. Kim et al. 2014. Different susceptibilities of bacterial community to silver nanoparticles in wastewater treatment systems. *Journal of Environmental Science and Health, Part A* 49 (6):685–693.

Kaegi, R., B. Sinnet, S. Zuleeg et al. 2010. Release of silver nanoparticles from outdoor facades. *Environmental Pollution* 158 (9):2900–2905.

Kaegi, R., A. Voegelin, B. Sinnet et al. 2011. Behavior of metallic silver nanoparticles in a pilot wastewater treatment plant. *Environmental Science & Technology* 45 (9):3902–3908.

Kalman, J., K. B. Paul, F. R. Khan, V. Stone, and T. F. Fernandes. 2015. Characterisation of bioaccumulation dynamics of three differently coated silver nanoparticles and aqueous silver in a simple freshwater food chain. *Environmental Chemistry* 12 (6):662–672.

Katsumiti, A., D. Gilliland, I. Arostegui, and M. P. Cajaraville. 2015. Mechanisms of toxicity of Ag nanoparticles in comparison to bulk and ionic Ag on mussel hemocytes and gill cells. *PLOS ONE* 10 (6):e0129039.

Katuli, K. K., A. Massarsky, A. Hadadi, and Z. Pourmehran. 2014. Silver nanoparticles inhibit the gill Na^+/K^+-ATPase and erythrocyte AChE activities and induce the stress response in adult zebrafish (*Danio rerio*). *Ecotoxicology and Environmental Safety* 106:173–180.

Keller, A. A., and A. Lazareva. 2014. Predicted releases of engineered nanomaterials: From global to regional to local. *Environmental Science & Technology Letters* 1 (1):65–70.

Khan, F. R., S. K. Misra, J. García-Alonso et al. 2012. Bioaccumulation dynamics and modeling in an estuarine invertebrate following aqueous exposure to nanosized and dissolved silver. *Environmental Science & Technology* 46 (14):7621–7628.

Khan, Farhan R, Kai B Paul, Agnieszka D Dybowska et al. 2015. Accumulation dynamics and acute toxicity of silver nanoparticles to Daphnia magna and Lumbriculus variegatus: Implications for metal modeling approaches. *Environmental Science & Technology* 49 (7):4389–4397.

Khan, S. S., P. Srivatsan, N. Vaishnavi, A. Mukherjee, and N. Chandrasekaran. 2011. Interaction of silver nanoparticles (SNPs) with bacterial extracellular proteins (ECPs) and its adsorption isotherms and kinetics. *Journal of Hazardous Materials* 192 (1):299–306.

Kiener, T. K., I. Selptsova-Friedrich, and W. Hunziker. 2008. Tjp3/zo-3 is critical for epidermal barrier function in zebrafish embryos. *Developmental Biology* 316 (1):36–49.

Kim, I., B. T. Lee, H. A. Kim et al. 2016. Citrate coated silver nanoparticles change heavy metal toxicities and bioaccumulation of *Daphnia magna*. *Chemosphere* 143:99–105.

Kim, J. H., D. L. Cho, G. J. Kim, B. Gao, and H. K. Shon. 2011. Titania nanomaterials produced from Ti-salt flocculated sludge in water treatment. *Catalysis Surveys from Asia* 15 (2):117–126.

Kim, J. Y., K. T. Kim, B. G. Lee, B. J. Lim, and S. D. Kim. 2013. Developmental toxicity of *Japanese medaka* embryos by silver nanoparticles and released ions in the presence of humic acid. *Ecotoxicology and Environmental Safety* 92:57–63.

Kim, S. and D. Y. Ryu. 2013. Silver nanoparticle-induced oxidative stress, genotoxicity and apoptosis in cultured cells and animal tissues. *Journal of Applied Toxicology* 33 (2):78–89.

Klaine, S. J., P. J. J. Alvarez, G. E. Batley et al. 2008. Nanomaterials in the environment: Behavior, fate, bioavailability, and effects. *Environmental Toxicology and Chemistry* 27 (9):1825–1851.

Krishnaraj, C., E. G. Jagan, S. Rajasekar et al. 2010. Synthesis of silver nanoparticles using *Acalypha indica* leaf extracts and its antibacterial activity against water borne pathogens. *Colloids and Surfaces B: Biointerfaces* 76 (1):50–56.

Kumari, M., S. Pandey, V. P. Giri et al. 2016. Tailoring shape and size of biogenic silver nanoparticles to enhance antimicrobial efficacy against MDR bacteria. *Microbial Pathogenesis* 105:346–355.

Kwak, J. I., R. X. Cui, S. H. Nam et al. 2016. Multispecies toxicity test for silver nanoparticles to derive hazardous concentration based on species sensitivity distribution for the protection of aquatic ecosystems. *Nanotoxicology* 10 (5):521–530.

Kwok, K. W., M. Auffan, A. R. Badireddy et al. 2012. Uptake of silver nanoparticles and toxicity to early life stages of Japanese medaka (*Oryzias latipes*): Effect of coating materials. *Aquatic Toxicology* 120–121:59–66.

Kwok, K. W. H., W. Dong, S. M. Marinakos et al. 2016. Silver nanoparticle toxicity is related to coating materials and disruption of sodium concentration regulation. *Nanotoxicology* 10 (9):1306–1317.

Kwon, D., H. W. Nho, and T. H. Yoon. 2014. X-ray and electron microscopy studies on the biodistribution and biomodification of iron oxide nanoparticles in *Daphnia magna*. *Colloids and Surfaces B: Biointerfaces* 122:384–389.

Langdon, K. A., M. J. McLaughlin, J. K. Kirby, and G. Merrington. 2014. The effect of soil properties on the toxicity of silver to the soil nitrification process. *Environmental Toxicology and Chemistry* 33 (5):1170–1178.

Lara, H. H., N. V. Ayala-Núñez, L. Ixtepan-Turrent, and C. Rodríguez Padilla. 2010. Bactericidal effect of silver nanoparticles against multidrug-resistant bacteria. *World Journal of Microbiology and Biotechnology* 26 (4):615–621.

Lau, B. L. T., W. C. Hockaday, K. Ikuma, O. Furman, and A. W. Decho. 2013. A preliminary assessment of the interactions between the capping agents of silver nanoparticles and environmental organics. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 435:22–27.

Lee, K. J., P. D. Nallathamby, L. M. Browning, C. J. Osgood, and X. H. N. Xu. 2007. *In vivo* imaging of transport and biocompatibility of single silver nanoparticles in early development of zebrafish embryos. *ACS Nano* 1 (2):133–143.

Leonardo, T., E. Farhi, S. Pouget et al. 2016. Silver accumulation in the green microalga *Coccomyxa actinabiotis*: Toxicity, *in situ* speciation, and localization investigated using synchrotron XAS, XRD, and TEM. *Environmental Science & Technology* 50 (1):359–367.

Levard, C., E. M. Hotze, G. V. Lowry, and G. E. Brown. 2012. Environmental transformations of silver nanoparticles: Impact on stability and toxicity. *Environmental Science & Technology* 46 (13):6900–6914.

Levard, C., S. Mitra, T. Yang et al. 2013. Effect of chloride on the dissolution rate of silver nanoparticles and toxicity to *E. coli*. *Environmental Science & Technology* 47 (11):5738–5745.

Levard, C., B. C. Reinsch, F. M. Michel et al. 2011. Sulfidation processes of PVP-coated silver nanoparticles in aqueous solution: Impact on dissolution rate. *Environmental Science & Technology* 45 (12):5260–5266.

Li, C. C., Y. J. Wang, F. Dang, and D. M. Zhou. 2016. Mechanistic understanding of reduced AgNP phytotoxicity induced by extracellular polymeric substances. *Journal of Hazardous Materials* 308:21–28.

Li, F. M., Z. Liang, X. Zheng et al. 2015. Toxicity of nano-TiO2 on algae and the site of reactive oxygen species production. *Aquatic Toxicology* 158:1–13.

Li, H., A. Turner, and M. T. Brown. 2013. Accumulation of aqueous and nanoparticulate silver by the marine gastropod *Littorina littorea*. *Water, Air, & Soil Pollution* 224 (1):1354–1363.

Li, W. R., X. B. Xie, Q. S. Shi et al. 2010a. Antibacterial activity and mechanism of silver nanoparticles on *Escherichia coli*. *Applied Microbiology and Biotechnology* 85 (4):1115–1122.

Li, X., J. J. Lenhart, and H. W. Walker. 2010b. Dissolution-accompanied aggregation kinetics of silver nanoparticles. *Langmuir* 26 (21):16690–16698.

Li, X., J. J. Lenhart, and H. W. Walker. 2012. Aggregation kinetics and dissolution of coated silver nanoparticles. *Langmuir* 28 (2):1095–1104.

Liu, J. and R. H. Hurt. 2010. Ion release kinetics and particle persistence in aqueous nanosilver colloids. *Environmental Science & Technology* 44 (6):2169–2175.

Liu, J., Y. S. Hwang, and J. J. Lenhart. 2015. Heteroaggregation of bare silver nanoparticles with clay minerals. *Environmental Science: Nano* 2 (5):528–540.

Liu, J., K. G. Pennell, and R. H. Hurt. 2011. Kinetics and mechanisms of nanosilver oxysulfidation. *Environmental Science & Technology* 45 (17):7345–7353.

Lok, C. N., C. M. Ho, R. Chen et al. 2007. Silver nanoparticles: Partial oxidation and antibacterial activities. *Journal of Biological Inorganic Chemistry* 12 (4):527–534.

Lok, C. N., C. M. Ho, R. Chen et al. 2006. Proteomic analysis of the mode of antibacterial action of silver nanoparticles. *Journal of Proteome Research* 5 (4):916–924.

Lopes, S., C. Pinheiro, A. M. V. M. Soares, and S. Loureiro. 2016. Joint toxicity prediction of nanoparticles and ionic counterparts: Simulating toxicity under a fate scenario. *Journal of Hazardous Materials* 320:1–9.

Lowry, G. V., B. P. Espinasse, A. R. Badireddy et al. 2012. Long-term transformation and fate of manufactured Ag nanoparticles in a simulated large scale freshwater emergent wetland. *Environmental Science & Technology* 46 (13):7027–7036.

Macken, A., H. J. Byrne, and K. V. Thomas. 2012. Effects of salinity on the toxicity of ionic silver and Ag-PVP nanoparticles to *Tisbe battagliai* and *Ceramium tenuicorne*. *Ecotoxicology and Environmental Safety* 86:101–110.

Magesky, A. and É. Pelletier. 2015. Toxicity mechanisms of ionic silver and polymer-coated silver nanoparticles with interactions of functionalized carbon nanotubes on early development stages of sea urchin. *Aquatic Toxicology* 167:106–123.

Magesky, A., C. A. O. Ribeiro, L. Beaulieu, and É. Pelletier. 2017. Silver nanoparticles and dissolved silver activate contrasting immune responses and stress-induced Hsp expression in sea urchin. *Environmental Toxicology and Chemistry* 36:1872–1886.

Magesky, A., C. A. O. Ribeiro, and É. Pelletier. 2016. Physiological effects and cellular responses of metamorphic larvae and juveniles of sea urchin exposed to ionic and nanoparticulate silver. *Aquatic Toxicology* 174:208–227.

Marambio-Jones, C., and E. M. V. Hoek. 2010. A review of the antibacterial effects of silver nanomaterials and potential implications for human health and the environment. *Journal of Nanoparticle Research* 12 (5):1531–1551.

Marques, B. F., L. F. Cordeiro, L. W. Kist et al. 2013. Toxicological effects induced by the nanomaterials fullerene and nanosilver in the polychaeta *Laeonereis acuta* (Nereididae) and in the bacteria communities living at their surface. *Marine Environmental Research* 89:53–62.

Martínez-Castañón, G. A., N. Niño-Martínez, F. Martínez-Gutierrez, J. R. Martínez-Mendoza, and F. Ruiz. 2008. Synthesis and antibacterial activity of silver nanoparticles with different sizes. *Journal of Nanoparticle Research* 10 (8):1343–1348.

Martínez-Gutierrez, F., E. P. Thi, J. M. Silverman et al. 2012. Antibacterial activity, inflammatory response, coagulation and cytotoxicity effects of silver nanoparticles. *Nanomedicine: Nanotechnology, Biology and Medicine* 8 (3):328–336.

Masrahi, A., A. R. VandeVoort, and Y. Arai. 2014. Effects of silver nanoparticle on soil-nitrification processes. *Archives of Environmental Contamination and Toxicology* 66 (4):504–513.

Mattsson, K., K. Adolfsson, M. T. Ekvall et al. 2016. Translocation of 40 nm diameter nanowires through the intestinal epithelium of *Daphnia magna*. *Nanotoxicology* 10 (8):1160–1167.

Maurer-Jones, M. A., I. L. Gunsolus, C. J. Murphy, and C. L. Haynes. 2013. Toxicity of engineered nanoparticles in the environment. *Analytical Chemistry* 85 (6):3036–3049.

Maurer, L. L. and J. N. Meyer. 2016. A systematic review of evidence for silver nanoparticle-induced mitochondrial toxicity. *Environmental Science: Nano* 3 (2):311–322.

McCarthy, M. P., D. L. Carroll, and A. H. Ringwood. 2013. Tissue specific responses of oysters, *Crassostrea virginica*, to silver nanoparticles. *Aquatic Toxicology* 138–139:123–128.

McNaught, A. D. and A. Wilkinson. 2014. *Compendium of Chemical Terminology (the Gold Book)*. 2nd ed. Oxford: Blackwell Scientific Publications.

McShan, D., P. C. Ray, and H. Yu. 2014. Molecular toxicity mechanism of nanosilver. *Journal of Food and Drug Analysis* 22 (1):116–127.

Miao, A. J., K. A. Schwehr, C. Xu et al. 2009. The algal toxicity of silver engineered nanoparticles and detoxification by exopolymeric substances. *Environmental Pollution* 157 (11):3034–3041.

Millour, M., K. Doiron, K. Lemarchand, and J. P. Gagné. 2015. Does the bacterial media culture chemistry affect the stability of nanoparticles in nanotoxicity assays? *Journal of Xenobiotics* 5 (5772):34–36.

Mishra, S. and H. B. Singh. 2015. Biosynthesized silver nanoparticles as a nanoweapon against phytopathogens: Exploring their scope and potential in agriculture. *Applied Microbiology and Biotechnology* 99 (3):1097–1107.

Mitrano, D. M., S. Motellier, S. Clavaguera, and B. Nowack. 2015. Review of nanomaterial aging and transformations through the life cycle of nano-enhanced products. *Environment International* 77:132–147.

Moreno-Garrido, I., S. Pérez, and J. Blasco. 2015. Toxicity of silver and gold nanoparticles on marine microalgae. *Marine Environmental Research* 111:60–73.

Morones, J. R., J. L. Elechiguerra, A. Camacho et al. 2005. The bactericidal effect of silver nanoparticles. *Nanotechnology* 16 (10):2346–2353.

Mosselhy, D. A., W. He, D. Li, Y. Meng, and Qingling Feng. 2016. Silver nanoparticles: *In vivo* toxicity in zebrafish embryos and a comparison to silver nitrate. *Journal of Nanoparticle Research* 18 (8):222.

Murray, L., M. D. Rennie, E. C. Enders, K. Pleskach, and J. D. Martin. 2016. Effect of nanosilver on cortisol release and morphometrics in rainbow trout (*Oncorhynchus mykiss*). *Environmental Toxicology and Chemistry*.

Navarro, E., F. Piccapietra, B. Wagner et al. 2008. Toxicity of silver nanoparticles to *Chlamydomonas reinhardtii*. *Environmental Science & Technology* 42 (23):8959–8964.

Nel, A. E., L. Mädler, D. Velegol et al. 2009. Understanding biophysicochemical interactions at the nano-bio interface. *Nature Materials* 8 (7):543–557.

Oprsal, J., L. Blaha, M. Pouzar et al. 2015. Assessment of silver nanoparticle toxicity for common carp (*Cyprinus carpio*) fish embryos using a novel method controlling the agglomeration in the aquatic media. *Environmental Science and Pollution Research* 22 (23):19124–19132.

Osborne, O. J., S. Lin, C. H. Chang et al. 2015. Organ-specific and size-dependent Ag nanoparticle toxicity in gills and intestines of adult zebrafish. *ACS Nano* 9 (10):9573–9584.

Ostaszewska, T., M. Chojnacki, M. Kamaszewski, and E. Sawosz-Chwalibóg. 2016. Histopathological effects of silver and copper nanoparticles on the epidermis, gills, and liver of *Siberian sturgeon*. *Environmental Science and Pollution Research* 23 (2):1621–1633.

Oukarroum, A., L. Barhoumi, L. Pirastru, and D. Dewez. 2013. Silver nanoparticle toxicity effect on growth and cellular viability of the aquatic plant Lemna gibba. *Environmental Toxicology and Chemistry* 32 (4):902–907.

Oukarroum, A., S. Bras, F. Perreault, and R. Popovic. 2012a. Inhibitory effects of silver nanoparticles in two green algae, *Chlorella vulgaris* and *Dunaliella tertiolecta*. *Ecotoxicology and Environmental Safety* 78:80–85.

Oukarroum, A., S. Polchtchikov, F. Perreault, and R. Popovic. 2012b. Temperature influence on silver nanoparticles inhibitory effect on photosystem II photochemistry in two green algae, *Chlorella vulgaris* and *Dunaliella tertiolecta*. *Environmental Science and Pollution Research* 19 (5):1755–1762.

Park, E. J., J. Yi, Y. Kim, K. Choi, and K. Park. 2010. Silver nanoparticles induce cytotoxicity by a Trojan-horse type mechanism. *Toxicology in Vitro* 24 (3):872–878.

Peretyazhko, T. S. 2014. Size-controlled dissolution of silver nanoparticles at neutral and acidic pH conditions: Kinetics and size changes. *Environmental Science & Technology* 48:11954–11961.

Peters, L. E., M. MacKinnon, T. Van Meer, M. R. van den Heuvel, and D. G. Dixon. 2007. Effects of oil sands process-affected waters and naphthenic acids on yellow perch (*Perca flavescens*) and Japanese medaka (*Orizias latipes*) embryonic development. *Chemosphere* 67 (11):2177–2183.

Petersen, E. J., T. B. Henry, J. Zhao et al. 2014. Identification and avoidance of potential artifacts and misinterpretations in nanomaterial ecotoxicity measurements. *Environmental Science & Technology* 48 (8):4226–4246.

Petosa, A. R., D. P. Jaisi, I. R. Quevedo, M. Elimelech, and N. Tufenkji. 2010. Aggregation and deposition of engineered nanomaterials in aquatic environments: Role of physicochemical interactions. *Environmental Science & Technology* 44 (17):6532–6549.

Philippe, A. and G. E. Schaumann. 2014. Interactions of dissolved organic matter with natural and engineered inorganic colloids: A review. *Environmental Science & Technology* 48 (16):8946–8962.

Piccapietra, F., C. G. Allue, L. Sigg, and R. Behra. 2012. Intracellular silver accumulation in *Chlamydomonas reinhardtii* upon exposure to carbonate coated silver nanoparticles and silver nitrate. *Environmental Science & Technology* 46 (13):7390–7397.

Piccinno, F., F. Gottschalk, S. Seeger, and B. Nowack. 2012. Industrial production quantities and uses of ten engineered nanomaterials in Europe and the world. *Journal of Nanoparticle Research* 14 (9):1109.

Piticharoenphun, S., L. Šiller, M. L. Lemloh et al. 2012. Agglomeration of silver nanoparticles in sea urchin. *International Journal of Environmental Pollution and Remediation* 1 (1):44–50.

Pomeroy, L. R., P. J. B. le Williams, F. Azam, and J. E. Hobbie. 2007. The microbial loop. *Oceanography* 20 (2):28–33.

Poynton, H. C., J. M. Lazorchak, C. A. Impellitteri et al. 2012. Toxicogenomic responses of nanotoxicity in *Daphnia magna* exposed to silver nitrate and coated silver nanoparticles. *Environmental Science & Technology* 46 (11):6288–6296.

Prabhu, S., and E. K. Poulose. 2012. Silver nanoparticles: Mechanism of antimicrobial action, synthesis, medical applications, and toxicity effects. *International Nano Letters* 2 (1):32.

Pradhan, A., S. Seena, C Pascoal, and F. Cássio. 2011. Can metal nanoparticles be a threat to microbial decomposers of plant litter in streams? *Microbial Ecology* 62 (1):58–68.

Qian, H., K. Zhu, H. Lu et al. 2016. Contrasting silver nanoparticle toxicity and detoxification strategies in *Microcystis aeruginosa* and *Chlorella vulgaris*: New insights from proteomic and physiological analyses. *Science of the Total Environment* 572:1213–1221.

Radzig, M. A., V. A. Nadtochenko, O. A. Koksharova et al. 2013. Antibacterial effects of silver nanoparticles on gram-negative bacteria: Influence on the growth and biofilms formation, mechanisms of action. *Colloids and Surfaces B: Biointerfaces* 102:300–306.

Raffi, M., F. Hussain, T. M. Bhatti et al. 2008. Antibacterial characterization of silver nanoparticles against *E. coli* ATCC-15224. *Journal of Materials Science and Technology* 24 (2):192–196.

Rai, M. K., S. D. Deshmukh, A. P. Ingle, and A. K. Gade. 2012. Silver nanoparticles: The powerful nanoweapon against multidrug-resistant bacteria. *Journal of Applied Microbiology* 112:841–852.

Ramskov, T., V. E. Forbes, D. Gilliland, and H. Selck. 2015. Accumulation and effects of sediment-associated silver nanoparticles to sediment-dwelling invertebrates. *Aquatic Toxicology* 166:96–105.

Reichelt-Brushett, A. J., and P. L. Harrison. 2005. The effect of selected trace metals on the fertilization success of several scleractinian coral species. *Coral Reefs* 24 (4):524–534.

Ribeiro, F., J. A. Gallego-Urrea, K. Jurkschat et al. 2014. Silver nanoparticles and silver nitrate induce high toxicity to *Pseudokirchneriella subcapitata*, *Daphnia magna* and *Danio rerio*. *Science of the Total Environment* 466–467:232–241.

Ribeiro, F., C. A. M. Van Gestel, M. D. Pavlaki et al. 2017. Bioaccumulation of silver in *Daphnia magna*: Waterborne and dietary exposure to nanoparticles and dissolved silver. *Science of the Total Environment* 574:1633–1639.

Ringwood, A. H., M. McCarthy, T. C. Bates, and D. L. Carroll. 2010. The effects of silver nanoparticles on oyster embryos. *Marine Environmental Research* 69:S49–S51.

Sakka, Y., L. M. Skjolding, A. Mackevica, J. Filser, and A. Baun. 2016. Behavior and chronic toxicity of two differently stabilized silver nanoparticles to *Daphnia magna*. *Aquatic Toxicology* 177:526–535.

Samarajeewa, A. D., J. R. Velicogna, J. I. Princz et al. 2017. Effect of silver nano-particles on soil microbial growth, activity and community diversity in a sandy loam soil. *Environmental Pollution* 220, Part A:504–513.

SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks). 2014. *Nanosilver: Safety, Health and Environmental Effects and Role in Antimicrobial Resistance*. Luxembourg.

Schiavo, S., N. Duroudier, E. Bilbao et al. 2017. Effects of PVP/PEI coated and uncoated silver NPs and PVP/PEI coating agent on three species of marine microalgae. *Science of the Total Environment* 577:45–53.

Shah, V., J. Jones, J. Dickman, and S. Greenman. 2014. Response of soil bacterial community to metal nanoparticles in biosolids. *Journal of Hazardous Materials* 274:399–403.

Shahverdi, A. R., A. Fakhimi, H. R. Shahverdi, and S. Minaian. 2007. Synthesis and effect of silver nanoparticles on the antibacterial activity of different antibiotics against Staphylococcus aureus and Escherichia coli. *Nanomedicine* 3 (2):168–171.

Shanthi, S., B. D. Jayaseelan, P. Velusamy et al. 2016. Biosynthesis of silver nanoparticles using a probiotic *Bacillus licheniformis* Dahb1 and their antibiofilm activity and toxicity effects in *Ceriodaphnia cornuta*. *Microbial Pathogenesis* 93:70–77.

Sheng, Z. and Y. Liu. 2011. Effects of silver nanoparticles on wastewater biofilms. *Water Research* 45 (18):6039–6050.

Shon, H. K., S. Vigneswaran, I. S. Kim et al. 2007. Preparation of titanium dioxide (TiO_2) from sludge produced by titanium tetrachloride ($TiCl_4$) flocculation of wastewater. *Environmental Science & Technology* 41 (4):1372–1377.

Shrivastava, S., T. Bera, A. Roy et al. 2007. Characterization of enhanced antibacterial effects of novel silver nanoparticles. *Nanotechnology* 18 (22):225103.

Šiller, L., M. L. Lemloh, S. Piticharoenphun et al. 2013. Silver nanoparticle toxicity in sea urchin *Paracentrotus lividus*. *Environmental Pollution* 178:498–502.

Silver, S., L. T. Phung, and G. Silver. 2006. Silver as biocides in burn and wound dressings and bacterial resistance to silver compounds. *Journal of Industrial Microbiology and Biotechnology* 33 (7):627–634.

Smolders, R., M. Baillieul, and R. Blust. 2005. Relationship between the energy status of *Daphnia magna* and its sensitivity to environmental stress. *Aquatic Toxicology* 73 (2):155–170.

Soenen, S. J., W. J. Parak, J. Rejman, and B. Manshian. 2015. (Intra)cellular stability of inorganic nanoparticles: Effects on cytotoxicity, particle functionality, and biomedical applications. *Chemical Reviews* 115 (5):2109–2135.

Som, C., M. Berges, Q. Chaudhry et al. 2010. The importance of life cycle concepts for the development of safe nanoproducts. *Toxicology* 269 (2):160–169.

Somorjai, G. A. 1994. *Introduction of Surface Chemistry and Catalysis*. New York: Wiley Interscience.

Sondi, I. and B. Salopek-Sondi. 2004. Silver nanoparticles as antimicrobial agent: A case study on *E. coli* as a model for Gram-negative bacteria. *Journal of Colloid and Interface Science* 275 (1):177–182.

Sørensen, S. N., H. C. H. Lützhøft, R. Rasmussen, and A. Baun. 2016. Acute and chronic effects from pulse exposure of *D. magna* to silver and copper oxide nanoparticles. *Aquatic Toxicology* 180:209–217.

Sotiriou, G. A., A. Meyer, J. T. N. Knijnenburg, S. Panke, and S. E. Pratsinis. 2012. Quantifying the origin of released Ag^+ ions from nanosilver. *Langmuir* 28 (45):15929–15936.

Stensberg, M. C., R. Madangopal, G. Yale et al. 2014. Silver nanoparticle-specific mitotoxicity in *Daphnia magna*. *Nanotoxicology* 8 (8):833–842.

Sun, X., Z. Sheng, and Y. Liu. 2013. Effects of silver nanoparticles on microbial community structure in activated sludge. *Science of the Total Environment* 443:828–835.

Suwa, R., C. Kataoka, and S. Kashiwada. 2014. Effects of silver nanocolloids on early life stages of the scleractinian coral *Acropora japonica*. *Marine Environmental Research* 99:198–203.

Talesa, V., G. B. Principato, E. Giovannini, M. V. Giovanni, and G. Rosi. 1993. Dimeric forms of cholinesterase in *Sipunculus nudus*. *European Journal of Biochemistry* 215 (2):267–275.

Thurman, E. M. 1985. Amount of organic carbon in natural waters. In, edited by E. M. Thurman, 7–65. Hingham: Kluwer Academic Publishers.

Tremblay, L., and J. P. Gagné. 2009. Organic matter distribution and reactivity in the waters of a large estuarine system. *Marine Chemistry* 116 (1–4):1–12.

Ulm, L., A. Krivohlavek, D. Jurašin et al. 2015. Response of biochemical biomarkers in the aquatic crustacean *Daphnia magna* exposed to silver nanoparticles. *Environmental Science and Pollution Research* 22 (24):19990–19999.

UNECE (United Nations Economic Commission for Europe). 2013. Globally harmonized system of classification and labelling of chemicals (GHS) #9789210547451. New York and Geneva: Secretariat of United Nations.

USEPA (US Environmental Protection Agency). 2009. Emerging contaminants-nanomaterials fact sheet. EPA 505-F-509-011. Washington, DC: Office of Solid Waste and Emergency Response.

van Aerle, R., A. Lange, A. Moorhouse et al. 2013. Molecular mechanisms of toxicity of silver nanoparticles in zebrafish embryos. *Environmental Science & Technology* 47 (14):8005–8014.

Vance, M. E., T. Kuiken, E. P. Vejerano et al. 2015. Nanotechnology in the real world: Redeveloping the nanomaterial consumer products inventory. *Beilstein Journal of Nanotechnology* 6:1769–1780.

Verwey, E. J. W. and J. Th G. Overbeek. 1948. *Theory of the Stability of Lyophobic Colloids*. Amsterdam: Elsevier.

Vijayakumar, S., B. Malaikozhundan, P. Ramasamy, and B. Vaseeharan. 2016. Assessment of biopolymer stabilized silver nanoparticle for their ecotoxicity on *Ceriodaphnia cornuta* and antibiofilm activity. *Journal of Environmental Chemical Engineering* 4 (2):2076–2083.

Völker, C., C. Boedicker, J. Daubenthaler, M. Oetken, and J. Oehlmann. 2013. Comparative toxicity assessment of nanosilver on three *Daphnia* species in acute, chronic and multi-generation experiments. *PLOS ONE* 8 (10):e75026.

von Moos, N. and V. I. Slaveykova. 2014. Oxidative stress induced by inorganic nanoparticles in bacteria and aquatic microalgae—State of the art and knowledge gaps. *Nanotoxicology* 8 (6):605–630.

Wang, H., K. T. Ho, K. G. Scheckel et al. 2014. Toxicity, bioaccumulation, and biotransformation of silver nanoparticles in marine organisms. *Environmental Science & Technology* 48 (23):13711–13717.

Wang, Z., L. Zhang, J. Zhao, and B. Xing. 2016. Environmental processes and toxicity of metallic nanoparticles in aquatic systems as affected by natural organic matter. *Environmental Science: Nano* 3 (2):240–255.

Wise, J. P., Sr., B. C. Goodale, S. S. Wise et al. 2010. Silver nanospheres are cytotoxic and genotoxic to fish cells. *Aquatic Toxicology* 97 (1):34–41.

Wu, Y. and Q. Zhou. 2012. Dose- and time-related changes in aerobic metabolism, chorionic disruption, and oxidative stress in embryonic medaka (*Oryzias latipes*): Underlying mechanisms for silver nanoparticle developmental toxicity. *Aquatic Toxicology* 124–125:238–246.

Wu, Y. and Q. Zhou. 2013. Silver nanoparticles cause oxidative damage and histological changes in medaka (*Oryzias latipes*) after 14 days of exposure. *Environmental Toxicology and Chemistry* 32 (1):165–173.

Wu, Y., Q. Zhou, H. Li et al. 2010. Effects of silver nanoparticles on the development and histopathology biomarkers of Japanese medaka (*Oryzias latipes*) using the partial-life test. *Aquatic Toxicology* 100 (2):160–167.

Xin, Q., J. M. Rotchell, J. Cheng, J. Yi, and Q. Zhang. 2015. Silver nanoparticles affect the neural development of zebrafish embryos. *Journal of Applied Toxicology* 35 (12):1481–1492.

Xiu, Z. M., Q. B. Zhang, H. L. Puppala, V. L. Colvin, and P. J. J. Alvarez. 2012. Negligible particle-specific antibacterial activity of silver nanoparticles. *Nano Letters* 12 (8):4271–4275.

Xu, X. H. N., W. J. Brownlow, S. V. Kyriacou, Q. Wan, and J. J. Viola. 2004. Real-time probing of membrane transport in living microbial cells using single nanoparticle optics and living cell imaging. *Biochemistry* 43 (32):10400–10413.

Yang, Y., J. Quensen, J. Mathieu et al. 2014. Pyrosequencing reveals higher impact of silver nanoparticles than Ag^+ on the microbial community structure of activated sludge. *Water Research* 48:317–325.

Yang, Y., J. Wang, Z. Xiu, and P. J. J. Alvarez. 2013. Impacts of silver nanoparticles on cellular and transcriptional activity of nitrogen-cycling bacteria. *Environmental Toxicology and Chemistry* 32 (7):1488–1494.

Yu, W. L. and M. Borkovec. 2002. Distinguishing heteroaggregation from homoaggregation in mixed binary particle suspensions by multiangle static and dynamic light scattering. *Journal of Physical Chemistry B* 106 (51):13106–13110.

Zhang, C., Z. Hu, and B. Deng. 2016a. Silver nanoparticles in aquatic environments: Physiochemical behavior and antimicrobial mechanisms. *Water Research* 88:403–427.

Zhang, L., Y. He, N. Goswami et al. 2016b. Uptake and effect of highly fluorescent silver nanoclusters on *Scenedesmus obliquus*. *Chemosphere* 153:322–331.

Zhang, L., J. Li, K. Yang, J. Liu, and D. Lin. 2016c. Physicochemical transformation and algal toxicity of engineered nanoparticles in surface water samples. *Environmental Pollution* 211:132–140.

Zhang, X., C. W. Yang, H. Q. Yu, and G. P. Sheng. 2016d. Light-induced reduction of silver ions to silver nanoparticles in aquatic environments by microbial extracellular polymeric substances (EPS). *Water Research* 106:242–248.

Zhou, D., A. I. Abdel-Fattah, and A. A. Keller. 2012. Clay particles destabilize engineered nanoparticles in aqueous environments. *Environmental Science & Technology* 46 (14):7520–7526.

Zhou, Q., W. Liu, Y. Long, C. Sun, and G. Jiang. 2015. Toxicological effects and mechanisms of silver nanoparticles. In *Silver Nanoparticles in the Environment*, edited by J. Liu and G. Jiang, 109–138. Berlin Heidelberg: Springer-Verlag.

Zhu, M., G. Nie, H. Meng, and T. Xia. 2012. Physicochemical properties determine nanomaterial cellular uptake, transport, and fate. *Accounts of Chemical Research* 46 (3):622–631.

Zou, X., P. Li, Q. Huang, and H. Zhang. 2016. The different response mechanisms of *Wolffia globosa*: Light-induced silver nanoparticle toxicity. *Aquatic Toxicology* 176:97–105.

Zouzelka, R., P. Cihakova, J. R. Ambrozova, and J. Rathousky. 2016. Combined biocidal action of silver nanoparticles and ions against Chlorococcales (*Scenedesmus quadricauda*, *Chlorella vulgaris*) and filamentous algae (Klebsormidium sp.). *Environmental Science and Pollution Research* 23 (9):8317–8326.

Zuykov, M., É. Pelletier, and S. Demers. 2011. Colloidal complexed silver and silver nanoparticles in extrapallial fluid of *Mytilus edulis*. *Marine Environmental Research* 71 (1):17–21.

10

Nanotoxicity on Human and Plant Pathogenic Microbes and Aquatic Organisms

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10.1 Introduction

Nanotechnology industry has gained considerable attention in recent times leading to a great diversity of nanoproducts in the market. Inadequate information over nanoparticle producers and actually produced quantities has limited the estimation of the global production of nanoparticles. The estimation of nanoparticle exactly present in the environment is a big challenge which is further complicated by the generation of natural nanoparticles in the environment. A rough estimate is that the global annual production of nanoparticles will be 1000–10,000 t compared to several hundred tons in 2001. The growth in nanotechnology industry has also raised several concerns regarding its fate in biological systems and impact on environment. A separate branch of science concerning nanoparticle ecotoxicology has emerged to evaluate the hazards of nanoparticles to humans, plants, and microbes (Oberdorster et al. 2005). Some nanoparticles have been approved by the Food and Drug Administration for commercial applications, yet many other nanoparticles have been reported to accumulate and cause mild to severe nanotoxicity. Heavy metals, pesticides, and ultrafine particles are known to accumulate in ecosystem, yet the extrapolation of the toxicity of these bulk-sized particles against their nanocounterparts has not been straightforward. The problem relies in the fact that the physicochemical properties of these nanosized particles greatly vary with that those of bulk-sized particles. The present chapter is thus focused on the evaluation of nanoparticle toxicity with reference to its effects on humans, plant pathogenic microbes, and aquatic organisms. The chapter briefly focuses on the characterization of physicochemical properties of nanoparticles that are inherent to its toxicity. The chapter describes the mechanism of nanoparticle toxicity as applicable to humans, plant pathogenic microorganisms, and aquatic organisms through cited examples.

10.1.1 Nanoparticle Type, Source, and Exposure

The development of nanotechnology has resulted in a growing public debate on the toxicity and environmental impact of direct and indirect exposures to nanoparticles (Brayner 2008; Panda et al. 2011). Nanoparticles can be divided into two main classes: natural and anthropogenic nanoparticles. Natural nanoparticles have been present in environment long before the era of nanotechnology started. Examples of natural nanoparticles include soil colloids, carbon nanotubes, fullerenes, airborne nanocrystals of sea salts, and biogenic magnetite (Nowack and Bucheli 2007). Anthropogenic nanoparticles are further divided into two types: incidental and manufactured nanoparticles. Incidental nanoparticles are inadvertently produced in human-made processes (carbon black, nanotubes, and fullerenes) (Nowack and Bucheli 2007). Manufactured nanoparticles are those that are intentionally produced. Spills, transport, and disposal of waste from nanoparticle manufacturing units lead to contamination of soils with nanoparticles. Thereafter, these nanoparticles reach the water bodies through effluent discharge, seepage to groundwater, wind or water runoffs. Nanoparticle exposure to humans can thus occur through multiple routes such as dermal, pulmonary, injection, nasal, and oral. Anthropogenic activities may lead these nanoparticles to soil, where soil microorganisms, including plant pathogenic microorganisms, and plants are exposed to them. Aquatic system acts as sink

for these nanoparticles; hence, nanotoxicity evaluation on aquatic organisms is also required as they may get bioaccumulated in fish and thus enter the food chain causing further toxicity to humans and animals. Although a number of publications upon toxicity evaluation of nanoparticles have been carried out, the pace of safety evaluations has lagged far behind their development.

10.1.2 Characterization of Nanoparticles

Test nanoparticles must be characterized before toxicity assessment because the exact reason for their toxicity is not known. Thus, an extensive characterization for *shape*, *size*, *surface area*, *surface chemistry*, *surface charge*, *crystallinity*, *porosity*, *agglomeration state*, and *solubility* is recommended. *Dynamic light scattering* (DLS), *electron microscopy* (*scanning electron microscopy* [SEM] and *transmission electron microscopy* [TEM]), and *atomic force microscopy* (AFM) are the most widely used methods for studying the size distribution of nanoparticles. The *Brunauer–Emmett–Teller* (BET) method has been the method used for calculation of surface areas of solid nanoparticles through physical adsorption of gas molecules onto their surface. The BET surface represents the surface area which is freely available for adsorbing a liquid nitrogen monolayer; the amount of nitrogen released is measured upon vaporizing that layer. Thus, the primary particle diameter is then calculated from specific surface area and the density of the particles. The disadvantage of the method is that it assumes a monodispersed system of average-sized spheres neglecting the size distribution of the particles, which is a key parameter in size-dependent toxicity assessment (Weibel et al. 2005). Electron microscopy is the most widely used technique that directly measures *particle size*, *size distribution*, and *morphology*, the disadvantage being time-consuming approach which requires a sufficient number of particles to be analyzed before a sound statistical assessment is carried out. Also a dry form of the particle is measured, prepared through vacuum treatment, which somewhat alters its properties (Powers et al. 2007). An *atomic force microscope* in comparison is a cost-effective instrument with many advantages in nanoparticle characterization. It uses a cantilever with a very thin probe that oscillates over the surface of the sample offering three-dimensional visualization with vertical resolutions of less than 0.1 nm and *X–Y* resolutions of around 1 nm. AFM is used to evaluate *size*, *surface texture*, *morphology*, and *roughness* (Gupta et al. 2005). AFM is advantageous over TEM for measurements are taken in ambient air and liquid dispersions compared to samples characterized in vacuum using the latter technique (Scalf and West 2006). DLS is a time-dependent method based on the scattering intensity of light produced by particles in Brownian motion. The *size* of the particle is measured using the *Stokes–Einstein relation*. The hydrodynamic size obtained by DLS is generally greater than the size measured by techniques, such as TEM and BET (Scalf and West 2006). In DLS measurements, particles tend to aggregate; hence, the method gives the size of clustered particles rather than individual ones. DLS has the advantage of measuring the hydrodynamic diameter under exposure conditions and thus gives an account of the nanoparticle stability with respect to time and medium. Murdock et al. (2008) used DLS to study the *in vitro* toxicity assessment of nanoparticles on the dispersion state, exposure medium, presence of serum, and the time between sample preparation and exposure. The *nanoparticle tracking and analysis* (NTA) method of *particle size distribution* is based on the Brownian motion of nanoparticles. It allows the

visualization of individual nanoparticles with simultaneous analysis of its Brownian motion. The particle size distribution can be obtained with higher resolution. It gives a better understanding of nanoparticle aggregation than DLS while avoiding any intensity bias toward large particles. NTA can thus be used to identify and characterize nanoparticle aggregates/agglomerates (Montes-Burgos et al. 2010). Auger electron spectroscopy, X-ray photoelectron spectroscopy, secondary ion mass spectroscopy, AFM, and scanning tunneling microscopy are some analytical methods which provide information about *elemental composition*, *topography*, and *surface structure* (Baer et al. 2010).

10.1.3 Uptake and Internalization of Nanoparticles in Live Cells

To assess nanoparticle toxicity, it is essential to understand the mechanism of uptake of nanoparticles and its localization within the nanoparticles. Endocytosis and phagocytosis are the two important mechanisms that exist in cells for the uptake of nutrients from their surroundings. An endocytic pathway has been suggested for the uptake and internalization of nanoparticles within live cells (Garnett and Kallinteri et al. 2006; Yacobi et al. 2010; Greulich et al. 2011). Phagocytosis may then play an important role in the distribution of nanoparticles to different part of an organism. Nanoparticle interaction with biological system that is responsible for observed toxicity can be either physical or chemical. Physical factors governing observed nanotoxicity includes size and surface properties. The physical mechanism of nanoparticle toxicity involves interaction with the membrane leading to its disruption and thus facilitating uptake of nanoparticle and transportation to cellular organelles (Navarro et al. 2008; Leroueil et al. 2008; Hussain et al. 2005; Ovrevik et al. 2004). Another physical mechanism of nanotoxicity involves interaction with macromolecules affecting protein folding, aggregation, and fibrillation (Hauck et al. 2008; Chen and von Mikecz 2005). The chemical mechanism of nanoparticle toxicity involves production of reactive oxygen species (ROS); disruption of membrane potential; and release and dissolution of toxic ions, oxidative stress, and surfactant properties (Nel et al. 2006; Auffan et al. 2008; Xia et al. 2008; Kamat et al. 2000; Cottingham et al. 2002).

Several techniques have been used to study interaction and uptake of nanoparticles with cellular systems. TEM has been used to study the cellular internalization of nanoparticles. High-resolution TEM provides information on membrane invaginations, vesicle formation, and localization of nanoparticles (Motskin et al. 2009). It helps in understanding the ultrastructural changes occurring in cells after nanoparticle uptake (Song et al. 2010). However, TEM is only a qualitative tool for assessing nanoparticle uptake and is usually confined to imaging a few cells due to the complicated sample preparation and image analysis involved. Backscattered electron images from SEM are used to visualize high-energy electrons that were reflected or backscattered from the specimen after elastic scattering interactions with sample atoms (Pelka et al. 2009). This enables the detection of bright nanoparticles against the cellular dark background. Nanoparticle uptake has been confirmed through elemental analysis of the samples using *energy-dispersive X-ray spectroscopy*, *inductively coupled plasma mass spectroscopy*, and energy filtered TEM (Kuhnel et al. 2009; Dubey et al. 2015; Porter et al. 2007; Tang et al. 2009). *Flow cytometry* is a simple, easy, sensitive, cost-effective, and noninvasive approach used to study nanoparticle uptake in

mammalian cells (Xu et al. 2009). The drawback of this technique is that it cannot be used to localize or study the fate of nanoparticles inside the cells.

10.1.4 Effect of Nanoparticles on Toxicity Assays

Nanoparticles display several unique physicochemical properties, which interfere with normal test systems, and thus, traditional toxicity assays cannot be used as such (Kroll et al. 2009). Single-walled carbon nanotubes (SWCNTs) have been reported to interact with a variety of indicator dyes commonly used in cytotoxicity assays, e.g., 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide (MTT), Coomassie blue, alamar blue, and neutral red assay. The nanotubes interact with formazan crystals and stabilize their chemical structure preventing their solubilization. However, Monteiro-Riviere et al. (2009) suggested that carbon black alone can interact with the dye to cleave tetrazolium ring and cause a false-positive reaction. This interference with the MTT assay has also been reported for silica and ultrafine superparamagnetic iron oxide nanoparticles (Doak et al. 2009). Nanomaterials were also reported to interfere with annexin V/propidium iodide binding due to high adsorption capacities (Monteiro-Riviere et al. 2009). Doak et al. (2009) used a cell-free system to demonstrate the interference of fluorescence emission of dichlorofluorescein in dextran-coated iron oxide nanoparticles. Optical properties of nanoparticles also change the results as was reported with sodium titanate nanoparticles (Davis et al. 2007).

10.1.4.1 Nanoparticle Toxicity to Humans

The interaction of nanoparticles with living organisms especially humans have gained increasing importance and have become a significant area of research. There has been a growing concern regarding the ill effects it may cause on human health with the increase in its production and exposure. Nanotoxicology studies have revealed that certain nanomaterials, previously considered safe for human use are now known to exert adverse effects on human health. Studies have implicated that none of the particles are inert and can exert toxicity to exposed cells even at low concentration (Ferin 2004). Silver is one such element whose antimicrobial actions are well documented, yet it exerts higher toxicity compared to asbestos to human cells (Soto et al. 2005). Human beings' exposure to nanoparticles has been implicated with reduced macrophage clearance, bioaccumulation, increased proliferation of epithelial cells, tumor formation, oxidative stress, inflammation, fibrosis, and emphysema (Ferin 2004; Borm et al. 2004; Oberdorster et al. 1994; Dasenbrock et al. 1996; Driscoll et al. 1996; Nikula et al. 1995). Nanoparticles can directly interact and damage the DNA of the cell upon penetrating it or may damage the DNA through the production of ROS and may thus result in the development of cancer (Karlsson 2010; Alkilany and Murphy 2010; Shukla et al. 2011; Toyokuni 1998). Several nanoparticles have been reported to exhibit cytotoxicity to human cells (Kang et al. 2012; Sharma et al. 2011; Mittal et al. 2011; Kumar et al. 2011). Hence, a comprehensive evaluation of nanoparticle toxicity on human health is essentially required. Both *in vitro* and *in vivo* assays have been employed for the same. *In vitro* assays that are cheaper, ethically acceptable, and devoid of systemic variations are more often employed on tissue specific

cell line to conclude on the effects of these nanoparticles. However, *in vivo* effects of these nanoparticles involve highly complex and codependent pathways that would be impossible to study through a single cell-based *in vitro* assay.

10.2 *In Vivo* Nanoparticle Toxicology: Impact on Human Health

In vivo studies of nanoparticle toxicity have been carried out to understand their complex behavior in all intact organs and physiological mechanisms. It has been difficult to accurately quantify the internalization and distribution of nanoparticles *in vivo* in nanoparticle-exposed biological organisms. Hence, living intact organisms are preferred for the assessment of the dynamic trafficking of nanosized particles coupled with modern analytical tools of biotechnology such as microfluidics and microelectrochemistry. *In vivo* assays are generally carried out in model organisms such as mice, rats, rabbits, or fish. These animal models are the most representative system for predicting the impact of nanoparticles on human health. The selection of suitable model organism and route of exposure is critical for the evaluation of nanotoxicity which provides us with important data on alteration in serum chemistry and tissue morphology along with tissue specificity or overall distribution of nanoparticles upon their exposure. *In vivo* assays are advantageous with the experimental setup to evaluate uptake mechanisms, distribution, clearance, and toxicity mechanisms of these nanoparticles (Seaton et al. 2010). Examples of engineered nanoparticles used in industrial processes include titanium dioxide (Mueller and Nowack. 2008), zinc oxide (Osmond and McCall 2010), silver (Ahamed et al. 2010), silicon dioxide (Napierska et al. 2010), cerium dioxide (Park et al. 2008), carbon nanotubes (Aitken et al. 2006), and gold nanoparticles (Sperling et al. 2008). Nanoparticle toxicity studies have been carried out on such industrial nanoparticles along with studies on nanodrugs to evaluate the associated health hazards to industrial workers and subpopulation that are exposed to them. However, these studies face many challenges to aptly assess important parameters of nanoparticle toxicity following exposure and have also been criticized on ethical grounds. The observed toxicity of nanoparticle depends on the pharmacokinetics (absorption, distribution, metabolism, excretion) and pharmacodynamic behavior. The toxicity effects of nanoparticles on human health have been studied in context with the route of nanoparticle exposure and its translocation to different organs. Humans can be exposed to natural or human-made nanoparticles through a variety of routes including inhalation, oral, dermal, or injection. Nanoparticles can be absorbed via olfactory bulb following inhalation and are then translocated to the heart, liver, and kidney through circulating body fluids (Takenaka et al. 2001). Nanoparticles owing to their small size have been known to cross the blood–brain barrier as well as the blood–placenta barrier. Nanoparticles are also widely used in agriculture for plant protection and, despite proper washing and rinsing of these fruits, may lead to oral administration and absorption through the gastrointestinal (GI) tract. Nanoparticle residues have been reported to be present in liver, spleen, heart, and brain tissues through systemic circulation following the digestion and GI tract absorption of orally administered nanoparticles. Table 10.1 summarizes the adverse health effects of exposure to different nanoparticles on human beings.

TABLE 10.1

Nanoparticle Toxicity to Humans and Associated Health Hazards

Nanoparticles	Human Health Hazard	References
Airborne nanoparticles such as zirconium dioxide, iron oxide, copper oxide, zinc oxide, aluminium oxide, silica, asbestos	*Neurodegenerative diseases* Parkinson disease, Alzheimer disease	Liu et al. 2006; Campbell et al. 2005; Quintana et al. 2006; Donaldson et al. 2005; Antonini et al. 2006
	Cardiovascular diseases Anemia	Donaldson and Stone 2003; Schulz et al. 2005
	Pancreatitis	Riediker et al. 2004
	Rheumatoid arthritis; scleroderma; systemic lupus erythematosus	Fubini and Hubbard 2003; Noonan et al. 2006; Pfau et al. 2005
Dermally absorbed nanoparticles in cosmetics: carbon nanotubes, titanium dioxide, silver nanoparticles	Kaposi's sarcoma; podoconiosis	Montella et al. 1997; Rehn et al. 2003
	Pulmonary inflammation	Oberdorster et al. 1992
	Cytotoxicity to fibroblast cells and keratinocytes	Dunn and Edwards 2004; Poon and Burd 2004
Orally ingested nanoparticles of zirconium, calcium, silicon, gypsum, carbon, ceramic filosilicates, silver, titanium dioxide, aluminium silicates	Colon cancer	Gatti 2004
	Crohn's disease	Lomer et al. 2002; Powell et al. 1996
Injected polystyrene nanoparticles	Thrombosis	Hoet et al. 2004; Vermylen et al. 2005; Nemmar et al. 2002b
Gold nanoparticles	Fetus nanotoxicity	Myllynen et al. 2008
Fullerenes	Fetus nanotoxicity	Tsuchiya et al. 1996

10.2.1 Nanoparticle Toxicity following Respiratory Exposure

Airborne nanoparticles enter the human body through inhalation, and despite the protective mucociliary escalation, it is able to translocate via endocytosis, not only to the liver, spleen, heart, and other body organs but also to the central nervous system via the olfactory bulb after crossing the blood–brain barrier (Yacobi et al. 2010; Donaldson et al. 2002; Geiser 2010). Airborne nanoparticles can enter the lymphatic circulation and can hamper the immune response by causing toxicity to immune cells (Lucarelli et al. 2004). Inhaled nanoparticles such as zirconium dioxide alter the reactivity of the macrophages to infecting antigens by hyperactivating these phagocytic cells leading to severe inflammation (Lucarelli et al. 2004). Contrary to the action of zirconium dioxide which increases the expression of pathogen receptors (bacterial or viral), many nanoparticles such as titanium dioxide and silicon dioxide repress the expression of these receptors leading to low immune response. Chronic inflammation via ROS production and aggravated phagocytosis has been reported in lungs and other organs (Oberdorster 2002; Donaldson et al. 2010). These result in disease conditions that are often associated with unregulated or impaired basic cellular processes such as proliferation, metabolism, and apoptosis.

Neurodegenrative diseases such as Parkinson and Alzheimer diseases are a result of oxidative damage of macromolecules caused by the bioaccumulation of iron and

few other metals such as copper, zinc, and aluminum (Liu et al. 2006; Campbell et al. 2005). Brain cells accumulate iron as it is necessary for neuronal function yet the increased concentration of iron is toxic to cells. Inhaled metal oxides of size less than 50 nm have been reported to cross the blood brain barrier and thus accumulate in brain cells leading to oxidative damage and breakdown of myelin coat that result in neurodegenerative diseases (Quintana et al. 2006; Donaldson et al. 2005). *In vivo* studies of nasal and brain tissue in canines upon exposure to inhaled air pollutants and autopsy report of human brain tissues provide pathological evidences similar to that observed with Alzheimer disease (Peters et al. 2006; Calderon-Garciduenas et al. 2003). Alzehimer disease has also been associated with the uptake of aluminum nanoparticles (Antonini et al. 2006). Conclusive studies of nanoparticle toxicity in neurodegenerative diseases has been hindered due to the evolving nature of neurological diseases and the associated factors such as age, genetics, stress, associated memory loss about the nature, and duration of exposed toxicant (Antonini et al. 2006). Nanoparticle induced protein misfolding, and fibrillation results in damage to brain tissues (Linse et al. 2007; Oberdorster et al. 2009). *Cardiovascular diseases* are suggested to be the result of pulmonary inflammation that triggered the release of a number of cytokines following the inhalation of nanoparticulate material (Donaldson and Stone 2003; Schulz et al. 2005). Furthermore, biodistribution studies have demonstrated the translocation of nanoparticles following lymphatic vessels to systemic blood circulation that can directly lead to the observed disease conditions (Nemmar et al. 2001; Schwartz and Morris 1995). An example of such mechanism is nonendocytic and nonphagocytic uptake of inhaled nanoparticles by red blood cells that hampers the oxygen transportation resulting in anemia (Schulz et al. 2005).

The following are the common health hazards associated with inhaled nanoparticles:

- Uncontrolled proliferation and suppressed apoptosis, leading to cancer
- Uncontrolled apoptosis in premature cells, leading to neurodegenerative diseases (Peters 2005; Antonini et al. 2006)
- Production of ROS, inflammation, oxidative stress, and damage to macromolecules, leading to several diseases such as cardiovascular disease and pancreatitis (Riediker et al. 2004)
- Autoimmune diseases such as rheumatoid arthritis, scleroderma, and systemic lupus erythematosus, which have been associated with severe inflammation reaction caused by silica and asbestos nanoparticles (Fubini and Hubbard 2003; Noonan et al. 2006; Pfau et al. 2005)

10.2.2 Nanoparticle Toxicity following Dermal Exposure

Dermal exposure to nanoparticles is routinely observed with industrial workers, which may cause occupational hazards. Nanoparticles used in consumer products such as cosmetics can gain excess to human body through dermal exposure (Crosera et al. 2009). Nanoparticles such as fullerenes and quantum dots have been reported to exhibit transdermal absorption, whereas data on nanoparticle such as titanium dioxide are still inconclusive (Sperling et al. 2008; Stern and McNeil 2008). Studies have reported the internalization of carbon nanotubes (multiwalled carbon nanotubes [MWCNTs]) by human epidermal keratinocytes (Monteiro-Riviere et al. 2005).

Diseases such as Kaposi's sarcoma and podoconiosis have been associated with the lymphatic uptake of nanoparticles following dermal route of exposure (Montella et al. 1997). Titanium dioxide nanoparticles have been widely used in cosmetics and have thus been evaluated for its toxicity. These nanoparticles are known to reflect and scatter ultraviolet (UV) rays, and they can absorb significant UV radiation. However, in aqueous media, these nanoparticles have been reported to contribute in the production of ROS following UV irradiation that can cause significant damage to cellular macromolecules (Gurr et al. 2005; Dunford et al. 1997). Although inflammatory effects and genotoxicity of these nanoparticles have not been observed in many studies in the absence of UV radiation, few other *in vivo* studies have resulted in pulmonary inflammation in rats (Rehn et al. 2003; Oberdorster et al. 1992). Silver nanoparticles have been used in biomedical applications for their antibacterial and anti-inflammatory effects, yet they also have been implicated with cytotoxicity to fibroblast cells and keratinocytes (Dunn and Edwards-Jones 2004; Poon and Burd 2004).

10.2.3 Nanoparticle Toxicity following Oral Exposure

Nanoparticles used in food packaging and processing and as additives are taken up via caveole or clathrin-mediated endocytic pathway and absorbed through GI tract (Powell et al. 2010). The oral administration of nanoparticles resulting in the presence of nanoparticles in GI tract for absorption and biodistribution may lead to several disorder and diseases. It has been observed that the GI barrier may not be effective against particle size lower than 20 μm aiding nanoparticle absorption and toxicity. However, the observed nanoparticle toxicity in GI tract is somewhat reduced owing to the presence and interaction of complex mixture of different ingested particles and released gut enzymes (Hoet et al. 2004). This is in correlation with the *in vitro* toxicity studies of nanoparticles wherein reduced cytotoxicity is observed in medium with high fetal bovine serum concentration. Colon cancer, ulcerative colitis, and Crohn's disease have been associated with nanoparticle toxicity. Nanoparticles have been associated with colon cancer as they have been routinely observed in colon tissues of cancer patient as against to their absence in healthy individuals. Bulk size zirconium, calcium, silicon, gypsum, carbon, ceramic filosilicates, silver, and stainless steel have been present in colon mucosa and are not considered toxic; however, their nanosized counterparts cause cytotoxicity that may lead to colon cancer (Gatti 2004). Crohn's disease have also been associated with the uptake and increased presence of nanoparticles (Lomer et al. 2002). Nanoparticles such as titanium dioxide (100–200 nm), environmental silicates (100–700 nm), and aluminum silicates (100–400 nm) were reported to be taken up by macrophages in local lymphatic tissues of human gut and result in Crohn's disease (Lomer et al. 2002; Powell et al. 1996). Abnormal response including inflammation following the consumption of food additives with dietary nanoparticles have been suggested as the causative factor for the disease (Lomer et al. 2004). However, the end response to exogenous nanoparticle and its association with Crohn's disease may vary in different subjects owing to the genetic predisposition (Lomer et al. 2002).

10.2.4 Nanoparticle Toxicity following Injection

Nanoparticles are found in blood circulation following intravenous injection or during systemic circulation and biodistribution. These nanoparticles can then be translocated

to reticuloendothelial system and may thus exert disease conditions to the cells and tissues involved. Limited studies have been reported over the adverse health effects of nanoparticle exposure to organs such as the liver, spleen, or kidney. Yet conclusive biodistribution studies report the translocation of nanoparticles to these organs, and it can be presumed that they may lead to liver and kidney diseases. The size and charge of nanoparticles have been the governing factor in the adverse health effects caused by injected nanoparticles. The size and coating of nanoparticles governs its protein coating and hypersensitivity reaction owing to the opsonization of nanoparticles because complementation pathway has been indicated (Reijnders 2006). Nanoparticles in blood circulation have been associated with thrombosis (Nemmar et al. 2002a,b). Injected polystyrene and diesel exhaust particles of size below 60 nm exhibit size-dependent rapid clotting of blood in arteries and veins within an hour (Hoet et al. 2004; Vermylen et al. 2005; Nemmar et al. 2002b). Few studies are available which have shown different kidney and liver diseases of unknown origin being associated with the presence of nanoparticles in these organs (Gatti and Rivasi 2002; Gatti et al. 2004). Patients suffering from liver and kidney diseases have been correlated to the presence of particle debris in the organs of healthy patients are suggestive of the nanoparticle associated cytotoxicity to liver and kidney cells. The nanotoxicity may lead to the formation of particle debris resulting in disease condition ranging from fever to swelling in the organs or even result in renal failure (Gatti and Rivasi 2002; Ballestri et al. 2001). Gold nanoparticles entering systemic blood circulation have been found to exert fetus nanotoxicity (Myllynen et al. 2008) as they cross the blood–placenta barrier. Fullerenes were also found to be lethal to mouse embryos (Tsuchiya et al. 1996).

10.3 *In Vitro* Study of Nanoparticle Toxicity: Cellular Mechanisms

In vitro culture systems have been increasingly used in toxicology research for evaluating effects of various chemicals, pesticides, industrial wastes, and nanoparticles. Animal cell culture has substantially grown as an essential *in vitro* biological system in cell and molecular biology in recent years. Toxicological information at cellular and molecular levels can be efficiently acquired with cell cultures (Bols et al. 2005), and thus, they have been extensively utilized to study the toxicity of nanoparticles (Buzea et al. 2007; Segner et al. 1998). However, few studies have suggested against the *in vitro* screening stating that these do not reflect the original effects of nanosized materials (Warheit et al. 2009). In comparison to *in vivo* assays, *in vitro* toxicity tests are faster, convenient, less expensive, and devoid of any ethical issues. The poor dispersion of nanoparticles during the *in vivo* exposure of nanoparticles negatively affects its biological distribution and activity leading to misleading results (Buford et al. 2007). Hence, *in vitro* toxicity tests have been the first choice for most researchers working with nanomaterials. Although, *in vitro* assays may not completely replace animal testing, it could help ensure that live specimens are used when it is absolutely necessary. The risk assessment of different nanomaterials being in early stages, most of the nanotoxicity studies have therefore been carried out using classical *in vitro* toxicity assays.

Nanoparticles are known to produce ROS that can directly impact the integrity of cell through damage to macromolecules, including processes such as DNA damage,

lipid peroxidation, and protein carbonylation (Unfried et al. 2007; Li et al. 2008). The production of ROS such as hydroxyl ions and superoxide anions is considered as the most important mechanism for the observed toxicity which leads to inflammation, oxidative stress, and cell death or apoptosis (Lewinski et al. 2008; Singh et al. 2010). Proteins are the building block of life by functioning as enzymes, signaling molecules, or structural components of the cell. Damage to proteins disrupts vital cellular processes. Nanoparticles exhibit similar size and magnitude to that of a protein molecule and are able to oxidize the side chain of polypeptide chain to bring about protein carbonylation. Nanoparticle-induced oxidation of protein alters its native conformation resulting in altered or destroyed function. Furthermore, the active conformation of protein may also be altered due to the protein fibrillation and misfolding where nanoparticles may interact with polypeptide chains exhibiting chaperone-like activity (Takahashi et al. 2011; Ishii et al. 2003; Akiyoshi et al. 1999; Wu et al. 2008; Wagner et al. 2010; Aili et al. 2008). Misfolded proteins may disrupt the cell signaling process and have thus been associated with neurodegenerative diseases (Bucciantini et al. 2002). DNA contains the hereditary information, and earliest *in vitro* studies were focused on understanding the interaction of nanoparticles on it. Also, the interaction of nanoparticles with DNA gained further importance with the conclusive reports of localization of uptaken nanoparticles in nucleus of cell. Genotoxicity studies of nanoparticles have been carried out and have resulted in both reversible DNA damage (Comet assay) and irreversible DNA damage (micronucleus test) (Barnes et al. 2008; Karlsson et al. 2010). Nanoparticles are thought to directly intercalate with the DNA or through electrochemical interaction (Xie et al. 2009; Mehrabi and Wilson 2007). The production of ROS and release of free ions play a key role in DNA damage. Apart from direct intercalation or the physical and/or electrochemical interaction with nanoparticles, ROS is again believed to play a key role in observed gentoxicity for which the nanoparticles even need not necessarily enter the nucleus (Xie et al. 2009; Mehrabi and Wilson 2007; Bhabra et al. 2009; Myllynen 2009). ROS triggers the upregulation of transcription factors such as NF-κB or AP-1 that affect proinflammatory responses in cell. A number of enzymatic and nonenzymatic responses against the nanoparticle action are affected within the cell. These biological responses can occur even before the nanoparticles are taken up or while the uptake of nanoparticle occurs. Frustrated phagocytosis is one such process that was observed with MWCNTs when cells fail to engulf the nanoparticles.

10.3.1 *In Vitro* Studies of Nanoparticle Toxicity

A large number of studies have been conducted to explain the effect of different nanoparticles on different cell culture-based systems. *In vitro* studies have revealed that nanomaterial can damage membranes through the production of ROS (Rodea-Palomares et al. 2011) or by changing its stability and morphology (Ginzburg and Balijepalli 2007) in a size- and dose-dependent manner. Studies with carbon nanotubes (Zhu et al. 2006a) and fullerenes (Foley et al. 2002) indicate that mitochondria are the major site of their attack that disrupts its function leading to apoptosis. Other nanoparticles such as titanium dioxide, carbon nanotubes, silver, and polysterene also exhibit the ability to induce apoptosis by targeting mitochondria (Hussain et al. 2005; Xia et al. 2006; Jia et al. 2005). Another organelle that is mostly targeted by

nanoparticle is lysosomes as cells try to digest or exocytose the internalized nanoparticles (Greulich et al. 2011; Al-Rawi et al. 2011). A number of studies have provided the evidence of nanoparticle uptake and localization within the nucleus (Williams et al. 2009; Nabiev et al. 2007; Hoshino et al. 2004). Smaller nanoparticles may gain entry through nuclear pores, whereas large size nanoparticles are taken up by receptor mediated transport. Titanium dioxide nanoparticles have been demonstrated to produce ROS following UV irradiation that results in the *in vitro* suppression of tumor growth in cancerous human bladder cell system (Kubota et al. 1994). These nanoparticles are widely used in sunscreens but have been shown to cause genotoxicity through *in vitro* and *in vivo* experiments (Serpone et al. 2001; Dunford et al. 1997). The genotoxicity potential of titanium dioxide nanoparticles was also evaluated in human peripheral blood lymphocyte cells (Kang et al. 2008). Comet assay and micronucleus test resulted in dose-dependent genotoxicity of the nanoparticles. Similar results were also obtained by Wang et al. (2007) in human lymphoblastoid cell line (WIL2–NS). The phosphorylation of histone H2AX in adenocarcinoma epithelial cell line (A549) also complemented the observed gentoxicity potential of titanium dioxide. Zinc oxide nanoparticles are also widely used in cosmetics and were evaluated for their genotoxicity potential using human epidermal keratinocytes. Results showed significant dose-dependent cytotoxicity and genotoxicity (Sharma et al. 2011). Similar results for the cytotoxicity potential of zinc oxide nanoparticles were also demonstrated with human liver cells (Hep G2) using comet assay. Nanoparticles have also been reported to cause *in vitro* proinflammatory effects in human endothelial cells (Peters et al. 2004). Copper nanoparticles (CuNPs) owing to their redox sensitivity were employed to investigate the role played by these ions and their contribution toward ROS elicitation leading to toxic effects. The toxicity of silver nanoparticles were evaluated on human fibrosarcoma, HT-1080, and skin carcinoma, A431, cell lines which resulted in an increase in lipid peroxidation, and a decrease in activity of both enzymatic (superoxide dismutase) and nonenzymatic (reduced glutathione) antioxidant potential (Arora et al. 2008). Gold nanoparticles were also evaluated for oxidative stress parameters in human lung fibroblast cells which also resulted in significant levels of lipid peroxidation compared to control indicating the associated oxidative stress (Li et al. 2010). Nanoparticle-induced ROS-independent pathways were also reported to cause cellular toxicity. Zinc oxide nanoparticles were studied for ROS production, which did not contribute to toxicity (Fernandez-Cruz et al. 2013). There is still a doubt if oxidative stress associated with nanomaterials is the direct cause of cytotoxicity or a secondary effect of cellular damage. A coincubation strategy was used to challenge liver cells with high ROS levels following CuNP exposure while activating aryl hydrocarbon receptor (AhR) gene battery of cellular antioxidant defense using the ligand ß-naphthoflavone at the same time. Phase I cytochrome P450s, phase II detoxification enzymes (glutathione *S*-transferases), and antioxidants (glutathione [GSH]) resulted in complete reversal of oxidative damage and redox homeostasis, yet no reduction in cytotoxicity was observed. A nonoxidative stress-mediated cellular damage resulting from nanomaterials was implicated. Au nanoparticles have been reported to generate reactive nitrogen species in blood, which leads to oxidative stress (Jia et al. 2009). Nickel ferrite nanoparticles have also been shown to induce oxidative stress mediated apoptosis in human lung epithelial cells (Ahamed et al. 2011).

10.4 Nanoparticle Toxicity to Plant Pathogenic Microbes

Nanotechnologies have already emerged as an advantageous and safe technology in different fields such as healthcare, environmental technology, and food industry. Hitherto, their application in agriculture industry has not been yet utilized to its potential. However, nanotechnology research in crop improvement (production and protection) is gaining momentum worldwide. The development of nanotechnology in areas of agricultural biotechnology has led the way for efficient and sustainable agricultural practices, particularly by increasing crop protection against different plant pathogens (Nair and Kumar 2013). Nanotechnology approaches have been employed for several applications in agriculture that includes improvement in seed germination, increased plant growth, detection of plant pathogen, detection of pesticide/herbicide residues, and protection against plant pathogens (Khot et al. 2012). Plant pathogens result in a decrease of crop yield along with poor quality of plant products. Traditional approaches such as plant breeding, development, and application of new pesticides, proper sanitation, and integrated pest management are being used for crop improvement and plant protection. Nanotechnologies are emerging as advantageous tools to administer and control different fertilizers, herbicides, and pesticides in an effective and safe way (Rai and Ingle 2012). Different nanoparticles and biocomposite nanomaterials such as gold, silver, copper, zinc, magnetized iron, titanium, aluminum, and cerium have been successfully applied to derive products for application in environmental technology, food industry, water purification, medicine, and electronics (Bradley et al. 2011; Zambrano-Zaragoza et al. 2011; Lee et al. 2010; Qureshi et al. 2009). These nanoparticles have also been used in crop protection to enhance agricultural productivity (Emamifar et al. 2010; Sharon et al. 2010; Nair et al. 2010; Bouwmeester et al. 2009). However, the unique physiochemical properties of nanoparticles leading to the toxicity studies that warrant their unregulated application in consumer products prove advantageous in the field of plant protection. Nanoparticle-associated toxicity can be utilized against the variety of microbial plant pathogens. Antimicrobial properties of many nanoparticles is now well documented that can be used to increase the yield and quality of plant products by suppressing or preventing the growth of plant pathogens. In general, five different mechanisms of actions have been highlighted for the antimicrobial action of these nanoparticles. Metallic nanoparticles are known to interact with microbial membrane disrupting its function and permeability. Nanoparticles release toxic ions that disrupt membrane integrity and energy metabolism leading to cytotoxicity and death. Furthermore, these metallic nanoparticles also mediate the production of ROS that causes oxidative stress and cellular and DNA damage (Lemire et al. 2013; Zeng et al. 2007). Different studies with nanoparticles strongly suggest for their effective and eco-friendly application against insects, plant pathogens, and pests (Choudhury et al. 2010; Liu 2006). Table 10.2 summarizes the nanoparticles that exhibit potent nanotoxicity against the variety of plant pathogenic bacteria and fungal strains. In the following section, we will now focus on the application of nanoparticle toxicity as an advantageous tool for protection against a variety of plant pathogens.

TABLE 10.2

Nanoparticles with Potent Antimicrobial Activity against Plant Pathogenic Microbes

Nanoparticles	Plant Pathogenic Microbes	References
Gold	*E. coli, B. subtilis, A. tumefaciens*	Wang et al. 2012c
Copper oxide	*P. fluorescens, V. parahaemolyticus, A. hydrophila*	Liu et al. 2009
	Fusarium oxysporum	Liu et al. 2009
	A. solani	Dong and Yang 2011
	A. alternate	Saharan et al. 2013
Iron	*E. coli, B. subtilis, A. tumefaciens*	Wang et al. 2012c
Zinc and zinc titanate nanoparticles	*A. niger*	Ruffolo et al. 2010
	P. expansum	He et al. 2010
Sulfur	*A. niger*	Choudhury et al. 2010
Silicon	*M. grisea*	Liu et al. 2012a
Fullerene C60	*E. coli, B. subtilis, A. tumefaciens*	Wang et al. 2012c
Silver	*B. sorokiniana*	Kasprowicz et al. 2010
	M. grisea	Jo et al. 2009
	Candida spp.	Li et al. 2012
	T. mentagrophytes	Panacek et al. 2006 Min et al. 2009
	C. albicans, Trichoderma sp, *F. graminearum*	Dimkpa et al. 2013
	E. coli, B. subtilis, A. tumefaciens	Wang et al. 2012c
Chitosan-coated silver	*A. alternata, B. cinerea, C. lunata, M. phaseolina, R. solani, S. sclerotiorum*	Krishnaraj et al. 2012
Silver-silica	*Raffaelea* sp.	Woo et al. 2009
	S. cepivorum	Jung et al. 2010
	C. gloeosporioides	Aguilar-Mendez et al. 2010
	F. oxysporum	Musarrat et al. 2010; Kasprowicz et al. 2010
	F. culmorum	
	Botrytis spp., *Blumeria* spp., *Colletotrichum* spp., *Magnaporthe* spp., *Phytophthora* spp., *Pythium* spp., *Rhizoctonia* spp., *Sphaerotheca* spp.	O'Neill et al. 2003; Shankar et al. 2003; Yao et al. 2004
	B. cinerea	Oh et al. 2006
Silver sulfide	*A. niger*	Fateixa et al. 2009

10.4.1 Antibacterial Activity of Nanoparticles

Nanoparticles exert their antimicrobial action either by *interacting with microbial membranes* or by *oxidation of macromolecules*. Nanoparticles interact with microbial membranes and results in altered membrane permeability and damage. When they enter the bacterial cell, generate ROS leading to the oxidation of macromolecules affecting critical cellular processes that result in cell death (Marambio-Jones and Hoek 2010; Musee et al. 2011). *E. coli*, *S. aureus*, and *B. subtilis* have been utilized as model microorganisms to establish the antimicrobial properties of nanoparticles (Sondi and Salopek-Sondi 2004; Chou et al. 2005; Yoon et al. 2008; Huh and Kwon 2011). Several nanoparticles such as silver (Morones et al. 2005), SWCNTs

(Kang et al. 2007), fullerene C60 (Lyon et al. 2006), titanium dioxide (Zan et al. 2007) have shown significant antimicrobial properties against the monoculture of these model bacterial species. Several studies have been reported establishing the role of nanoparticles in protection against plant pathogens. Gold, silver, iron, and fullerene C60 nanoparticles were evaluated for their toxicity to pant pathogenic bacteria such as *E. coli*, *B. subtilis*, and *A. tumefaciens*. Silver nanoparticles exhibit potent bactericidal action on all the tested bacterial strains, and fullerene (C60) inhibited the growth of all the bacterial strains. However, with increased exposure time, the antibacterial activity of fullerene (C60) diminished. In contrast to them, gold and iron nanoparticles failed to demonstrate antimicrobial activity (Wang et al. 2012c). Surface charge and size are the governing factors of their antimicrobial potential (Wang et al. 2012c). Dose-dependent increase in the antibacterial activity of nano-copper montmorillonite was demonstrated by Liu et al. (2009). It showed potent bactericidal against strains of *P. fluorescens*, *V. parahaemolyticus*, and *A. hydrophila*. However, limited bactericidal efficiency of only about 25% was observed against *B. subtilis* and *L. acidophilus*. The antibacterial action was again dependent on dose and temperature (Liu et al. 2009). Nano-copper oxide nanoparticles also showed significant antibacterial activity against *Fusarium oxysporum* that causes root rot in pepper.

10.4.2 Antifungal Activity of Nanoparticles

Fungi cause the most serious diseases in plants and constitute the largest group of plant pathogenic microbes. Hence, protection against fungi is very much desired to enhance the yield and quality of plant products. Traditional approaches have been proven to be expensive and cause damage to our ecosystem wherein the application of nanoparticles to provide protection against these pathogens is more cost effective and eco-friendly. The nanotechnological approach has been focused to develop efficient antimicrobial compound and delivery system to overcome lack of synthetic fungicides. Nanoparticles made of zinc, silica, titanium, copper, etc., exhibit potent antifungal activity. Zinc oxide and zinc titanate nanoparticles showed strong growth inhibition of fungal plant pathogen *A. niger* (Ruffolo et al. 2010). These nanoparticles have also shown the inhibition of spore germination and suppression/deformation of fungal hyphae leading to death of *P. expansum* (He et al. 2010). The antifungal activity of sulfur nanoparticles was compared to elemental particle against *A. niger*, which showed increased growth inhibitory activity for nanosulfur (Choudhury et al. 2010). Copper nanoparticles coated with chitosan also exhibit high inhibition of fungal growth in pathogenic *A. alternate* (Saharan et al. 2013). Silicon has been reported to self-assemble into plants in form of biomineralized silicon dioxide imparting resistance against fungal pathogens (Wang et al. 2001). The inhibition of fungal hyphae growth on nanoparticle-treated rice varieties Nongda 18 (resistant variety) and Mongolian (susceptible variety) was compared that resulted in the growth inhibition of *M. grisea* fungal pathogen on both varieties compared with control groups. It established the efficiency of silicon nanoparticles in the protection of rice varities from *M. grisea* infection (Liu et al. 2012a). Further studies have reported a decrease in fungal disease occurrence by 31% in Nongda 18 and 51% in Mongolian rice variety and an increase in photosynthesis (Liu et al. 2012b). Nanoparticles of silica combined with silver (NSSs) have been evaluated against fungal pathogens that cause powdery mildew disease in pumpkin (Park et al. 2006). It showed antifungal activity

at a concentration, much lower (3 parts per million [ppm]) than the concentration that is harmless for beneficial bacteria (10 ppm). However, the controlled application of NSSs is suggested as it can kill all beneficial bacteria at a concentration of 100 ppm. These nanoparticles were also safe to use, as studies on cucumber and pansy leaves following its application did not result in phytotoxicity or chemical injury (Park et al. 2006). The antifungal activity of silica–silver nanoparticle was found to increase with decreasing size of nanoparticles as nanoparticles of size less than 5 nm can easily be transported through protoplasm membrane (Wainwright et al. 1986). In this combination, silver is thought to act as the disinfecting agent, whereas the silica plays the protective role by forming a physical barrier preventing recurrence of diseases (Kim et al. 2002). Silver–silica nanoparticle formulation is environmentally safe, cost–effective, and even beneficial to human health can be considered as a suitable alternative to costly fungicides that can offer protection to several other plant pathogens including *Botrytis* spp., *Blumeria* spp., *Colletotrichum* spp., *Magnaporthe* spp., *Phytophthora* spp., *Pythium* spp., *Rhizoctonia* spp., and *Sphaerotheca* spp. (O'Neill et al. 2003; Shankar et al. 2003; Yao et al. 2004). Mangnesium oxide nanoparticle also strongly interacts with microbial membrane that allows it to exert significant antimicrobial activity (Huang et al. 2005). Copper nanoparticles exhibit more toxicity against fungal pathogens compared to known fungicide bayistin (Kanhed et al. 2014). Nano-copper oxide nanoparticles have resulted in significant antimicrobial activity against *A. solani* that causes early blight disease in tomato (Dong and Yang 2011).

10.4.2.1 Silver Nanoparticle: Protection against Phytofungal Diseases

Silver nanoparticles have been shown to exhibit most potent antimicrobial activity and are suggested for the management of plant diseases (Roe et al. 2008; Kim et al. 2008; Oh et al. 2006). Silver nanoparticle toxicity has been evaluated against different clinical fungal isolates such as *Candida* spp. and *T. mentagrophytes* obtained from the American Type Culture Collection (Li et al. 2012; Panacek et al. 2006; Min et al. 2009). Silver nanoparticles have been also shown to exhibit fungal toxicity against 14 sheath blights causing fungal pathogens where it strongly inhibits the growth of fungal hyphae (Liu and Xu 2008). Nanosilver also strongly inhibited the spore germination of the nine test fungal pathogens. In case of *C. graminicola* fungal strain, the nanosilver inhibition of seed germination was even stronger than the inhibition of the fungal hyphae. However, linear dose and response relationship against *B. cinera* (kenaf), *S. rolfsii* (tobacco), and *P. digitatum* (citrus) was not observed (Liu and Xu 2008). Silver nanoparticles act against plant pathogens through a variety of mechanisms such as inhibition of mycelia growth, decrease in mycelia respiration, and increase in membrane permeability that result in the apoptosis of mycelium cells. Silver nanoparticles at a concentration of 200 mg/L are also effective in inhibiting the growth of ryegrass fungal pathogen (Jo et al. 2009). Similar growth inhibitory and disease preventive activity was observed against *C. albicans*, *Trichoderma* sp., and *F. graminearum* following nanosilver treatment (Dimkpa et al. 2013). Silver nanoparticle has also been reported to be effective against controlling the infection of *B. sorokiniana* and *M. grisea* (Kasprowicz et al. 2010; Jo et al. 2009). One hundred ppm of silver nanoparticles exhibited spore germination and growth of cucumber and pumpkin fungal pathogens (Lamsal et al. 2010). Silver nanoparticles

were also used in combination with other materials to provide enhanced antifungal activity. Silver silicon dioxide nanoparticles were reported to inhibit the growth of *B. cinerea* (Oh et al. 2006). Silver nanoparticles coupled with antifungal fluconazole exhibited increased antifungal activity in disk diffusion assay against fungal strains such as *P. herbarum*, *P. glomerata*, *C. albicans*, *F. semitectum*, and *Trichoderma* sp. (Gajbhiye et al. 2009). Silver sulfide nanoparticles inhibited *A. niger* growth (Fateixa et al. 2009). Many more research papers have been published establishing the antifungal activity of silver nanoparticles against plant pathogens such as ambrosia fungus *Raffaelea* sp., white rot-causing fungus *S. cepivorum* in onion, anthracnose causing *C. gloeosporioides* in fruits, *F. oxysporum*, and *F. culmorum* (Woo et al. 2009; Jung et al. 2010; Aguilar-Mendez et al. 2010; Musarrat et al. 2010; Kasprowicz et al. 2010). The surface modification of nanoparticles can increase the efficiency of these nanoparticles as was observed with chitosan coating of silver nanoparticles. Chitosan-coated silver nanoparticles showed strong and increased inhibitory activity in comparison to uncoated silver nanoparticles against a variety of plant pathogenic fungal strains including *A. alternata*, *B. cinerea*, *C. lunata*, *M. phaseolina*, *R. solani*, and *S. sclerotiorum* (Krishnaraj et al. 2012).

10.5 Nanoparticle Toxicity to Aquatic Organisms

The aquatic environment acts as a sink for a wide variety of natural and manufactured nanoparticles (Table 10.1). These nanoparticles can enter water bodies via bathing and sewage effluents (Daughton and Ternes 1999; Handy and Shaw 2007; Ternes et al. 2004) leading to increased chances of nanoparticle exposure to aquatic organisms. Research experiments carried out with aquatic organisms in liquid media containing nanoparticles resulted in a number of adverse effects (Lovern and Klaper 2006; Templeton et al. 2006; Roberts et al. 2007) that includes change in their physiological mechanism, abnormal behavioral patterns, respiratory and oxidative stresses, organ pathologies, decrease in fertility, and increase in their mortality. Nanotoxicity observed in aquatic organisms depends on its concentration and liquid media used for nanoparticle dispersion (Oberdorster et al. 2006; Zhu et al. 2006b). Nanoparticle toxicity has been compared to their bulk size particles on the basis of size, degree of aggregation, chemical composition, solubility, oxidation statu,s and cell type (Pujalte et al. 2011). *In vivo* studies of nanoparticle exposure results in higher sensitivity of these nanoparticles in aquatic organisms (Federici et al. 2007). Polyunsaturated fatty acids (PUFAs) are of utmost importance to aquatic organism as it helps in maintaining membrane fluidity at lower temperature (Monserrat et al. 2007). These PUFAs are more prone to oxidation, and thus, higher degree of peroxidation is observed in aquatic organisms compared to mammalian cells (Monserrat et al. 2007). Further nanotoxicity is caused due to the oxidation of proteins in the form of protein carbonyl contents. The oxidation of protein predominantly occurs via metal-catalyzed oxidation and secondarily via oxidized macromolecules produced by ROS, which could be a possible explanation for high protein damage observed in the nanoparticle-treated cells (Monserrat et al. 2007). Furthermore, the level of nonenzymatic antioxidant (tGSH) and activity of antioxidant enzymes such as superoxide dismutase (SOD) and catalase have been studied as biomarkers for antioxidant defense system in animals.

These are important components of cellular defense against oxidative stress. A change in tGSH level or antioxidant enzyme activity has been reported that is considered as an adaptive response of cells against nanoparticle induced oxidative stress.

10.5.1 Uptake of Nanoparticles by Aquatic Organisms

Research studies pertaining to the fate of nanoparticles in water bodies and their penetration into cells of aquatic organisms have been increasing in the past decade (Kuznetsova et al. 2014; Dubey et al. 2015; Taju et al. 2014). Nanoparticles such as titanium dioxide are released in water bodies through facade paints (Kaegi et al. 2008). These nanoparticles tend to aggregate in aqueous environment, and their poor solubility limits their actual uptake by cells (Maynard et al. 2004; Brant et al. 2005; Piotrovsky and Kiselev 2006). However, these nanoparticles can still exert sublethal toxicity due to indirect systemic toxic effects caused by the surface adhesion of nanoparticles rather than uptake into tissues, as was demonstrated by Federici et al. (2007). Fullerenes also exhibit low mobility in soil and aqueous media (Lecoanet and Weisner 2004; Brant et al. 2005), yet studies have reported their internalization into cellular environment (Oberdorster 2007; Jain et al. 2007).

10.5.1.1 Uptake Studies of Carbon Nanoparticles

Studies with aquatic invertebrates such as amoeba (*E. histolytica*), cladocerans (*D. magna*), copepods (*A. tenuiremis*), and infusoria (*T. pyriformis* and *S. mytilus*) have successfully demonstrated the uptake of carbon nanoparticles with food particles, yet their biodistribution into different organs is yet to be concluded (Zhu et al. 2009; Elias et al. 2007; Roberts et al. 2007; Zhu et al. 2006b,c; Oberdorster E. et al. 2006; Templeton et al. 2006). Aquatic oligochaete *L. variegatus* and terrestrial oligochate *E. foetida* were shown to uptake carbon nanotubes that were not detected in tissues other than GI tract (Petersen et al. 2008a,b). The uptake of nanoparticles through penetration into aquatic organisms depends on the structure and surface modifications of the nanoparticles. Fullerenes (C60) penetrated zebrafish embryos, whereas hydroxylated fullerenes and agglomerates of SWCNTs failed to penetrate them through the chorion (Isaacson et al. 2007; Cheng et al. 2007). Aquatic species such as *O. mykiss*, *A. mexicanum* larvae, and *X. laevis* tadpoles were demonstrated to take up nanoparticles through exposure to liquid media containing carbon nanotubes, which were found in the gills and gut (Smith et al. 2007; Mouchet et al. 2007, 2008). These nanoparticles may have been taken up via gills, which provide a thin entry route to nanoparticles, and are described to have toxic effects on these pulmonary targets (Nemmar et al. 2002c; BeruBe et al. 2007).

10.5.1.2 Uptake Studies of Metal-Based Nanoparticles

Studies with uptake of metallic nanoparticles demonstrated the uptake of copper (Griffit et al. 2007) and silver nanoparticles through the gills and chorion of zebra fish (Lee et al. 2007). Studies with metallic oxide nanoparticles such as aluminum oxide, titanium dioxide, and zinc oxide demonstrated the presence of these nanoparticles in the gut of daphnia following the 48-hour exposure of nanoparticle suspension (Zhu et

al. 2009). Titanium dioxide nanoparticles were found to accumulate in daphnia (Zhu et al. 2010), whereas no accumulation was observed in rainbow trout (Federici et al. 2007; Johnston et al. 2010). Titanium dioxide when injected into rainbow trout fish was biodistributed to different organs and were retained to over a period of 90 days (Scown et al. 2009). Similarly, titanium and tin were detected in different tissues of common carp (muscle and liver) and guppy (gills, spleen and gut), respectively, following the exposure to nanoparticle suspensions (Sun et al. 2007; Krysanov et al. 2009). Zebrafish exposed to cerium oxide nanoparticles resulted in its distribution to the liver, whereas exposure to zinc oxide nanoparticles failed to demonstrate the accumulation or biodistribution of nanoparticles (Johnston et al. 2010). Fluorescent quantum dots taken up by *D. magna* were shown to be distributed in different organs of daphnia (Ingle et al. 2008). Cadmium selenide quantum dots were also reported to penetrate into the cytoplasm and nuclei of African clawed frog blastomeres (Dubertret et al. 2002).

Hence, despite the contradictory studies, it is now well established that these nanoparticles can penetrate into the aquatic organisms and may thus exert toxicity. Recently, several papers have been published on nanomaterial toxicity to aquatic organisms using acute toxicity assays, but the results are quite ambiguous and contradictory. Various studies with engineered nanomaterials have focused on their effects on gene expression, cell proliferation, oxidative stress, DNA damage, fertility, and embryotoxicity of these nanoparticles (Klaine et al. 2008; Handy et al. 2008; Warheit et al. 2007). Nanoparticle toxicity to the exposed aquatic organisms is summarized in Table 10.3.

10.5.2 Nanoparticle Toxicity in Fish

Nanoparticles have been described to have toxic effects on pulmonary targets (Nemmar et al. 2002c; BeruBe et al. 2007). Gills are the respiratory organs of the fish and thus allows for studying pulmonary nanotoxicity (Dubey et al. 2015). Nanoparticle uptake leads to bioaccumulation and inflammation in gill and gut injury, even at low concentration (Federici et al. 2007). The gills and liver are the two major organs that have been studied to demonstrate nanoparticle toxicity to fish. Nanoparticles have been demonstrated to be present in blood and distributed to different organs such as liver, brain, and testis of medaka fish (Noack et al. 2000). Several *in vivo* and *in vitro* studies have demonstrated dose-dependent nanotoxicity to fish cells. Gill cell lines have been utilized for the evaluation of toxicity caused by aquatic pollutants, and good correlations with data observed from live fish studies have been reported (Dayeh et al. 2002). Studies of carbon nanoparticles (0.1–0.5 mg/L) on pulmonary targets in fish demonstrated dose-dependent respiratory pathologies and an aggressive behavioral pattern in rainbow trout fish (Smith et al. 2007). Significant correlation between *in vivo* and *in vitro* toxicities of silver nanoparticles to gill cell lines of *L. rohita* and *C. catla* has been reported (Taju et al. 2014). *In vivo* assays in zebra fish and rainbow trout have demonstrated an increase in lipid peroxidation in gill cells following exposure to titanium dioxide nanoparticles (Xiong et al. 2011; Federici et al. 2007). It was proposed that titanium dioxide nanoparticles in direct contact with gill cells generate ROS in presence of light (Federici et al. 2007). Dubey et al. (2015) developed a gill cell line from *W. attu* and demonstrated the internalization of titanium

TABLE 10.3

Nanoparticle Toxicity to Aquatic Organisms

Nanoparticle	Organism	Toxicity Mechanism	References
Carbon	*O. mykiss*	Respiratory pathologies, aggressive behavioral pattern	Smith et al. 2007
	F. serratus	Reproductive and developmental toxicity	Nielsen et al. 2008
Titanium dioxide	*D. rario, O. mykiss*	Lipid peroxidation in gill tissue	Xiong et al. 2011; Federici et al. 2007
	D. magna	Bioaccumulation, oxidative stress and marginal mortility reduced growth and reproduction	Ates et al. 2013; Zhu et al. 2010
Iron oxide	*D. rario* *T. pseudonana* *I. galban* *D. tertiolecta* *C. vulgaris*	Delayed embryo hatching, Deformed embryo Embryo mortality Growth inhibition Decrease in the concentration of chlorophyll a, carbon dioxide absorption and net photosynthetic rate	Xiaoxiao et al. 2012; Arturo et al. 2012; Xiaoxiao et al. 2012
Silver	*D. rario*	Larval abnormalities Increased embryo mortality	Lee et al. 2007
Zinc oxide	*P. subcapitata*	Oxidative stress	Franklin et al. 2007
Copper oxide	*S. plana*	Oxidative stress	Buffet et al. 2011
Gold citrate	*M. edulis*	Bioaccumulation of gold that resulted in oxidative stress	Tedesco et al. 2010
Cadmium quantum dots	*C. reinhardtii, P. subcapitata*	Membrane damage	Bouldin et al. 2008
	E. complanata	Lipid peroxidation, and genotoxicity in gills and digestive glands along with a reduction in hemocyte viability	Gagne et al. 2008
Fullerenes (C60)	*P. promelas, M. salmoides*	Lipid peroxidation and oxidative damage observed in gills, brain and liver tissue	Oberdorster et al. 2006; Oberdorster 2004; Zhu et al. 2006a
	D. magna	Reduce fertility output Altered behavior patterns Increased mortality	Oberdorster et al. 2006; Lovern et al. 2007; Lovern, Klaper, 2006
	A. tonsa	Cytotoxicity	Baun et al. 2008
	M. edulis	Release of lysozymes, oxidative stress, production of nitric oxide, respiratory burst hematocytes cytotoxicity	Canesi et al. 2012
	C. virginica	Lysosomal destabilization of digestive glands, abnormal development of oyster larvae	Canesi et al. 2012
Copper and silver	*D. magna*	Reduced growth and reproduction	Griffit et al. 2007

dioxide and zinc oxide nanoparticles in gill cells localized in cytoplasm and around nucleus. Exposure to fullerenes of fathead minnow, *P. promelas*, and largemouth bass, *M. salmoides*, resulted in lipid peroxidation and oxidative damage to cells of gills, brain, and liver (Oberdorster et al. 2006; Oberdorster 2004; Zhu et al. 2006a). Unmodified fullerenes at a low concentration failed to show significant toxicity, when aqueous dispersion was prepared through sonication. However, modified fullerenes (tetrahydrofuran) were found to alter gene expression and exert acute toxic compared to unmodified fullerenes (Henry et al. 2007; Usenko et al. 2007, 2008; Isaacson et al. 2007; Zhu X. et al. 2007). Copper nanoparticles were demonstrated to be cytotoxic to zebrafish gill cells.

Embryotoxicity to zebrafish was demonstrated with iron oxide nanoparticles that showed delayed embryo hatching, deformed embryo, and embryo mortality (Xiaoxiao et al. 2012). Aggregated copper nanoparticles adhered to the surface of fish embryos altering the surface properties and interfering with chorionic enzymes that delayed embryo hatching. Silver nanoparticles were also demonstrated to exhibit embryo toxicity to zebrafish (Lee et al. 2007). Dose-dependent increase in larval abnormalities and increase in embryo mortality were observed (Lee et al. 2007). Nanosized oxides of metal (aluminum, zinc and titanium) were also studied for embryo toxicity in zebrafish in which zinc oxide nanoparticles were toxic to fish embryos (Zhu et al. 2008). These nanoparticles are known to exert toxicity to fish physiology. Titanium dioxide nanoparticles failed to demonstrate significant toxicity in many studies, yet sublethal effects such as biochemical changes, organ pathology, and respiratory abnormalities were reported in rainbow trout (Federici et al. 2007). Hydrated tin dioxide nanoparticles also did not result in significant toxicity to guppies for short exposure times (Krysanov et al. 2009). G4 polyaminoamide nanoparticles at sublethal concentrations were able to demonstrate growth inhibition, cytotoxicity, and embryo toxicity in zebrafish, whereas dendrimers with carboxyl functional group failed to demonstrate nanotoxicity in *D. rario* (King et al. 2007).

10.5.3 Nanoparticle Toxicity in Phytoplanktons

Phytoplanktons are known as the primary producers in the aquatic environment. The bioaccumulation of nanoparticles leading to their toxic effects on phytoplanktons would thus affect the entire aquatic ecosystem. It has been observed that nanoparticles can be easily taken up by these phytoplanktons along with nutrients from the aquatic environments depending on the shape, size, and morphology of these nanoparticles (Scarano and Morelli 2003). Nanoparticles can exist in the form of stable nanoparticle crystals on the surface of phytoplanktons and may thus be taken up by these organisms under suitable conditions. Formation of metallic nanocrystals has been observed in marine phytoplankton exposed to cadmium (Scarano and Morelli 2003). Phytoplanktons, such as *T. pseudonana*. *I galban*, and *D. tertiolecta*, are susceptible to iron nanotoxicity where it was reported to inhibit the growth of these marine phytoplankton (Keller et al. 2012). The effects are alleviated with the bioaccumulation of these nanoparticles that can enter the food chain of aquatic ecosystem and can cause further nanotoxicity to consumer organisms (Keller et al. 2012). Nanoparticle toxicity studies with phototrophic microbes have reported that the nanoparticles can disrupt the energy metabolism. Algae are photoautotrophic organisms that convert solar

energy to chemical energy by converting glucose to carbon dioxide while releasing oxygen to the atmosphere. Chlorophylls a, b, and c are the major components of the phytoplankton photosynthetic apparatus. Iron oxide nanoparticles resulted in dose-dependent decrease in the concentration of chlorophyll a, carbon dioxide absorption, and net photosynthetic rate with increasing concentration of the nanoparticle oxide in *C. vulgaris* (Xiaoxiao et al. 2012). Characteristic markers of oxidative stress such as malondialdehyde (MDA) and GSH concentration were found to increase in a dose-dependent manner. The extent of lipid peroxidation leading to oxidative membrane damage was assessed using MDA content that increased with increasing concentration of metal nanooxide. Similar effects were also observed with GSH concentration that is a marker of nonenzymatic antioxidant capacity of an organism (Xiaoxiao et al. 2012). However, studies on the evaluation of toxicity exerted by metal oxide nanoparticle yielded variable results. Metal oxides such as aluminum oxide, titanium dioxide, cerium oxide, and zirconium dioxide failed to exert significant toxic effects on photosynthesis in green alga, *P. subcapitata* (Velzeboer et al. 2008), whereas zinc oxide and iron oxide nanoparticles have resulted in dose-dependent inhibition of growth in green algae (Franklin et al. 2007). Similar toxic effects had also been observed with carbon nanoparticles and quantum dots. Quantum dots made up of cadmium nanoparticles have been demonstrated to cause membrane damage in green algae *C. reinhardtii* and *P. subcapitata* wherein the normal crescent or sickle-shaped cell structure of unexposed cells changed intospherical shape (Bouldin et al. 2008). Carbon nanoparticle was demonstrated to exert reproductive and developmental toxicity in marine macroalgae *F. serratus* (Nielsen et al. 2008). These nanoparticles result in reduced fertility and defects in polar body axis alignment (Nielsen et al. 2008).

10.5.4 Nanoparticle Toxicity in Crustaceans

Crustaceans are commonly found in all aquatic ecosystems and are able to ingest nanoparticles from the surrounding aqueous environment through their filter-based feeder mechanism (Adams et al. 2006). Particle size is the major governing factor for the uptake and bioaccumulation of nanoparticles in these crustaceans. The surface charge (hydrophobicity) of the nanoparticles is the other important factor that impacts the observed toxicity effects as they can readily interact and adhere to the negatively charged cell surface of the crustacean (Zhu et al. 2009). *D. magna*, a common freshwater cladoceran, has been used as a model organism for toxicity studies (US EPA 1994). Smaller nanoparticles of size below 50 nm diameter are more readily taken up by the filter feeder barrier of *D. magna*, whereas larger particles are generally rejected and removed by the action of postabdominal claw. Fullerenes have been shown to reduce the fertility output and altered mounting in daphnids. Dose-dependent nanotoxicity was observed in daphnids wherein an increased mortality was observed with increasing concentration of fullerenes nanoparticles (Oberdorster et al. 2006). Hydrophobic fullerene (C60) caused their toxicity by adhering to the exoskeleton and antennae of the marine copepod, *A. tonsa* (Baun et al. 2008). Toxicity studies of fullerenes (C60) were done on *D. magna* which resulted in altered behavior patterns and increased mortality even at a very low concentration of 0.25 µl/L (Lovern et al. 2007; Lovern and Klaper 2006). Brine shrimp, *A salina*, also have filter feeder mechanism for nanoparticle uptake similar to that in *D. magna* and can thus easily ingest nanoparticles of size below 50 nm diameter, resulting in its associated toxic

effects. Furthermore, this zooplankton is used in aquaculture for feeding larval fish and can thus lead to the bioaccumulation of ingested nanoparticles (Ates et al. 2013). Toxicity studies with metallic nanoparticle titanium dioxide in zooplanktons resulted in its bioaccumulation, oxidative stress, and marginal mortility (Ates et al. 2013). Similar results were observed with titanium dioxide nanoparticle in daphnias where low toxicity was observed in acute assays; however, increased toxicity was observed with increased exposure time. It was demonstrated that with acute toxicity studies, titanium dioxide nanoparticles showed only marginal effects even at a concentration of 100 mg/L that changed to reduced growth and reproduction with chronic exposure at a concentration of only 0.5–5mg/L (Zhu et al. 2010). Similar toxic effects were also observed with metallic nanoparticles such as copper and silver in daphnias (Griffit et al. 2007).

10.5.5 Nanoparticle Toxicity in Mollusks

Mollusks are abundantly found in both marine and freshwater environments and have been extensively utilized for biomonitoring of environmental perturbations (Canesi et al. 2012). These bivalves have developed endocytotic and phagocytotic filter feeder mechanisms for the uptake of nano- and microsized materials from the aqueous environment. Moore (2006) proposed that mollusks are one of the representative groups that should be analyzed for the toxic effects of nanoparticles. Mollusks are routinely exposed to nanoparticles, and chronic exposure results due to the mollusks' ability to act as a suspension filter for microbes, microalgae, particulate matter, sediments, and natural nanoparticles (Canesi et al. 2012).

In correlation with the low toxicity of nanoparticles on aquatic organisms in acute toxicity assays whereas chronic exposure resulted in high nanotoxicity, the edible bivalve mollusks were required to be evaluated for chronic exposure (Zhu et al. 2010). Nanotoxicity-induced release of lysozymes, oxidative stress, production of nitric oxide, and respiratory burst are common toxic effects of nanoparticles (Canesi et al. 2012). Lysosomes in cells of digestive glands and hemocytes are two major targets for nanoparticles in bivalve mollusks (Canesi et al. 2012). Uptake and internalization studies in blue mussels and cockles have demonstrated the endocytic uptake of nanoparticles that are then translocated to the digestive glands (Moore 2006). Carbon nanoparticles such as fullerene (C60) induced cytotoxicity in *M. edulis* hematocytes (Canesi et al. 2012). Nanoparticles agglomerates are more readily ingested by certain mollusks such as mussels and oysters (Ward and Kach 2009). Another study of fullerenes (C60) toxicity in *C. virginica* oysters resulted in nanoparticle-induced lysosomal destabilization of digestive glands and abnormal development of oyster larvae (Canesi et al. 2012). Cadmium quantum dots were accumulated by freshwater mollusks *E. complanata* that resulted in lipid peroxidation and genotoxicity in gills and digestive glands along with a reduction in hemocyte viability (Gagne et al. 2008). Nanoparticle induced oxidative stress has been implicated for the observed nanotoxicity. It was demonstrated with the study of metallic copper oxide nanoparticles on marine mollusk, *S. plana*, which induced oxidative stress biomarkers. Nanoparticle-induced increases in the activity of antioxidant enzymes such as SOD, catalase, and GST were also observed (Buffet et al. 2011). Gold citrate nanoparticle studies on *M. edulis* also resulted in the bioaccumulation of gold that resulted in oxidative stress condition (Tedesco et al. 2010).

10.6 Conclusion

The nanotechnology industry is growing, leading to an increase in the exposure of biological organisms to different nanomaterials. Humans are regularly exposed to these nanomaterials through respiratory, dermal, oral, or injection routes. Industrial workers and subpopulation are routinely exposed to the nanomaterials produced along with the general population who are exposed to the nanoparticles present in the polluted air and in consumer products. These nanoparticles with their small size and unique surface properties penetrate into the exposed cells and their retention leads to several disease conditions. Nanoparticles are known to be able cross blood–brain barrier and have been described to cause neurodegenerative diseases such as Alzheimer's and Parkinson's disease. Their presence in systemic circulation also result in several circulatory- and blood-related diseases such as thrombosis; pancreatitis; Kaposi's sarcoma; podoconiosis; ulcerative colitis; Crohn's disease; and autoimmune diseases such as rheumatoid arthritis, scleroderma, and systemic lupus erythematosus. The ability to cross the blood placenta barrier further leads to embryo toxicity and mortality. At cellular level, these nanoparticles are known to generate ROS and release their toxic ions that cause damage to macromolecules of the cells. This results in inflammation, oxidative stress, genetoxicity, and cytotoxicity. Through these mechanisms, they are also able to suppress the apoptotic signals and thus results in proliferative growth of cells leading to cancer. The inherent toxicity of nanomaterials on live cells has been utilized to provide protection against plant pathogenic microbes. The antimicrobial activity of these nanoparticles have been demonstrated at a concentration that is harmless to plants as no phytotoxicity or chemical injury is observed at the test concentrations. These nanoparticles thus exhibit antimicrobial activity by interacting with membrane, altering their transport and oxidation of macromolecules, and disrupting the energy metabolism by damaging the membrane-associated enzymes. These nanoparticles have thus been successfully used to control several bacterial and fungal diseases. Several toxic effects of nanoparticles on aquatic organisms have been observed that include change in physiological mechanism, behavior pattern, respiratory distress, oxidative stress, organ pathologies, decrease in fertility, and an increase in mortality. Dose-dependent nanotoxicity has been observed with crustaceans, phytoplanktons, mollusks, and fish. Nanotoxicity studies conducted with these organisms have generated awareness toward its toxic effects and thus warrants against its unregulated application and release to environment.

REFERENCES

Adams, L.K., Lyon, D.Y., McIntosh, A., and Alvarez, P.J.J. 2006. Comparative ecotoxicity of nano-scale titanium dioxide, silicon dioxide and zinc oxide water suspensions. *Water Res* 40:3527–3532.

Aguilar-Mendez, M.A., San Martın-Martınez, E., Ortega-Arroyo, L., Cobian-Portillo, G., and Sanchez-Espindola, E. 2010. Synthesis and characterization of silver nanoparticles: Effect on phytopathogen *Colletotrichum gloesporioides*. *J Nanopart Res* 13:2525–2532.

Ahamed, M., Akhtar, M.J., Siddiqui, M.A. et al. 2011. Oxidative stress mediated apoptosis induced by nickel ferrite nanoparticles in cultured A549 cells. *Toxicology* 283:101–108.

Ahamed, M., Alsalhi, M.S., and Siddiqui, M.K. 2010. Silver nanoparticle applications and human health. *Clin Chim Acta* 411:1841–1848.

Aili, D., Enander, K., Rydberg, J. et al. 2008. Folding induced assembly of polypeptide decorated gold nanoparticles. *J Am Chem Soc* 130:5780–5788.

Aitken, R.J., Chaudhry, M.Q., Boxall, A.B., and Hull, M. 2006. Manufacture and use of nanomaterials: Current status in the UK and global trends. *Occup Med* 56:300–306.

Akiyoshi, K., Sasaki, Y., and Sunamoto, J. 1999. Molecular chaperone-like activity of hydrogel nanoparticles of hydrophobized pullulan: Thermal stabilization with refolding of carbonic anhydrase B. *Bioconjug Chem* 10:321–324.

Alkilany, A.M., and Murphy, C.J. 2010. Toxicity and cellular uptake of gold nanoparticles: What we have learned so far? *J Nanopart Res* 12:2313–2333.

Al-Rawi, M., Diabate, S., and Weiss, C. 2011. Uptake and intracellular localization of submicron and nano-sized SiO(2) particles in HeLa cells. *Arch Toxicol* 85:813–826.

Antonini, J.M., Santamaria, A.B., Jenkins, N.T., Albini, E., and Lucchini, R. 2006. Fate of manganese associated with the inhalation of welding fumes: Potential neurological effects. *Neurotoxicol* 27:304–310.

Arora, S., Jain, J., Rajwade, J.M., and Paknikar, K.M. 2008. Cellular responses induced by silver nanoparticles: In vitro studies. *Toxicol Lett* 179:93–100.

Ates, M., Daniels, J., and Arslan, Z. 2013. Effects of aqueous suspensions of titanium dioxide nanoparticles on *Artemia salina*: Assessment of nanoparticle aggregation, accumulation, and toxicity. *Environ Monit Assess* 185:3339–3348.

Auffan, M.L., Achouak, W., Rose, J.R. et al. 2008. Relation between the redox state of iron-based nanoparticles and their cytotoxicity toward *Escherichia coli*. *Environ Sci Technol* 42:6730–6735.

Baer, D.R., Gaspar, D.J., Nachimuthu, P., Techane, S.D., and Castner, D.G. 2010. Application of surface chemical analysis tools for characterization of nanoparticles. *Anal Bioanal Chem* 396(3):983–1002.

Ballestri, M., Baraldi, A., Gatti, A.M. et al. 2001. Liver and kidney foreign bodies granulomatosis in a patient with malocclusion, bruxism, and worn dental prostheses. *Gastroenterology* 121:1234–1238.

Barnes, C.A., Elsaesser, A., Arkusz, J. et al. 2008. Reproducible comet assay of amorphous silica nanoparticles detects no genotoxicity. *Nano Lett* 8:3069–3074.

Baun, A., Hartmann, N.B., Grieger, K., and Kisk, K.O. 2008. Ecotoxicity of engineered nanoparticles to aquatic invertebrates: A brief review and recommendations for future toxicity testing. *Ecotoxicology* 17:387–395.

BeruBe, K., Balharry, D., Sexton, K., Koshy, L., and Jones, T. 2007. Combustion-derived nanoparticles: Mechanisms of pulmonary toxicity. *Clin Exp Pharmacol Physiol* 34(10):1044–1050.

Bhabra, G., Sood, A., Fisher, B. et al. 2009. Nanoparticles can cause DNA damage across a cellular barrier. *Nat Nano* 4:876–883.

Bols, N.C., Dayeh, V.R., Lee, L.E.J., and Schirmer, K. 2005. Use of fish cell lines in the toxicology and ecotoxicology of fish. In: *Biochem Molecular Biology of Fishes Vol. 6: Environmental Toxicology*, ed. Moon, T.W. and Mommsen, T.P., 43–84. Amsterdam: Elsevier Science.

Borm, P.J.A., Schins, R.P.F., and Albrecht, C. 2004. Inhaled particles and lung cancer: Part B: Paradigms and risk assessment. *Int J Cancer* 110:3–14.

Bouldin, J.L., Ingle, T.M., Sengupta, A., Alexander, R., Hannigan, R.E., and Buchanan, R.A. 2008. Aqueous toxicity and food chain transfer of quantum dots in freshwater algae and *Ceriodaphnia dubia*. *Environ Toxicol Chem* 27(9):1958–1963.

Bouwmeester, H., Dekkers, S., Noordam, M.Y. et al. 2009. Review of health safety aspects of nanotechnologies in food production. *Regul Toxicol Pharmacol* 53:52–62.

Bradley, E.L., Castle, L., and Chaudhry, Q. 2011. Applications of nanomaterials in food packaging with a consideration of opportunities for developing countries. *Trends Food Sci Technol* 22:604–610.

Brant, J., Lecoanet, H., and Wiesner, M.R. 2005. Aggregation and deposition characteristics of fullerene nanoparticles in aqueous systems. *J. Nanopart. Res* 7:545–553.

Brayner, R. 2008. The toxicological impact of nanoparticles. *Nanotoday* 3:48–55.

Bucciantini, M., Giannoni, E., Chiti, F. et al. 2002. Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. *Nature* 416:507–511.

Buffet, P.E., Tankoua, O.F., Pan, J.F. et al. 2011. Behavioural and biochemical responses of two marine invertebrates *Scrobicularia plana* and *Hediste diversicolor* to copper oxide nanoparticles. *Chemosphere* 84:166–174.

Buford, M.C., Hamilton, R.F. Jr., and Holian, A. 2007. A comparison of dispersing media for various engineered carbon nanoparticles. *Part Fibre Toxicol* 4:6.

Buzea, C., Pacheco Blandino, I.I., and Robbie, K. 2007. Nanomaterials and nanoparticles: Sources and toxicity. *Biointerphases* 2(4):MR17–MR172.

Calderon-Garciduenas, L., Maranpot, R.R., Torres-Jardon, R. et al. 2003. DNA damage in nasal and brain tissues of canines exposed to air pollutants is associated with evidence of chronic brain inflammation and neurodegeneration. *Toxicol Pathol* 31:524–538.

Campbell, A., Oldham, M., Becaria, A. et al. 2005. Particulate matter in polluted air may increase biomarkers of inflammation in mouse brain. *Neurotoxicol* 26:133–140.

Canesi, L., Ciacci, C., Fabbri, R., Marcomini, A., Pojana, G., and Gallo, G. 2012. Bivalve molluscs as a unique target group for nanoparticle toxicity. *Mar Environ Res* 76:16–21.

Chen, M. and von Mikecz, A. 2005. Formation of nucleoplasmic protein aggregates impairs nuclear function in response to Silicon dioxide nanoparticles. *Exp Cell Res* 305:51–62.

Cheng, J., Flahaut, E., and Cheng, S.H. 2007. Effect of carbon nanotubes on developing zebrafish (*Danio rerio*) embryos. *Env Toxicol Chem* 26(4):708–716.

Chou, W.L., Yu, D.G., and Yang, M.C. 2005. The preparation and characterization of silver-loading cellulose acetate hollow fiber membrane for water treatment. *Polym Adv Tech* 16:600–607.

Choudhury, S.R., Nair, K.K., Kumar, R. et al. 2010. Nanosulfur: A potent fungicide against food pathogen, *Aspergillus niger*. *AIP Conf Proc* 1276:154–157.

Cottingham, M.G., Hollinshead, M.S., and Vaux, D.J. 2002. Amyloid fibril formation by a synthetic peptide from a region of human acetylcholinesterase that is homologous to the Alzheimer's amyloid-beta peptide. *Biochemistry* 41:13539–13547.

Crosera, M., Bovenzi, M., Maina, G. et al. 2009. Nanoparticle dermal absorption and toxicity: A review of the literature. *Int Arch Occup Environ Health* 82:1043–1055.

Dasenbrock, C., Peters, L., Creutzenberg, O., and Heinrich, U. 1996. The carcinogenic potency of carbon particles with and without PAH after repeated intratracheal administration in the rat. *Toxicol Lett* 88:15–21.

Daughton, C. and Ternes, T. 1999. Pharmaceuticals and personal care products in the environment: Agents of subtle change? *Environ Health Persp Suppl* 6:907–938.

Davis, R.R., Lockwood, P.E., Hobbs, D.T. et al. 2007. *In vitro* biological effects of sodium titanate materials. *J Biomed Mater Res B Appl Biomater* 83(2):505–511.

Dayeh, V.R., Schirmer, K., and Bols, N.C. 2002. Applying whole-water samples directly to fish cell cultures in order to evaluate the toxicity of industrial effluents. *Water Res* 36:3727–3738.

Dimkpa, C.O., McLean, J.E., Britt, D.W., and Anderson, A.J. 2013. Antifungal activity of Zinc oxide nanoparticles and their interactive effect with a biocontrol bacterium on growth antagonism of the plant pathogen *Fusarium graminearum*. *Biometals* 26(6):913–924.

Doak, S.H., Griffiths, S.M., Manshian, B. et al. 2009. Confounding experimental considerations in nanogenotoxicology. *Mutagenesis* 24(4):285–293.

Donaldson, K. and Stone, V. 2003. Current hypotheses on the mechanisms of toxicity of ultrafine particles. *Ann Ist Super Sanita* 39:405–410.

Donaldson, K., Brown, D., Clouter, A. et al. 2002. The pulmonary toxicology of ultrafine particles. *J Aerosol Med* 15:213–220.

Donaldson, K., Murphy, F.A., Duffin, R., and Poland, C.A. 2010. Asbestos, carbon nanotubes and the pleural mesothelium: A review of the hypothesis regarding the role of long fibre retention in the parietal pleura, inflammation and mesothelioma. *Part Fibre Toxicol* 7:5.

Donaldson, K., Tran, L., Jimenez, L.A. et al. 2005. Combustion-derived nanoparticles: A review of their toxicology following inhalation exposure. *Part Fibre Toxicol* 2:10.

Dong, H.Z. and Yang, J.L. 2011. Study on the inhibitory activities of nano-CuO to three kinds of vegetable bacteria. *N Hortic (Plant Protect)* 22:129–130.

Driscoll, K.E., Carter, J.M., Howard, B.W. et al. 1996. Pulmonary inflammation, chemokine, and mutagenic responses in rats after subchronic inhalation of carbon black. *Toxicol Appl Pharmacol* 136:327–380.

Dubertret, B., Skourides, P., Norris, D.J. et al. 2002. *In vivo* imaging of quantum dots encapsulated in phospholipid micelles. *Science* 298:1759–1762.

Dubey, A., Goswami, M., Yadav, K., and Chaudhary, D. 2015. Oxidative stress and nanotoxicity induced by titanium dioxide and zinc oxide on WAG cell line. *Plos One* 10(5):e0127493.

Dunford, R., Salinaro, A., Cai, L. et al. 1997. Chemical oxidation and DNA damage catalysed by inorganic sunscreen ingredients. *FEBS Lett* 418:87–90.

Dunn, K. and Edwards-Jones, V. 2004. The role of Acticoat with nanocrystalline silver in the management of burns. *Burns* 30:S1–S9.

Elias, A.L., Carrero-Sanchez, J.C., Terrones, H. et al. 2007. Viability studies of pure carbon and nitrogen doped nanotubes with *Entamoeba histolytica*: From amoebicidal to biocompatible structures. *Small* 3(10):1723–1729.

Emamifar, A., Kadivar, M., Shahedi, M., and Soleimanian-Zad, S. 2010. Evaluation of nanocomposite packaging containing Ag and zinc oxide on shelf life of fresh orange juice. *Innov Food Sci Emerg Technol* 11:742–748.

Fateixa, S., Neves, M.C., Almeida, A., Oliveira, J., and Trindade, T. 2009. Anti-fungal activity of silicon dioxide/Ag_2S nanocomposites against *Aspergillus niger*. *Colloids Surfaces B* 74:304–308.

Federici, G., Shaw, B., and Handy, R. 2007. Toxicity of titanium dioxide nanoparticles to rainbow trout (*Oncorhynchus mykiss*): Gill injury, oxidative stress, and other physiological effects. *Aquat Toxicol* 84:415–430.

Ferin, J. 2004. Pulmonary retention and clearance of particles. *Toxicol Lett* 72:121–125.

Fernandez-Cruz, D., Gomez, C.G., and Babin, M. 2013. *In vitro* evaluation of cellular responses induced by zinc oxide nanoparticles, zinc ions and bulk zinc oxide in fish cells. *Sci Total Environ* 452–453:262–274.

Foley, S., Crowley, C., Smaihi, M. et al. 2002. Cellular localisation of a water-soluble fullerene derivative. *Biochem Biophys Res Commun* 294:116–119.

Franklin, N.M., Rogers, N.J., Apte, S.C. et al. 2007. Comparative toxicity of nanoparticulate zinc oxide, bulk Zinc oxide, and $ZnCl_2$ to a freshwater microalga (*Pseudokirchneriella subcapitata*): The importance of particle solubility. *Environ Sci Technol* 41(24):8484–8490.

Fubini, B., and Hubbard, A. 2003. Serial review: Role of reactive oxygen and nitrogen species (ROS/RNS) in lung injury and diseases. *Free Rad Biol Med* 34:1507–1516.

Gagne, F., Auclair, J., Turcotte, P. et al. 2008. Ecotoxicity of CdTe quantum dots to freshwater mussel: Impacts on immune system, oxidative stress and genotoxicity. *Aquatic Toxicology* 86:333–340.

Gajbhiye, M., Kesharwani, J., Ingle, A., Gade, A., and Rai, M. 2009. Fungus mediated synthesis of silver nanoparticles and their activity against pathogenic fungi in combination with fluconazole. *Nanomedicine* 5:382–386.

Garnett, M.C. and Kallinteri, P. 2006. Nanomedicines and nanotoxicology: Some physiological principles. *Occup Med* 56:307–311.

Gatti, A.M. 2004. Biocompatibility of micro- and nano-particles in the colon: Part II. *Biomater* 25:385–392.

Gatti, A.M. and Rivasi, F. 2002. Biocompatibility of micro- and nanoparticles: Part I: In liver and kidney. *Biomater* 23:2381–2387.

Gatti, A.M., Montanari, S., Monari, E., Gambarelli, A., Capitani, F., and Parisini, B. 2004. Detection of micro- and nano-sized biocompatible particles in the blood. *J Mater Sci Mater Med* 15:469–472.

Geiser, M. 2010. Update on macrophage clearance of inhaled micro and nanoparticles. *J Aerosol Med Pulm Drug Deliv* 23:207–217.

Ginzburg, V.V. and Balijepalli, S. 2007. Modeling the thermodynamics of the interaction of nanoparticles with cell membranes, *Nano Lett* 7:3716–3722.

Greulich, C., Diendorf, J., Simon, T., Eggeler, G., Epple, M., and Koller, M. 2011. Uptake and intracellular distribution of silver nanoparticles in human mesenchymal stem cells. *Acta Biomater* 7:347–354.

Griffitt, R.J., Weil, R., Hyndman, K.A. et al. 2007. Exposure to copper nanoparticles causes gill injury and acute lethality in zebrafish (*Danio rerio*). *Env Sci Techn* 41(23):8178–8186.

Gupta, S., Brouwer, P., Bandyopadhyay, S. et al. 2005. TEM/AFM investigation of size and surface properties of nanocrystalline ceria. *J Nanosci Nanotechnol* 5(7):1101–1107.

Gurr, J.R., Wang, A.S., Chen, C.H., and Jan, K.Y. 2005. Ultrafine titanium dioxide particles in the absence of photoactivation can induce oxidative damage to human bronchial epithelial cells. *Toxicology* 213:66–73.

Handy, R., and Shaw, B. 2007. Toxic effects of nanoparticles and nanomaterials: Implications for public health, risk assessment and the public perception of nanotechnology. *Health Risk Soc* 9:125–44.

Handy, R.D., Kammer, F., Lead, J.R. et al. 2008. The ecotoxicology and chemistry of manufactured nanoparticles. *Ecotoxicology* 17(4):287–314.

Hauck, T.S., Ghazani, A.A., and Chan, W.C.W. 2008. Assessing the effect of surface chemistry on gold nanorod uptake, toxicity, and gene expression in mammalian cells. *Small* 4:153–159.

He, L., Liu, Y., Mustapha, A., and Lin, M. 2010. Antifungal activity of zinc oxide nanoparticles against *Botrytis cinerea* and *Penicillium expansum*. *Microb Res* 166: 207–215.

Henry, T.B., Menn, F.M., Fleming, J.T. et al. 2007 Attributing effects of aqueous C60 nano aggregates to tetrahydrofuran decomposition products in larval zebrafish by assessment of gene expression. *Env Health Perspect* 115:1059–1065.

Hoet, P.H.M., Bruske-Hohlfeld, I., and Salata, O.V. 2004. Nanoparticles—Known and unknown health risks. *J Nanobiotechnol* 2:12–27.

Hoshino, A., Fujioka, K., Oku, T. et al. 2004. Quantum dots targeted to the assigned organelle in living cells. *Microbiol Immunol* 48:985–994.

Huang, L., Li, D.Q., Lin, Y.J., Wei, M., Evans, D.G., and Duan, X. 2005. Controllable preparation of nano-MgO and investigation of its bactericidal properties, *J Inorg Biochem* 99(5):986–993.

Huh, A.J. and Kwon, Y.J. 2011. Nanoantibiotics: A new paradigm for treating infectious diseases using nanomaterials in the antibiotics resistant era. *J Control Release* 156:128–145.

Hussain, S.M., Hess, K.L., Gearhart, J.M., Geiss, K.T., and Schlager, J.J. 2005. *In vitro* toxicity of nanoparticles in BRL 3A rat liver cells. *Toxicol In Vitro* 19:975–983.

Ingle, T.M., Alexander, R., Bouldin, J., and Buchanan, R.A. 2008. Absorption of semiconductor nanocrystals by the aquatic invertebrate *Ceriodaphnia dubia*. *Bull Env Contam Toxicol* 81:249–252.

Isaacson, C.W., Usenko, C.Y., Tanguay, R.L., and Field, J.A. 2007. Quantification of fullerenes by LC/ESI-MS and its application to *in vivo* toxicity assays. *Analyt Chem* 79(23):9091–9097.

Ishii, D., Kinbara, K., Ishida, Y. et al. 2003. Chaperonin mediated stabilization and ATP-triggered release of semiconductor nanoparticles. *Nature* 423:628–632.

Jain, A.K., Mehra, N.K., Lodhi, N. et al. 2007. Carbon nanotubes and their toxicity. *Nanotoxicology* 1(3):167–197.

Jia, G., Wang, H., Yan, L. et al. 2005. Cytotoxicity of carbon nanomaterials: Single-wall nanotube, multi-wall nanotube, and fullerene. *Environ Sci Technol* 39:1378–1383.

Jia, H.Y., Liu, Y., Zhang, X.J. et al. 2009. Potential oxidative stress of gold nanoparticles by induced-NO releasing in serum. *J Am Chem Soc* 131:40–41.

Jo, Y.K., Kim, B.H., and Jung, G. 2009. Antifungal activity of silver ions and nanoparticles on phytopathogenic fungi. *Plant Dis* 93(10):1037–1043.

Johnston, B.D., Scown, T.M., Moger, J. et al. 2010. Bioavailability of nanoscale metal oxides titanium dioxide, CeO_2, and zinc oxide to fish. *Environ Sci Technol* 44(3):1144–1151.

Jung, J.H., Kim, S.W., Min, J.S., Kim, Y.J., Lamsal, K., and Kim, K.S. 2010. The effect of nano-silver liquid against the white rot of the green onion caused by *Sclerotium cepivorum*. *Mycobiology* 38(1):39–45.

Kaegi, R., Ulrich, A., Sinnet, B. et al. 2008. Synthetic titanium dioxide nanoparticle emission from exterior facades into the aquatic environment. *Env Pollut* 156(2):233–239.

Kamat, J.P., Devasagayam, T.P., Priyadarsini, K.I., and Mohan, H. 2000. Reactive oxygen species mediated membrane damage induced by fullerene derivatives and its possible biological implications. *Toxicology* 155:55–61.

Kang, S., Pinault, M., Pfefferle, L.D. et al. 2007. Single-walled carbon nanotubes exhibit strong antimicrobial activity. *Langmuir* 23:8670–8673.

Kang, S.J., Kim, B.M., Lee, Y.J., and Chung, H.W. 2008. Titanium dioxide nanoparticles trigger p53-mediated damage response in peripheral blood lymphocytes. *Environ Mol Mutagen* 49:399–405.

Kang, S.J., Ryoo, I.G., Lee, Y.J., and Kwak, M.K. 2012. Role of the Nrf2-heme oxygenase-1 pathway in silver nanoparticle-mediated cytotoxicity. *Toxicol Appl Pharmacol* 258:89–98.

Kanhed, P. et al. 2014. *In vitro* antifungal efficacy of copper nanoparticles against selected crop pathogenic fungi. *Mater Lett* 115:13–17.

Karlsson, H.L. 2010. The comet assay in nanotoxicology research. *Anal Bioanal Chem* 398:651–666.

Kasprowicz, M.J., Kozio, M., and Gorczyca, A. 2010. The effect of silver nanoparticles on phytopathogenic spores of *Fusarium culmorum*. *Can J Microbiol* 56:247–253.

Keller, A.A. Garner, K. Miller, R.J., and Lenihan, H.S. 2012. Toxicity of nano-zero valent iron to freshwater and marine organisms. *Plos One* 7:1–10.

Khot, L.R., Sankaran, S., Maja, J.M., Ehsani, R., and Schuster, E.W. 2012. Applications of nanomaterials in agricultural production and crop protection: A review. *Crop Prot* 35:64–70.

Kim, H., Kang, H., Chu, G., and Byun, H. 2008. Antifungal effectiveness of nanosilver colloid against rose powdery mildew in greenhouses. *Solid State Phenom* 135:15–18.

Kim, S.G., Kim, K.W., Park, E.U., and Choi, D. 2002. Silicon induced cell wall fortification of rice leaves: A possible cellular mechanism of nanotechnology in agriculture enhanced host resistance to blast. *Phytopathology* 92:1095–1103.

King, H.T.C., Dengler, E., Gao, W.J. et al. 2007. Developmental toxicity of low generation PAMAM dendrimers in zebrafish. *Toxicol Appl Pharm* 225:70–79.

Klaine, S.J., Alvarez, P.J., Batley, G.E. et al. 2008. Nanomaterials in the environment: Behavior, fate, bioavailability, and effects. *Env Toxicol Chem* 27(9):1825–1851.

Krishnaraj, C., Ramachandran, R., Mohan, K., and Kalaichelvan, P.T. 2012. Optimization for rapid synthesis of silver nanoparticles and its effect on phytopathogenic fungi. *Spectrochimica Acta Part A* 93:95–99.

Kroll, A., Pillukat, M.H., Hahn, D., and Schnekenburger, J. 2009. Current in vitro methods in nanoparticle risk assessment: Limitations and challenges. *Eur J Pharm Biopharm* 72(2):370–377.

Krysanov, E.Y., Demidova, T.B., Pelgunova, L.A. et al. 2009. Effect of nanoparticles of hydrated stannun dioxide ($SnO_2 \times H_2O$) on guppi (*Poecilia reticulata* Peters, 1860). *Dokl Akad Nauk* 426(6):844–846.

Kubota, Y., Shuin, T., Kawasaki, C. et al. 1994. Photokilling of T-24 human bladder cancer cells with titanium dioxide. *Br J Cancer* 70:1107–1111.

Kuhnel, D., Busch, W., Meiszner, T. et al. 2009. Agglomeration of tungsten carbide nanoparticles in exposure medium does not prevent uptake and toxicity toward a rainbow trout gill cell line. *Aquat Toxicol* 93(2–3):91–99.

Kumar, A., Pandey, A.K., Singh, S.S., Shanker, R., and Dhawan, A. 2011. Engineered zinc oxide and titanium dioxide nanoparticles induce oxidative stress and DNA damage leading to reduced viability of Escherichia coli. *Free Radic Biol Med* 51:1872–1881.

Kuznetsova, G.P., Larina, O.V., Petushkova, N.A. et al. 2014. Effects of fullerene C60 on proteomic profile of *Danio Rerio* fish embryos. *Bull Exp Biol Med* 156(5):694–698.

Lamsal, K., Kim, S.W., Jung, J.H., Kim, Y.S., Kim K.S., and Lee, Y.S. 2010. Inhibition effects of silver nanoparticles against powdery mildews on cucumber and pumpkin. *Microbiology* 39(1):26–32.

Lecoanet, H.F., and Wiesner, M.R. 2004. Velocity effects on fullerene and oxide nanoparticle deposition in porous media. *Environ Sci Technol* 38(16):4377–4382.

Lee, J., Mahendra, S., and Alvarez, P.J.J. 2010. Nanomaterials in the construction industry: A review of their applications and environmental health and safety considerations. *ACS Nano* 4:3580–3590.

Lee, K.J., Nallathamby, P.D., Browning, L.M. et al. 2007. *In vivo* imaging of transport and biocompatibility of single silver nanoparticles in early development of zebrafish embryos. *ACS Nano* 1(2)133–143.

Lemire, J.A., Harrison, J.J., and Turner, R.J. 2013. Antimicrobial activity of metals: Mechanisms, molecular targets and applications. *Nat Rev Microbiol* 11(6):371–384.

Leroueil, P.R., Berry, S.A., Duthie, K. et al. 2008. Wide varieties of cationic nanoparticles induce defects in supported lipid bilayers. *Nano Lett* 8:420–424.

Lewinski, N., Colvin, V., and Drezek, R. 2008. Cytotoxicity of nanoparticles. *Small* 4(1):26–49.

Li, G., He, D., Qian, Y. et al. 2012. Fungus-mediated green synthesis of silver nanoparticles using *Aspergillus terreus*. *Int J Mol Sci* 13:466–476.

Li, J.J., Hartono, D., Ong, C.N., Bay, B.H., and Yung, L.Y.L. 2010. Autophagy and oxidative stress associated with gold nanoparticles. *Biomaterials* 31:5996–6003.

Li, N., Xia, T., and Nel, A.E. 2008. The role of oxidative stress in ambient particulate matter induced lung diseases and its implications in the toxicity of engineered nanoparticles. *Free Radic Biol Med* 44:1689–1699.

Linse, S., Cabaleiro-Lago, C., Xue, W.F. et al. 2007. Nucleation of protein fibrillation by nanoparticles. *Proc Natl Acad Sci U S A* 104:8691–8696.

Liu, G., Mena, P., Harris, P.R.L., Rolston, R.K., Perry, G., and Smith, M.A. 2006. Nanoparticle iron chelators: A new therapeutic approach in Alzheimer disease and other neurologic disorders associated with trace metal imbalance. *Neurosci Lett* 406:189–193.

Liu, H.F. and Xu, W.Y. 2008. The inhibition effect of nano-silver on plant pathogenic fungi. Dissertation for master's degree, Fujian Agriculture and Forestry University, Fuzhou.

Liu, J.B., Chang, H.B., Ma, J.Y., Tang, S.S., and Qi, J.Z. 2012b. Effects of nano-silica on rice's resistance to *Magnaporthe oryzae* and on rice growth. *J Jilin Agric Univ* 2(157–161):165.

Liu, J.B., Gao, C., Gao, J., Ma, J.Y., and Chang, H.B. 2012a. Effects of silicon on the ultrastructure of *Oryza sativa* leaves infected by *Magnaporthe grisea*. *J South China Agric Univ* 1:40–43.

Liu, W.T. 2006. Nanoparticles and their biological and environmental applications. *J Biosci Bioeng* 102:1–7.

Liu, P.W., Guo, T., and Wei, H. 2009. Bactericidal effects of copper-bearing nano-montmorillonite on three aquatic pathogenic bacteria and two intestinal available bacteria in vitro. *J Shanghai Ocean Univ* 5:520–526.

Lomer, M.C.E., Hutchinson, C., Volkert, S. et al. 2004. Dietary sources of inorganic microparticles and their intake in healthy subjects and patients with Crohn's disease. *British J Nutrition* 92:947–955.

Lomer, M.C.E., Thompson, R.P.H., and Powell, J.J. 2002. Fine and ultrafine particles of the diet: Influence on the mucosal immune response and association with Crohn's disease. *Proc Nutrition Soc* 61:123–130.

Lovern, S.B. and Klaper, R. 2006. *Daphnia magna* mortality when exposed to titanium dioxide and fullerene (C60) nanoparticles. *Env Toxicol Chem* 25(4):1132–1137.

Lovern, S.B., Strickler, J.R., and Klaper, R. 2007. Behavioral and physiological changes in *Daphnia magna* when exposed to nanoparticle suspensions (titanium dioxide, nanoC60, and C60HxC70Hx). *Environ Sci Technol* 41(12):4465–4470.

Lucarelli, M., Gatti, A.M., Savarino, G. et al. 2004. Innate defence functions of macrophages can be biased by nano-sized ceramics metallic particles. *Eur Cytokine Netw* 15:339–346.

Lyon, D.Y., Adams, L.K., Falkner, J.C. et al. 2006. Antibacterial activity of fullerene water suspensions: Effects of preparation method and particle size. *Environ Sci Technol* 40:4360–4366.

Marambio-Jones, C. and Hoek, E.M.V. 2010. A review of the antibacterial effects of silver nanomaterials and potential implications for human and the environment. *J Nanopart Res* 12:1531–1551.

Maynard, A.D., Baron, P.A., Foley, M. et al. 2004. Exposure to carbon nanotube material: Aerosol release during the handling of unrefined single walled carbon nanotube material. *J Toxicol Env Health A* 67:87–107.

Mehrabi, M. and Wilson, R. 2007. Intercalating gold nanoparticles as universal labels for DNA detection. *Small* 3:1491–1495.

Min, J.S., Kim, K.S., Kim, S.W. et al. 2009. Effects of colloidal silver nanoparticles on sclerotium-forming phytopathogenic fungi. *Plant Pathol J* 25:376–380.

Mittal, S., Sharma, V., Vallabani, N.V., Kulshrestha, S., Dhawan, A., and Pandey, A.K. 2011. Toxicity evaluation of carbon nanotubes in normal human bronchial epithelial cells. *J Biomed Nanotechnol* 7:108–109.

Monserrat, J.M., Martinez, P.E., Geracitano, L.A. et al. 2007. Pollution biomarkers in estuarine animals: Critical review and new perspectives. *Comp Biochem Physiol C Toxicol Pharmacol* 146:221–234.

Monteiro-Riviere, N.A., Inman, A.O., and Zhang, L.W. 2009. Limitations and relative utility of screening assays to assess engineered nanoparticle toxicity in a human cell line. *Toxicol Appl Pharmacol* 234(2):222–235.

Monteiro-Riviere, N.A., Nemanich, R.J., Inman, A.O., Wang, Y.Y., and Riviere, J.E. 2005. Multi-walled carbon nanotube interactions with human epidermal keratinocytes. *Toxicol Lett* 155:377–384.

Montella, M., Franceschi, S., Geddes da Filicaia, M. et al. 1997. Classical Kaposi sarcoma and volcanic soil in southern Italy: A case-control study. *Epidemiol Prev* 21:114–117.

Montes-Burgos, I., Walczyk, D., Hole, P., Smith, J., Lynch, I., and Dawson, K.A. 2010. Characterisation of nanoparticle size and state prior to nanotoxicological studies. *J Nanopart Res* 12:47–53.

Moore, M.N. 2006. Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? *Environ Int* 32:967–976.

Morones, J.R., Elechiguerra, J.L., Camacho, A. et al. 2005. The bactericidal effect of silver nanoparticles. *Nanotechnology* 16:2346–2353.

Motskin, M., Wright, D.M., Muller, K. et al. 2009. Hydroxyapatite nano and microparticles: Correlation of particle properties with cytotoxicity and biostability. *Biomaterials* 30(19):3307–3317.

Mouchet, F., Landois, P., Flahaut, E. et al. 2007. Assessment of the potential *in vivo* ecotoxicity of double walled carbon nanotubes (DWNTs) in water, using the amphibian *Ambystoma mexicanum*. *Nanotoxicology* 1(2):149–156.

Mouchet, F., Landois, P., Sarremejeana, E. et al. 2008. Characterisation and *in vivo* ecotoxicity evaluation of double-wall carbon nanotubes in larvae of the amphibian *Xenopus laevis*. *Aquat Toxicol* 87(2):127–137.

Mueller, N.C. and Nowack, B. 2008. Exposure modeling of engineered nanoparticles in the environment. *Environ Sci Technol* 42:4447–4453.

Murdock, R.C., Braydich-Stolle, L., Schrand, A.M., Schlager, J.J., and Hussain, S.M. 2008. Characterization of nanomaterial dispersion in solution prior to in vitro exposure using dynamic light scattering technique. *Toxicol Sci* 101(2):239–253.

Musarrat, J., Dwivedi, S., Singh, B.R., Al-Khedhairy, A.A., and Azam Naqvi, A. 2010. A production of antimicrobial silver nanoparticles in water extracts of the fungus *Amylomyces rouxii* strain KSU-09. *Biores Technol* 101:8772–8776.

Musee, N., Thwalaa, M., and Nota, N. 2011. The antibacterial effects of engineered nanomaterials: Implications for wastewater treatment plants. *J Environ Monit* 13:1164–1183.

Myllynen, P. 2009. Nanotoxicology: Damaging DNA from a distance. *Nat Nanotechnol* 4:795–796.

Myllynen, P.K., Loughran, M.J., Howard, C.V., Sormunen, R., Walsh, A.A., and Vahakangas, K.H. 2008. Kinetics of gold nanoparticles in the human placenta. *Reprod Toxicol* 26:130–137.

Nabiev, I., Mitchell, S., Davies, A. et al. 2007. Nonfunctionalized nanocrystals can exploit a cell's active transport machinery delivering them to specific nuclear and cytoplasmic compartments. *Nano Lett* 7:3452–3461.

Nair, R. and Kumar, D.S. 2013. Chapter 10: Plant diseases-control and remedy through nanotechnology. In: *Crop Improvement under Adverse Conditions*, ed. Tuteja, N., and Gill, S.S., 231–243. New York: Springer-Verlag.

Nair, R., Varghese, S.H., Nair, B.G., Maekawa, T., Yoshida, Y., and Kumar, D.S. 2010. Nanoparticulate material delivery to plants. *Plant Sci* 179:154–163.

Napierska, D., Thomassen, L.C., Lison, D., Martens, J.A., and Hoet P.H. 2010. The nanosilica hazard: Another variable entity. *Part Fibre Toxicol* 7:39.

Navarro, E., Baun, A., Behra, R. et al. 2008. Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi. *Ecotoxicology* 17:372–386.

Nel, A., Xia, T., Madler, L., and Li, N. 2006. Toxic potential of materials at the nanolevel. *Science* 311:622–627.

Nemmar, A., Hoet, P.H., Vanquickenborne, B. et al. 2002c. Passage of inhaled particles into the blood circulation in humans. *Circulation* 105(4):411–414.

Nemmar, A., Hoylaerts, M.F., Hoet, P.H.M. et al. 2002a. Ultrafine particles affect experimental thrombosis in an *in vivo* hamster model. *Am J Respir Crit Care Med* 166:998–1004.

Nemmar, A., Nemery, B., Hoylaerts, M.F., and Vaermylen, J. 2002b. Air pollution and thrombosis: An experimental approach. *Pathophysiol Haemost Thromb* 32:349–350.

Nemmar, A., Vanbilloen, H., Hoylaerts, M.F., Hoet, P.H., Verbruggen, A., and Nemery, B. 2001. Passage of intratracheally instilled ultrafine particles from the lung into the systemic circulation in hamster. *Am J Respir Crit Care Med* 164:1665–1668.

Nielsen, H.D., Berry, L.S., Stone, V., Burridge, T.R., and Fernandes, T.F. 2008. Interactions between carbon black nanoparticles and the brown algae *Fucus serratus*: Inhibition of fertilization and zygotic development. *Nanotechnology* 2:88–97.

Nikula, K.J., Snipes, M.B., Barr, E.B., Griffith, W.C., Henderson, R.F., and Mauderly, J.L. 1995. Comparative pulmonary toxicities and carcinogenicities of chronically inhaled diesel exhaust and carbon black in F344 rats. *Fundam Appl Toxicol* 25:80–94.

Noack, A.G., Grant, C.D., and Chittleborough, D.J. 2000. Colloid movement through stable soils of low cation-exchange capacity. *Environ Sci Technol* 34:2490–2497.

Noonan, C.W., Pfau, J.C., Larson, T.C., and Spence, M.R. 2006. Nested case-control study of autoimmune disease in an asbestos-exposed population. *Envir Health Persp* 114:1243–1247.

Nowack, B. and Bucheli, T.D. 2007. Occurrence, behavior and effects of nanoparticles in the environment. *Environ Pollut* 150(1):5–22.

O'Neill, M., Vine, M.G., Beezer, G. et al. 2003. Antimicrobial properties of silver containing wound dressings: A microcalorimertic study. *Int J Pharm* 263:61–68.

Oberdorster, E. 2004. Manufactured nanomaterials (fullerenes, C60) induce oxidative stress in the brain of juvenile largemouth Bass. *Environ Health Perspect* 112:1058–1062.

Oberdorster, G. 2002. Toxicokinetics and effects of fibrous and nonfibrous particles. *Inhal Toxicol* 14:29–56.

Oberdorster, G., 2007. Biokinetics and effects of nanoparticles. In: *Nanotechnology-Toxicological Issues and Environmental Safety*, ed. Simeonova, P.P., Opopol, N., and Luster, M.I., 15–51. Dordrecht: Springer

Oberdorster, E., Zhu, S., Blickley, T.M. et al. 2006. Ecotoxicology of carbon-based engineered nanoparticles: Effects of fullerene (C60) on aquatic organisms. *Carbon* 44:1112–1120.

Oberdorster, G., Elder, A., and Rinderknecht, A. 2009. Nanoparticles and the brain: Cause for concern? *J Nanosci Nanotechnol* 9:4996–5007.

Oberdorster, G., Ferin, J., and Lehnert, B.E. 1994. Correlation between particle size, *in vivo* particle persistence, and lung injury. *Environ Health Persp* 102:173–179.

Oberdorster, G., Ferin, J., Gelein, R., Soderholm, S.C., and Finkelstein, J. 1992. Role of the alveolar macrophage in lung injury: Studies with ultrafine particles. *Environ Health Perspect* 97:193–199.

Oberdorster, G., Oberdorster, E., and Oberdorster, J. 2005. Nanotoxicology: An emerging discipline evolving from studies of ultrafine particles. *Environ Health Perspect* 113:823–839.

Oh, S.D., Lee, S., Choi, S.H. et al. 2006. Synthesis of Ag and Ag-silicon dioxide nanoparticles by γ-irradiation and their antibacterial and antifungal efficiency against *Salmonella enteric* serovar *Typhimurium* and *Botrytis cinerea*. *Colloids Surf A* 275:228–233.

Osmond, M.J. and McCall, M.J. 2010. Zinc oxide nanoparticles in modern sunscreens: An analysis of potential exposure and hazard. *Nanotoxicology* 4:15–41.

Ovrevik, J., Lag, M., Schwarze, P., and Refsnes, M. 2004. p38 and Src-ERK1/2 pathways regulate crystalline silica-induced chemokine release in pulmonary epithelial cells. *Toxicol Sci* 81:480–490.

Panacek, A., Kolar, M., Vecerova, R. et al. 2006. A new composition of nanosized silica-silver for control of various plant diseases. *Plant Pathol J* 22: 295–302.

Panda, K.K., Achary, V.M.M., Krishnaveni, R. et al. 2011. *In vitro* biosynthesis and genotoxicity bioassay of silver nanoparticles using plants. *Toxicol In Vitro* 25:1097–1105.

Park, B., Donaldson, K., Duffin, R. et al. 2008. Hazard and risk assessment of a nanoparticulate cerium oxide-based diesel fuel additive—A case study. *Inhal Toxicol* 20:547–566.

Park, H.J., Kim, S.H., Kim, H.J., and Choi, S.H. 2006. A new composition of nanosized silica-silver for control of various plant diseases. *Plant Pathol J* 22:295–302.

Pelka, J., Gehrke, H., Esselen, M. et al. 2009. Cellular uptake of platinum nanoparticles in human colon carcinoma cells and their impact on cellular redox systems and DNA integrity. *Chem Res Toxicol* 22(4):649–659.

Peters, A. 2005. Particulate matter and heart disease: Evidence from epidemiological studies. *Toxicol Appl Pharmacol* 207:S477–S482.

Peters, A., Veronesi, B., Calderon-Garciduenas, L. et al. 2006. Translocation and potential neurological effects of fine and ultrafine particles: A critical update. *Part Fibre Toxicol* 3:13.

Peters, K., Unger, R.E., Kirkpatrick, C.J., Gatti, A.M., and Monari, E. 2004. Effects of nano-scaled particles on endothelial cell function in vitro: Studies on viability, proliferation and inflammation. *J Mater Sci Mater Med* 15:321–325.

Petersen, E.J., Huang, Q., and Weber, W.J. 2008a Bioaccumulation of radio labeled carbon nanotubes by *Eisenia foetida*. *Environ Sci Technol* 42(8):3090–3095.

Petersen, E.J., Huang, Q., and Weber, W.J. 2008b. Ecological uptake and depuration of carbon nanotubes by *Lumbriculus variegatus*. *Env Health Perspect* 116(4):496–500.

Pfau, J.C., Sentissi, J.J., Weller, G., and Putnam, E.A. 2005. Assessment of autoimmune responses associated with asbestos exposure in Libby, Montana, USA. *Environ Health Perspect* 113:25–30.

Piotrovskii, L.B. and Kiselev, O.I. 2006. *Fullereny v Biologii (Fullerenes in Biology)*, St. Petersburg: Rostok.

Poon, V.K. and Burd, A. 2004. *In vitro* cytotoxicity of silver: Implication for clinical wound care. *Burns* 30:140–147.

Porter, A.E., Gass, M., Muller, K., Skepper, J.N., Midgley, P., and Welland, M. 2007. Visualizing the uptake of C60 to the cytoplasm and nucleus of human monocyte-derived macrophage cells using energy-filtered transmission electron microscopy and electron tomography. *Environ Sci Technol* 41(8):3012–3017.

Powell, J.J., Ainley, C.C., Harvey, R.S. et al. 1996. Characterization of inorganic microparticles in pigment cells of human gut associated lymphoid tissue. *Gut* 38:390–395.

Powell, J.J., Faria, N., Thomas-McKay, E., and Pele, L.C. 2010. Origin and fate of dietary nanoparticles and microparticles in the gastrointestinal tract. *J Autoimmun* 34:J226–J233.

Powers, K., Palazuelos, M., Moudgil, B., and Roberts, S. 2007. Characterization of the size, shape and state of dispersion of nanoparticles for toxicological studies. *Nanotoxicology* 1(1):42–51.

Pujalte, I., Passagne, I., Brouillaud, B. et al. 2011. Cytotoxicity and oxidative stress induced by different metallic nanoparticles on human kidney cells. *Part Fibre Toxicol* 8:10.

Quintana, C., Bellefqih, S., Laval, Y.J. et al. 2006. Study of the localization of iron, ferritin, and hemosiderin in Alzheimer's disease hippocampus by analytical microscopy at the subcellular level. *J Struct Biol* 153:42–54.

Qureshi, A., Kang, W.P., Davidson, J.L., and Gurbuz, Y. 2009. Review on carbon-derived, solid-state, micro and nano sensors for electrochemical sensing applications. *Diam Relat Mater* 18:1401–1420.

Rai, M., and Ingle, A. 2012. Role of nanotechnology in agriculture with special reference to management of insect pests. *Appl Microbiol Biotechnol* 94:287–293.

Rehn, B., Seiler, F., Rehn, S., Bruch, J., and Maier, M. 2003. Investigations on the inflammatory and genotoxic lung effects of two types of titanium dioxide: Untreated and surface treated. *Toxicol Appl Pharmacol* 189:84–95.

Reijnders, L. 2006. Cleaner nanotechnology and hazard reduction of manufactured nanoparticles. *J Clean Prod* 14:124–133.

Riediker, M., Devlin, R.B., Griggs, T.R. et al. 2004. Cardiovascular effects in patrol officers are associated with fine particulate matter from brake wear and engine emissions. *Part Fibre Toxicol* 4:2.

Roberts, A.P., Mount, A.S., Seda, B. et al. 2007. *In vivo* biomodification of lipid coated carbon nanotubes by *Daphnia magna*. *Environ Sci Technol* 41(8):3025–3029.

Rodea-Palomares, Boltes, K., Fernandez-Pinas, F. et al. 2011. Physicochemical characterization and ecotoxicological assessment of CeO_2 nanoparticles using two aquatic microorganisms. *Toxicol Sci* 119:135–145.

Roe, D., Karandikar, B., Bonn-Savage, N., Gibbins, B., and Roullet, J.B. 2008. Antimicrobial surface functionalization of plastic catheters by silver nanoparticles. *J Antimicrob Chemother* 61:869–876.

Ruffolo, S.A., La Russa, M.F., Malagodi, M., Oliviero Rossi, C., Palermo, A.M., and Crisci, G.M. 2010. Zinc oxide and $ZnTiO_3$ nanopowders for antimicrobial stone coating. *Appl Phys A* 100:829–834.

Saharan, V., Mehrotra, A., Khatik, R., Rawal, P., Sharma, S.S., and Pal, A. 2013. Synthesis of chitosan based nanoparticles and their *in vitro* evaluation against phytopathogenic fungi. *Int J Biol Macromol* 62:677–683.

Scalf, J. and West, P. 2006. *Part I: Introduction to Nanoparticles Characterization with AFM.* Santa Clara, CA: Pacific Nanotechnology.

Scarano, G., and Morelli, E. 2003. Properties of phytochelatin-coated CdS nanocrystallites formed in a marine phytoplanktonic alga (*Phaeodactylum tricornutum*, Bohlin) in response to. *Can J Plant Sci* 165:803–810.

Schulz, H., Hardewr, V., Ibald-Mulkli, A. et al. 2005. Cardiovascular effects of fine and ultrafine particles. *J Aerosol Med* 18:1–22.

Schwartz, J., and Morris, R. 1995. Air pollution and hospital admissions for cardiovascular disease in Detroit, Michigan. *Am J Epidemiol* 142:23–35.

Scown, T.M., van Aerle, R., Johnston, B.D. et al. 2009. High doses of intravenously administered titanium dioxide nanoparticles accumulate in the kidneys of rainbow trout but with no observable impairment of renal function. *Toxicol Sci* 109(2):372–380.

Seaton, L., Tran, R., Aitken, K., and Donaldson. 2010. Nanoparticles, human health hazard and regulation. *J R Soc Interface* 7:S119–S129.

Segner, H. 1998. Fish cell lines as a tool in aquatic toxicology. *EXS* 86:1–38.

Serpone, N., Salinaro, A., and Emeline, A. 2001. Deleterious effects of sunscreen titanium dioxide nanoparticles on DNA: Efforts to limit DNA damage by particle surface modification. *Proc SPIE* 4258:86–98.

Shankar, S.S., Ahmad, A., and Sastry, M. 2003. Gerariium leaf assisted biosynthesis of silver nanoparticles. *Biotechnol Prog* 19:1627–1631.

Sharma, V., Singh, S.K., Anderson, D., Tobin, D.J., and Dhawan, A. 2011. Zinc oxide nanoparticle induced genotoxicity in primary human epidermal keratinocytes. *J Nanosci Nanotechnol* 11:3782–3788.

Sharon, M., Choudhary, A., and Kumar, R. 2010. Nanotechnology in agricultural diseases and food safety. *J Phytol* 4:83–92.

Shukla, R.K., Kumar, A., Pandey, A.K., Singh, S.S., and Dhawan, A. 2011. Titanium dioxide nanoparticles induce oxidative stress-mediated apoptosis in human keratinocyte cells. *J Biomed Nanotechnol* 7:100–101.

Singh, S., DBritto, V., Prabhune, A.A., Ramana, C.V., Dhawan, A., and Prasad, B.L.V. 2010. Cytotoxic and genotoxic assessment of glycolipid-reduced and -capped gold and silver nanoparticles. *New J Chem* 34(2):294–301.

Smith, C.J., Shawa, B.J., and Handy, R.D. 2007. Toxicity of single walled carbon nanotubes to rainbow trout (*Oncorhynchus mykiss*): Respiratory toxicity, organ pathologies, and other physiological effects. *Aquat Toxicol* 82(2):94–109.

Sondi, I., and Salopek-Sondi, B. 2004. Silver nanoparticles as antimicrobial agent: A case study on *E. coli* as a model for Gram-negative bacteria. *J Colloid Interface Sci* 275, 177–182.

Song, M.M., Song, W.J., Bi, H., Wang, J., Wu, W.L., Sun, J., and Yu, M. 2010. Cytotoxicity and cellular uptake of iron nanowires. *Biomaterials* 31(7):1509–1517

Soto, K.F., Carrasco, A., Powell, T.G., Garza, K.M., and Murr, L.E. 2005. Comparative *in vitro* cytotoxicity assessment of some manufactured nanoparticulate materials characterized by transmission electron microscopy. *J Nanoparticle Res* 7:145–169.

Sperling, R.A., Rivera Gil, P., Zhang, F., and Zanella, M., and Parak, W.J. 2008. Biological applications of gold nanoparticles. *Chem Soc Rev* 37:1896–1908.

Stern, S.T., and McNeil, S.E. 2008. Nanotechnology safety concerns revisited. *Toxicol Sci* 101:4–21.

Sun, H., Zhang, X., Niu, Q. et al. 2007. Enhanced accumulation of arsenate in carp in the presence of titanium dioxide nanoparticles. *Water Air Soil Pollut* 178:245–254.

Taju, G., Majeed, A., Nambi, K.S.N., and Hameed, A.S.S. 2014. *In vitro* assay for the toxicity of silver nanoparticles using heart and gill cell lines of *Catla catla* and gill cell line of *Labeo rohita*. *Comp Biochem Physiol C* 161:41–52.

Takahashi, H., Sawada, S., and Akiyoshi, K. 2011. Amphiphilic polysaccharide nanoballs: A new building block for nanogel biomedical engineering and artificial chaperones. *ACS Nano* 5:337–345.

Takenaka, S., Karg, E., Roth, C. et al. 2001. Pulmonary and systemic distribution of inhaled ultrafine silver particles in rats. *Environ Health Persp* 109:547–551.

Tang, J., Xiong, L., Wang, S. et al. 2009. Distribution, translocation and accumulation of silver nanoparticles in rats. *J Nanosci Nanotechnol* 9(8):4924–4932.

Tedesco, S., Doyle, H., Blasco, J., Redmond, G., and Sheehan, D. 2010. Oxidative stress and toxicity of gold nanoparticles in *Mytilus edulis*. *Aquat Toxicol* 100:178–186.

Templeton, R.C., Ferguson, P.L., Washburn, K.M. et al. 2006. Life cycle effects of single walled carbon nanotubes (SWNTs) on an estuarine meiobenthic copepod. *Environ Sci Technol* 40(23):7387–7393.

Ternes, T., Joss, A., and Siegrist, H. 2004. Peer reviewed: Scrutinizing pharmaceuticals and personal care products in wastewater treatment. *Environ Sci Technol* 38:392–399.

Toyokuni, S. 1998. Oxidative stress and cancer: The role of rodox regulation. *Biotherapy* 11:147–154.

Tsuchiya, T., Oguri, I., Yamakoshi, Y.N., and Miyata, N. 1996. Novel harmful effects of fullerene on mouse embryos *in vitro* and *in vivo. FEBS Lett* 393:139–145.

Unfried, K., Albrecht, C., Klotz, L.O., Von Mikecz, A., Grether-Beck, S., and Schins, R.P.F. 2007. Cellular responses to nanoparticles: Target structures and mechanisms. *Nanotoxicology* 1:52–71.

US EPA (US Environmental Protection Agency). 1994. 10-Day Chronic-*Daphnia magna* or *Daphnia pulex*, SOP #2028. Available from http://www.ert.org/products/2028.PDF.

Usenko, C.Y., Harper, S.L., and Tanguay, R.L. 2007. *In vivo* evaluation of carbon fullerene toxicity using embryonic zebrafish. *Carbon* 45(9):1891–1898.

Usenko, C.Y., Harper, S.L., and Tanguay, R.L. 2008. Fullerene C60 exposure elicits an oxidative stress response in embryonic zebrafish. *Toxicol Appl Pharmacol* 229(1):44–55.

Velzeboer, I., Hendriks, A.J., Ragas, A.M., and van de Meent, D. 2008. Aquatic ecotoxicity tests of some nanomaterials. *Env Toxicol Chem* 27(9):1942–1947.

Vermylen, J., Nemmar, A., Nemery, B., and Hoylaerts, F. 2005. Ambient air pollution and acute myocardial infarction. *J Thromb Haemost* 3:1955–1961.

Wagner, S.C., Roskamp, M., Pallerla, M., Araghi, R.R., Schlecht, S., and Koksch, B. 2010. Nanoparticle-induced folding and fibril formation of coiled-coil-based model peptides. *Small* 6:1321–1328.

Wainwright, M., Grayston, S.J., and deJong, P. 1986. Adsorption of insoluble compounds by mycelium of the fungus *Mucor flavus. Enzym Micro Technol* 8:597–600.

Wang, J.J., Sanderson, B.J., and Wang, H. 2007. Cyto and genotoxicity of ultrafine titanium dioxide particles in cultured human lymphoblastoid cells. *Mutat Res* 28:99–106.

Wang, L.H., Wang, Y.H., Zhou, Y.L., Duan, X.Y., Li, M., and Zhang, F.S. 2001. Relationship between nanostructure Silicon dioxide and occurrence of plant fungi. *J Huazhong Agric Univ* 6:593–597.

Wang, W.J., Xu, S.F., Kong, F.D., Tang, T.S., Huang, Y.F., Bai, Q.Y., and Zheng, T. 2012c. Studies on nano-colloidal of antibodies gold dot immuno-filtration assay for detection of infectious bovine rhinotracheitis. *Chin Vet Sci* 8:831–836.

Ward, J.E., and Kach, D.J. 2009. Marine aggregates facilitate ingestion of nanoparticles by suspension feeding bivalves. *Mar Environ Res* 68:137–142.

Warheit, D.B., Hoke, R.A., Finlay, C. et al. 2007. Development of a base set of toxicity tests using ultrafine titanium dioxide particles as a component of nanoparticle risk management. *Toxicol Lett* 171(3):99–110.

Warheit, D.B., Sayes, C.M., and Reed, K.L. 2009. Nanoscale and fine zinc oxide particles: Can in vitro assays accurately forecast lung hazards following inhalation exposures? *Environ Sci Technol* 43(20):7939–7945.

Weibel, A., Bouchet, R., Boulch, F., and Knauth, P. 2005. The big problem of small particles: A comparison of methods for determination of particle size in nanocrystalline anatase powders. *Chem Mater* 17(9):2378–2385.

Williams, Y., Sukhanova, A., Nowostawska, M. et al. 2009. Probing cell-type specific intracellular nanoscale barriers using size-tuned quantum dots. *Small* 5:2581–2588.

Woo, K.S., Kim, K.S., Lamsal, K. et al. 2009. An *in vitro* study of the antifungal effect of silver nanoparticles on oak wilt pathogen *Raffaelea* sp. *J Microbiol Biotechnol* 19:760–764.

Wu, W.H., Sun, X., Yu, Y.P. et al. 2008. Titanium dioxide nanoparticles promote beta-amyloid fibrillation in vitro. *Biochem Biophys Res Commun* 373:315–318.

Xia, T., Kovochich, M., Brant, J. et al. 2006. Comparison of the abilities of ambient and manufactured nanoparticles to induce cellular toxicity according to an oxidative stress paradigm. *Nano Lett* 6:1794–1807.

Xiaoxiao, C., Xing, Z., Rui, L. et al. 2012. Photosynthetic toxicity and oxidative damage induced by nano- Fe3O4 on Chlorella vulgaris in aquatic environment, Open Journal of Ecology 2(1): 21–28.

Xia, T., Kovochich, M., Liong, M. et al. 2008. Comparison of the mechanism of toxicity of zinc oxide and cerium oxide nanoparticles based on dissolution and oxidative stress properties. *ACS Nano* 2:2121–2134.

Xie, W., Wang, L., Zhang, Y. et al. 2009. Nuclear targeted nanoprobe for single living cell detection by surface-enhanced Raman scattering. *Bioconjug Chem* 20:768–773.

Xiong, D., Fang, T., Yu, L., Sima, X., and Zhu, W. 2011. Effects of nano-scale titanium dioxide, zinc oxide and their bulk counterparts on zebrafish: Acute toxicity, oxidative stress and oxidative damage. *Sci Total Environ* 409:1444–1452.

Xu, A., Chai, Y., Nohmi, T., and Hei, T.K. 2009. Genotoxic responses to titanium dioxide nanoparticles and fullerene in gpt delta transgenic MEF cells. *Part Fibre Toxicol* 6:3.

Yacobi, N.R., Malmstadt, N., Fazlollahi, F. et al. 2010. Mechanisms of alveolar epithelial translocation of a defined population of nanoparticles. *Am J Respir Cell Mol Biol* 42:604–614.

Yao, C.P., Wang, L., Yu, M., Zee, S.Y., and Yip, W.K. 2004. Differential expression of three genes encoding an ethylene receptor in rice during development, and in response to indole-3-acetic acid and silver ions. *J Exp Bot* 55:547–555.

Yoon, K., Byeon, J., Park, J. et al. 2008. Antimicrobial characteristics of silver aerosol nanoparticles against *Bacillus subtilis* bioaerosols. *Environ Eng Sci* 25:289–293.

Zambrano-Zaragoza, M.L., Mercado-Silva, E., Gutierrez-Cortez, E., and Castano Tostado, E.D. 2011. Quintanar-Guerrero optimization of nanocapsules preparation by the emulsion diffusion method for food applications. *LWT-Food Sci Technol* 44:1362–1368.

Zan, L., Fa, W., Peng, T.P. et al. 2007. Photocatalysis effect of nanometer titanium dioxide and titanium dioxide-coated ceramic plate on hepatitis B virus. *J Photochem Photobiol B* 86:165–169.

Zeng, F., Hou, C., Wu, S.Z., Liu, X.X., Tong, Z., and Yu, S.N. 2007. Silver nanoparticles directly formed on natural macroporous matrix and their anti-microbial activities. *Nanotechnology* 18:1–8.

Zhu, S., Oberdorster, E., and Haasch, M.L. 2006a. Toxicity of an engineered nanoparticle (fullerene, C60) in two aquatic species, daphnia and fathead minnow. *Mar Environ Res* 62:S5–S9.

Zhu, X., Chang, Y., and Chen, Y. 2010. Toxicity and bioaccumulation of titanium dioxide nanoparticle aggregates in *Daphnia magna*. *Chemosphere* 78(3):209–215.

Zhu, Y., Ran, T., Li, Y. et al. 2006c. Dependence of the cytotoxicity of multi walled carbon nanotubes on the culture medium. *Nanotecnology* 17:4668–4674.

Zhu, X., Zhu, L., Chen, Y., and Tian, S. 2009. Acute toxicities of six manufactured nanomaterial suspensions to *Daphnia magna*. *J Nanopart Res* 11:67–75.

Zhu, X., Zhu, L., Duan, Z. et al. 2008. Comparative toxicity of several metal oxide nanoparticle aqueous suspensions to zebrafish (*Danio rerio*) early developmental stage. *J Env Sci Health* 43(3)278–284.

Zhu, X., Zhu, L., Li, Y. et al. 2007. Developmental toxicity in zebrafish (*Danio rerio*) embryos after exposure to manufactured nanomaterials buckminsterfullerene aggregates (nC60) and fullerol. *Env Toxicol Chem* 26(5):976–979.

Zhu, Y., Zhao, Q., Li, Y., Cai, X., and Li, W. 2006b. The interaction and toxicity of multi-walled carbon nanotubes with *Stylonychia mytilus*. *J Nanosci Nanotechnol* 6:1357–1364.



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11

Methods of In Vitro *and* In Vivo *Nanotoxicity Evaluation in Plants*

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11.1 Introduction

The burgeoning field of nanotechnology has propelled an upsurge in the use of various natural and engineered nanomaterials for industrial, clinical, and environmental applications. The nanoscale size range of 1–100 nm and large surface area-to-volume ratio are the fundamental characteristic features of nanoparticles, making them a worldwide multibillion-dollar phenomenon. They differ from their bulk counterparts in terms of the physicochemical properties of size, shape, surface chemistry, charge, dissolution, pH, and conductivity. Hence, nanomaterials pose higher toxicological implications compared to their bulk forms (Buzea et al., 2007). The global annual production of nanomaterials reached 1000 t in 2004, and by 2015, the nanotechnology industry is estimated to attain a staggering $ 1 trillion (Maynard et al., 2006; EPA and Office of the Science Advisor, 2007). There are already more than 1600 nanobased consumer products, and hundreds are expected to appear in the global markets in the years to come (Rejeski and Lekas, 2008; EPA Federal Facilities Restoration and Reuse Office, 2014). At least 44 elements of the periodic table are being synthesized in nanoforms such as tubes, clays, and dots for various uses by more than 140 companies worldwide (Chen et al., 2004). Among them, the most widely produced metal nanomaterials include TiO_2, ZnO, Al_2O_3, Fe, and Ag nanoparticles, while carbon-based nanoparticles include single-walled (SW) and multiwalled (MW) carbon nanotubes (CNTs).

On the other hand, the aforementioned metal and carbon-based nanomaterials have been enlisted as emerging environmental threats by the US Environmental Protection Agency (US EPA) due to their potential toxicological impact on different biological systems leading to nanopollution (EPA Federal Facilities Restoration and Reuse Office, 2014). Literature survey (PubMed; 1982–2016) revealed an increase in publications on nanotoxicity profiling as a whole and genotoxicity in particular. However, reports on nanotoxicity in plant systems are scarce (Figure 11.1). The biological uptake, transport, accumulation, excretion, and overall fate of nanoparticles directly influence their toxicity in plants. In this regard, much work is being carried out on their uptake/internalization, biodistribution, and subsequent downstream toxicodynamics. Plants being primary producers are susceptible to nanotoxicological ramifications due to high bioavailability of metal and carbon-based nanomaterials in natural sediments, agricultural soils, and aquatic environments. Based on the guidelines by the Organization for Economic Cooperation and Development (OECD, 2006), the testing of potentially hazardous chemical compounds in plant systems is the first essential step of toxicity testing to assess their impact on environmental health. Particle characterization is a prerequisite for nanotoxicity evaluation as toxicity depends on particle morphology, surface topography/chemistry, size, shape, chemical structure, and colloidal stability. Preliminary cytogenotoxicity analysis is succeeded by the detailed mechanistic study of DNA damage and the mode of cell death induced by nanoparticles (Figure 11.2). This chapter encompasses the most essential methods for the evaluation of nanoparticle fate, cytotoxicity, genotoxicity, and oxidative stress as an underlying mechanism of nanotoxicity in various model plant systems.

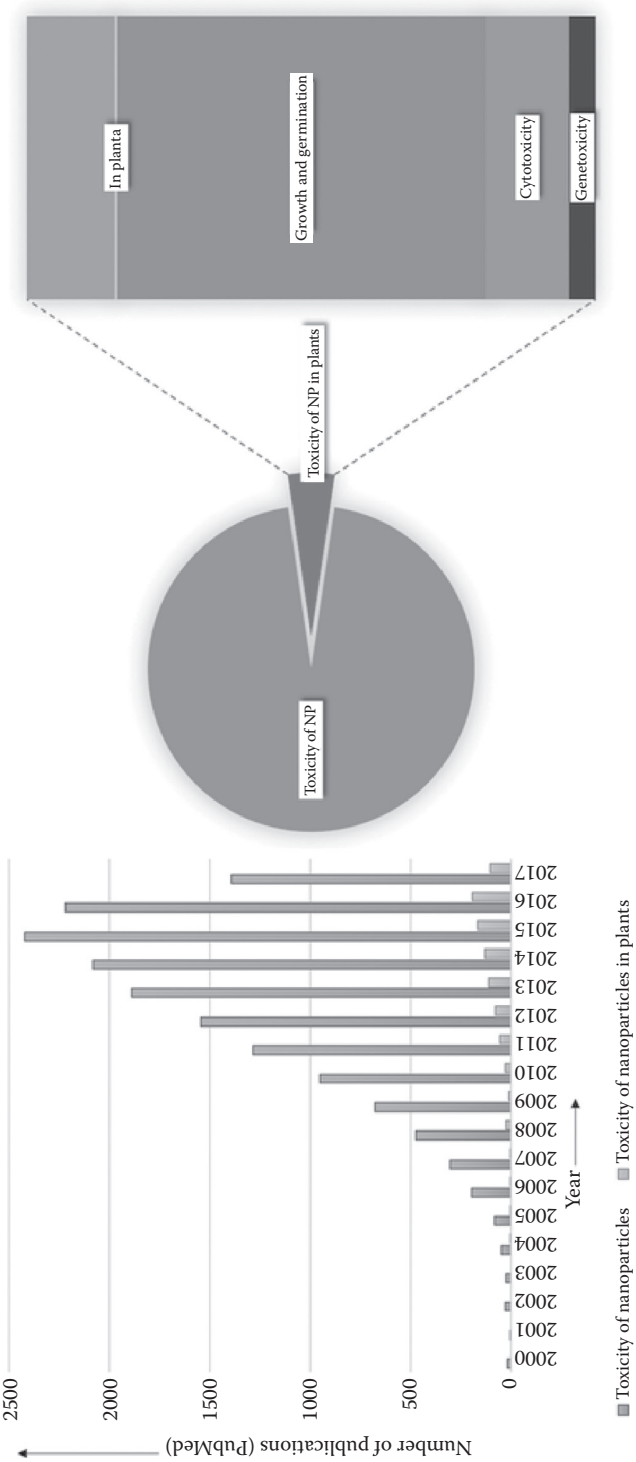


FIGURE 11.1 Literature survey on the toxicity of nanoparticles (source: PubMed; searched on April 8, 2017). The graph indicates the number of hits on toxicity of nanoparticles and toxicity of nanoparticles in plants. The pie chart shows a meager fraction of *in planta* nanotoxicity studies.

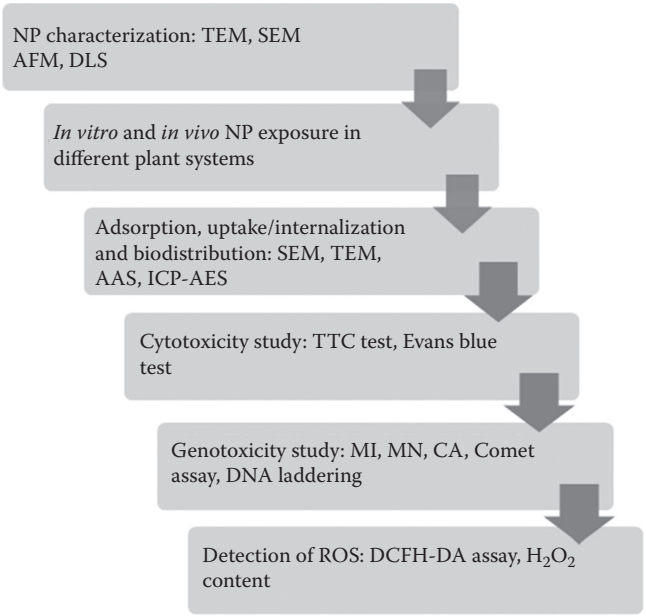


FIGURE 11.2 Schematic representation of basic nanotoxicology methods. Physicochemical characterization using TEM, SEM, AFM, and DLS are prerequisites of nanotoxicology investigations using plant systems. This is followed by dosage selection and exposure of the nanoparticles both *in vitro* and *in vivo*. The fate of the nanoparticles within the plant systems is assessed by SEM (adsorption), TEM (biodistribution), and AAS/ICP-AES (uptake/internalization) to correlate with the toxicity studies. Cytotoxicity is assessed by TTC and Evans blue tests for cell viability. Genotoxicity evaluation methods include cytogenetic parameters of MI, MN frequency, and CAs. DNA strand breaks are studied by comet assay and DNA laddering. As an indirect mechanism of toxicity, the generation of ROS is detected by fluorescent staining (DCFH-DA assay) and spectrophotometric estimation of H_2O_2 content. SEM, scanning electron microscopy; TEM, transmission electron microscopy; AFM, atomic force microscopy; DLS, dynamic light scattering; AAS, atomic absorption spectroscopy; ICP-AES, inductively coupled plasma atomic emission spectroscopy; MI, mitotic index; MN, micronuclei; CA, chromosome aberrations; NP, nanoparticle.

11.2 Nanomaterials and Model Plant Systems

11.2.1 Nanomaterials Needing Urgent Safety Appraisal

Among the numerous engineered nanoparticles synthesized to meet the growing demands of the world population, specific nonmetallic, metallic, and carbon-based nanomaterials have been enlisted by various regulatory bodies for urgent safety appraisal (Costantini et al., 2001; EPA Federal Facilities Restoration and Reuse Office, 2014; OECD, 2016). In 2010, the Organization for Economic Cooperation and Development stressed on a priority list of nanomaterials including titanium dioxide (TiO_2), cerium oxide (CeO_2), zinc oxide (ZnO), silver (Ag), iron (Fe), aluminum oxide (Al_2O_3), silicon dioxide (SiO_2), gold (Au), fullerenes (C_{60}), SWCNTs, and MWCNTs (Servin and White, 2016). A few years later, the US EPA enlisted various nanomaterials including TiO_2, CeO_2, nanoscale zerovalent iron particles (nZVI), Ag, C_{60},

SWCNT, and MWCNT as emerging environmental threats (EPA Federal Facilities Restoration and Reuse Office, 2014). Based on the sources, nanomaterials of ecotoxicological implications have been categorized into three groups composed of natural, incidental, and engineered forms (EPA Federal Facilities Restoration and Reuse Office, 2014). Naturally occurring nanoparticles including different forms of iron such as magnetite (Fe_3O_4) and maghemite (Fe_2O_3) occur in the earth's crust and are generated from volcanic lightning strikes and fires (Singh et al., 2010). The natural occurrence of other carbon-based nanomaterials such as nanotubes (single or multiwalled) can also be attributed to the same sources (EPA Federal Facilities Restoration and Reuse Office, 2014). Interestingly, naturally occurring fullerenes/buckyballs (C60, C20, C70) can be found in geological materials such as meteors, which are affected by high-energy phenomena such as lightning strikes (Trpkovic et al., 2012). Anthropogenic emissions of most metallic, nonmetallic, and carbon-based nanoparticles designed to meet the growing industrial and consumer needs are classified in the incidental and engineered nanoparticle exposure category. Some of the most widely used engineered nanomaterials are oxides of cerium (CeO_2), titanium (TiO_2), zinc (ZnO), iron (Fe_3O_4, Fe_2O_3), silver (Ag), and nZVI iron (nZVI Fe^0) nanoparticles. Among them, TiO_2 and CeO_2 nanoparticles are most extensively used, followed by ZnO, silver, and iron-based nanoparticles. TiO_2 nanoparticles are extensively used in numerous food and personal care products such as pharmaceuticals, cosmetics, toothpastes, pigments, glazes, enamels, plastics, paper, and fibers. The global production of TiO_2 had increased to 5000 t in 2010 and is projected to exponentially rise until 2025 with the ever-increasing demand (Weir et al., 2012). The major application of CeO_2 nanoparticles is in environmental remediation as diesel additives to reduce carbon monoxide, polycyclic aromatic hydrocarbons, and particulate matter effluence from vehicle exhausts (Erdakos et al., 2014). In United States alone, the projected emission in CeO_2 nanoparticles has been estimated to rise to 1750 t/year in the near future (Erdakos et al., 2014). ZnO nanoparticles are used in cosmetics (ultraviolet [UV] protectors), paints, and coating materials. Hence, the production of ZnO nanoparticles has escalated to 528 t/year (Zafar et al., 2016). The unique properties of Ag nanoparticles enable diverse applications in biomedicine, cosmetology, food, pharmacy, textile, construction, power, and electronic devices. The global market for Ag nanoparticles is projected to escalate to ~800 t by 2025 (Pulit-Prociak and Banach, 2016). Currently, the Ag nanoparticle market stands at 320–500 t/ annum (Pulit-Prociak and Banach, 2016; Kasprowicz et al., 2016). Among the carbon-based nanomaterials, CNTs are widely applied in industry and biomedicine. The annual production is estimated to be ~400 t in the European Union itself. The global production market of CNTs is $ 0.67 billion. Within the CNT market, MWCNTs hold the maximum share of $ 0.63 billion, which was predicted to escalate to $ 1.1 billion by 2016 (Sun et al., 2014; Ghosh et al., 2015). Among the various types of iron nanoparticles, the rising use of nZVI in the removal of organic and inorganic contaminants from soil, ground, and surface water has resulted in ~70 field applications. By the end of 2015, the global market for nanoremediation of wastewater and groundwater was estimated to reach $2.9 billion, within which nZVI hold a sizeable fraction (Bardos et al., 2011; Mueller et al., 2012; Tosco et al., 2014). On the other hand, the iron nanoparticle industry is estimated to be positioned at a staggering €20–40 million, which roughly corresponds to 100 t due to their uses in fuel cells, oxygen sensors, ceramics, optoelectronic devices, diagnostics, and therapy (Singh et al., 2010; European Commission, 2012). Most phytotoxicity

studies on all the preceding nanoparticles have focused on alterations in the physiological parameters of germination and plant growth as an effect of nanoparticle uptake, internalization, and cellular distribution (López-Moreno et al., 2010a; Rico et al., 2013; Song et al., 2013; Burke et al., 2015; Wang et al., 2016; Zafar et al., 2016). In the past decade, studies on genotoxicity and cytotoxicity of these nanomaterials have gained momentum in different plant systems (López-Moreno et al., 2010b; Ghosh et al., 2015, 2016, 2017).

11.2.2 Model Plant Systems Used for Nanotoxicity Testing

Plants are the primary producers of terrestrial and aquatic ecosystems. They form critical components of ecosystem functioning and play a pivotal role in the food supply integrity to subsequent trophic levels. Owing to their sessile life cycles, they are the primary organisms of the food chain to be susceptible to nanomaterial-induced adversities (Ma et al., 2015). However, the interaction of nanomaterials with plant systems is not fully understood. The use of model plants in nanotoxicity testing was popularized during the initial years of the twenty-first century. Among the diverse plant systems, model plants have emerged as the most popular candidates for nanotoxicity testing. *Arabidopsis thaliana* and *Nicotiana* sp. are two most widely used model systems due to their completely sequenced genomes (Bevan and Walsh, 2005; Sierro et al., 2014). Other plant systems such as *Allium cepa*, *Vicia faba*, and tobacco BY-2 cells are gaining popularity in recent years (Poborilova et al., 2013; Ghosh et al., 2016; Ahmed et al., 2017). This can be attributed to the ease of seed germination, plant growth, and maintenance. However, the use of tobacco BY-2 cells in nanotoxicity evaluation is rather rare. The highly synchronized and less complex characteristics of tobacco BY-2 cell suspensions enable enhanced sensitivity to nanotoxicity testing. Hence, the present chapter encompasses the methods of nanotoxicity testing in the aforementioned model plant systems.

11.3 Characterization of Nanoparticles

11.3.1 Preparation of Nanoparticle Dispersions

The solubility of most metallic, nonmetallic, and carbon-based nanomaterials is contentious. The dissolution of nanoparticles into the constituent metal ions in various solvents used for nanotoxicity testing is a known phenomenon (Kittler et al., 2010; Reed et al., 2012; Ahmed et al., 2017; Ghosh et al., 2017). Numerous reports evince the successful dispersion of cerium (CeO_2), titanium (TiO_2), zinc (ZnO), iron (Fe_3O_4, Fe_2O_3), silver (Ag), and nZVI (Fe^0) nanoparticles in test solvents by ultrasonic vibration (Ghosh et al., 2010, 2017; Gui et al., 2015; Ahmed et al., 2017).

Nanomaterials for phytotoxicity studies are usually synthesized or procured in powdered form. Most *in vivo* phytotoxicity analyses rely on MilliQ water suspensions of test nanomaterials. On the other hand, *in vitro* nanotoxicity assessments on plant cell suspensions involve stable dispersions of nanomaterials in the culture media prior to treatment. Furthermore, the media should be devoid of chelating agents such as ethylenediaminetetraacetic acid (EDTA) or phosphate groups (Poborilova et al., 2013). The solvents must be filter sterilized to avoid contamination with external particulate

and/or organic matter. Ultrasonic vibrations for at least 20–30 min, at 100–200 W and 20–30 kHz, are widely implemented to create colloidal suspensions of nanoparticles evenly dispersed throughout the solvent. Two main techniques of ultrasonication, viz., bath and probe, have been developed to prevent agglomeration (loosely arranged group of nanoparticles) and/or aggregation (tightly bound mass of particles). In most cases, probe ultrasonication result in more stable dispersions than bath (Jiang et al., 2009). Therefore, the ultrasonication of each treatment concentration is a crucial step to prevent nanoparticles from aggregation/agglomeration.

Ultrasonicated suspensions of MWCNT (10 µg/mL) may be used as positive control for all nanotoxicity analyses *in planta* and *in vitro* due of its easy penetrability into plant cells and affirmable reproducibility of cytogenotoxicity response results (Ghosh et al., 2011, 2015).

11.3.2 Physicochemical Characterization

Physicochemical characteristics chiefly determine the nanoparticle–nanoparticle and nanoparticle–solvent interactions (Jiang et al., 2009). The properties of size, shape, surface topography, charge, conductivity, and polydispersity influence their phytotoxicity (Ghosh et al., 2017). In water suspensions or dispersions in media where the main constituent of the medium is water, a hydronium ion shell formation takes place. This results in increased particle diameter, which is termed as *hydrodynamic diameter* (Jiang et al., 2009). The primary particle characteristics are determined by transmission electron microscopy (TEM), scanning electron microscopy (SEM), and atomic force microscopy (AFM). Dynamic light scattering (DLS) and zeta potential analyses determine the hydrodynamic characteristics of nanoparticles in suspension to confer an idea about their colloidal stability (Figure 11.3). Highly dilute (0.1–1 µg/mL),

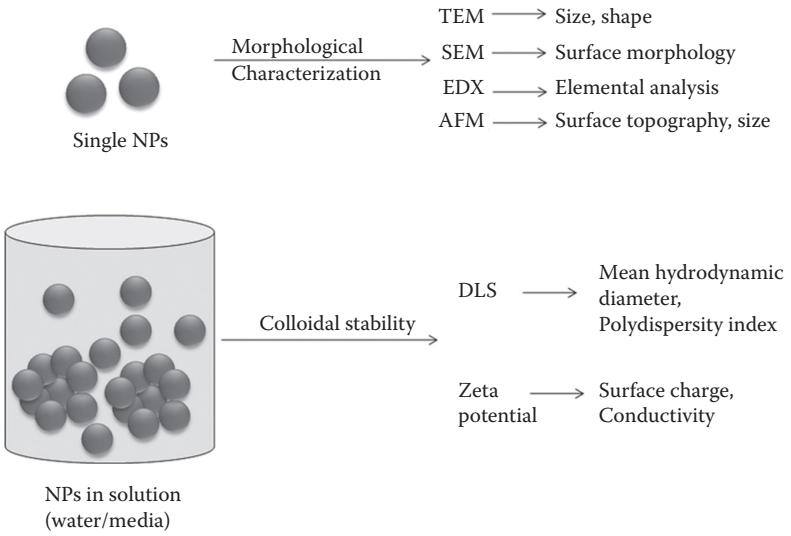


FIGURE 11.3 Nanoparticle characterization methods. The primary characteristics are studied by TEM, SEM, EDX, and AFM. The hydrodynamic characteristics to determine colloidal stability are analyzed by DLS and zeta potential assessment. NP, nanoparticle.

ultrasonicated suspensions of nanoparticles must be used for the preceding primary and hydrodynamic characterizations.

The morphology, size (primary diameter and diameter of agglomerates/aggregates), and shape are determined by TEM. Highly dilute samples (5 μL) are loaded on support grids (carbon-based type B, copper mesh 300) and analyzed by TEM (JEM-2100 LaB6, 200 kV, Jeol, Peabody, Massachusetts, United States). The surface morphology of the nanoparticles is studied by SEM (Carl Zeiss Pvt, Oberkochen, Germany) after loading the samples in powdered form onto a sample holder platform and sputter coating with gold or platinum. Energy dispersive X-ray spectroscopy (EDX) (Model 51–ADD0011, Oxford Instruments, Germany) coupled with TEM and SEM confirms the elemental purity of the samples by quantification (atomic or weight%) of the presence of constituent elements. AFM study provides a detailed understanding of the size and surface topography of each nanoparticle. The samples (10 μl) are drop casted on clean, thin, and smooth mica sheets and scanned by AFM (Veeco Instruments Inc., New York, United States) usually at a frequency of 300 kHz in the tapping mode.

As hydrodynamic characterization is a prerequisite for all nanotoxicity evaluations, DLS (90Plus Particle Size Analyzer, Brookhaven Instruments Corporation, Holtsville, New York, United States) and zeta potential (Malvern Instruments, Sl. No. MAL1053771, Worcestershire, United Kingdom) studies are performed to yield data on mean hydrodynamic diameter, polydispersity index (extent of agglomeration), zeta potential (surface charge in solution), and conductivity using Zetasizer ver. 7.10 software.

11.4 Plant Growth Conditions

11.4.1 *In Vivo* Test Systems

For *in vivo* nanotoxicity evaluation, *A. thaliana* (Geisler-Lee et al., 2012; Syu et al., 2014), *N. tabaccum* (Ghosh et al., 2010; Burklew et al., 2012), *A. cepa* (Ghosh et al., 2017; Ahmed et al., 2017), and *V. faba* (Patlolla et al., 2012; Ghosh et al., 2016) are the most popular *in planta* systems.

A. thaliana seeds are surface sterilized with 70% ethanol, washed in sterile MilliQ water, and further surface sterilized in 1–8% solutions of common household bleach (sodium hypochlorite; NaClO) in water (v/v) for 5–10 min and rinsed thrice with sterile MilliQ water (Geisler-Lee et al., 2012; Ma et al., 2013). This is usually followed by nanoparticle treatment in liquid, semisolid, or solid tissue culture media for germination studies. The liquid media usually is composed of one-fourth strength of Hoagland medium, and nanoparticles may be dispersed in it for seed treatment in 24 well plates (Geisler-Lee et al., 2012). Semisolid media constitute half strength of Murashige and Skoog (MS) medium (MS basal medium supplemented with vitamins and 1% sucrose in deionized water at pH 5.7), along with 3g/L phytoblend agar (Ma et al., 2013). The solid medium comprises MS (½) medium, 20% sucrose, 0.05% MES buffer (pH 5.4) and 0.8–0.9% agar (Kim et al., 2014). The sterilized seeds are stratified in the same medium at 4°C overnight prior to nanoparticle treatment. Seeds are incubated in the nanoparticle-containing media in growth chambers with cycles of 16 hours of light and 8 hours of dark, and a temperature of 18–22°C is maintained (Ma et al., 2013).

In the case of *N. tabaccum*, seeds are imbibed in water overnight and allowed to germinate in autoclaved soil kept in well-watered earthen pots (30 cm diameter) at 25°C ± 2°C. Nanoparticle treatment is hydroponically carried out when the plantlets reach the fourth leaf stage (Ghosh et al., 2010).

For *A. cepa*, the outer scaly leaves are removed and equal-sized bulbs are allowed to germinate (25°C; 12-hour light and dark cycle) in sterilized moist sand. The bulbs are treated with various concentrations of nanoparticles when the roots attain a length of 2–3 cm (Ghosh et al., 2017).

Prior to nanotoxicity studies in *V. faba*, healthy seeds are imbibed in distilled water for 24 hours and allowed to germinate in petri plates (25°C) containing moist filter papers for 3–4 days. When the primary root reaches a length of 2–3 cm, it is transferred to sterile moist sand for 7 days (25°C; 12-hour light and dark cycle) until the shoots attain double nodal stage (Patlolla et al., 2012; Ghosh et al., 2016).

A. thaliana seeds are exposed to nanoparticles in liquid, semisolid, or solid media. Nanoparticle treatment is preferable in semisolid or solid medium to avoid precipitation or aggregation (Lee et al., 2010). For *in planta* treatments, germinated seedlings are transferred to fresh plates containing media with test concentrations of nanoparticles (Syu et al., 2014). All hydroponic treatments for *N. tabaccum*, *A. cepa*, and *V. faba* are performed in well-aerated glass vials at 25°C. *A. cepa* bulbs are treated with different concentrations of nanoparticles diluted in sterile deionized double distilled water for 3 or 24 hours (Ghosh et al., 2016, 2017). *N. tabacum* plantlets at the fourth leaf stage are treated with different concentrations of nanoparticles for 3 hours and transferred to double distilled water and kept for 24 hours (Ghosh et al., 2010). *V. faba* seedlings are treated for 3 hours with nanoparticles at various concentrations and transferred to double distilled water and kept for 24 hours (Ghosh et al., 2016).

11.4.2 *In Vitro* Cell Suspensions

Tobacco BY-2 cells suspensions are emerging *in vitro* systems for the analysis of nanotoxicity at the cellular level (Krystofova et al. 2013; Poborilova et al. 2013; Loukanov et al. 2016). The cells are multiplied in half MS liquid medium containing 3% (w/v) sucrose and 0.2 mg/L 2,4-dichlorophenoxyaetic acid (0.2 mg/L) according to Moriyasu and Ohsumi (1996). The media can also be supplemented with KH_2PO_4 (0.2 g/L) and thiamine (1 mg/L) along with sucrose and 2,4,-dichlorophenoxyaetic acid (Nagata et al. 1992). The cultures are maintained by the passage of 2–3 mL of cells in stationary phase to fresh liquid MS media (80 mL) every week. The cultures are kept under constant rotation at 100 revolutions per minute (rpm) at 25°C ± 1°C (Moriyasu and Ohsumi 1996). Logarithmic phase cells are treated with nanoparticles in the same liquid MS media devoid of chelating agents (EDTA and phosphate) for either 12, 24, 36, 48, 72, and 96 hours (Poborilova et al. 2013) or for 5 days (Krystofova et al. 2013).

11.5 Methods of Adsorption, Internalization, and Biodistribution Study

The fate of nanoparticles depends on their adherence to biological surfaces, uptake through the cell wall and cell membrane, internalization and biodistribution within

the cell and plant body (Ma et al., 2015). The following methods elucidate various means of sample preparation for studying the adsorption, uptake/internalization, and biodistribution in plant systems.

An important factor that regulates the phytotoxicity of metal nanoparticles is ion dissolution into the solvent (Misra et al., 2012; Maurer-Jones et al., 2013). The nanoparticle suspensions at all working concentrations can be subjected to centrifugation at 1000 g for 15 min followed by filtration of the supernatants through 0.2 μm filters. The resultant filtrate is estimated for the respective amount of metal ions leeched into the solvent by inductively coupled plasma atomic absorption spectrometry (ICP-AES) (Arcos Simultaneous ICP Spectrometer, Spectro Analytical Instruments GmbH, Kleve, Germany) or atomic absorption spectrometry (AAS) (AAnalyst 400, Perkin-Elmer, Waltham, Massachusetts, United States) (Ghosh et al., 2017). Appropriate dilutions are made, and the values are expressed in parts per million. Metal nanoparticless of Zn, Ag, and Fe essentially require such dissolution studies (Ghosh et al., 2016).

The dissolution study is followed by the evaluation of nanoparticle adsorption on the plant surface exposed to various treatment concentrations of nanoparticles. This is observed by SEM, and the amount of adsorbed nanoparticles is quantified by EDX (Wang et al., 2016; Ahmed et al., 2017; Ghosh et al., 2017). Treated samples are fixed with 2.5% glutaraldehyde and 0.4% paraformaldehyde in 0.1 M sodium phosphate buffer at pH 7.2. They are dehydrated in an ethanol series and 1% osmium tetroxide (OsO_4) is used as postfixative. The dehydrated and fixed samples are mounted on double-side sticky carbon tapes placed on aluminum stubs and sputter coated prior to observation by SEM (Ghosh et al., 2017). Another method of SEM sample preparation involves rapid freezing of the treated plant tissues. Nanoparticle-exposed fresh roots are mounted on aluminum stubs covered with double-side sticky carbon tapes, and a Quorum Tech CryoTransfer system is used to prepare the samples for SEM. The aluminum stubs mounted with the samples are placed on a vacuum transfer rod under continuous freezing environment. The samples are then frozen in a stream of liquid nitrogen and transferred to a cool stage under vacuum for cryo-SEM observation (Ghosh et al., 2015). Visualization is done without coating using a Zeiss EVO-MA 10 scanning electron microscope (Carl Zeiss Pvt Ltd., Oberkochen, Germany).

Cellular internalization and biodistribution of nanoparticles within treated plant tissues are best visualized by TEM (Kim et al., 2014; Ghosh et al., 2015). Plant samples (root or shoot) treated with various nanoparticles are excised from the bulbs or seedlings, washed, and fixed in Karnovsky's solution overnight at 4°C. They are then washed in 0.1 M cacodylate buffer and treated with 2% aqueous osmium tetroxide (OsO_4) in 0.2 M cacodylate buffer at 4°C for 2 hours. Subsequent dehydration is carried out in different grades of ethanol where the samples are placed in each grade twice for 15 min at 4°C. The dehydrated samples are incubated in propylene oxide for 15 min, transferred to epoxy resin–propylene oxide (1:1) and kept overnight. They are embedded in fresh epoxy resin and kept for 3–4 hours and reembedded in fresh epoxy resin prepared in polyethylene capsule blocks. These blocks are cured at 60°C for 48 hours and ultramicrotome sections (with or without lead citrate stain) are prepared. The sections are mounted onto support grids (carbon-based type B, copper mesh 300) and analyzed by TEM (JEM-2100 LaB6, 200 kV, Jeol, Peabody, Massachusetts, United States).

The amount of nanoparticles adsorbed and internalized can be further quantified using ICP-AES or AAS analyses. Numerous methods of digestion of plant tissues have evolved over the past few decades. The least cumbersome among them is the nitric–perchloric acid digestion method of Miller (1998). In this method, 500 mg of treated plant tissue (roots or shoots) are placed in 6 mL concentrated nitric acid overnight at room temperature for the predigestion of organic content. This is followed by digestion for 1 hour at 150°C in a fume hood. The samples are allowed to cool down to room temperature and 2 mL of perchloric acid is slowly added for the complete oxidation of organic matter. Digestion is carried out for 2 hours at 200°C. The samples are again cooled to room temperature and filtered using 2 μm filter disks. Deionized water is used to make up the volume to 25 mL, and the samples are analyzed by ICP-AES or AAS.

The principles of *in vitro* nanotoxicity methods carried out using tobacco BY-2 cell suspensions are homologous to that carried out *in vivo*, with moderate differences in test conditions. Although nanotoxicity studies using tobacco BY-2 cells are scarce, available literature provides specific methods (Poborilova et al. 2013) for the estimation of uptake and internalization for specific nanoparticles. Poborilova et al. (2013) quantified the presence of aluminum nanoparticles within treated tobacco BY-2 cells and aluminum ions in the culture media by morin staining. The formation of fluorescent Al-morin complexes is detected by fluorescence microscopy. Moreover, the bioimaging of tobacco BY-2 cells upon treatment with photosensitizer conjugated ultrasmall carbon nanodots can be done to study their uptake by the endocytosis pathway using fluorescence microscopy (Loukanov et al. 2016).

11.6 Assessment of Phytotoxicity

11.6.1 Germination and Growth Parameters

For all *in vivo* studies, the root and shoot lengths of control and nanoparticle treated plants are measured (five longest and five shortest per plant) and photographed. The average root and shoot lengths are calculated and graphically represented along with representative images (Kim et al., 2014). The tolerance index (%) of the roots can be calculated (Ahmed et al., 2017) by the following formula:

$$\text{Tolerance index (\%)} = \frac{\text{Length of roots in the treated sets}}{\text{Length of roots of negative control}} \times 100$$

Numerous reports elucidate the inhibition of root growth in *A. cepa* bulbs treated with TiO_2 (Ghosh et al., 2010) and ZnO (Ahmed et al., 2017). On the other hand, increase in root length was observed in Arabidopsis treated with nZVI (Kim et al., 2014). These findings were further confirmed by Ghosh et al., (2017), where nZVI promoted root and shoot growth in *A. cepa* bulbs. The detrimental effects of CeNPs at high concentrations were mirrored by decreased plant growth by up to 85% in *A. thaliana* (Ma et al., 2013). In *N. tabaccum*, the overall plant growth in terms of root length, germination rate, and average biomass declined in response to TiO_2 nanoparticle exposure (Frazier et al., 2014).

The analysis of increase or decrease in cell density as a vital parameter is carried to assess phytotoxicity in tobacco BY2 cells exposed to nanoparticles *in vitro* (Poborilova et al. 2013). Cells are counted at precise time intervals using a hemocytometer, and the density is calculated from at least 10 random fields for each treatment set. High concentrations of Al_2O_3 nanoparticles stalled the growth of BY-2 cells as observed by 54% decrease in cell density (Poborilova et al. 2013).

11.6.2 Cell Viability Studies

An integral aspect of nanotoxicity studies in plant systems is the assessment of cell viability. This involves the use of vital stains such as Evan's blue, as well as cellular metabolic activity markers such as triphenyl tetrazolium chloride (TTC). While Evans blue dye is excluded by the intact cell membranes of living cells, dead cells are stained due to their membrane permeability (Baker and Mock, 1994). For *in planta* analyses, root tips are excised and immersed in 0.25% aqueous Evan's blue solution and incubated for 15 min. The excess dye is washed with distilled water thrice, and the tips are photographed for qualitative analysis of cell death. The stained root tips are placed in fresh microcentrifuge tubes containing *N*,*N*-dimethylformamide (500 µL). The tips are incubated in *N*,*N*-dimethylformamide for 30 min to 1 hour until all the internalized dye is solubilized. The optical density of the resultant solutions is recorded at 600 nm using a spectrophotometer. This decisive end point was used to affirm the cytotoxicity of MWCNT (Ghosh et al., 2015), nZVI (Ghosh et al., 2017), ZnO (Ghosh et al., 2016), and Ag nanoparticles (Panda et al., 2011) in *A. cepa*.

TTC, on the other hand, relies on the mitochondrial enzyme activities. It is acted upon by mitochondrial oxidoreductase enzymes to form characteristic red formazan products (Chang et al., 1999). Excised root tips are immersed in 0.5% aqueous solutions of TTC and incubated for 15 min at 37°C. The tips are photographed and immersed in 95% ethanol for complete solubilization of the red formazan, and the absorbance is recorded at 490 nm. TTC assay revealed the onset of mitochondrial dysfunction in *A. cepa* treated with nZVI (Ghosh et al., 2017), MWCNT (Ghosh et al., 2015), ZnO (Ahmed et al., 2017), Al_2O_3 (De et al., 2016), and CuO (Deng et al., 2016) nanoparticles.

In case of nanotoxicity studies using tobacco BY-2 cells, a highly confirmatory end point for viability is fluorescein diacetate (FDA)/propidium iodide (PI) dual staining. FDA is metabolized by living cells into fluorescin which emits green fluorescence as a mark of viability. Dead/dying cells take up PI through damaged cell membranes leading to its intercalation within DNA to emit red fluorescence (Babula et al., 2012). Nanoparticle-treated cell suspensions are incubated with FDA (2.4 µM/L) and PI (30 µM/L) for 5 min at 25°C. The cells are washed with phosphate buffer saline and viewed by fluorescence microscopy. Cell viability is assessed from at least 1000 cells and graphically represented. BY-2 cells treated with Al_2O_3 (Poborilova et al., 2013) and iron nanoparticles (Krystofova et al., 2013) showed rise in red fluorescence of PI indicating loss of cell viability. Hence, these end points can be cumulatively categorized as the first step to toxicity testing of nanomaterials *in planta* and *in vitro*.

11.7 Genotoxicity Methods

A wide range of genotoxicity bioassays is available for the direct detection of genetic alterations of mutations and chromosomal aberrations. Indirect methods include the assessment of DNA adducts or DNA strand breaks which can be performed by single-cell gel electrophoresis or comet assay (Mukherjee and Gichner, 2009). Nanoparticles are known to induce genotoxicity by two main mechanisms. The first mode of DNA damage is by direct DNA strand breaks either mechanically or by direct chemical interactions with DNA, histones, and other nuclear proteins. The second approach involves indirect DNA damage by the generation of reactive oxygen species (ROS), which may, in turn, impair the DNA repair machinery (Donaldson et al., 2010; Magdolenova et al., 2014). Moreover, dissociated metal ions in nanoparticle suspensions may diffuse through the plant cell membranes and exacerbate genotoxic responses (Maurer-Jones et al., 2013; Ghosh et al., 2017). Both modes of damage can be studied by the cytogenetic parameters to evaluate alterations at the chromosomal level. Single and double strand breaks in DNA can be studied by alkaline comet assay and DNA laddering (Figure 11.4).

11.7.1 Cytogenetic Studies

The cytogenetic parameters of mitotic index (MI), chromosome aberrations (CAs), and micronuclei (MN) frequency are analyzed by the method of Mukherjee and Sharma (1988) with modifications (Ghosh et al., 2010). Root tips of *A. cepa* and *V. faba* treated with nanoparticles are excised (10–15) from five replica plants per treatment concentration. They were fixed in a solution of acetic acid: ethanol in 1:3 ratio for 24 hours. Subsequent hydrolysis is carried out in 45% acetic acid for 15 min. Following hydrolysis, the tips are stained in 2% aceto-orcein: 1(N) HCl (9:1 ratio) for

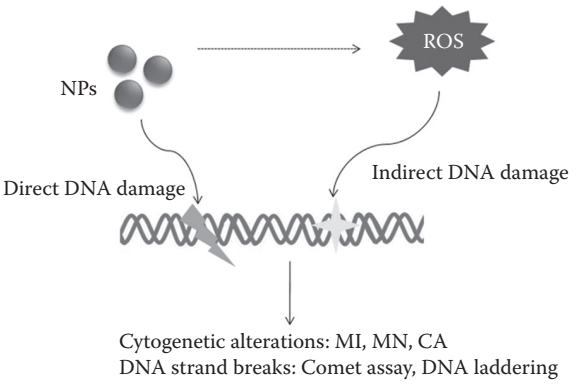


FIGURE 11.4 End points used for the assessment of genotoxicity of nanoparticles. Direct and indirect genotoxicities are evaluated by the cytogenetic endpoints of MI, MN frequency, and CAs. The extent of DNA strand breaks are studied by comet assay at the level of single cells and DNA laddering at the level of whole genomes. ROS, reactive oxygen species; NP, nanoparticle.

45 min. The meristematic region (M) and the quiescent (F1) region situated above the meristematic region are excised separately on clean grease free glass slides with sharp razor blades. Separate slides are prepared for M and F1 regions. The excised portions are placed in a drop of 45% acetic acid and cover slips are mounted on them. Squashing is performed by the application of gentle pressure over the cover slips in a circular manner for even spreading of the cells. The slides are sealed with wax or nail varnish and observed under a light microscope (Leica, Germany). At least 3000 cells are scored per treatment concentration. The parameters of MI as a measure of number of cells in mitosis per 1000 cells and CA as the percentage of cells per 1000 cells that contained abnormalities such as breaks (metaphase, anaphase), sticky bridges (anaphase, telophase), laggards (anaphase), early separation (anaphase), and vagrants are analyzed from the M regions. Nuclear aberrations such as multinucleate, binucleate cells, nuclear bubs, notches, and lobulated nuclei are similarly scored. The MN frequency in terms of the number of MN per 1000 cells is assessed from the F1 regions. All the preceding end points are sensitive biomarkers for the detection of environmental mutagenesis (Leme and Marin-Morales, 2009). These parameters affirmed the genotoxicity of TiO_2 (Ghosh et al., 2010; Pakrashi et al., 2014), Ag (Kumari et al., 2009), MWCNT (Ghosh et al., 2011, 2015), nZVI (Ghosh et al., 2017), and ZnO (Kumari et al., 2011; Ghosh et al., 2016) nanoparticles in *A. cepa* and Ag nanoparticles in *V. faba* (Patlolla et al., 2012).

11.7.2 Single-Cell Gel Electrophoresis (Comet Assay)

The extent of DNA damage is analyzed by comet assay according to the method of Gichner et al. (2006) with the following modifications (Ghosh et al., 2010). Slides with 1/4 frosted ends are thoroughly washed to make them grease free and base coated with 1% normal melting point agarose to make the first layer. The slides are allowed to dry overnight. Excised root or leaf tissues from plants treated with nanoparticles are placed on sterile disposable polystyrene-coated 60 mm petriplates containing 400 μL of 400 mM Tris buffer (pH 7.5) kept on ice packs for 2 min to keep the tissues turgid. The roots are finely chopped using new sharp razor blades to facilitate nuclei isolation. Two hundred μL of the obtained nuclei suspension is added to 200 μL of 1% low-melting point agarose (LMPA) and thoroughly mixed by pipetting. Eighty μL of this suspension is placed on the base coated slides excluding the frosted end, and a coverslip (22 mm × 50 mm) is mounted to make the second layer. Three replicate slides are prepared. The second layer containing the nuclei is cooled and allowed to solidify (4°C) for 15 min. Following solidification, the coverslips are removed, and a third layer containing 0.5% LMPA is placed on the solidified second layer, and coverslips are mounted. The third layer is allowed to solidify at 4°C. Following the solidification of the third layer, the coverslips are removed, and the slides were placed in a gel electrophoresis tank containing alkaline electrophoresis buffer, which is composed of of 300 mM NaOH and 1 mM EDTA; pH >13 prepared in cold double distilled water and the DNA was allowed to unwind at 4°C for 20 min in the case of *A. cepa*; 15 min for *N. tabaccum*, and 10 min for *V. faba*. Gel electrophoresis is performed for 30 min in *A. cepa*, 20 min in *N. tabaccum*, and 15 min in case of *V. faba*, at 26 V and 300 mA at 4°C. The slides are washed in cold double distilled water and neutralized in 400 mM Tris buffer (pH 7.5) for 5 min followed by washing in cold double distilled water.

The slides are air dried at room temperature and stored at 4°C for further scoring. The slides are stained with 20 µg/mL of ethidium bromide and rinsed with distilled water to remove excess stain. Slides are observed under a fluorescent microscope (Leica, Wetzlar, Germany) equipped with appropriate filter (N 2.1). A charge-coupled device camera is connected to the microscope, and images of the nuclei are captured (×100). The comet parameter % tail DNA from 300 nuclei per concentration (100 nuclei per slide) is calculated using the image analysis software Komet 5.5 (Kinetic Imaging; Andor Technology, Nottingham, United Kingdom). Four median values of 25 nuclei per 100 nuclei are taken from each slide, and the data are statistically analyzed.

The comet assay is a powerful tool in the assessment of nanoparticle-induced DNA damage *in planta* at the level of single cells. The three plant species of *A. cepa*, *N. tabaccum*, and *V. faba* are most widely employed for genotoxicity studies using this end point. This is attributed to their ease of cultivation, high sensitivity to mutagens, and high reproducibility of results (Gichner et al., 2016). Moreover, the use of *A. cepa* and *V. faba* as test systems provides the advantage of corroborating the genotoxicity data obtained by cytogenetic parameters and comet assay from the same roots. Comet assay revealed significant genotoxicity of TiO_2 (Ghosh et al., 2010; Demir et al., 2014), MWCNT (Ghosh et al., 2011, 2015), nZVI (Ghosh et al., 2017), ZnO (Demir et al., 2014; Ghosh et al., 2016) in *A. cepa* roots. In *N. tabaccum* leaves, the genotoxicity of TiO_2 (Ghosh et al., 2010) and Ag (Ghosh et al., 2012) nanoparticles was reported. Other studies involving tomato plants exposed to Ni nanoparticles (Faisal et al., 2013) and eggplant treated with Co nanoparticles (Faisal et al., 2016) utilized this versatile tool in the assessment of DNA strand breaks.

11.7.3 DNA Laddering

A modified cetrimonium bromide (CTAB) method (Doyle and Doyle, 1987) is followed for the extraction of genomic DNA from the nanoparticle treated plants (Ghosh et al., 2015). One hundred mg of nanoparticle-treated plant tissue (root or shoot or leaf) is excised from each treated concentration and stored in −20°C for further use. The tissue is homogenized in CTAB buffer (100 mM Tris buffer [pH 8], 25 mM EDTA, 2 M NaCl, 3% polyvinylpyrrolidone, and 3% CTAB) followed by incubation at 65°C in a water bath for 20 min. The incubated mixture is cooled to room temperature and an equal volume of chloroform–isoamyl alcohol (24:1) is added. The mixture is centrifuged for 5 min at 12000 rpm. The resultant clear upper aqueous phase is transferred to a new microfuge tube, two-thirds volume of ice-cold 2-propanol is added and incubated for 30 min at −20°C. The suspensions are centrifuged at 10,000 rpm for 10 min, and the resultant pellet is washed in 75% ethanol twice. The nucleic acid pellet is air dried in a sterile laminar airflow, dissolved in Tris–EDTA buffer (10 mM Tris buffer; pH 8, and 1 mM EDTA) and stored at 4°C for further use. RNase treatment is done by incubating the nucleic acid solutions in 10 mg/mL RNase A for 30 min at 37°C. The purity of the isolated DNA is estimated by measuring the ratio of absorbance at 260:280 nm. DNA laddering is performed by resolving the isolated DNA on an agarose gel of 2.5% in 1× TAE (Tris acetate EDTA) buffer at 100 V. A 100 bp ladder is used for reference. Following electrophoresis, the gel is stained with ethidium bromide (0.5 µg/mL) and photographed in a UV transilluminator-based gel documentation system (E-Gel®

Imager, ThermoFisher Scientific, Bengaluru, India). This method is one of the classical end points to qualitatively assess whole genome DNA breaks using gel electrophoresis. The occurrence of DNA fragments that migrate to form a characteristic ladder-like pattern designates programmed cell death (Ghosh et al., 2015). Carbon-based nanoparticles such as MWCNT have been reported to inculcate the formation of such DNA fragmentation in *A. cepa* (Ghosh et al., 2011, 2015). Characteristic DNA fragmentation was also observed in *A. cepa* and *N. tabaccum* treated with TiO_2 nanoparticles (Ghosh et al., 2010).

11.7.4 Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridinetriphosphate–Nick Labeling Assay

DNA fragmentation by nanoparticles is confirmed by the terminal deoxynucleotidyl transferase-mediated deoxyuridinetriphosphate–nick labeling assay, which labels the terminal ends of fragmented DNA strands (Poborilova et al. 2013). This confirmatory test was utilized to assess Al_2O_3 nanoparticle-induced DNA damage in tobacco BY-2 cell suspensions using tetramethylrhodamine-red fluorochrome mediated *in situ* cell death detection kit (Roche, Germany) (Poborilova et al. 2013). The treatment sets include untreated and nanoparticle-treated BY-2 cells, and positive (DNAse-1 treated) and negative (cells treated with labeling solution without terminal transferase) controls are maintained. However, to our knowledge, no other genotoxicity studies have been performed using BY-2 cells treated with nanoparticles.

11.8 Detection of ROS

Numerous studies have elucidated the generation of ROS as an indirect mechanism of DNA damage induced by nanoparticles in different plant systems (Ma et al., 2015; Faisal et al., 2016). The identification of the different types of ROS is crucial to the assessment of downstream toxicity responses. The following sections elucidate the qualitative and quantitative methods of ROS estimation in plant systems under nanoparticle stress.

11.8.1 Detection of OH•, ROO• Radicals, and H_2O_2 by DCFH-DA Assay

The production of ROS is analyzed by the hydrophobic non-fluorescent probe dichlorodihydro-fluorescein diacetate (DCFH-DA). It rapidly penetrates into the cells and is hydrolyzed by intracellular esterases to produce dichlorofluorescin (DCFH). DCFH is oxidized by intracellular ROS to its fluorescent two-electron product 2′,7′dichlorofluorescein (DCF) that is quantified by flow cytometry (Faisal et al., 2016), fluorescence microscopy, and confocal laser scanning microscopy (Ghosh et al., 2017). Treated root tips of *A. cepa* and *V. faba* are excised, stained with 25 µM DCFH-DA for 15 min in the dark, and washed thrice in phosphate buffer saline. The tips are observed by fluorescence or confocal laser scanning microscopy using an appropriate excitation filter (Alexa Fluor 488™). Images are taken using a Z-stack method where a number of optical sections are sliced through the tips in high resolution without

disturbing their morphology. The fluorescence intensity can be quantified using ImageJ or FV31S-SW Viewer software.

A confirmatory method for the detection of hydroxyl, peroxyl radicals, and H_2O_2 relies on the fluorescence spectrophotometric quantification of DCF fluorescence. Excised root tips ($n = 5$; 1 cm in length) are homogenized in 1 mL of cold phosphate buffer saline with the aid of prechilled mortar and pestles. The homogenates are subjected to centrifugation for 10 min at 14000 rpm and at a temperature of 4°C. The appropriate dilution of stock concentration of DCFH-DA is added to the supernatant to attain a final concentration of 0.25 μM. The solutions are incubated at 35°C ± 1°C for 15 min in the dark. Fluorescence intensity is measured at specific excitation (485 nm) and emission (530 nm) wavelengths using a fluorescence spectrophotometer (Hitachi, F-7000, Tokyo, Japan).

Numerous nanotoxicity studies in plant systems employed this staining method to estimate the amount of ROS generated. The high ROS specificity of DCFH-DA is advantageous in its implementation to any biological system ranging from animal to plant cells. This method yielded positive data on ROS generation by TiO_2 (Pakrashi et al., 2014), MWCNT (Ghosh et al., 2015), nZVI (Ghosh et al., 2017), ZnO (Ghosh et al., 2016; Ahmed et al., 2017), and Ag (Panda et al., 2011) nanoparticles in *A. cepa*. ROS was also detected in Arabidopsis (Kim et al., 2014) and rice (Wang et al., 2016) treated with nZVI, tomato exposed to nickel (Faisal et al., 2013), and eggplant treated with cobalt nanoparticles (Faisal et al., 2016).

In tobacco BY-2 cells, the staining method described by Poborolova et al., (2013) is similar to that described earlier with slight differences. The treated cells are incubated in 10 μM DCFH-DA solutions in MS medium for 1 hour in the dark. Image procurement and quantification of fluorescence intensity is same as that stated earlier. This method yielded conclusive data on high ROS generation in BY-2 cells in response to Al_2O_3 nanoparticles (Poborolova et al., 2013).

11.8.2 Estimation of H_2O_2 Content

The method of Loreto and Velikova (2001) is followed to quantify the H_2O_2 content in *A. cepa* roots treated with nanoparticles. One g of treated root samples is homogenized in 0.1% (w/v) trichloroacetic acid (3 mL) at 4°C followed by centrifugation for 15 min at 12,000 rpm at the same temperature. The equal volume of the supernatant is added to 10 mM sodium phosphate buffer (pH 7.0) and 1 M potassium iodide (2 mL). The absorbance of the resultant solution is recorded at 390 nm using a spectrophotometer. H_2O_2 can also be qualitatively estimated by staining root tips with 1% 3,3-diaminobenzidine (pH 3.8) in darkness for 10 min (Murali Achary and Panda, 2010). The tips are then illuminated for 30 min in cool fluorescent light for the appearance of characteristic brown color of H_2O_2 and photographed. CeO_2 nanoparticles at high concentrations induced H_2O_2 generation in rice (Rico et al., 2013). In *A. cepa*, high H_2O_2 content was noted upon treatment with nZVI (Ghosh et al., 2017), ZnO (Ghosh et al., 2016), and Ag nanoparticles (Panda et al., 2011). The H_2O_2 content of tobacco BY-2 cells can be estimated by titanium tetrachloride ($TiCl_4$) method, which depends on the formation of colored Ti complexes, indicating the presence of H_2O_2. The absorbance is read at 410 nm. Poborilova et al. (2013) reported the rise in H_2O_2 content in BY-2 cells treated with Al_2O_3 nanoparticles.

11.9 Conclusions and Future Perspectives

The overall implications of the experimental methods described in this chapter substantiate the toxic ramifications of environmentally relevant nanomaterials in plant systems. These methods yield confirmatory data with high reproducibility. However, adequate knowledge focusing on *in vivo* and *in vitro* nanotoxicity is deficient in plant systems. Available literature suggests the manifestation of chromosome/nuclear aberrations, MN, and DNA strand breaks culminating in cytotoxicity. Such effects are ascribed to the uptake, internalization, and biodistribution of nanoparticles within plant cells. Moreover, the dissolution of ions from metal nanoparticles into the exposure solvents exacerbates their toxicity in plant cells. Physical interactions of nanomaterials with cellular biomolecules and organelles cause direct damages. On the other hand, the overproduction of reactive oxygen intermediates such as hydroxyl, peroxyl radicals, and H_2O_2 upon the interaction of nanoparticles with cellular components are indirect mechanisms of cytogenotoxicity in plants. Considering the crucial role of plants in terrestrial and aquatic ecosystems, further mechanistic analyses in terms of DNA damage repair and mode of cell death are warranted in model and edible plants using environmentally realistic concentrations and conditions. Furthermore, there is an immense dearth of *in vitro* nanotoxicity investigations using plant cell suspensions. Owing to their high sensitivity and greater surface area of exposure, plant cell suspensions are excellent candidates for the analysis of the mode of action of nanoparticles at the cellular level. Therefore, this chapter paves the way for further application of the methods described to assess nanotoxicity in diverse plant systems both *in planta* and *in vitro.*

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REFERENCES

Ahmed, Bilal, Sourabh Dwivedi, Malik Zainul Abdin, Ameer Azam, Majed Al-Shaeri, Mohammad Saghir Khan, Quaiser Saquib, Abdulaziz A Al-Khedhairy, and Javed Musarrat. 2017. Mitochondrial and chromosomal damage induced by oxidative stress in Zn^{2+} ions, ZnO-bulk and ZnO-NPs treated *Allium cepa* roots. *Scientific Reports* 7 (January): 40685.

Babula, Petr, Ondrej Vodicka, Vojtech Adam, Marie Kummerova, Ladislav Havel, Jan Hosek, Ivo Provaznik, Helena Skutkova, Miroslava Beklova, and Rene Kizek. 2012. Effect of fluoranthene on plant cell model: Tobacco BY-2 suspension culture. *Environmental and Experimental Botany* 78 (May): 117–26.

Baker, C. Jacyn, and Norton M. Mock. 1994. An improved method for monitoring cell death in cell suspension and leaf disc assays using evan's blue. *Plant Cell, Tissue and Organ Culture* 39 (1): 7–12.

Bardos, Paul, Brian Bone, Daniel Elliott, Niels Hartog, and John Henstock. 2011. *A Risk/Benefit Approach to the Application of Iron Nanoparticles for the Remediation of Contaminated Sites in the Environment*. Department for Environment, Food and Rural Affairs. London. 1–111.

Bevan, Michael, and Sean Walsh. 2005. The *Arabidopsis* genome: A foundation for plant research. *Genome Research* 15 (12): 1632–42.

Burke, David, Nicole Pietrasiak, Shu Situ, Eric Abenojar, Mya Porche, Pawel Kraj, Yutthana Lakliang, and Anna Samia. 2015. Iron oxide and titanium dioxide nanoparticle effects on plant performance and root associated microbes. *International Journal of Molecular Sciences* 16 (10): 23630–50.

Burklew, Caitlin E., Jordan Ashlock, William B. Winfrey, and Baohong Zhang. 2012. Effects of aluminum oxide nanoparticles on the growth, development, and microRNA expression of tobacco (*Nicotiana tabacum*). *PLoS One* 7 (5): e34783.

Buzea, Cristina, Ivan I. Pacheco, and Kevin Robbie. 2007. Nanomaterials and nanoparticles: Sources and toxicity. *Biointerphases* 2 (4): MR17–MR71.

Chang, Wan-Chun, Ming-Hwa Chen, and Tse-Min Lee. 1999. 2,3,5-Triphenyltetrazolium reduction in the viability assay of *Ulva fasciata* (Chlorophyta) in response to salinity stress. *Botanical Bulletin—Academia Sinica* 403: 207–12.

Chen, Yongsheng, Paul Westernhoff, John C. Crittenden, and David Capco. 2004. *Fate, Transport, Transformation, and Toxicity of Nanomaterials in Conventional Drinking Water Treatment Processes.* Accessed August 3. https://archive.epa.gov/ncer/publications/web/pdf/epa_grantee_meeting_talk_08-19-04-yongsheng.pdf.

Costantini, Maria G., Steve Cadle, J. Michael Davis, Norbert Englert, David Kittelson, Andreas Mayer, John McAughey et al. 2001. *Evaluation of Human Health Risk from Cerium Added to Diesel Fuel*. https://www.healtheffects.org/system/files/Cerium.pdf.

De, Arpita, Manoswini Chakrabarti, Ilika Ghosh, and Anita Mukherjee. 2016. Evaluation of genotoxicity and oxidative stress of aluminium oxide nanoparticles and its bulk form in *Allium cepa*. *The Nucleus* 59 (3): 219–25.

Demir, Eşref, Nuray Kaya, and Bülent Kaya. 2014. Genotoxic effects of zinc oxide and titanium dioxide nanoparticles on root meristem cells of *Allium cepa* by comet assay. *Turkish Journal of Biology* 38: 31–9.

Deng, Fei, Shuling Wang, and Hua Xin. 2016. Toxicity of CuO nanoparticles to structure and metabolic activity of *Allium cepa* root tips. *Bulletin of Environmental Contamination and Toxicology* 97 (5): 702–8.

Donaldson, Ken, Craig A. Poland, and Roel P. F. Schins. 2010. Possible genotoxic mechanisms of nanoparticles: Criteria for improved test strategies. *Nanotoxicology* 4 (4): 414–20.

Doyle, Jeff J., and Jane L. Doyle. 1987. A Rapid DNA Isolation Procedure for Small Quantities of Fresh Leaf Tissue. *Phytochemical Bulletin* 19. http://www.citeulike.org/user/echinotrix/article/678648.

EPA (Environmental Protection Agency) and Office of the Science Advisor. 2007. *Nanotechnology White Paper.* https://www.epa.gov/sites/production/files/2015-01/documents/nanotechnology_whitepaper.pdf.

EPA Federal Facilities Restoration and Reuse Office. 2014. *Technical Fact Sheet—Nanomaterials.* https://www.epa.gov/sites/production/files/2014-03/documents/ffrrofactsheet_emergingcontaminant_nanomaterials_jan2014_final.pdf.

Erdakos, Garnet B., Prakash V. Bhave, George A. Pouliot, Heather Simon, and Rohit Mathur. 2014. Predicting the effects of nanoscale cerium additives in diesel fuel on regional-scale air quality. *Environmental Science & Technology* 48 (21): 12775–82.

European Commission. 2012. *Types and Uses of Nanomaterials, Including Safety Aspects*. European Commission, Brussels, SWD(2012) 288. 2012. https://ec.europa.eu/health//sites/health/files/nanotechnology/docs/swd_2012_288_en.pdf.

Faisal, Mohammad, Quaiser Saquib, Abdulrahman A. Alatar, Abdulaziz A. Al-Khedhairy, Mukhtar Ahmed, Sabiha M. Ansari, Hend A. Alwathnani, Sourabh Dwivedi, Javed Musarrat, and Shelly Praveen. 2016. Cobalt oxide nanoparticles aggravate DNA damage and cell death in eggplant via mitochondrial swelling and NO signaling pathway. *Biological Research* 49 (1): 20.

Faisal, Mohammad, Quaiser Saquib, Abdulrahman A. Alatar, Abdulaziz A. Al-Khedhairy, Ahmad K. Hegazy, and Javed Musarrat. 2013. Phytotoxic hazards of NiO-nanoparticles in tomato: A study on mechanism of cell death. *Journal of Hazardous Materials* 250–251 (April): 318–32.

Frazier, Taylor P., Caitlin E. Burklew, and Baohong Zhang. 2014. Titanium dioxide nanoparticles affect the growth and microRNA expression of tobacco (*Nicotiana tabacum*). *Functional & Integrative Genomics* 14 (1): 75–83.

Geisler-Lee, Jane, Qiang Wang, Ying Yao, Wen Zhang, Matt Geisler, Kungang Li, Ying Huang, Yongsheng Chen, Andrei Kolmakov, and Xingmao Ma. 2012. Phytotoxicity, accumulation and transport of silver nanoparticles by *Arabidopsis thaliana*. *Nanotoxicology* 7 (3): 323–37.

Ghosh, Ilika, Amitava Mukherjee, and Anita Mukherjee. 2017. In planta genotoxicity of nZVI: Influence of colloidal stability on uptake, DNA damage, oxidative stress and cell death. *Mutagenesis* 32 (3): 371–87.

Ghosh, Manosij, Maumita Bandyopadhyay, and Anita Mukherjee. 2010. Genotoxicity of titanium dioxide (TiO_2) nanoparticles at two trophic levels: Plant and human lymphocytes. *Chemosphere* 81 (10): 1253–62.

Ghosh, Manosij, Sreetama Bhadra, Aremu Adegoke, Maumita Bandyopadhyay, and Anita Mukherjee. 2015. MWCNT uptake in *Allium cepa* root cells induces cytotoxic and genotoxic responses and results in DNA hyper-methylation. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 774: 49–58.

Ghosh, Manosij, Anirban Chakraborty, Maumita Bandyopadhyay, and Anita Mukherjee. 2011. Multi-walled carbon nanotubes (MWCNT): Induction of DNA damage in plant and mammalian cells. *Journal of Hazardous Materials* 197 (December): 327–36.

Ghosh, Manosij, Manivannan J, Sonali Sinha, Anirban Chakraborty, Sanjaya Kumar Mallick, Maumita Bandyopadhyay, and Anita Mukherjee. 2012. In vitro and in vivo genotoxicity of silver nanoparticles. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 749 (1): 60–69.

Ghosh, Manosij, Aditi Jana, Sonali Sinha, Manivannan Jothiramajayam, Anish Nag, Anirban Chakraborty, Amitava Mukherjee, and Anita Mukherjee. 2016. Effects of ZnO nanoparticles in plants: Cytotoxicity, genotoxicity, deregulation of antioxidant defenses, and cell-cycle arrest. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 807: 25–32.

Gichner, Tomáš, Anita Mukherjee, and Jiří Velemínský. 2006. DNA staining with the fluorochromes EtBr, DAPI and YOYO-1 in the comet assay with tobacco plants after treatment with ethyl methanesulphonate, hyperthermia and DNase-I. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 605 (1): 17–21.

Gichner, Tomas, Irena Znidar, Elizabeth D. Wagner, and Michael J. Plewa. 2016. Chapter 4. The use of higher plants in the comet assay. In *The Comet Assay in Toxicology (Vol. 30)*, ed. Dhawan, Alok and Diana Anderson. Royal Society of Chemistry Publishing, Cambridge, United Kingdom, 112–33.

Gui, Xin, Zhiyong Zhang, Shutong Liu, Yuhui Ma, Peng Zhang, Xiao He, Yuanyuan Li et al. 2015. Fate and phytotoxicity of CeO_2 nanoparticles on lettuce cultured in the potting soil environment. *PLoS One* 10 (8): e0134261.

Jiang, Jingkun, Günter Oberdörster, and Pratim Biswas. 2009. Characterization of size, surface charge, and agglomeration state of nanoparticle dispersions for toxicological studies. *Journal of Nanoparticle Research* 11 (1): 77–89.

Kasprowicz, Marek, Anna Gorczyca, and Piotr Janas. 2016. Production of silver nanoparticles using high voltage arc discharge method. *Current Nanoscience* 12 (6): 747–53.

Kim, Jae-Hwan, Yongjik Lee, Eun-Ju Kim, Sungmin Gu, Eun Ju Sohn, Young Sook Seo, Hyun Joo An, and Yoon-Seok Chang. 2014. Exposure of iron nanoparticles to *Arabidopsis thaliana* enhances root elongation by triggering cell wall loosening. *Environmental Science & Technology* 48 (6): 3477–85.

Kittler, S., C. Greulich, J. Diendorf, M. Köller, and M. Epple. 2010. Toxicity of silver nanoparticles increases during storage because of slow dissolution under release of silver ions. *Chemistry of Materials* 22 (16): 4548–54.

Krystofova, Olga, Jiri Sochor, Ondrej Zitka, Petr Babula, Vit Kudrle, Vojtech Adam, and Rene Kizek. 2013. Effect of magnetic nanoparticles on tobacco BY-2 cell suspension culture. *International Journal of Environmental Research and Public Health* 10 (1): 47–71.

Kumari, Mamta, S. Sudheer Khan, Sunandan Pakrashi, Amitava Mukherjee, and Natarajan Chandrasekaran. 2011. Cytogenetic and genotoxic effects of zinc oxide nanoparticles on root cells of *Allium cepa*. *Journal of Hazardous Materials* 190 (1–3): 613–21.

Kumari, Mamta, A. Mukherjee, and N. Chandrasekaran. 2009. Genotoxicity of silver nanoparticles in *Allium cepa*. *Science of the Total Environment* 407 (19): 5243–46.

Lee, Chang Woo, Shaily Mahendra, Katherine Zodrow, Dong Li, Yu-Chang Tsai, Janet Braam, and Pedro J. J. Alvarez. 2010. Developmental phytotoxicity of metal oxide nanoparticles to *Arabidopsis thaliana*. *Environmental Toxicology and Chemistry* 29 (3): 669–75.

Leme, Daniela Morais, and Maria Aparecida Marin-Morales. 2009. *Allium cepa* test in environmental monitoring: A review on its application. *Mutation Research/Reviews in Mutation Research* 682 (1): 71–81.

López-Moreno, Martha L., Guadalupe de la Rosa, José Á. Hernández-Viezcas, Hiram Castillo-Michel, Cristian E. Botez, José R. Peralta-Videa, and Jorge L. Gardea-Torresdey. 2010a. Evidence of the differential biotransformation and genotoxicity of ZnO and CeO_2 nanoparticles on soybean (*Glycine max*) plants. *Environmental Science & Technology* 44 (19): 7315–20.

López-Moreno, Martha L., Guadalupe de la Rosa, José A. Hernández-Viezcas, José R. Peralta-Videa, and Jorge L. Gardea-Torresdey. 2010b. X-ray absorption spectroscopy (XAS) corroboration of the uptake and storage of CeO_2 nanoparticles and assessment of their differential toxicity in four edible plant species. *Journal of Agricultural and Food Chemistry* 58 (6): 3689–93.

Loreto, Francesco and Violeta Velikova. 2001. Isoprene produced by leaves protects the photosynthetic apparatus against ozone damage, quenches ozone products, and reduces lipid peroxidation of cellular membranes. *Plant Physiology* 127 (4): 1781–7.

Loukanov, Alexandre, Ryota Sekiya, Midori Yoshikawa, Naritaka Kobayashi, Yuji Moriyasu, and Seiichiro Nakabayashi. 2016. Photosensitizer-conjugated ultrasmall carbon nanodots as multifunctional fluorescent probes for bioimaging. *The Journal of Physical Chemistry C* 120 (29): 15867–74.

Ma, Chuanxin, Sudesh Chhikara, Baoshan Xing, Craig Musante, Jason C. White, and Om Parkash Dhankher. 2013. Physiological and molecular response of *Arabidopsis thaliana* (L.) to nanoparticle cerium and indium oxide exposure. *ACS Sustainable Chemistry & Engineering* 1 (7): 768–78.

Ma, Chuanxin, Jason C. White, Om Parkash Dhankher, and Baoshan Xing. 2015. Metal-based nanotoxicity and detoxification pathways in higher plants. *Environmental Science & Technology* 49 (12): 7109–22.

Magdolenova, Zuzana, Andrew Collins, Ashutosh Kumar, Alok Dhawan, Vicki Stone, and Maria Dusinska. 2014. Mechanisms of genotoxicity: A review of *in vitro* and *in vivo* studies with engineered nanoparticles. *Nanotoxicology* 8 (3): 233–78.

Maurer-Jones, Melissa A., Maral P. S. Mousavi, Li D. Chen, Philippe Bühlmann, Christy L. Haynes, P. R. Scheuerman, D. P. Allison et al. 2013. Characterization of silver ion dissolution from silver nanoparticles using fluorous-phase ion-selective electrodes and assessment of resultant toxicity to *Shewanella oneidensis*. *Chemical Science* 4 (6): 2564.

Maynard, Andrew D., Robert J. Aitken, Tilman Butz, Vicki Colvin, Ken Donaldson, Günter Oberdörster, Martin A. Philbert et al. 2006. Safe handling of nanotechnology. *Nature* 444 (7117): 267–69.

Miller, Robert. 1998. Nitric-perchloric acid wet digestion in an open vessel. In *Handbook of Reference Methods for Plant Analysis*, ed. Kalra, Yash, P. CRC Press. Boca Raton, FL, 57–60.

Misra, Superb K., Agnieszka Dybowska, Deborah Berhanu, Samuel N. Luoma, and Eugenia Valsami-Jones. 2012. The complexity of nanoparticle dissolution and its importance in nanotoxicological studies. *Science of the Total Environment* 438 (November): 225–32.

Moriyasu, Yuji, and Yoshinori Ohsumi. 1996. Autophagy in tobacco suspension-cultured cells in response to sucrose starvation. *Plant Physiology* 111 (4): 1233–41.

Mueller, Nicole C., Jürgen Braun, Johannes Bruns, Miroslav Černík, Peter Rissing, David Rickerby, and Bernd Nowack. 2012. Application of nanoscale zero valent iron (NZVI) for groundwater remediation in Europe. *Environmental Science and Pollution Research* 19 (2): 550–58.

Mukherjee, Anita and Thomas Gichner. 2009. *Research Methods in Plant Sciences: Allelopathy* (Eds: Sampietro, D. A. and S. S. Narwal), Studium Press, LLC. Houston, TX, 97–108. http://www.ueb.cas.cz/en/category/source-titles/research-methods-plant-sciences-allelopathy-eds-d-sampietro-and-s-s-narwal.

Mukherjee, Anita, and Archana Sharma. 1988. Effects of cadmium and selenium on cell division and chromosomal aberrations in *Allium sativum* L. *Water, Air, and Soil Pollution* 37 (3–4): 433–38.

Murali Achary, V. Mohan, and Brahma B. Panda. 2010. Aluminium-induced DNA damage and adaptive response to genotoxic stress in plant cells are mediated through reactive oxygen intermediates. *Mutagenesis* 25 (2): 201–9.

Nagata, Toshiyuki, Yasuyuki Nemoto, and Seiichiro Hasezawa. 1992. Tobacco BY-2 cell line as the 'HeLa' cell in the cell biology of higher plants. *International Review of Cytol*ogy 132, 1–30.

OECD (Organization for Economic Cooperation and Development). *Test No. 208: Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test*. 2006. OECD Guidelines for the Testing of Chemicals, Section 2. OECD Publishing, Paris, France.

OECD Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology. 2016. Categorisation of Manufactured Nanomaterials Workshop Report Series on the Safety of Manufactured Nanomaterials. Accessed on August 3, 2017. http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2016)9&doclanguage=en.

Pakrashi, Sunandan, Nitin Jain, Swayamprava Dalai, Jerobin Jayakumar, Prathna Thanjavur Chandrasekaran, Ashok M. Raichur, Natarajan Chandrasekaran, and Amitava Mukherjee. 2014. In vivo genotoxicity assessment of titanium dioxide nanoparticles by *Allium cepa* root tip assay at high exposure concentrations. *PLoS ONE* 9 (2): e87789.

Panda, Kamal K., V. Mohan M. Achary, R. Krishnaveni, Bijaya K. Padhi, Sachindra N. Sarangi, Surendra N. Sahu, and Brahma B. Panda. 2011. In vitro biosynthesis and genotoxicity bioassay of silver nanoparticles using plants. *Toxicology in Vitro* 25 (5): 1097–1105.

Patlolla, Anita K, Ashley Berry, LaBethani May, and Paul B Tchounwou. 2012. Genotoxicity of silver nanoparticles in *Vicia faba*: A pilot study on the environmental monitoring of nanoparticles. *International Journal of Environmental Research and Public Health* 9 (5): 1649–62.

Poborilova, Zuzana, Radka Opatrilova, and Petr Babula. 2013. Toxicity of aluminium oxide nanoparticles demonstrated using a BY-2 plant cell suspension culture model. *Environmental and Experimental Botany* 91 (July): 1–11.

Pulit-Prociak, Jolanta and Marcin Banach. 2016. Silver nanoparticles—A material of the future…? *Open Chemistry* 14 (1): 651–68.

Reed, Robert B., David A. Ladner, Christopher P. Higgins, Paul Westerhoff, and James F. Ranville. 2012. Solubility of nano-zinc oxide in environmentally and biologically important matrices. *Environmental Toxicology and Chemistry* 31 (1): 93–99.

Rejeski, David and Deanna Lekas. 2008. Nanotechnology field observations: Scouting the new industrial west. *Journal of Cleaner Production* 16 (8–9): 1014–17.

Rico, Cyren M., Jie Hong, Maria Isabel Morales, Lijuan Zhao, Ana Cecilia Barrios, Jian-Ying Zhang, Jose R. Peralta-Videa, and Jorge L. Gardea-Torresdey. 2013. Effect of cerium oxide nanoparticles on rice: A study involving the antioxidant defense system and in vivo fluorescence imaging. *Environmental Science & Technology* 47 (11): 5635–42.

Servin, Alia D., and Jason C. White. 2016. Nanotechnology in agriculture: Next steps for understanding engineered nanoparticle exposure and risk. *NanoImpact* 1 (January): 9–12.

Sierro, Nicolas, James N D Battey, Sonia Ouadi, Nicolas Bakaher, Lucien Bovet, Adrian Willig, Simon Goepfert, Manuel C Peitsch, and Nikolai V Ivanov. 2014. The tobacco genome sequence and its comparison with those of tomato and potato. *Nature Communications* 5 (May): 3833.

Singh, Neenu, Gareth J.S. Jenkins, Romisa Asadi, and Shareen H. Doak. 2010. Potential toxicity of superparamagnetic iron oxide nanoparticles (SPION). *Nano Reviews* 1 (1): 5358.

Song, Uhram, Minjoo Shin, Gisuk Lee, Jinkyu Roh, Younghun Kim, and Eun Ju Lee. 2013. Functional analysis of TiO_2 nanoparticle toxicity in three plant species. *Biological Trace Element Research* 155 (1): 93–103.

Sun, Tian Yin, Fadri Gottschalk, Konrad Hungerbühler, and Bernd Nowack. 2014. Comprehensive probabilistic modelling of environmental emissions of engineered nanomaterials. *Environmental Pollution* 185 (February): 69–76.

Syu, You-yu, Jui-Hung Hung, Jui-Chang Chen, and Huey-wen Chuang. 2014. Impacts of size and shape of silver nanoparticles on *Arabidopsis* plant growth and gene expression. *Plant Physiology and Biochemistry* 83 (October): 57–64.

Tosco, Tiziana, Marco Petrangeli Papini, Carolina Cruz Viggi, and Rajandrea Sethi. 2014. Nanoscale zerovalent iron particles for groundwater remediation: A review. *Journal of Cleaner Production* 77 (August): 10–21.

Trpkovic, Andreja, Biljana Todorovic-Markovic, and Vladimir Trajkovic. 2012. Toxicity of pristine versus functionalized fullerenes: Mechanisms of cell damage and the role of oxidative stress. *Archives of Toxicology* 86 (12): 1809–27.

Wang, Jie, Zhanqiang Fang, Wen Cheng, Xiaomin Yan, Pokeung Eric Tsang, and Dongye Zhao. 2016. Higher concentrations of nanoscale zero-valent iron (nZVI) in soil induced rice chlorosis due to inhibited active iron transportation. *Environmental Pollution* 210 (March): 338–45.

Weir, Alex, Paul Westerhoff, Lars Fabricius, Kiril Hristovski, and Natalie von Goetz. 2012. Titanium dioxide nanoparticles in food and personal care products. *Environmental Science & Technology* 46 (4): 2242–50.

Zafar, Hira, Attarad Ali, Joham S. Ali, Ihsan U. Haq, and Muhammad Zia. 2016. Effect of ZnO nanoparticles on *Brassica nigra* seedlings and stem explants: Growth dynamics and antioxidative response. *Frontiers in Plant Science* 7 (April): 535.

12

In Vitro *and* In Vivo *Nanotoxicity Evaluation in Plants*

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12.1 Introduction

The nanoparticle and nanotechnology field is a fast-growing research niche (Maynard et al. 2006). Nanoparticles are particles with at least one dimension less than 100 nm. *Nanotechnology* is a collective term that implies the capacity to work with materials at the nanometer scale. Nanotechnology thus has potential applications in a wide range of sectors, from energy (production, catalysis, storage), materials (lubricants, abrasives, paints, tires, and sportsware), electronics (chips and screens), optics, and remediation (pollution absorption, water filtering and disinfection), to food (additives and packaging), cosmetics (skin lotions and sunscreen), and medicine (diagnostics and drug delivery). This width reflects a diversity of materials that are being or will be used in the different applications (Buzea et al. 2007).

Nanomaterials possess different properties compared to the same material in its coarser or bulk form (Walker and Bucher 2009). Once a material is reduced below 100 nm in size, its components demonstrate unusual features based on quantum mechanics, rather than macroscopic Newtonian mechanics, which influence a variety of material properties such as conductivity, heat transfer, melting temperature, optical properties, and magnetization (Nowack and Bucheli 2007). Taking the advantages of these singular properties in order to develop new products is the main purpose of nanotechnology, and that is why it is regarded as "the next industrial revolution" (Oberdorster et al. 2005). Nanotechnology is a multidisciplinary field, as it combines the knowledge from different disciplines: chemistry, physics, and biology among others (Handy et al. 2008). Surface chemistry is also of great importance to the properties of nanoparticles. The decreasing size causes their surface effects to become more significant, due to an increase in the volume fraction of surface atoms, which determines, in some instances, their special properties (Aitken et al. 2006).

One of the most frequently used procedures involving the preparation of nanoparticles is the use of capping stabilizing agents or surfactants, which help prevent nanoparticle aggregation and Ostwald ripening (Farre et al. 2011). In such cases, stabilizers not only preserve nanoparticles size but also play a crucial role in controlling the shape of the nanoparticles (Das and Ansari 2009). Several studies exist on the toxicological properties of nanoparticles. Several studies have shown that some of them can pass through the various protective barriers of living organisms. The inhaled nanoparticles can end up in the bloodstream after passing through all the respiratory or gastrointestinal protective mechanisms. They are then distributed in the various organs and accumulate at specific sites. They can travel along the olfactory nerves and directly penetrate into the brain, just as they can pass through cell barriers. Another special feature of nanoparticles is that their toxicity seems to be linked to their surface. Nanoparticles are so tiny that small quantities (expressed in terms of mass) could have major toxic effects, because of their large surface (Maynard et al. 2006). Soluble nanoparticle toxic effects are linked to their different components, regardless of the initial size of the particle. These effects are well known and are not described here. However, the situation is totally different for insoluble or very low-solubility nanoparticles (Donaldson et al. 2004).

The first feature of nanoparticles is their pulmonary deposition mode, whereby the particles will be deposited throughout the pulmonary system. It has been clearly shown that mucociliary clearance and phagocytosis are well-documented pulmonary

clearance mechanisms for micrometric particles (Donaldson et al. 2004). Because of their size, nanometric particles can enter the extrapulmonary organs. This involves the migration of solid particles through the epithelial layers and through the nerve endings, along the neuronal axons to the central nervous system. Thus, insoluble nanoparticles pass through the respiratory or gastrointestinal protective mechanisms and are distributed to the various organs throughout the body, including the brain. They are stored in the cells and end up in the bloodstream. These properties are currently being extensively studied in pharmacology, because nanoparticles could be used as vectors to transport drugs to targeted sites in the body (Maynard et al. 2006).

Nanometric particles does not account for the number of particles or the specific surface for a given mass of particles (Donaldson et al. 2004). It is wiser to state the dose according to the characteristics of the nanoparticles (number and surface) instead of their mass. Nanoparticles have a natural tendency to agglomerate, meaning that they group together to form much larger particles (Biswas and Wu 2005).

Most industrial applications require unagglomerated particles. Aggregated masses of nanoparticles can be difficult to cleave into their individual primary particles. Thus, despite having small primary nanoparticles, the formation of large aggregates (100 nm) due to particle coalescence can obviate the point of creating a high surface-area powder (Lidén 2011). In such a context, producers use different postsynthesis strategies to prevent aggregation or stimulate deaggregation. The nanoparticle surface is modified for the intended application, often by coating the particle with a chemical (Tervonen et al. 2009). These surface changes can have a major impact on nanoparticle toxicity or safety. The manufacturer and the user must consider this reality, which can totally alter the toxicity of a specific product. Little information exists on the impact of these surface properties, except in pharmacology (Maynard et al. 2006).

12.2 Types of Nanoparticles

The types of nanoparticles whose ecotoxic effects are being dealt with in this paper are (1) fullerenes (grouping Buckminster fullerenes, carbon nanotubes [CNTs], nanocones, etc.); (2) metal nanoparticles (e.g., elemental Ag, Au, and Fe); (3) oxides (or binary compounds when including carbides, nitrides, etc.), e.g., TiO_2 and Fe oxides; (4) complex compounds (alloys, composites, nanofluids, etc., consisting of two or more elements), e.g., cobalt and zinc iron oxide; (5) quantum dots (or q-dots); and (6) organic polymers (dendrimers, polystyrene, etc.) (Buzea et al. 2007).

12.3 Behavior of Nanoparticles

Ecotoxicity studies of nanoparticles are scarce and difficult to compare, and data from experiments under simplified conditions indicate that some nanoparticles are toxic to a number of organisms, even in very low amounts. This concerns fullerenes, silver nanoparticles, and q-dots and, to a lesser degree, CNTs and nanoparticles of Cu, ZnO, TiO_2, and SiO_2. To determine if nanoparticles represent a risk to organisms and the environment, crucial information is lacking regarding mobility, transfer, and uptake as affected by environmental matrices (Buzea et al. 2007). Nanoparticles

once released into the environment organisms are likely to be exposed in different ways. Human toxicity studies, with a main focus on workers environments, primarily consider not only dispersion and uptake from air, but also direct uptake through ingestion; dermal exposure; and, in some special cases related to medical use, injections. Environmental issues also concern air, as particulate matter dispersed in air will fall out by gravity once they are condensed or aggregated and thus reach a certain size. Both in this and other scenarios for spreading nanoparticles, water may serve as a transport medium and a temporary reservoir for nanoparticles. Yet the ultimate recipients for any nonvolatile compound or particle spreading in the environment will be sediments and soils (Buzea et al. 2007).

12.3.1 Mobility of Nanoparticles

Nanomaterials end up in the environment, that is, in water, sediments, and soil (The Royal Society & The Royal Academy of Engineering, Nanoscience and Nanotechnologies 2004). Three aspects seem important when assessing the impact of nanoparticles as pollutants ending up in the environment: (1) mobility (transport and transfer)—their ability to move from one place to another (e.g., from a spill site to an uncontaminated site) or from one recipient to another (e.g., from soil to drinking water or food plants); (2) ecotoxicity—the possible harm that nanoparticles can cause to organisms living in water, sediments, and soils that they enter; and (3) modification—how and to which extent nanoparticles are modified by contact with the environment (and the consequences of such modifications on ecotoxicity and mobility) (Buzea et al. 2007). A major determinant for particle mobility is the stability of its suspension. If destabilized, a particle suspension will aggregate, which, in turn, may lead to massive deposition (Motzer 2008). Several factors that affect nanoparticle surface potential may destabilize a suspension, including pH changes, increased ion concentration, and dilution or degradation of stabilizing agents (surfactants or coatings) (Klaine et al. 2008). On the other hand, nanoparticles have large surface area–to-volume ratios and potentially high sorption capacities for other aqueous species such as natural organic matter (Flahaut 2010). Environmental factors such as pH and ionic strength (Hardman 2006) together with the physical–chemical properties and structure and concentration of nanoparticles (US EPA Office of Inspector General 2011) may determine whether they are bound within or transported out of soils and sediments.

12.3.2 Redox Transformations

Another interesting issue is how redox transformations may influence the transformation and fate of engineered nanoparticles (Zhang and Wang 1997). Redox reactions occur in a wide range of environments and are important for the degradation of organic matter, the generation of energy by chemolithotrophic organisms, and the precipitation and dissolution reactions that influence the sequestration and mobility of metals. Due to chemical composition, surface charge, and high specific surface area, some nanoparticles may have a large capacity to adsorb both inorganic and organic pollutants. One important topic is therefore how nanoparticles may influence mobility, bioavailability, and degradation of potentially harmful compounds in the environment such as polycyclic aromatic hydrocarbons (PAHs), heavy metals, and so forth (Buzea et al. 2007).

12.3.3 Degradability

Nanoparticles are more likely to accumulate in the environment than to disappear. Mineral nanoparticles are more or less prone to weathering and dissolution, and some mineral nanoparticles, such as ZnO, are known to dissolve over time when exposed to common environmental conditions. Aggregates of mineral nanoparticles that are formed during preparation in an aqueous environment may be easily broken up mechanically, as has been demonstrated with Fe_2O_3 (Auffan 2010). Organic polymer-based or polymer-like nanoparticles, such as starch derivatives and dendrimers, are highly biodegradable and are rapidly degraded both in environment and within organisms. Similarly, organic coatings and surfactants that are used to disperse nanoparticles for various applications are easily degradable in the environment (Buzea et al. 2007).

12.3.4 Fate and Behavior of Nanoparticles in the Environment

12.3.4.1 Air

There is a natural background of nanoparticles (ultrafine particles—smaller than 0.1 μm) in the atmosphere, and despite little work having been done on ecological systems, it is known that when compared to anthropogenic sources and potential released manufactured nanoparticles, the natural background present in the air is considerably low (Klaine et al. 2008). Nanoparticles have short residence time. However, they attach to accumulation mode particles and remain in the atmosphere as long as the accumulation mode particles participate in the atmospheric chemistry and physics. Diesel nanoparticles are one of the concerning nanoparticles released into the atmosphere. These nanoparticles contribute to global warming since they absorb light and endanger human health. When ultrafine particles are directly released from the source, they are called primary particles; volatile nanoparticle (secondary nanoparticle) composed by nucleation mode may be formed in the atmosphere by photochemical processes. The key species in the atmosphere are sulfuric acid, nitric acid, and organic gases (Biswas and Wu 2005; Nowack and Bucheli 2007). Some conditions (e.g., temperature, residence time, dilution rate, dilution ratio, and concentration of carbon particles) are necessary for production of these secondary nanoparticles (Abdul-Khalek et al. 1999). Therefore, this event will result in an increasing of particle number concentration above their natural concentration present in the atmosphere; moreover, these nucleation particles may continue to grow by vapor condensation and coagulation, which result in atmospheric optical effects, building soiling, and climate change (Biswas and Wu 2005; Nowack and Bucheli 2007).

12.3.4.2 Water

Industrial products and wastes tend to end up in waterways, increasing the possibility of nanoparticle contamination, although almost no current data on behavior and fate of nanoparticle in aquatic systems are available (Nowack and Bucheli 2007). Colloids, particles in the 1 nm to 1 μm size range, are macromolecules and molecular assemblies. They include organic (e.g., primarily humic substances [HSs] and fibrillar material, usually protein and polysaccharide exudates from microbes) and inorganic

matter (e.g., Fe, Mn, Al, and Si oxides and thus analogous to manufactured metal oxide nanoparticle), and biological material (e.g., bacteria) (Nowack and Bucheli 2007; Klaine et al. 2008).

Colloidal fate and behavior are determined by aggregation. Colloids tend to aggregate to particles (>1 μm), and their transport depends on the first one, being denominated sedimentation. Like metals, manufactured nanoparticles tend to aggregate and subsequently settle. This process has positive effects on the purification of water bodies, because this behavior results in a transfer of nanoparticle from water column to sediments, resulting in a pollutant loss. Despite this possible pollutant loss, natural colloids interact with nanoparticle, and this will affect the nanoparticle behavior, being more difficult to predict the nanoparticle effect on the aquatic system. Knowing nanoparticle size, surface charges, and chemical reactivity, it is possible to predict some potential biological effects in the aqueous environment, such as generation of reactive oxygen species (ROS), phototoxicity, and photocatalytic activity and interaction with soluble pollutants (Auffan 2010), but due to the aquatic system complexity, this physical behavior is hard to predict.

Oceans exhibit changes in physicochemical characteristics with depth (e.g., temperature) that may influence aggregation and colloid chemistry. Analogously to freshwater, aggregates of nanoparticles may sink into the ocean floor, as well as accumulate at the interface between cold and warm currents or be recycled by biota. This may threaten pelagic species since they feed at these zones, while deposition in the sediment may present a risk of exposure to benthic species. Nanoparticles may also accumulate on the surface microlayers of the oceans, presenting a route of aerosol exposure risk to marine birds and mammals, as well as the organisms living on the surface microlayer (Kennedy et al. 2004). Oberdorster et al. (2006) exposed freshwater crustaceans *Daphnia magna*; the marine copepod *Hyalella azteca*; and fish, such as *Pimephales promelas* and *Oryzias latipes*, to C60 (fullerenes), and toxicity was reported. Kashiwada (2006) reported an increase in toxicity with the salinity enhancement, along with a greater tendency for the particles to form aggregates, when eggs of Japanese medaka were exposed to fluorescent nanoparticle (30 mg L^{-1}) at different salinities. At salinities similar to the ones found in normal seawater, accumulation decreased, but egg mortality remained high. Therefore, it is not possible to predict or evaluate the impact of Nanoparticle on aquatic systems; however, ecotoxicological studies show that aquatic organisms, both unicellular and animals, are affected by nanoparticle toxicity (Nowack and Bucheli 2007).

12.3.4.3 Soil

Soil and sediments are the ultimate sinks of nanoparticles, and whether directly or indirectly released (e.g., via sewage treatment plants, aerial deposition, or waste handling), nanoparticles will end up in soil; this system may present the most significant exposure avenue for assessing environmental risk. Soil being one of the main sinks, it raises concern about the entry of nanoparticles into food webs and human access to contaminated agriculture (Unrine et al. 2010). In terrestrial ecosystems, natural nanoparticles are present (soil colloids), such as organic matter, clays, iron oxides, and other minerals that play an important role in biogeochemical processes. Therefore, the introduction of manufactured nanoparticles in this ecosystem may change the

profile of the natural nanoparticles and interfere with soil development (pedogenesis) or behavior (e.g., crusting and dispersion) (Klaine et al. 2008). The fate of nanoparticles in terrestrial ecosystems is difficult to elucidate, and there is few data that report the transport of nanoparticles through soils. Nanoparticles are made to have different properties than their similar bulk and macroscopic material, and these differences may change the fate and effects of nanoparticle in terrestrial environment (Klaine et al. 2008) The high surface area, reactivity, size, aggregation, and absorbance are important determinants of their adhesion to soil, and thus, their mobility and transport (Borm et al. 2006). The bioavailability of nanoparticles also depends on specific properties of each nanoparticle, in addition to composition of the soil matrix, porosity, hydraulic conductivity, groundwater gradient and flow velocity, and geochemical properties (e.g., dissolved oxygen; pH; ionic strength; and concentrations of nitrate, nitrite, and sulfate) (Nowack and Bucheli 2007; Klaine et al. 2008). Unrine et al. (2010) reported absorption, biodistribution through tissues, and statistically significant decrease in reproduction of earthworms *Eisenia fetida* when exposed to Au nanoparticles. This study demonstrates the importance of considering food chain accumulation and trophic transfer, but information that nanoparticles end up in higher-level organisms is extremely limited (Zhu et al. 2008). Nanoparticles have been extensively used as antimicrobial agents against pathogenic bacteria, but this abusive use is leading to negative consequences against the populations of soil microbes that play beneficial roles in the environment, such as promoting plant growth, element cycling, and degradation of pollutants. The nanoparticles of Ag, CuO, and ZnO showed both toxicity on pathogenic bacteria (e.g., *Escherichia coli* and *Staphylococcus aureus*) and beneficial effects on microbes, such as *Pseudomonas putida*, which has bioremediation potential and is a strong root colonizer (Molina et al. 2006). These nanoparticles showed higher toxicity when compared to their equivalent bulk materials (Hardman 2006). Therefore, terrestrial systems being the larger and most reactive sink for nanoparticles, more research and standardized testing protocols are needed for fate/behavior and food web accumulation of nanoparticles.

12.4 Ecotoxicity of Nanoparticles

The development of nanotechnologies has introduced important amounts of manufactured nanoparticles into the environment, including those in the ambient air and water. In order to protect human health and wildlife from the potential adverse effects of a broad range of nanomaterials, an increasing number of studies have focused on the assessment of the toxicity of the nanoparticles commonly used in industry (Navarro et al. 2008). Primary geogenic nanoparticles may form as aerosols from, for example, sprays of salts and sulfates mostly from the sea or as sulfate aerosols from volcanic emissions, or even as forest fires (soot and/or PAHs) (Motzer 2008). Moreover, nanoparticles may be introduced in the environment by anthropic reasons, being introduced, for example, intentionally to remediate groundwater and contaminated soil (Klaine et al. 2008; Flahaut 2010) or also unintentionally by several sources, as research and clinical settings and solid or liquid waste streams production facilities. The biggest risk of release of this material may occur during transportation to good manufacturers and by intentional releases for environmental application (Flahaut 2010).

The toxicity and degradation of these compounds in the environment cannot be accurately assessed yet because it depends on the nanoparticle type, physicochemical properties, and the environment media in which they partition and the respective conditions. So, this is a field that requires intense research. For example, it is recognized that research needs to be performed in the following fields: (1) detection of nanoparticles in the environment, (2) measurement of emissions of nanoparticles, (3) life cycle of nanoparticles in the environment, (3) toxicity of nanoparticles to the environment, and (f) immediate and longer impact on environment (e.g., http://www.nanocap.eu/). The concern of nanoparticle exposure is due to some important issues, such as the half-lives of these materials that may be months to possibly years, the increase on the concentration of anthropic substances since nanoparticles are often produced in a large scale, and because this material is expected to be accumulated and transformed by time and local environmental conditions (Hardman 2006; Flahaut 2010).

The current knowledge about nanoparticles and the challenges of nanotoxicity assessment lead to a lack of effective regulation of nanoparticle use. Some governmental entities, such as the Environmental Protection Agency (EPA), have been conducting efforts in order to not only comprehend the properties of nanoparticles and their potential risks for human health and the environment but also to regulate the use, storage, and disposal of these materials to allow a safe and sustainable nanotechnology development. Although there are no specific environmental laws, nanoparticles cut across some existing EPA regulations. For example, several nanomaterials are considered chemical substances under the Toxic Substances Control Act, and pesticides containing nanotechnology products are regulated under the Federal Insecticide, Fungicide, and Rodenticide Act (see http://www.epa.gov/nanoscience/). Still, a recent report from the EPA's (2011) Office of Inspector General stated that ". . . EPA does not currently have sufficient information or processes to effectively manage the human health and environmental risks of nanomaterials . . ." and that ". . . EPA has the statutory authority to regulate nanomaterials but currently lacks the environmental and human health exposure and toxicological data to do so effectively." According to this report, some proposed policies have failed, and others that await approval, are facing significant barriers to their effectiveness (US EPA 2011).

Oxidative stress in terms of ROS generation is a parameter that is convenient to measure in the context of toxicity and ecotoxicity, because cells respond to oxidative stress by mounting a number of protective responses that can easily be measured as enzymatic or genetic expression responses (Zhang 2003). Several in vitro studies on the toxicity of nanoparticles have shown the generation of ROS, for example, by TiO_2 and fullerenes (Banfield and Zhang 2001). On the other hand, some authors have found that nanoparticle, including, for example, fullerenes, may also protect against oxidative stress (Quinn et al. 2005). This apparent dichotomy underlines the need for research on nanoparticle–cell interactions and mechanistic aspects of nanoparticles metabolism in organisms and specific cells.

Many nanoparticles are photochemically active in the sense that they generate excited electrons when exposed to light (e.g., TiO_2, ZnO, SiO_2, and fullerenes). In the presence of oxygen, these electrons can form superoxide radicals by direct electron transfer (Elliot and Zhang 2001). Thus, situations where organisms are simultaneously exposed to nanoparticles and light (and, in particular, ultraviolet light, which is more energetic than visible light) are of particular concern in an ecotoxicity context.

Ecotoxicity measurements are conducted on different trophic levels including microorganisms, plants, invertebrates, and vertebrates, and test systems have been standardized for some organisms and for some exposure conditions. The standardized protocols specify some physical and physiological conditions (medium composition, age of organisms, etc.) as well as exposure times (e.g., for acute or chronic toxicity) and which end points to measure (growth, fecundity, activity of enzymes, expression of genes, etc.). Microorganisms (mainly not only bacteria, but also fungi, protozoa, and algae) have the advantage that they are ubiquitous and highly diverse (filling a range of habitats and functions), small (permitting miniaturized tests), and with short generation times (permitting rapid tests) (Buzea et al. 2007).

Apart from microorganisms, there are many very useful organisms from different environments that may be used in ecotoxicity testing. It may be interesting to use organisms of different trophic levels (from different steps in a food chain), from primary producers to grazers and predators, as some environmental pollutants may accumulate in the food chain (biomagnification). In soil, organisms may also be selected based on specific modes of exposure (contact, ingestion, and prey preferences), specific habitats (surface, shallow, or deep subsurface, aerated or anoxic environments, etc.), or specific functions (denitrification, bioperturbation, etc.). In either water or soil, organisms may also be chosen because they are important for carrying out an ecological process, for example, related to biogeochemical cycling of elements (Buzea et al. 2007).

Ecotoxicity strictly means toxicity to environmental relevant organisms, while the term *bioassay* implies that toxicity or stress caused by a compound has been measured in an environmental matrix pertinent to the habitats where the organism lives in nature. Exposing a fish to nanoparticles in pure water (may be with some added salts) may thus be an ecotoxicity test, while exposing it to nanoparticle in water containing salts, dissolved organic carbon, and other colloidal materials would constitute a bioassay (Buzea et al. 2007). The modification of nanoparticles after entering in contact with environmental matrix constituents, such as ions, natural colloids, and other charged surfaces, is likely to not only affect mobility, aggregation, and so forth, but also modify toxicity characteristics.

Like traditional toxicology, ecotoxicology may also use a wide range of physiological and genetic end points, but in addition, ecotoxic organisms may be assayed on a functional level, which adds to the complexity of such investigations. Most studies on the adverse effects of nanoparticles have used very pure systems with few possible interactions between nanoparticles and matrix constituents affecting bioavailability. Thus, the conclusions that may be drawn from these results must acknowledge the fact that environmental constituents in water, soil, and sediments (dissolved organic matter, condensed HSs, clays, etc.) are likely to affect bioavailability and thus toxicity of nanoparticles (Buzea et al. 2007).

Nanoparticles safety doubts have been underlined, and their use has come under some scrutiny by both private and public institutions, regarding in particular the possible hazards associated with nanoparticles either deliberately or inadvertently produced (Abdul-Khalek et al. 1999). A massive industrial production of nanoparticles in the near future may result in the appearance of both nanoparticles and the waste generated during their production in various environments, yielding the possibility that humans could be exposed to these nanoparticles through inhalation, dermal contact, or ingestion and absorption through the digestive tract. When considering the

environmental risks of nanoparticles, a paradox arises when one understands that potentially dangerous nanoparticles also have the potential to produce more environmentally friendly processes, the so-called green chemistry, and can be used to deal with environmental contaminants (Handy et al. 2008; Das and Ansari 2009). An example of that is the use of engineered nanoparticles for water treatment and groundwater remediation, which has not only been proven to be efficient but has also raised concerns for human exposure to nanoparticles contained in the treated water. In order to guarantee the safe use of nanoparticles, some aspects must be taken into account: knowledge, detection, and prevention. An investigation into whether a substance is dangerous or not involves a determination of the inherent toxicity of the material, the manner of its interaction with living cells, and the effect of exposure time. It should be noted that the doses or exposure concentrations used in in vitro and in vivo toxicological studies are most often extraordinarily high in comparison with possible accidental human exposure (Abdul-Khalek et al. 1999; Tervonen et al. 2009).

Consequently, more research is needed before generalized statements can be made regarding nanoparticle ecotoxicology. Currently, the prevention of the escape of nanoparticles to the environment is the best approach under consideration. In this sense, the embedding of nanoparticles into organic or inorganic matrices reduces their mobility and prevents their appearance in the environment (Kennedy et al. 2004). The use of nanocomposites such as these might be the simplest way to increase the safety of nanoparticles. A complementary approach to ensure the safety of nanoparticles is to use magnetic nanoparticles in their design. Magnetic nanoparticles are of great interest for researchers from a wide range of disciplines due to their useful properties and reaction to a magnetic field (Oberdorster et al. 2006). In fact, polymeric materials containing magnetic nanoparticles with certain functionalities (e.g., catalytically active or bactericide) can find numerous technological applications. Their popularity lies in the fact that magnetic nanoparticles can be easily recovered if leakage from the nanocomposite occurs by using simple magnetic traps (Kashiwada 2006).

12.5 Toxicity of Nanoparticles on Plant Growth

The bulk production of nanoparticles often leads to their indiscriminate release in nature through industrial wastewaters (Brunner et al. 2006). A majority of the manufactured nanoparticles contain heavy metals. Thus, soil and water contamination with metallic nanoparticles have become an important environmental issue. Nanoparticles interact with the plants and results in the uptake and accumulation that affect their fate and transport in the ecosystem. Moreover, nanoparticles could remain attached to the plant surface and impart physical and chemical damages to the plant organs. Usually, nanoparticles enter the plant root system through the lateral root junctions and reach the xylem through the cortex and the pericycle (Dietz and Herth 2011). Notably, nanoparticle entry into the plant can be stopped by the cell wall. The specific properties of cell wall allowing the transport of nanoparticles could be attributed to the pore size of cell wall (Fleischer et al. 1999). The nanoparticles that are in the size range within the cell wall pore size could effectively cross the cell wall and reach the plasma membrane (Navarro et al. 2008). The rate of entry depends on the size and surface properties of nanoparticles. Indeed, the smaller nanoparticles

can easily enter into plant cells. In contrast, larger nanoparticles, being unable to enter the cells, cannot affect the cell metabolic pathways (Verano-Braga et al. 2008). Larger nanoparticles can only penetrate through the hydathodes, flower stigmas, and stomata. The mechanism of interaction between nanoparticles and plants could be chemical or physical.

Chemical interactions involve the production of ROS (Nel et al. 2006), disturbance of ion cell membrane transport activity (Auffan et al. 2008), oxidative damage (Foley et al. 2002), and lipid peroxidation (Kamat et al. 2000). Following entry into the plant cells, nanoparticles after mixing behave as metal ions, react with sulfhydryl and carboxyl groups, and ultimately alter the protein activity. However, while conducting engineered nanomaterial-mediated ecotoxicity study, much attention needs to be paid toward various artifacts, which often leads to misinterpretations of results (Petersen et al. 2014). These potential factors include toxic impurities in nanomaterials, their proper storage, and dispersion in testing medium. Moreover, nanomaterials exert indirect toxicity, which affects plant growth and development through nutrient depletion with passage of time and estimation of nanomaterials dispersal in organisms. In addition, nanomaterials face different changes (viz., settling, dissolution, agglomeration, etc.) during the exposure period, which is difficult to accurately measure. Due to increased surface area and properties, nanomaterials readily adsorb organic molecules and inorganic ions from the nutrient medium, resulting in indirect toxicity symptoms including chlorosis and wilting (Slomberg and Schoenfisch 2012). Moreover, during nanomaterial exposure, organic acid in plant root exudates decreases the pH of the media, thus altering nutrient supply and nanomaterials properties (Marschner 1995). Inefficiency to explore the influence of these factors can direct to an inappropriate explanation of phytotoxicity and ultimately a fabricated impact of nanomaterials (Petersen et al. 2014).

12.5.1 Carbon-Based Nanomaterials

Increased applications of engineered carbon-based nanomaterials create concerns about their toxicity to humans and animals (Panessa-Warren et al. 2006; Pacurari 2008). Such carbon nanomaterials (such as single-walled CNTs [SWCNTs], multiwalled CNT [MWCNTs, and carbon buckyballs) also found increased applications in the field of agriculture and food. This raised questions regarding the safety of using such nanomaterials with crops.

12.5.1.1 Single-Walled Carbon Nanotubes

12.5.1.1.1 In Vitro Evaluation

Various studies showed contradictory results on the phytotoxicity of carbon nanomaterials in plants. The effects of functionalized SWCNTs (fCNTs; functionalized with poly-3-aminobenzenesulfonic for high dispersibility) and nonfunctionalized SWCNTs (CNTs) on root elongation of six different crop species, cabbage (*Brassica oleracea*), carrot (*Daucus carota*), cucumber (*Cucumis sativus*), lettuce (*Lactuca sativa*), onion (*Allium cepa*), and tomato (*Solanum lycopersicum*) were studied to understand their toxicity to crops (Canas et al. 2008). CNT-enhanced root elongation in onion and cucumber and nanotubes sheets were formed by both fCNTs and CNTs

on cucumber root surface due to their interaction with root surface; however, none entered into the roots. Cabbage and carrot were not affected by either form of nanotubes. Root elongation in lettuce was inhibited with fCNTs, and tomato was found to be most sensitive for CNTs with significant root length reduction.

In another research, laboratory and greenhouse studies were conducted to study the effect of SWCNTs on the germination and seedling growth of tomato (*Lycopersicum esculentum* Mill. cv. 'Falcato'), onion (*Allium cepa* L. cv. 'Yellow Sweet Spanish'), turnip (*Brassica rapa* L. cv. 'Toria'), and radish (*Raphanus sativus* L. cv. 'Small radish'). Seeds were germinated in four concentrations of SWCNTs (0, 10, 20, and 40 mg L^{-1}) in petri dishes under laboratory conditions. The same concentrations were used in a greenhouse to study the response of seedling growth to SWCNTs. SWCNTs at 40 mg L^{-1} had a deleterious and toxic effect on onion and radish seed germination. In the greenhouse experiment, the fresh weight of radish seedlings decreased as the SWCNT concentration increased (Haghighi and Teixeira da Silva 2014).

Also, rice seeds were cocultured with CNTs of different concentrations. The rice seedling growth situation was observed including germination percentage rates and length of seeding stem and root. Experiment results showed that when the concentration increased to 150 g mL^{-1}, the root length, root activity, and stem length decreased in comparison with the value of 100 g mL^{-1}, but still slightly higher than the control, although the root and stem lengths were shorter than the control. The results demonstrated, for the first time, that CNTs could promote rice seed germination and root growth at lower concentrations and may have toxic effects at high concentrations.

Lin et al. (2009) found the postponed or delayed flowering and yield in *Oryza sativa* on exposure of 400 mg L^{-1} SWCNTs.

12.5.1.1.2 In Vivo Evaluation

The ability of carbon nanomaterials to penetrate the cell wall and cell membrane of intact plant cells were also recently reported. *Nicotiana tabacum* L. BY-2 cells were used as models for the uptake of oxidized SWCNTs (less than 500 nm length) labeled with fluorescein isothiocyanate (FITC) (Liu et al. 2009). It was observed that FITC alone was not easily taken up by the cells, whereas SWCNT/FITC was effectively translocated into intact cells by fluid phase endocytosis (Samaj et al. 2004). This result clearly pointed out the ability of SWCNTs to penetrate the cell wall of plant cells. Further studies showed the ability of nanotubes to carry single-stranded DNA into intact plant cells by incubating the BY-2 cells in a medium with SWCNT/ssDNA-FITC (Liu et al. 2009). A pronounced difference was observed in the distribution of SWCNT/FITC and SWCNT/ssDNA-FITC in the cell. The former was localized in the vacuolar region, and latter, in the cytoplasmic strands, thus showing their ability to deliver different components to different cellular compartments. However, the mode of entry of nanotubes through the cell wall remains mysterious.

Arabidopsis and rice leaf protoplasts were exposed to SWCNTs and examined cell viability, DNA damage, ROS species generation, and related gene expression. Also the effects of nanoparticle on *Arabidopsis* leaves after injecting a SWCNT solution were tested. SWCNTs had an adverse effect on protoplasts and leaves through oxidative stress, leading to a certain amount of programmed cell death (Shen et al. 2010).

Also it was reported that SWCNTs exhibit dual-phase regulation to Arabidopsis mesophyll cells exposed to different concentration of SWCNTs. The mesophyll protoplasts were prepared by enzyme digestion and incubated with 15, 25, 50, and 100 μg mL^{-1} SWCNTs for 48 hours and were then observed by optical microscopy and transmission electron microscopy; the ROS generation was measured. Partial protoplasts were stained with propidium iodide and 4′,6-diamidino-2-phenylindole, partial protoplasts were incubated with fluorescein isothiocyanate-labeled SWCNTs and observed by fluorescence microscopy. Results showed that SWCNTs could traverse both the plant cell wall and cell membrane. With less than or equal to 50 μg mL^{-1} in the culture medium, SWCNTs stimulated plant cells to grow out trichome clusters on their surface; with more than 50 μg mL^{-1} SWCNTs in the culture medium, SWCNTs exhibited obvious toxic effects to the protoplasts such as increasing generation of ROS, inducing changes in protoplast morphology, changing green leaves into yellow, and inducing necrosis and apoptosis of protoplast cells (Yuan et al. 2011).

Yan et al. (2013) demonstrated the upregulation of *SLR1* and *RTCS* genes and, on the other hand, the downregulation of *RTH1* and *RTH3* genes in maize root tissue on SWCNT exposure.

12.5.1.2 Multiwalled Carbon Nanotubes

12.5.1.2.1 In Vitro Evaluation

In zucchini plants, no negative effects were observed on seed germination and root elongation in the tested range of MWCNTs, whereas a decrease in the biomass of plants was observed during further growth in the presence of SWCNTs (Stampoulis et al. 2009). In another work, two agronomic plant species were evaluated for the phytotoxicity of MWCNTs using germination and seedling growth of *Brassica juncea* and *Phaseolus mungo.* Both *B. juncea* and *P. mungo* seeds showed 100% germination with the application of CNTs, which indicated their nonhazardous nature for the germination of the seeds. As the *B. juncea* seed was grown with CNTs at 10, 20, and 40 g mL^{-1}, the enhancement of root growth was evidenced up to 138%, 202%, and 135%, respectively, compared to the control. In the case of *B. juncea*, the heights of the shoot and root were not affected at all by the studied concentrations of CNTs; however, phytotoxicity was evidenced at 40 g mL^{-1} CNTs by optical microscopy of the hairy root system, since a severe reduction in both the number of root hairs and their length was observed (Ghodake et al. 2010). Hence, the response of plants to nanomaterials varies with the type of plant species, their growth stages, and the nature of nanomaterials since the studies showed contradictory effects of even the same nanomaterial in different plants at different developmental stages.

The systemic toxicity and potential influence of 0–1000 mg L^{-1} MWCNTs on red spinach were also explored. The MWCNT-exposed plants exhibited growth inhibition and cell death after 15 days of hydroponic culture. The MWCNTs had adverse effects on root and leaf morphology, as observed by scanning electron microscopy and transmission electron microscopy (TEM). Raman spectroscopy detected the MWCNTs in leaves. Biomarkers of nanoparticle toxicity, ROS, and cell damage in the red spinach were greatly increased 15 days postexposure to the MWCNTs (Begum and Fugetsu 2012).

The functionalized MWCNT (f-MWCNT) material was examined for toxicity effects using unicellular marine green alga *Dunaliella tertiolecta. D. tertiolecta* was exposed to f-MWCNT, which had been preequilibrated with culture media for 24 hours. It was concluded that the f-MWCNTs were toxic to the marine alga *D. tertiolecta*. At concentrations of 1–10 mg L^{-1}, these f-MWCNT materials adversely affected algal growth, the photosystem II (PSII) photochemical process, and cellular glutathione redox status. The majority of the toxicity effects were likely associated with the large f-MWCNT aggregates, and the leachable or soluble components (the components that passed through 0.2 μm filters) from f-MWCNT were much less harmful but might still affect some of the PSII photochemical processes (Wei et al. 2010). Stampoulis et al. (2009) observed 60% reduction in the biomass of *Cucurbita pepo*, in the presence of 1000 mg L^{-1} MWCNTs. Similarly, Lin and Xing (2007) demonstrated morphological phytotoxicity of MWCNTs in lettuce. Similar effect was also observed by Begum and Fugetsu (2012) in red spinach by the same nanoparticles.

12.5.1.2.2 In Vivo Evaluation

Many studies investigated the potential toxicity of MWCNTs in plant cells. The interactions of MWCNTs with rice suspension cells had led to the aggregation of cells so that a self-defense response was observed among the cells itself to escape from the detrimental effects of MWCNTs (Tan and Fugetsu 2007). Some cells having immediate interaction with nanotubes act as a barrier, thus saving the rest of cells. The attachment of MWCNTs to the proteins and polysaccharides of cell wall results in a signaling cascade, which increased the production of certain compounds necessary for cell wall thickening. Such hypersensitive responses are common in plants during other biotic and abiotic stresses that adversely affect plant growth. Further studies by the same group reported the detachment of plasma membrane from the cell wall of those cells interacted with MWCNTs leading to cell death. The accumulation of ROS was induced by MWCNTs resulting in increased oxidative stress and decreased cell proliferation, which eventually led to complete cell death (Tan et al. 2009). The penetration of MWCNTs inside the cells was not reported in the preceding studies. As in rice suspension cells, MWCNTs were also found to be toxic for *Arabidopsis* T87 suspension cells (Lin et al. 2009). The cells cultured in media with loose agglomerates of MWCNTs (as received product without any further treatment) and fine-agglomerates of MWCNTs (obtained after ultrasonicating the loose agglomerates of MWCNTs) had decreased dry weight, less viability, lower chlorophyll content, and superoxide dismutase (SOD) activity which showed the detrimental effects of MWCNTs on *Arabidopsis* suspension cells. The fine agglomerates of MWCNTs inhibited cell growth more than the loose MWCNTs. This was explained by the fact that the plant cell cultures have greater tendencies to form clumps with variable number of cells. Loose agglomerates of nanotubes with larger size could not easily enter into such cell clumps, whereas the distribution of fine agglomerates in clumps is easier due to their small size and greater interaction with cell wall proteins and polysaccharides in an irreversible manner. This study clearly showed the size dependent toxicity of nanotubes on plant cells. The attachment of such extracellular materials on cell clumps led to hypersensitive responses, and cell death occurs by either apoptosis or necrosis.

Tan et al. (2009) reported that MWCNT induces ROS generations that eventually affect the rate of cell proliferation and, subsequently, the death of the plant cell.

Tan et al. (2009) reported that 10–30 nm MWCNT causes chromatin condensation and shrinkage of cells in *Oryza sativa* at concentrations of 20, 40, and 80 mg L^{-1}. Moreover, Lin and Xing (2007) also observed apoptosis in cells of lettuce exposed to MWCNT. Khodakovskaya et al. (2012) also demonstrated that MWCNTs were capable of upregulating the marker genes *NNtPIP1*, *NtLRX1*, and *CycB* that are responsible for water transport (aquaporin), formation of the cell wall, and cell divisions, respectively. In addition, Khodakovskaya et al. (2011) observed that presence of MWCNTs activates the aquaporin gene (*LeAqp2*).

12.5.1.3 Fullerenes

12.5.1.3.1 In Vitro Evaluation

The uptake, accumulation, and transmission of natural organic matter (NOM)-suspended carbon nanomaterials in rice plants were also reported. Fullerene C70 or MWCNTs were suspended in NOM solution to produce C70-NOM and MWCNTs-NOM to reduce the hydrophobicity of engineered nanomaterials. Rice seeds were germinated in different concentrations of C70-NOM or MWCNTs-NOM, and then the seedlings were grown in greenhouse until maturity. First-generation seeds were then used to raise the second-generation plants without any addition of nanomaterials. Microscopic images of various tissues of both first- and second-generation rice plants at different developmental stages showed the occurrence of C70 nanomaterials, whereas little MWCNTs was observed inside the cells. These results showed the transmission of C70 to the progeny through the seeds of first generation. C70 nanoparticle might enter the plant roots via osmotic pressure and capillary forces and enter through the cell wall pores and translocated through intercellular plasmadesmata. Since the aggregates were mostly found near the vascular system, there is a possibility of hindrance to the uptake of water and nutrients which is not good for the potential growth of the plants. Delay in flowering and reduced seed set was observed in plants incubated with both C70-NOM and MWCNTs-NOM at a concentration of 400 mg L^{-1}.

$C70(C(COOH)_2)_{4-8}$ nanoparticles reduce the elongation of root and stimulate negative geotropism in roots of *Arabidopsis thaliana*. The effect of water-soluble fullerene $C70(C(COOH)_2)_{4-8}$ on *Arabidopsis thaliana* plant growth was investigated, using the transgenic seedling lines expressing fluorescent makers. The retarded roots with shortened length and loss of root gravitropism were observed for seedlings grown in the fullerene-containing medium. Canas et al. (2008) and Chen et al. (2010) observed that in the presence of fullerol (C60(OH)), the root length of *Momordica charantia* (bitter melon) and *Allium cepa* reduced. It is unpredictable how different nanomaterials interact with plants and how they modify the genetic and molecular mechanisms of plants. Such an interaction varies with the type and time of exposure to nanomaterials, and hence, these points need to be considered while assessing nanotoxicity studies.

12.5.1.3.2 In Vivo Evaluation

It was demonstrated that the soot of fullerene disturbs the pathways of energy and transport of electron by repressing transcriptional genes.

Fluorescence imaging revealed the abnormalities of root tips of *Arabidopsis thaliana* in hormone distribution, cell division, microtubule organization, and mitochondrial activity (Liu et al. 2010).

12.5.2 Magnetic Nanoparticles

12.5.2.1 In Vitro Evaluation

The main advantage of using magnetic nanoparticle is that they allow a very specific localization of the particles to release their load, which is of great interest in the study of nanoparticulate delivery for plants. In recent years, great attention has been paid to genetic effects of ferrofluids that lead to chromosomal aberrations in young plants (Racuciu and Creanga 2009a). The influence of magnetic nanoparticle coated with tetramethylammonium hydroxide (as a stabilizing agent) on the growth of maize plants in early growth stages was studied (Racuciu and Creanga 2006). The "chlorophyll a" level was increased at low ferrofluid concentrations, while at higher concentrations, it was inhibited. In another work, water-based magnetic fluid obtained by coating magnetic nanoparticle with perchloric acid was added to germinated maize seeds (Racuciu and Creanga 2009b). A slight inhibitory effect was observed on the growth of the plantlets that led to brown spots on leaves at higher volume fractions of magnetic fluid. The excess iron treatment generated some oxidative stress in leaf cells, which, in turn, affected photosynthesis and led to decreased metabolic process rates. They also studied the oxidative stress induced by the ferrofluid concentration on living plant tissues. Maize seeds were germinated in the presence of magnetic fluid followed by exposure to electromagnetic field called LM-EMF samples. For LM-EMF samples, a decrease in assimilatory pigments was observed with increased volume fraction of magnetic fluid solution. Exposure to electromagnetic field produced some local heating on the applied region due to the electromagnetic field energy absorbed by internalized magnetic nanoparticles in plant tissues that, in turn, affect the redox reactions involved in photosynthesis process. A pronounced increase in the nucleic acid level was observed in LM-EMF samples due to the regeneration reactions of plant metabolism processes against the local heating of vegetal tissues produced by the electromagnetic field energy. There is also a chance for the magnetic nanoparticles to produce some magnetic influences on the enzymatic entities involved in different photosynthetic and developmental stages.

12.5.2.2 In Vivo Evaluation

The effect of nanoparticles on growth, proteosynthesis—total protein content and thiols—reduced (GSH) and oxidized (GSSG) glutathione, phytochelatins PC2-5, glutathione S-transferase (GST) activity, and antioxidant activity of BY-2 cells was studied. Significant changes in protein content, phytochelatins levels, and GST activity were observed in BY-2 cells treated with nanoparticles treatment. Results demonstrate the ability of nanoparticles to negatively affect metabolism and induce biosynthesis of protective compounds in a plant cell model represented by BY-2 cell suspension culture (Krystofova et al. 2013).

12.5.3 Metal- and Metal Oxide-Based Nanomaterials

12.5.3.1 Nano-TiO_2

12.5.3.1.1 In Vitro Evaluation

Raliya et al. (2015) indicated differences in root growth in tomato plants when exposed to TiO_2 nanoparticles via different routes such as soil and aerosolized foliar uptake. The authors hypothesized that the spray treatment would cause significant reduction in tomato root length of plant at all concentrations except at 1000 mg kg^{-1}. Asli and Neumann (2009) observed that 30 nm TiO_2 nanoparticles favor the growth of leaves in *Zea mays* stunted plant at concentrations of 30 and 1000 mg L^{-1}, while same nanoparticles of 35 nm size reduce the number of lateral roots in pea (Fan et al. 2014). Servin et al. (2013) observed that TiO_2 nanoparticles can cause altered chlorophyll content in cucumber. Jacob et al. (2013), working on *Phaseolus vulgaris*, demonstrated that TiO_2 nanoparticles lead to the alteration in chlorophyll content.

12.5.3.1.2 In Vivo Evaluation

In spinach seedlings, 0.25% of TiO_2 nanoparticles (colloidal) led the generation of oxidative stress in chloroplasts (Lei et al. 2008). Besides this, 2.8 nm TiO_2 (Alizarin red S) nanoparticles (20 μM) caused the elimination of microtubules in *Arabidopsis thaliana* (Wang et al. 2011). Moreno-Olivas et al. (2014) demonstrated that TiO_2 nanoparticles cause damage to the genomic DNA in *Cucurbita pepo.* Besides this, they also altered the expression of genes by their upregulation. Ghosh et al. (2010) have also observed that TiO_2 nanoparticles cause DNA damage in *Allium cepa* at 4 mM. In addition to this, at the same concentration of TiO_2 nanoparticles, bridge formations were found to be predominant at telophase–anaphase stage. Similarly, in *Nicotiana tabacum* (Ghosh et al. 2010) and *Zea mays* (Castiglione et al. 2011), DNA damages were observed at 2 and 10 mM TiO_2 nanoparticles, respectively. Moreover, negative impacts were also seen in the structures of chromosomes in *Zea mays* (Castiglione et al. 2011). Likewise, Wang et al. (2011) demonstrated that exposure of TiO_2 nanoparticles causes increase in number of tubulin monomers that ultimately affect the proteosome system in *Arabdiopsis thaliana*. However, the treatment of same nanoparticles in *Vicia narbonensis* causes chromosomal aberrations through the fragmentation of arms of chromosomes (Castiglione et al. 2011).

12.5.3.2 Aluminum-Based Nanoparticles

12.5.3.2.1 In Vitro Evaluation

Nanoaluminum has been widely used in various industries, and hence, there is greater chance for the interaction of such nanomaterials with higher plants that constitute a major portion of the ecosystem. Pure alumina nanoparticles (13 nm) without any modifications reduced root elongation in studied plants, corn (*Zea mays*), cucumber (*Cucumis sativus*), soybean (*Glycine max*), carrot (*Daucus carota*), and cabbage (*Brassica oleracea*) (Yang and Watts 2005), thus potentially retarding the growth of plants. However, it was surprisingly observed that when nanoparticles were loaded with phenanthrene (a major constituent of polycyclic aromatic hydrocarbons), their toxicity significantly decreased showing no adverse effects on roots of plants. This showed the relevance of proper surface modifications, which reduce the phytotoxicity of nanoparticles. Aluminum

concentration in rye grass was increased 2.5-fold above control tests, whereas no uptake of aluminum was observed in kidney beans, which inferred the difference in uptake and distribution efficiency of even the same kind of nanoparticles by different plants.

12.5.3.2.2 In Vivo Evaluation

In tobacco BY-2 cells, 8 nm of aluminum oxide nanoparticles caused reduction in the activities of both dehydrogenase and oxidoreductase enzymes (Poborilova et al. 2013).

12.5.3.3 Zinc-Based Nanoparticles

12.5.3.3.1 In Vitro Evaluation

The seed germination and root growth study of zucchini seeds in hydroponic solution containing ZnO nanoparticles showed no negative effects (Stampoulis et al. 2009), whereas the seed germination of rye grass and corn was inhibited by nanoscale zinc (nano-Zn [35 nm]) and zinc oxide (nano-ZnO [15–25 nm]), respectively (Lin and Xing 2007). The root growth of radish and rape incubated in nano-Zn suspension was significantly inhibited; however, such an inhibition was not detected while soaked in nano-ZnO suspension due to the selective permeability of seed coat. The same research team continued their work on the rhizosphere dissolution of ZnO nanoparticles to its contribution to phytotoxicity (Lin and Xing 2008). In another work, ryegrass plants were grown with ZnO nanoparticles and Zn^{2+} ions in nutrient solution, and it was observed that ZnO nanoparticles and Zn^{2+} had toxic effects at higher concentrations. Zn^{2+} ions were more toxic than ZnO nanoparticles resulting in yellowing and complete death of the plant at higher concentrations.

Mukherjee et al. (2014) demonstrated that ZnO nanoparticles at 125, 250, and 500 mg L^{-1} concentrations, decreased chlorophyll content in green peas (*Pisum sativum*).

12.5.3.3.2 In Vivo Evaluation

Ma et al. (2010) also observed that ZnO nanoparticles cause trafficking in intracellular spaces of cell wall pores (in case of apoplast pathways) and blockage in cell connections through obstruction in plasmadesmata. Zhao et al. (2013) observed phytotoxic effects of ZnO nanoparticles at a concentration of 1000 mg L^{-1} and demonstrated collapsed structure of vacuoles, cortical cells, epidermis, and roots. Electron microscopy images confirmed the uptake of ZnO nanoparticles and showed the damage of epidermal and cortical cells upon nanoparticle intake by cells. They also caused injury to the endodermal and vascular cells, which result ryegrass growth inhibition. After entering the cells, the nanoparticle transport from one cell to other through plasmadesmata. Sometimes, aggregation of nanoparticles may occur, resulting in blocking of pores and channels; hence, more research is needed to reduce the risk assessment and to clarify the ecotoxicity.

12.5.3.4 Copper (Cu) Nanoparticles

12.5.3.4.1 In Vitro Evaluation

The effects of Cu nanoparticles on the seedling growth of mung bean and wheat were studied using plant agar culture media for easy dispersion of nanoparticles without any precipitation (Lee et al. 2008). Mung bean was found to be more sensitive to Cu

nanoparticles than wheat and growth inhibition of seedlings was observed. TEM images confirmed the entry of copper nanoparticles across the cell membrane. The bioaccumulation of nanoparticles increased with its concentration in growth media and their bioavailability to the test plants was estimated by calculating the bioaccumulation factor. This study demonstrated plant agar test as a good protocol to test phytotoxicity of nanoparticles, which is hardly water soluble. Also, studies on the effects of Cu nanoparticles on the growth of zucchini plants showed reduced length of emerging roots (Stampoulis et al. 2009). Different flora and fauna respond differently to nanomaterials, and hence, it is necessary to evaluate the safe effective concentration of each group of nanoparticles before their application that reduce the risks of ecotoxicity to a great extent.

12.5.3.4.2 In Vivo Evaluation

Atha et al. (2012) found that copper oxide nanoparticles of size 100 nm cause damage in DNA in radish at concentrations of 10, 100, 500, and 1000 mg L^{-1} (Table 12.1). Similar results were also reported by Atha et al. (2012) in perennial ryegrass at concentrations of 10, 100, 500, and 1000 mg L^{-1} (Table 12.1). The studies investigating the impact of nanoscale copper particle types on plants have been summarized in Table 12.2.

12.5.3.5 Silver (Ag) Nanoparticles

12.5.3.5.1 In Vitro Evaluation

Ag nanoparticles have several antimicrobial functions to control various phytopathogens (Jo and Kim 2009). Studies on the seed germination and root growth of zucchini plants in hydroponic solution amended with Ag nanoparticles showed no negative effects, whereas a decrease in plant biomass and transpiration was observed on prolonging their growth in presence of Ag nanoparticles (Stampoulis et al. 2009). The cytotoxic and genotoxic impacts of Ag nanoparticles were studied using root tips of onion. It was investigated that Ag nanoparticles impaired the stages of cell division and caused cell disintegration (Kumari et al. 2009). All such studies throw light to the need for a more cytotoxic and genotoxic evaluations by considering the properties of nanoparticles, their uptake, translocation, and distribution in different plant tissues.

Nair and Chung (2014) demonstrated the phytotoxic effect of silver nanoparticles in *Oryza sativa* and concluded that on the exposure of 0.5 mg L^{-1} silver nanoparticles, root and shoot biomass significantly decreases, while further increasing the concentration also affects leaf area, contents of chlorophyll, and carotenoids. The impacts of Ag nanoparticles and ZnO nanoparticles on *Zea mays* L. were evaluated, and it was *Brassica oleracea* var. *capitata* L. were evaluated, and it was found that these two nanoparticles were capable of reducing the size of vacuoles, which eventually reduces the turgidity and size of the cell. Likewise, Hawthorne et al. (2012) reported similar results and showed that Ag nanoparticles at 205–750 mg L^{-1} cause biomass reduction up to 49–91% in *Cucurbita pepo.* Similarly, on the exposure of ZnO nanoparticles, shrinkage in the root tips was observed due to the disintegration of cortical and epidermal cells (Ma et al., 2010).

12.5.3.5.2 In Vivo Evaluation

Nair and Chung (2014a,b,c) have reported that the exposure of Ag nanoparticles leads to differential expressions of genes related to oxidative stress tolerance such as *MSD1*, *CSD1*, and *FSD* in rice (*Oryza sativa* L.) seedlings.

TABLE 12.1

Gene Expression in Response to Silver Nanoparticles

Plant (Cultivar)	Organ	Growth Stage	Nanoparticles (Particle Size, Dose)	Treatment Period	Technique	Major Findings	Reference
Wheat plants	Roots	5-day-old	Ag-NP 30 μM	3 weeks	Hydroponic culture	Genes involved in various functions including oxidative stress, defense against pathogens, and metal homeostasis were impacted in different ways depending upon the Ag source.	Pradas del Real et al. 2017
Soybean (*Glycine max* L. cv. Enrei)	Root including hypocotyl	2-day-old	Ag, ZnO, Al_2O_3 nanoparticles with flooding stress (0.5–500 ppm)	1–3 days	Gel-free (nano-LC MS/MS)	Al_2O_3 nanoparticles (50 ppm) promote seedling growth under flooding stress by regulating energy metabolism and cell death.	Pignocchi et al. 2006
Soybean (*Glycine max* L. cv. Enrei)	Root, cotyledon	2-day-old	Ag nanoparticles with flooding stress (2, 15, 50–80 nm; 0.2, 2, 20 ppm)	1–4 days	Gel-free (nano-LC MS/MS)	Ag nanoparticles (15 nm; 2 ppm) treatments facilitate seedling growth under flooding stress. Decreased abundance of glyoxalase II 3 and fermentation-related proteins: pyruvate decarboxylase 2 and alcohol dehydrogenase 1, indicating metabolic shift from fermentative pathways toward normal cellular processes.	Jacobs and Roe 2005

(*Continued*)

TABLE 12.1 (CONTINUED)

Gene Expression in Response to Silver Nanoparticles

Plant (Cultivar)	Organ	Growth Stage	Nanoparticles (Particle Size, Dose)	Treatment Period	Technique	Major Findings	Reference
Rice (*Oryza sativa* L. cv. IR651)	Root	10-day-old	Ag nanoparticles (30, 60 ppm)	20 days	Gel-based (2-DE, nano-LC/FT-ICR MS)	Increased abundance of proteins related to oxidative stress response pathway, Ca^{2+} regulation signaling, transcription, protein degradation, cell wall synthesis, cell division and apoptosis.	Nanjo et al. 2011
Eruca sativa (common name: rocket)	Root	Germinating seeds	Ag nanoparticles or $AgNO_3$ (0.1, 1, 10, 20, 100 ppm)	5 days	Gel-based (2-DE, nano-LC-nESI-MS/MS)	Alteration of some proteins related to the endoplasmic reticulum and vacuole, indicating these two organelles as the targets of the action of the Ag nanoparticles. Effects of Ag nanoparticles are not simply due to the release of Ag+.	Lee et al. 2009
Arabidopsis thaliana accession Columbia-0	Root	3-week-old	Ag (10–80 nm), TiO_2 (10–40 nm), MWNT	7 days	Microarray analysis	Exposure to nanoparticles repressed expression of phosphate-starvation and root-development genes.	Sedbrook et al. 2002
Arabidopsis thaliana (ecotype, Columbia) Wild, tir1, abi5 mutants	Seedling	4-day-old	Ag nanoparticles: triangular (47 nm), spherical (8 nm), decahedral (45 nm)	3 days	qRT-PCR	Ag nanoparticles induced ROS accumulation; interfered with ethylene biosynthesis; promoted root growth; triggered gene expression involved in cellular events; cell proliferation, metabolism, and hormone signaling pathways.	Solomon et al. 1997

(*Continued*)

TABLE 12.1 (CONTINUED)

Gene Expression in Response to Silver Nanoparticles

Plant (Cultivar)	Organ	Growth Stage	Nanoparticles (Particle Size, Dose)	Treatment Period	Technique	Major Findings	Reference
Arabidopsis thaliana (Col-0/ Redei-L211497)	Seedling	Germinating	Ag nanoparticles (20 nm; 5 ppm)	10 days	Microarray analysis	Upregulated genes primarily associated with metals and oxidative stress, while downregulated genes linked to biotic and hormonal stimuli.	Minic et al. 2007
Chlamydomonas reinhardtii (wild-type strain C137)	–	–	Ag (20 nm; 1 ppm), TiO_2 (5 nm; 1 ppm), ZnO (20 nm; 1 ppm), q-dots (6–10 nm, 0.12 ppm)	2 hours	RNA-seq analysis	Genes associated with photosynthesis were markedly decreased on exposure to TiO_2. Ag nanoparticles exposure led to the elevation of transcripts encoding components of cell wall and flagella.	Turktas et al. 2013

Source: Mahmoodzadeh, H. et al., Physiological Analysis of Silver Nanoparticles and $AgNO_3$ Effect to *Brassica napus* L., Msc Thesis of Plant Physiology, Department of Biology, Faculty of Science, Neyshabour Branch, Islamic Azad University, Tehran, 2017.

Abbreviations: 2-DE, Two-dimensional gel electrophoresis; nano-LC/FT ICR MS, nano-liquid chromatography/Fourier transform ion cyclotron resonance mass spectrometry; nano-LC MS/MS, nanoscale liquid chromatography coupled to tandem mass spectrometry.

TABLE 12.2

Studies Investigating the Impact of Nanoscale Copper Particle Types on Plants

Particle Size and Concentration(s)	Plant Species	End Point(s) Tested	Exposure Condition Period	Effect(s)/Remarks	Reference
Nano-CuO; size: 25–50 nm; concentrations:100, 500, or 1000 mg kg DW^{-1}	Sweet potato (*Ipomoea batatas*)	Crop yield and food safety	Field microcosms using substrate amended with three concentrations	Adverse effects on tuber biomass were observed only for the highest concentration of Cu applied. Cu accumulated in both the peel and flesh of the sweet potato tubers, with concentrations higher in the peel than the flesh for each element	Bradfield et al. 2017
Nano-CuO; size: <50 nm; concentrations: 5, 15, 30, 45, 60, 100, 200, 400, 600 mg L^{-1}	Soybean (*Glycine max*) and chickpea (*Cicer arietinum*) seedlings	Seed germination and root elongation	Petri dishes with filter paper soaked with distilled water-dissolved nano-CuO; 5 days	In both *Glycine max* and *Cicer arietinum*, germination was not checked up to 2000 mg L^{-1} nano-CuO, but the root growth was prevented above 500 mg L^{-1} nano-CuO; the elongation of the roots was severely inhibited with increasing concentration of nano-CuO compared to control; root necrosis was also observed	Adhikari et al. 2012
Nano-CuO; size: <100 nm; concentrations:10, 100, 500, and 1000 mg L^{-1}	*Raphanus sativus*	Plant root/shoot elongation and biomass	Petri dishes with filter paper soaked with distilled water-dissolved nano-CuO; 6 days	Strongly inhibited seedling growth over the entire treatment range; root growth was inhibited 97% and that of shoot growth was inhibited 79%	Atha et al. 2012

(*Continued*)

TABLE 12.2 (CONTINUED)

Studies Investigating the Impact of Nanoscale Copper Particle Types on Plants

Particle Size and Concentration(s)	Plant Species	End Point(s) Tested	Exposure Condition Period	Effect(s)/Remarks	Reference
Nano-CuO; size: <50 nm; concentration: 500 mg kg^{-1} s	*Triticum aestivum*	Root and shoot length, and number of roots; chlorophyll contents	Sand matrix; 14 days	Shoot length was significantly reduced by 13%; reduced root length by 59%; in contrast to plant length (root and shoot), nano-CuO caused proliferation of the number of the roots, significantly increasing the number of roots by 42%; roots exhibited brown necrotic lesions and were thinner and more brittle than the control plants; decreased chlorophyll levels compared to control	Dimkpa et al. 2012
Nano-CuO and nano-Cu; size: 50 nm; concentrations: 10, 50, 100, 500, and 1000 mg L^{-1}	*Cucumis sativus* seedlings	Biomass accumulation	Hydroponic culture; 5 days	The biomass level was 75% of that of control at 1000 mg L^{-1} of nano-CuO; the IC50 of nano-CuO was 376 mg L^{-1}; the biomass level was 33% of that of control at 1000 mg L^{-1} of nano-Cu; the IC50 of nano-Cu was 333 mg L^{-1}	Kim et al. 2012
Nano-Cu; size: <50 nm; concentrations: 100 and 500 mg L^{-1}	Zucchini (*Cucurbita pepo*)	Growth and photosynthetic traits	Hydroponics assay; 14 days	The biomass of plants exposed to 100 and 500 mg L^{-1} nano-Cu was 2.5 and 1.9 g, respectively; these values represented 93% and 99% reductions in normalized plant growth relative to untreated controls; transpiration volume was reduced by 51% in plants exposed to 100 mg nano-Cu L^{-1} and 61% in plants treated with 500 mg nano-Cu L^{-1}	Musante and White 2012

(*Continued*)

TABLE 12.2 (CONTINUED)

Studies Investigating the Impact of Nanoscale Copper Particle Types on Plants

Particle Size and Concentration(s)	Plant Species	End Point(s) Tested	Exposure Condition Period	Effect(s)/Remarks	Reference
Nano-CuO; size: 20–40 nm; concentrations: 2.0–100 mg L^{-1}	Maize (*Zea mays*) seedlings	Germination; root elongation; biomass	Hydroponic culture; 15 days	Inhibition of root elongation; chlorotic symptoms in seedlings; reduced biomass and root elongation	Wang et al. 2012
Nano-CuO; size: 30–50 nm; concentration: 1000 mg L^{-1}	Lettuce (*Lactuca sativa*), radish (*Raphanus sativus*), and *Cucumis sativus* seedlings	Seed germination and root elongation	Petri dishes with filterpaper soaked with distilled water-dissolved nano-CuO; 3 days	The measured effective con centrations (EC50) for seed germinations were 13 mg CuO L^{-1}), 398 CuO mg L^{-1}, and 228 mg CuO L^{-1} for seeds of *Lactuca sativa*, *Raphanus sativus*, and *Cucumis sativus*, respectively; role of the surface area-to-volume ratio of seeds in nano-CuO-mediated phytotoxicity was revealed; small seeds (that of *Lactuca sativa*) were the most sensitive to nano-CuO	Wu et al. 2012
Nano-CuO; size: <50 nm; concentration: 500 mg kg^{-1} sand	Wheat (*Triticum aestivum*)	Root phytotoxicity	Sand matrix; 14 days	Reduced root length by 64% from control levels; browning of the root surface	Dimkpa et al. 2013
Nano-CuO; size: <50 nm; concentrations: 50, 500, 2000, and 4000 mg L^{-1}	Buck wheat (*Fagopyrumesculentum*)	Seedling growth; root tissue morphology	Petri dishes with filter paper soaked with distilled water-dissolved nano-CuO; 7 days	High doses of nano-CuO (2000 and 4000 mg L^{-1}) significantly inhibited root length relative to that in the control seedling biomass decreased only in the treatments with 4000 mg L^{-1} of nano-CuO	Lee et al. 2013

(*Continued*)

TABLE 12.2 (CONTINUED)

Studies Investigating the Impact of Nanoscale Copper Particle Types on Plants

Particle Size and Concentration(s)	Plant Species	End Point(s) Tested	Exposure Condition Period	Effect(s)/Remarks	Reference
Nano-CuO; size: <50 nm; concentrations: 0.5, 1.0, and 1.5 mM	Rice (*Oryza sativa*) seedlings	Seed germination and seedlings growth; carotenoids content	Plastic trays with cotton pads soaked with double distilled water-dissolved nano-CuO; 14 days	Inhibition of seed germination and seedlings growth; declined carotenoids content	Shaw and Hossain 2013
Nano-CuO; size: <50 nm; concentrations: 100, 250, and 500 mg kg^{-1} sand	Bean (*Phaseolus vulgaris*)	Growth traits	Sand matrix; 7 days	Inhibition of growth was more apparent in roots (10–66%) than shoots (9–25%)	Dimkpa et al. 2015
Nano-CuO; concentrations: 100, 200, 400, and 600 mg L^{-1}	Cucumber (*Cucumis sativus*) seedlings	Seed germination and root elongation	Petri dishes with filter paper soaked with distilled water-dissolved nano-CuO; 7 days	Significant inhibition of seed germination and root elongation; differential expression of 34 proteins in *C. sativus* seeds where, the expression patterns of at least nine proteins, highly differential; one novel biomarker candidate protein (5977 *m/z*) was also identified	Moon et al. 2014
Nano-CuO; size: <50 nm; concentrations: 50, 100, 200, 400, and 500 mg L^{-1}	*Glycine max* seedlings	Shoot and root development, total chlorophyll content	Murashige and Skoog medium (1/2 strength); 14 days	Exposure to 500 mg L^{-1} of nano-CuO significantly reduced the shoot growth, weight, and total chlorophyll content; root length and fresh weights were significantly reduced at all concentrations of nano-CuO exposure	Nair and Chung 2014a

(*Continued*)

TABLE 12.2 (CONTINUED)

Studies Investigating the Impact of Nanoscale Copper Particle Types on Plants

Particle Size and Concentration(s)	Plant Species	End Point(s) Tested	Exposure Condition Period	Effect(s)/Remarks	Reference
Nano-CuO; size: 30 nm; concentrations: 0.5, 1.0, 2.0, 5.0, 10, 20, 50, and 100 mg L^{-1}	*Arabidopsis thaliana*	Plant biomass; total chlorophyll content; anthocyanin content; root elongation	Murashige and Skoog medium (1/2 strength); 21 days	Plant biomass and total chlorophyll content were significantly reduced under all the tested concentrations (2.0, 5.0, 10, 20, 50, and 100 mg L^{-1}; the anthocyanin content significantly increased upon exposure to 10, 20, 50, and 100 mg L^{-1}; significant reduction in root elongation was observed upon exposure to 0.5–100 mg L^{-1}; retarded primary root growth, enhanced lateral root formation, and loss of root gravitropism were also noted	Nair and Chung 2014b
Nano-CuO; Size: <50 nm; Concentrations: 0.5, 1.0, and 1.5 mM	Syrian barley (*Hordeum vulgare*)	Growth traits, chlorophyll fluorescence, and the contets of chlorophyll and epidermal flavonols	Plastic trays with cotton pads soaked with double distilled water-dissolved nano-CuO; 20 days	Significant gradual decreases in shoot length as well as shoot and root weight with increasing nano-CuO concentration; significant changes in the chlorophyll, epidermal flavonols contents; nanocopper-induced increase in quantum efficiency of photosystem (PSII; ΦPSII) was only recorded in highest nano-CuO concentration (1.5 mM); nano-CuO had no significant effects on maximal quantum yield of PSII (Fv/Fm) irrespective of nanocopper concentration	Shaw et al. 2014

(*Continued*)

TABLE 12.2 (CONTINUED)

Studies Investigating the Impact of Nanoscale Copper Particle Types on Plants

Particle Size and Concentration(s)	Plant Species	End Point(s) Tested	Exposure Condition Period	Effect(s)/Remarks	Reference
Nano-CuO; size: 20–30 nm; concentrations: 10 and 20 mg L^{-1}	*Lactuca sativa* seedlings	Plant root/shoot elongation and biomass; chlorophyll content	Hydroponic system using magenta boxes; 15 days	Reduced root length by 51%; no effects were observed in leaf length; reduced water content in roots by 61%; reduced biomass accumulation of roots and leaves by 69% and 52%, respectively; reduced chlorophyll content by 14% compared with the control	Trujillo-Reyes et al. 2014
Nano-CuO; size: 10–100 nm; nano-Cu; size: up to 10 μm; concentrations for both nanoparticle types: 5.0, 10, and 20 mg L^{-1}	*Lactuca sativa* and alfalfa (*Medicago sativa*)	Growth traits	Hydroponic system using magenta boxes; 15 days	The shortest root in *Lactuca sativa* (15.9 ± 2.4 cm) and *Medicago sativa* (16.2 ± 0.2 cm) occurred in plants treated with 20 mg L^{-1} nano-CuO and nano-Cu	Hong et al. 2015
Oxidative Stress and Its Metabolism					
Nano-CuO; size: <50 nm; concentration: 500 mg kg^{-1} sand	*Triticum aestivum*	Lipid peroxidation; oxidized glutathione; activity of peroxidase (POD) and catalase (CAT) enzymes	Sand matrix; 14 days	Elevated activity of POD and CAT in roots; more glutathione were present as the oxidized form, GSSG, in the roots than the shoots of the control plants on a fresh weight basis; the level of GSSG in the shoots of plants grown with nano-CuO significantly increased compared to control plants; increases in GSSG in roots were not significant	Dimkpa et al. 2012c

(*Continued*)

TABLE 12.2 (CONTINUED)

Studies Investigating the Impact of Nanoscale Copper Particle Types on Plants

Particle Size and Concentration(s)	Plant Species	End Point(s) Tested	Exposure Condition Period	Effect(s)/Remarks	Reference
Nano-CuO; size: 50 nm; concentrations: 10, 50, 100, 500, and 1000 mg L^{-1}	*Cucmis sativus*	Activity of ROS-metabolizing enzymes (SOD, CAT, and POD)	Hydroponic culture; 5 day	Enzyme activities of SOD, CAT, and POD in the root cells increased compared to that of control, where significant differences in their activity were noted at 100 mg L^{-1}; in particular, SOD and POD activities in root cells increased to higher than 50% of that of control, whereas CAT activity did not significantly change	Kim et al. 2012
Nano-CuO; size: <50 nm; concentrations: 0.5, 1.0, and 1.5 mM	Syrian barley (*Hordeum vulgare*)	Oxidative stress traits and metabolizing enzymes	Plastic trays with cotton pads soaked with double distilled water-dissolved nano-CuO; 20 days	Severe oxidative burst was revealed measured as H_2O_2 deposits evident in all stressed leaves irrespective of CuO concentration; intensity and the occurrence of spots were found to be more in high concentrations (1.0 and 1.5 mM) compared to 0.5mM nano-CuO; maximum cell death was observed with 1.0 and 1.5 mM nano-CuO exposed roots; maximum increase (~1.8-fold) in foliar lipid peroxidation (measured as malondialdehyde) was noticed in 1.5 mM CuO treatment irrespective of stress period; decreased GSH and the GSH/GSSG ratio; elevated SOD, ascorbate peroxidase (APX), and GR activity; declined activity of DHAR and MDHAR, and the recycling of AsA pool indicated as elevated DHA level	Shaw et al. 2014

(*Continued*)

TABLE 12.2 (CONTINUED)

Studies Investigating the Impact of Nanoscale Copper Particle Types on Plants

Particle Size and Concentration(s)	Plant Species	End Point(s) Tested	Exposure Condition Period	Effect(s)/Remarks	Reference
Nano-CuO; size: 20–30 nm; concentrations: 10 and 20 mg L^{-1}	*Lactuca sativa* seedlings	Activity of CAT and APX enzymes	Hydroponic system using magenta boxes; 15 days	At 10 mg L^{-1}, CAT and APX activities, respectively, increased and decreased in roots and leaves; however, the roots and leaves at 20 mg L^{-1} exhibited a lower CAT activity compared to the 10 mg L^{-1} treatment	Trujillo-Reyes et al. 2014
Nano-CuO; size:10–100 nm; nano-Cu; size: up to 10 μm; concentrations for both nanoparticle types: 5.0, 10, and 20 mg L^{-1}	*Lactuca sativa* and *Medicago sativa*	Assays for CAT and APX enzymes	Hydroponic system using magenta boxes; 15 days	CAT and APX activities were plant type- and plant organ-dependent irrespective of nano-Cu types; the downregulated and upregulated activity of, respectively, CAT and APX in *Lactuca sativa* and *Medicago sativa* roots indicated a differential ROS-generating	Hong et al. 2015
Nano-CuO; size: <100 nm; concentrations: 10, 100, 500, and 1000 mg L^{-1}	*Raphanus sativus*, perennial ryegrass (*Lolium perenne*) and annual ryegrass (*Lolium rigidum*)	DNA damage	Petri dishes with filter paper soaked with distilled water-dissolved nano-CuO; 6 days	Of the three plant species examined, *Lolium rigidum* was the most resistant to nano-CuO-induced DNA damage; under similar nano-CuO concentrations (10, 100, 500, and 1000 mg L^{-1}), the accumulation of Fapy Gua and 8-OH-Gua was E2 times lower and that of Fapy Ade was E10 times lower in *L. perenne* than in *R. sativus*; only high doses of nano-CuO were evidenced to cause the accumulation of Fapy Ade, Fapy Gua, and 8-OH-Gua in *L. rigidum*	Atha et al. 2012

(*Continued*)

TABLE 12.2 (CONTINUED)

Studies Investigating the Impact of Nanoscale Copper Particle Types on Plants

Particle Size and Concentration(s)	Plant Species	End Point(s) Tested	Exposure Condition Period	Effect(s)/Remarks	Reference
Nano-CuO; size: <50 nm; concentrations: 50, 500, 2000, and 4000 mg L^{-1}	Buck wheat (*Fagopyrum esculentum*)	Random amplified polymorphic (RAPD) DNA assays	Petri dishes with filter paper soaked with distilled water-dissolved nano-CuO; 7 days	RAPD assay conducted at high nano-CuO concentrations (2000 and 4000 mg L^{-1}) revealed four random 10-mer primers that generated specific and reproducible results; of 87 bands, 58 showed changes compared to the controls and ranged in size from 200 to 1600 base pairs; DNA damage effect displayed for averaged RAPD pattern by four random amplifications. The average Nei's genetic identity (NGI) of seedlings exposed to nano-CuO at 2000 mg L^{-1} significantly decreased; at the highest dose tested (4000 mg L^{-1}), the reduction in NGI was significantly more pronounced than that in the control for DNA damage; the band changes tend to increase with an increase in nano-CuO concentrations	Lee et al. 2013

(*Continued*)

TABLE 12.2 (CONTINUED)

Studies Investigating the Impact of Nanoscale Copper Particle Types on Plants

Particle Size and Concentration(s)	Plant Species	End Point(s) Tested	Exposure Condition Period	Effect(s)/Remarks	Reference
Nano-CuO; size: <50 nm; concentrations: 50, 100, 200, 400, and 500 mg L^{-1}	*Glycine max* seedlings	The mRNA expression of different genes involved in lignin biosynthesis, viz., phenylalanine ammonialyase (PAL), cinnamate 4-hydroxylase (C4H), cinnamyl alcohol dehydrogenase (CAD), peroxidase 2 (POD2), peroxidase 4 (POD4), and peroxidase 7 (POD7) was studied using real-time polymerase chain reaction	Murashige and Skoog medium (1/2 strength); 14 days	The expression levels of PAL, C4H, and CAD genes were significantly upregulated upon exposure to 100, 200, and 400 mg L^{-1}; significant upregulation in the expression levels of POD2 and POD4 genes was observed upon exposure to 100, 200, 400, and 500 mg L^{-1}; exposure to 200, 400, and 500 mg L^{-1} of nano-CuO resulted in significant upregulation of POD7 gene	Nair and Chung 2014a
Nano-CuO; size: 30 nm; concentrations: 0.5, 1.0, 2.0, 5.0, 10, 20, 50, and 100 mg L^{-1}	*Arabidopsis thaliana*	Cell viability	Murashige and Skoog medium (1/2 strength); 21 days	Root cell death was not observed in roots of plants exposed to 1.0 mg L^{-1}; however, propidium iodide staining showed a dose-dependent increase in cytotoxicity in lateral root tips of plants which were exposed to 2.0, 5.0, 10, and 20 mg L^{-1}	Nair and Chung 2014b

(*Continued*)

TABLE 12.2 (CONTINUED)

Studies Investigating the Impact of Nanoscale Copper Particle Types on Plants

Particle Size and Concentration(s)	Plant Species	End Point(s) Tested	Exposure Condition Period	Effect(s)/Remarks	Reference
Uptake/Accumulation and Localization					
Nano-CuO; size: 25–80 nm; concentrations: 5.0, 10, 100, 500, and 1000 mg L^{-1}	Triticum aestivum	Adsorption and uptake of nano-CuO on the root	Agar culture media; 48 hours	Some of nano-CuO were strongly adsorbed on the plant root surface, and part of them, by mechanical adhesion; the uptake and adsorption of nano-CuO increased with increasing exposure concentrations in the range of 5.0–200 mg L^{-1}; the amount of nano-CuO adsorption was always lower than that of their uptake	Zhou et al. 2011
Nano-CuO; size: <100 nm; concentrations: 10, 100, 500, and 1000 mg L^{-1}	*Raphanus sativus*, *Lolium perenne*, and *Lolium rigidum*	Accumulation and localization in root and shoot tissues	Petri dishes with filter paper soaked with distilled water-dissolved nano-CuO; 6 days	In the 10, 100, 500, and 1000 mg L^{-1} nano-CuO and bulk CuO, exposed *R. sativus*, the approximately three times greater total Cu accumulation in nano-CuO-treated shoot, compared to bulk CuO); for *L. perenne*, the measured back ground level of Cu was 15.8 µg Cu g^{-1} plant shoots, and the total Cu uptake from nano-CuO was only 1.5 times the background level; the measured background level of Cu in the *L. rigidum* shoots was 21.0 µg Cu g^{-1} plant shoots, and the total Cu uptake (24.0 723.1 µg Cu g^{-1}) plant shoots from nano-CuO was equivalent to the background level	Atha et al. 2012

(*Continued*)

TABLE 12.2 (CONTINUED)

Studies Investigating the Impact of Nanoscale Copper Particle Types on Plants

Particle Size and Concentration(s)	Plant Species	End Point(s) Tested	Exposure Condition Period	Effect(s)/Remarks	Reference
Nano-CuO; size: <50 nm; concentration: 500 mg kg^{-1} sand	*Triticum aestivum*	Accumulation and speciation in shoot	Sand matrix; 14 days	*T. aestivum* shoots exhibited 375 ± 115 mg Cu kg^{-1} shoot dry weight; the Xanes data showed that nano-CuO was detected in the shoots of *T. aestivum* seedlings grown from roots exposed to the nano-CuO; LC analysis of the Xanes spectra from plants grown with nano-CuO revealed that majority (64 ± 10%) of the Cu was in the original form as CuO and the rest (36 ± 10%) was bound to sulfur as a reduced Cu (I)–S species	Dimkpa et al. 2012c
Nano-CuO; size: 50 nm; concentrations: 10, 50, 100, 500, and 1,000 mg L^{-1}	*Cucmis sativus*	Bioaccumulation	Hydroponic culture; 5 days	Bioaccumulation of Cu was concentration dependent; the Cu concentration in *C. sativus* steeply increased at 100 mg L^{-1} of Cu, after which accumulated levels reached about 3000 μg g^{-1} at 1000 mg L^{-1} of Cu; TEM images of root tissues revealed that nano-CuO entered into the endodermis of the *C. sativus* root cells	Kim et al. 2012
Nano-Cu; size: <50 nm; concentrations: 100 and 500 mg L^{-1}	*Cucurbita pepo*	Uptake	Hydroponics assay; 14 days	Cu shoot content in nano-Cu treatments at 100 mg L^{-1} was 3.9 μg g^{-1} (wet weight), and at 500 mg L^{-1}, this value was 4.8 μg g^{-1}	Musante and White 2012

(*Continued*)

TABLE 12.2 (CONTINUED)

Studies Investigating the Impact of Nanoscale Copper Particle Types on Plants

Particle Size and Concentration(s)	Plant Species	End Point(s) Tested	Exposure Condition Period	Effect(s)/Remarks	Reference
Nano-CuO; size: 20–40 nm; concentration 2.0–100 mg L^{-1}	*Zea mays*	Uptake in roots and shoots	Hydroponic culture; 15 days	Plant tissue Cu contents increased with increasing nano-CuO concentrations; with the exception of 100 mg L^{-1}, there were no significant differences in root Cu content across all treatments; the Cu content in the roots of plants exposed to 100 mg L^{-1} was 3.6 times higher than that in control; similarly, Cu content in the shoots at 100 mg L^{-1} was 7 times higher than that in the control; nano-CuO existed not only inside the cell wall of epidermal cell in root tips but also in the intercellular space and cytoplasm of cortical cells as well as in the nuclei	Wang et al. 2012
Nano-CuO; size: 20–30 nm; concentrations: 10 and 20 mg L^{-1}	*Lactuca sativa*	Uptake	Hydroponic system using magenta boxes; 15 days	Root Cu concentration was 3362 mg kg^{-1} dry weight; 20 mg L^{-1} exposed plant leaves exhibited 376% increased Cu content when compared to the control	Trujillo-Reyes et al. 2014

(*Continued*)

TABLE 12.2 (CONTINUED)

Studies Investigating the Impact of Nanoscale Copper Particle Types on Plants

Particle Size and Concentration(s)	Plant Species	End Point(s) Tested	Exposure Condition Period	Effect(s)/Remarks	Reference
Nano-CuO; size: 10–100 nm; nano-Cu; size: up to 10 μm; concentrations for both nanoparticle types: 5.0, 10, and 20 mg L^{-1}	*Lactuca sativa* and *Medicago sativa*	Uptake of Cu	Hydroponic system using magenta boxes; 15 days	In *L. sativa* root, Cu accumulation from nano-Cu at 5.0 and 20 mg L^{-1} was higher, compared to the nano-CuO; in the case of *L. sativa* shoot, only nano-Cu at 10 and 20 mg L^{-1} significantly increased Cu accumulation, with respect to the control; exposure to nano-Cu at 20 mg L^{-1} produced significantly higher Cu concentration in the shoots of both *L. sativa* and *M. sativa*; under all treatments, *M. sativa* translocated about 3–5% of Cu from root to shoot, while only 0.5–0.6% was translocated in *L. sativa*	Hong et al. 2015

Source: Mahmoodzadeh, H. et al., Physiological Analysis of CuO Bulk and Nanoparticles to Castor (*Ricinus communis* L.), MSc Thesis of Plant Physiology, Department of Biology, Faculty of Science, Mashhad Branch, Islamic Azad University, Tehran, 2017.

The studies investigating the gene expression analyses in response to Ag nanoparticle stress have been summarized in Table 12.1.

12.5.3.6 Silica Nanoparticles

12.5.3.6.1 In Vitro Evaluation

To assess the aquatic ecosystem safety for silica (SiO_2) nanoparticles, the growth inhibition and photosynthetic pigment contents of *Scenedesmus obliquus* in logarithm growth phase exposed to SiO_2 nanoparticles and SiO_2 bulk particles (BPs) suspensions were measured. SiO_2 nanoparticles with 10–20 nm diameters were found to be toxic. The 20% effective concentration (EC20) values for 72 and 96 hours were 388.1 and 216.5 mg L^{-1}, respectively. The contents of chlorophyll significantly decreased under moderate and high concentration (50, 100, and 200 mg L^{-1}) of SiO_2 nanoparticles after 96-hour exposure, but the carotenoids did not. SiO_2 BPs were found to be nontoxic up to 200 mg L^{-1}. The toxicity of SiO_2 nanoparticles is probably due to their sorption onto the surface of algal cells. The results imply that there is potential harm to aquatic environment by using SiO_2 nanoparticles, and it should deserve special concern (Wei et al. 2010).

Effects of four metal oxide nanoparticles, aluminum oxide (nAl_2O_3), silicon dioxide ($nSiO_2$), magnetite (nFe_3O_4), and zinc oxide (nZnO), on the development of *Arabidopsis thaliana* (Mouse-ear cress) were studied. Three toxicity indicators (seed germination, root elongation, and number of leaves) were quantified following exposure to each nanoparticle at three concentrations: 400, 2000, and 4000 mg L^{-1}. Among these particles, nZnO was most phytotoxic, followed by nFe_3O_4, $nSiO_2$, and nAl_2O_3, which were not toxic (Lee et al. 2010).

In another research, the phytotoxicity of silica nanoparticles (Si nanoparticles) was evaluated as a function of particle size (14, 50, and 200 nm), concentration (250 and 1000 mg L^{-1}), and surface composition toward *Arabidopsis thaliana* plants hydroponically grown for 3 and 6 weeks. Reduced development and chlorosis were observed for plants exposed to highly negative Si nanoparticles (–20.3 and –31.9 mV for the 50 and 200 nm Si nanoparticles, respectively) regardless of particle concentration when not controlling pH of the hydroponic medium, which resulted in increased alkalinity. Particles were no longer toxic to the plants at either concentration upon calcination or removal of surface silanols from the Si nanoparticles surface or adjusting the pH of the growth medium to pH 5.8. The phytotoxic effects observed for the negatively charged 50 and 200 nm Si nanoparticles were attributed to pH effects and the adsorption of macro- and micronutrients to the silica surface (Slomberg and Schoenfisch 2012).

The pear seedlings were irrigated with different concentrations of nanosilica (0, 10, 100, 500, and 1000 mg L^{-1}) for 14 days. Although the performed experiments did not show any acute toxic effects of adding of SiO_2 nanoparticles in irrigation to wild pear plant, the finding should be confirmed with other experiments of longer duration and high exposure concentrations before a final conclusion in this issue can be made (Zarafshar et al. 2015). In another study, two field crops (*Zea mays* L. and *Phaseolus vulgaris* L.), two medicinal plants (*Hyssopus officinalis* L. and *Nigella sativa* L.), and two weeds (*Amaranthus retroflexus* L. and *Taraxacum officinale* F. H. Wigg) were separately treated with three concentrations of SiO_2 nanoparticles (400, 2000, and 4000 mg L^{-1}). The effects of these treatments on morphological and

biochemical characteristics of the plants were assessed. In weeds, as SiO_2 nanoparticle concentration increased from 400 to 4000 mg L^{-1}, germination, root and shoot lengths, fresh and dry weights, and photosynthetic pigments as well as total protein decreased. Total carbohydrates in all plants significantly decreased, except for *A. retroflexus* at 400 mg L^{-1} SiO_2 nanoparticles. In all plant species, with increasing SiO_2 nanoparticles concentration, proline content significantly increased (Sharifi-Rad et al. 2016).

12.5.3.6.2 In Vivo Evaluation

The size-dependent uptake of the SiO_2 nanoparticles by the plants was confirmed using TEM and inductively coupled plasma-optical emission spectroscopy with plant roots containing 32.0×10^{-3}, 1.85×10^{-3}, and 7.00×10^{-3} mg Si kg tissue^{-1} nm^{-3} (normalized for Si nanoparticles volume) for the 14, 50, and 200 nm Si nanoparticles, respectively, after 6 weeks exposure at 1000 parts per million (ppm) (pH 5.8). This study demonstrates that the silica scaffolds are not phytotoxic up to 1000 ppm despite significant uptake of the Si nanoparticles (14, 50, and 200 nm) into the root system of *A. thaliana*. (Slomberg and Schoenfisch 2012).

REFERENCES

Abdul-Khalek, I., D. Kittelson, and F. Brear. 1999. The influence of dilution conditions on diesel exhaust particle size distribution measurements. *SAE Technical Pape*r 1999-01-1142.

Adhikari, T., S. Kundu, A.K., Biswas et al. 2012. Effect of copper oxide nanoparticle on seed germination of selected crops. *J. Agric. Sci. Technol.* A2: 815–823.

Aitken, R.J., M.Q. Chaudhry, A.B.A. Boxall et al. 2006. Manufacture and use of nanomaterials: Current status in the UK and global trends. *Occup. Med.* 56(5): 300–306.

Asli, S. and P.M. Neumann. 2009. Colloidal suspensions of clay or titanium dioxide nanoparticles can inhibit leaf growth and transpiration via physical effects on root water transport. *Plant Cell Environ*. 32: 577–584.

Atha, D.H., H. Wang, E.J. Petersen et al. 2012. Copper oxide nanoparticle mediated DNA damage in terrestrial plant models. *Environ. Sci. Technol*. 46: 1819–1827.

Auffan, M., W. Achouak, J. Rose et al. 2008. Relation between the redox state of iron-based nanoparticles and their cytotoxicity toward *Escherichia coli*. *Environ. Sci. Technol*. 42: 6730–6735.

Auffan, M., J.Y. Bottero, C. Chaneac et al. 2010. Inorganic manufactured nanoparticles: How their physicochemical properties influence their biological effects in aqueous environments. *Nanomedicine* 5(6): 999–1007.

Banfield, J.F. and H. Zhang. 2001. Nanoparticles in the environment. *Rev. Mineral. Geochem*. 44: 1–58.

Begum, P. and B. Fugetsu. 2012. Phytotoxicity of multi-walled carbon nanotubes on red spinach (*Amaranthus tricolor* L.) and the role of ascorbic acid as an antioxidant. *J. Hazard. Mater.* 243: 212–222.

Biswas, P. and C.Y. Wu. 2005. Critical review: Nanoparticles and the environment. *J. Air Waste Manag. Assoc.* 55(6): 708–746.

Borm, P., F.C. Klaessig, T.D. Landry et al. 2006. Research strategies for safety evaluation of nanomaterials: Part V: Role of dissolution in biological fate and effects of nanoscale particles. *Toxicol. Sci*. 90(1): 23–32.

Bradfield, S.J., P. Kumar, J.C. White et al. 2017. Zinc, copper, or cerium accumulation from metal oxide nanoparticles or ions in sweet potato: Yield effects and projected dietary intake from consumption. *Plant Physiol. Biochem.* 110: 128–137.

Brunner, T.J., P. Wick, P. Manser et al. 2006. In vitro cytotoxicity of oxide nanoparticles: Comparison to asbestos, silica, and the effect of particle solubility. *Environ. Sci. Technol.* 40: 4374–4381.

Buzea, C., I.I. Pacheco, and K. Robbie. 2007. Nanomaterials and nanoparticles: Sources and toxicity. *Biointerphases* 2(4): 17–71.

Canas, J.E., M. Long, S. Nations et al. 2008. Effects of functionalized and non-functionalized single-walled carbon nanotubes on root elongation of select crop species. *Environ. Toxicol. Chem.* 27: 1922–1931.

Castiglione, M.R., L. Giorgetti, C. Geri et al. 2011. The effects of nano-TiO_2 on seed germination, development and mitosis of root tip cells of *Vicia narbonensis* L. and *Zea mays* L. *J. Nanopart. Res.* 13(6): 2443–2449.

Chen, R., T.A. Ratnikova, M.B. Stone et al. 2010. Differential uptake of carbon nanoparticles by plant and mammalian cells. *Small* 6(5): 612–617.

Das, I. and S.A. Ansari. 2009. Nanomaterials in science and technology. *J. Sci. Ind. Res.* 68(8): 657–667.

Dietz, K.J. and S. Herth. 2011. Plant nanotoxicology. *Trends Plant Sci.* 16: 582–589.

Dimkpa, C.O., J.E. McLean, D.E. Latta et al. 2012. CuO and ZnO nanoparticles: Phytotoxicity, metal speciation, and induction of oxidative stress in sand-grown wheat. *J. Nanopart. Res.* 14: 1125.

Dimkpa, C.O., D.E. Latta, J.E. McLean et al. 2013. Fate of CuO and ZnO nano- and microparticles in the plant environment. *Environ. Sci. Technol.* 47: 4734–4742.

Dimkpa, C., J. McLean, D. Britt et al. 2015. Nano-CuO and interaction with nano-ZnO or soil bacterium provide evidence for the interference of nanoparticles in metal nutrition of plants. *Ecotoxicology* 24: 119–129.

Donaldson, K., V. Stone, C.L. Tran et al. 2004. Nanotoxicology. *Occup. Environ. Med.* 61(9): 727–728.

Elliott, D.W. and W.X. Zhang. 2001. Field assessment of nanoscale bimetallic particles for groundwater treatment. *Environ. Sci. Technol.* 35(24): 4922–4926.

Fan, R., Y.C. Huang, M.A. Grusak et al. 2014. Effects of nano-TiO_2 on the agronomically-relevant Rhizobium–legume symbiosis. *Sci. Total Environ.* 466: 503–512.

Farre, M., J. Sanchis, and D. Barcelo. 2011. Analysis and assessment of the occurrence, the fate and the behavior of nanomaterials in the environment. *Trends Anal. Chem.* 30(3): 517–527.

Flahaut, E. 2010. Introduction to the special focus issue: Environmental toxicity of nanoparticles. *Nanomedicine* 5(6): 949–950.

Fleischer, A., M.A. O'Neill, and R. Ehwald. 1999. The pore size of non-graminaceous plant cell walls is rapidly decreased by borate ester cross-linking of the pectic polysaccharide rhamnogalacturonan II. *Plant Physiol.* 121: 829–838.

Foley, S., C. Crowley, M.Smaihi et al. 2002. Cellular localisation of a water-soluble fullerene derivative. *Biochem. Biophys. Res. Commun.* 294: 116–119.

Ghodake, G., Y.D. Seo, D. Park et al. 2010. Phytotoxicity of carbon nanotubes assessed by *Brassica juncea* and *Phaseolus mungo*. *J. Nanoelectron. Optoelectron.* 5: 157–160.

Ghosh, M., M. Bandyopadhyay, and A. Mukherjee. 2010. Genotoxicity of titanium dioxide (TiO_2) nanoparticles at two trophic levels: Plant and human lymphocytes. *Chemosphere* 81(10): 1253–1262.

Haghighi, M. and J.A. Teixeira da Silva. 2014. The effect of carbon nanotubes on the seed germination and seedling growth of four vegetable species. *J. Crop Sci. Biotech.* 17(4): 201–208.

Handy, R.D., R. Owen, and E. Valsami-Jones. 2008. The ecotoxicology of nanoparticles and nanomaterials: Current status, knowledge gaps, challenges, and future needs. *Ecotoxicology* 17(5): 315–325.

Hardman, R. 2006. A toxicologic review of quantum dots: Toxicity depends on physicochemical and environmental factors. *Environ. Health Perspect.* 114(2): 165–172.

Hawthorne, J., C. Musante, and S.K. Sinha. 2012. Accumulation and phytotoxicity of engineered nanoparticles to *Cucurbita pepo*. *Int. J. Phytoreme-Diat.* 14(4): 429–442.

Hong, J., C.M. Rico, L. Zhao et al. 2015. Toxic effects of copper-based nanoparticles or compounds to lettuce (*Lactuca sativa*) and alfalfa (*Medicago sativa*). *Environ. Sci. Process Impact.* 17: 177–185.

Jacob, D.L., J.D. Borchardt, L. Navaratnam et al. 2013. Uptake and translocation of Ti from nanoparticles in crops and wetland plants. *Int. J. Phytoremediat.* 15(2): 142–153.

Jacobs, J. and J.L. Roe. 2005. SKS6 a multicopper oxidase-like gene, participates in cotyledon vascular patterning during *Arabidopsis thaliana* development. *Planta* 222: 652–666.

Jo, Y.K. and B.H. Kim. 2009. Antifungal activity of silver ions and nanoparticles on phytopathogenic fungi. *Plant Dis.* 93: 1037–1043.

Kamat, J.P., T.P. Devasagayam, K.I. Priyadarsini et al. 2000. Reactive oxygen species mediated membrane damage induced by fullerene derivatives and its possible biological implications. *Toxicology* 155: 55–61.

Kashiwada, S. 2006. Distribution of nanoparticles in the seethrough medaka (*Oryzias latipes*). *Environ. Health Perspect.* 114(11): 1697–1702.

Kennedy, C.B., S.D. Scott, and F.G. Ferris. 2004. Hydrothermal phase stabilization of 2-line ferrihydrite by bacteria. *Chem. Geol.* 212(3–4): 269–277.

Khodakovskaya, M.V., K. de Silva, D.A. Nedosekin et al. 2011. Complex genetic, photothermal, and photoacoustic analysis of nanoparticle-plant interactions. *P. Natl. A. Sci.* 108(3): 1028–1033.

Khodakovskaya, M.V., K. de Silva, A.S. Biris et al. 2012. Carbon nanotubes induce growth enhancement of tobacco cells. *ACS Nano* 6(3): 2128–2135.

Kim, S., S. Lee, and I. Lee. 2012. Alteration of phytotoxicity and oxidant stress potential by metal oxide nanoparticles in *Cucumis sativus*. *Water Air Soil Pollut.* 223: 2799–2806.

Klaine, S.J., P.J.J. Alvarez, G.E. Batley et al. 2008. Nanomaterials in the environment: Behavior, fate, bioavailability, and effects. *Environ. Toxicol. Chem.* 27: 9: 1825–1851.

Krystofova, O., J. Sochor, and O. Zitka. 2013. Effect of magnetic nanoparticles on tobacco BY-2 cell suspension culture. *Int. J. Environ. Res. Publ. Health.* 10(1): 47–71.

Kumari, M., A. Mukherjee, and N. Chandrasekaran. 2009. Genotoxicity of silver nanoparticles in *Allium cepa*. *Sci. Total Environ.* 407: 5243–5246.

Lee, W.M., Y.J. An, H. Yoon et al. 2008. Toxicity and bioavailability of copper nanoparticles to terrestrial plants *Phaseolus radiatus* (mung bean) and *Triticum aestivum* (wheat); plant agar test for water-insoluble nanoparticles. *Environ. Toxicol. Chem.* 27: 1915–1921.

Lee, D.S., B.K. Kim, S.J. Kwon et al. 2009. *Arabidopsis* GDSL lipase 2 plays a role in pathogen defense via negative regulation of auxin signaling. *Biochem. Biophys. Res. Commun.* 379: 1038–1042.

Lee, C.W., S. Mahendra, K. Zodrow et al. 2010. Developmental phytotoxicity of metal oxide nanoparticles to *Arabidopsis thaliana*. *Environ Toxicol Chem*. 29(3): 669–75.

Lee, S., H. Chung, S. Kim et al. 2013. The genotoxic effect of ZnO and CuO nanoparticles on early growth of buckwheat, *Fagopyrum esculentum*. *Water Air Soil Pollut*. 224: 111.

Lei, Z., S. Mingyu, W. Xiao et al. 2008. Antioxidant stress is promoted by nano-anatase in spinach chloroplasts under UV-B radiation. *Biol. Trace Elem. Res*. 121(1): 69–79.

Lidén, G. 2011. The European commission tries to define nanomaterials. *Ann. Occup. Hyg*. 55(1): 1–5.

Marschner, H. 1995. Mineral. *Nutrition of Higher Plants*, 2nd ed.; Academic Press: San Diego, CA.

Lin, D. and B. Xing. 2007. Phytotoxicity of nanoparticles: Inhibition of seed germination and root growth. *Environ. Pollut*. 150: 243–250.

Lin, D. and B. Xing. 2008. Root uptake and phytotoxicity of ZnO nanoparticles. *Environ. Sci. Technol*. 42: 5580–5585.

Lin, C., B. Fugetsu, Y. Su et al. 2009. Studies on toxicity of multi-walled carbon nanotubes on *Arabidopsis* T87 suspension cells. *J. Hazard. Mater.* 170: 578–583.

Lin, S. 2009. Uptake, translocation, and transmission of carbon nanomaterials in rice plants. *Small* 5: 1128–1132.

Liu, Y., P. Laks, and P. Heiden. 2002. Controlled release of biocides in solid wood. Part 2. Efficacy against *Trametes versicolor* and *Gloeophyllum trabeum* wood decay fungi. *J. Appl. Polym. Sci.* 86: 608–614.

Liu, Q., B. Chen, Q. Wang et al. 2009. Carbon nanotubes as molecular transporters for walled plant cells. *Nano Lett*. 9: 1007–1010.

Ma, X., J. Geiser-Lee, Y. Deng et al. 2010. Interactions between engineered nanoparticles and plants: Phytotoxicity, uptake and accumulation. *Sci. Total. Environ*. 408(16): 3053–3061.

Mahmoodzadeh, H., A. Eshaghi, and T. Gholami. 2017a. Physiological Analysis of CuO Bulk and Nanoparticles to Castor (*Ricinus communis* L.). MSc Thesis of plant physiology. Department of Biology, Faculty of Science, Mashhad Branch, Islamic Azad University, Tehran.

Mahmoodzadeh, H., A. Safipour Afshar, and M. Sarabi. 2017b. Physiological Analysis of Silver Nanoparticles and $AgNO_3$ Effect to *Brassica napus* L. MSc Thesis of plant physiology. Department of Biology, Faculty of Science, Neyshabour Branch, Islamic Azad University, Tehran.

Maynard, A.D., R.J. Aitken, T. Butz et al. 2006. Safe handling of nanotechnology. *Nature* 444(7117): 267–269.

Minic, Z., E. Jamet, L. Négroni et al. 2007. A sub-proteome of *Arabidopsis thaliana* mature stems trapped on Concanavalin A is enriched in cell wall glycoside hydrolases. *J. Exp. Bot*. 58: 2503–2512.

Molina, M.A., J.L. Ramos, and M. Espinosa-Urgel. 2006. A two-partner secretion system is involved in seed and root colonization and iron uptake by *Pseudomonas putida* KT2440. *Environ. Microbiol.* 8(4): 639–647.

Moon, Y.S., E.S. Park, T.O. Kim et al. 2014. SELDI-TOF MS-based discovery of a biomarker in *Cucumis sativus* seeds exposed to CuO nanoparticles. *Environ. Toxicol. Pharmacol*. 38: 922–931.

Motzer, W.E. 2008. Nanomaterials: New emerging contaminants and their potential impact to water resources, http://www.grac.org/NanomaterialsandWaterResources.pdf.

Moreno-Olivas, F., V.U. Gant Jr., K.L. Johnson et al. 2014. Random amplified polymorphic DNA reveals that TiO_2 nanoparticles are genotoxic to *Cucurbita pepo*. *J. Zhejiang Univ. Sci. A*. 15: 618–623.

Mukherjee, A., J.R. Peralta-Videa, S. Bandyopadhyay et al. 2014. Physiological effects of nanoparticulate ZnO in green peas (*Pisum sativum* L.) cultivated in soil. *Metallomics* 6(1): 132–138.

Musante, C. and J.C. White. 2012. Toxicity of silver and copper to *Cucurbita pepo*: Differential effects of nano and bulk-size particles. *Environ. Toxicol*. 27: 510–517.

Nair, P.G. and I. Chung. 2014a. A mechanistic study on the toxic effect of copper oxide nanoparticles in soybean (*Glycine max* L.) root development and lignification of root cells. *Biol. Trace Element. Res*. 162: 342–352.

Nair, P.G. and I. Chung. 2014b. Impact of copper oxide nanoparticles exposure on *Arabidopsis thaliana* growth, root system development, root lignificaion, and molecular level changes. *Environ. Sci. Pollut. Res*. 21: 12709–12722.

Nair, P.M.G. and I.M. Chung. 2014c. Physiological and molecular level effects of silver nanoparticles exposure in rice (*Oryza sativa* L.) seedlings. *Chemosphere* 112: 105–113.

Nanjo, Y., K. Maruyama, H. Yasue et al. 2011. Transcriptional responses to flooding stress in roots including hypocotyl of soybean seedlings. *Plant Mol. Biol*. 77: 129–144.

Navarro, E., A. Baun, R. Behra et al. 2008. Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi. *Ecotoxicology* 17: 372–386.

Nel, A., T. Xia, L. Mädler et al. 2006. Toxic potential of materials at the nanolevel. *Science* 311: 622–627.

Nowack, B. and T.D. Bucheli. 2007. Occurrence, behavior and effects of nanoparticles in the environment. *Environ. Pollut*. 150(1): 5–22.

Oberdorster, G., A. Maynard, K. Donaldson et al. 2005. Principles for characterizing the potential human health effects from exposure to nanomaterials: Elements of a screening strategy. *Part. Fibre Toxicol*. 2(8).

Oberdorster, E., S. Zhu, T.M. Blickley et. al. 2006. Ecotoxicology of carbon-based engineered nanoparticles: Effects of fullerene (C60) on aquatic organisms. Carbon 44(6): 1112–1120.

Pacurari, M. 2008. Raw single-wall carbon nanotubes induce oxidative stress and activate MAPKs, AP-1, NF-KB and Akt in normal and malignant human mesothelial cells. *Environ. Health Perspect*. 116: 1211–1217.

Panessa-Warren, B.J., J.B. Warren, S.S. Wong et al. 2006. Biological cellular response to carbon nanoparticle toxicity. *J. Phys. Condens. Matter*. 18: S2185–S2201.

Petersen, E.J., T.B. Henry, J. Zhao et al. 2014. Identification and avoidance of potential artifacts and misinterpretations in nanomaterial ecotoxicity measurements. *Environ. Sci. Technol*. 48: 4226–4246.

Pignocchi, C., G. Kiddle, I. Hernandez et al. 2006. Ascorbate oxidase-dependent changes in the redox state of the apoplast modulate gene transcript accumulation leading to modified hormone signaling and orchestration of defense processes in tobacco. *Plant Physiol*. 141: 423–435.

Poborilova, Z., R. Opatrilova, and P. Babula. 2013. Toxicity of aluminium oxide nanoparticles demonstrated using a BY-2 plant cell suspension culture model. *Environ. Exp. Bot*. 91: 1–11.

Pradas del Real, A.E., V. Vidal, M. Carriere et al. 2017. Silver nanoparticles and wheat roots: A complex interplay. *Environ. Sci. Technol*. 51(10): 5774–5782.

Quinn, J., C. Geiger, C. Clausen et al. 2005. Field demonstration of DNAPL dehalogenation using emulsified zero-valent iron. *Environ. Sci. Technol*. 39(5): 1309–1318.

Racuciu, M. and D. Creanga. 2006. TMA-OH coated magnetic nanoparticles internalize in vegetal tissue. *Romanian J. Phys*. 52: 395–402.

Racuciu, M. and D. Creanga. 2009a. Cytogenetic changes induced by beta-cyclodextrin coated nanoparticles in plant seeds. *Romanian J. Phys*. 54: 125–131.

Racuciu, M. and D. Creanga. 2009b. Biocompatible magnetic fluid nanoparticles internalized in vegetal tissues. *Romanian J. Phys*. 54: 115–124.

Raliya, R., R. Nair, S. Chavalmane et al. 2015. Mechanistic evaluation of translocation and physiological impact of titanium dioxide and zinc oxide nanoparticles on the tomato (*Solanum lycopersicum* L.) plant. *Metallomics* 7(12): 1584–1594.

Samaj, J., F. Baluska, B. Voigt et al. 2004. Endocytosis, actin cytoskeleton, and signaling. *Plant Physiol*. 135: 1150–1161.

Sedbrook, J.C., K.L. Carroll, K.F. Hung et al. 2002. The *Arabidopsis* SKU5 gene encodes an extracellular glycosylphosphatidyl inositol-anchored glycoprotein involved in directional root growth. *Plant Cell* 14: 1635–1648.

Servin, A.D., M.I. Morales, H. Castillo-Michel et al. 2013. Synchrotron verification of TiO_2 accumulation in cucumber fruit: A possible pathway of TiO_2 nanoparticle transfer from soil into the food chain. *Environ. Sci. Technol*. 47(20): 11592–11598.

Sharifi-Rad, J., M. Sharifi-Rad, and J.A. Teixeira da Silva. 2016. Morphological, physiological and biochemical responses of crops (*Zea mays* L., *Phaseolus vulgaris* L.), medicinal plants (Hyssopus officinalis L., Nigella sativa L.), and weeds (*Amaranthus retroflexus* L., *Taraxacum officinale* F.H. Wigg) exposed to SiO_2 nanoparticles. *J. Agr. Sci. Tech*. 18: 1027–1040.

Shaw, A.K. and Z. Hossain. 2013. Impact of nano-CuO stress on rice (*Oryza sativa* L.) seedlings. *Chemosphere* 93: 906–915.

Shaw, A.K., S. Ghosh, H.M. Kalaji et al. 2014. Nano CuO stress induced modulation of antioxidative defense and photosynthetic performance of Syrian barley (*Hordeum vulgare* L.). *Environ. Exp. Bot*. 102: 37–47.

Shen, C., Q. Zhang, J. Li et al. 2010. Induction of programmed cell death in *Arabidopsis* and rice by single-wall carbon nanotubes. *Am. J. Bot*. 97(10): 1602–1609.

Slomberg, D.L. and M.H. Schoenfisch. 2010. Silica nanoparticle phytotoxicity to *Arabidopsis thaliana*. *Environ. Toxicol. Chem*. 29(6): 1399.

Slomberg, D.L. and M.H. Schoenfisch. 2012. Silica nanoparticle phytotoxicity to *Arabidopsis thaliana*. *Environ. Sci. Technol*. 46: 10247–10254.

Solomon, E.I., T.E. Machonkin, and U.M. Sundaram. 1997. Spectroscopy of multi-copper oxidases, in: Messerschmidt, A. (Ed.), *Multicopper Oxidases*, World Scientific: Singapore, pp. 103–127.

Stampoulis, D., S.K. Sinha, and J.C. White. 2009. Assay-dependent phytotoxicity of nanoparticles to plants. *Environ. Sci. Technol*. 43: 9473–9479.

Tan, X.M. and B. Fugetsu. 2007. Multi-walled carbon-nanotubes interact with cultured rice cells: Evidence of a self-defense response. *J. Biomed. Nanotechnol*. 3: 285–288.

Tan, X.M., C. Lin, and B. Fugetsu. 2009. Studies on toxicity of multi-walled carbon nanotubes on suspension rice cells. *Carbon* 47: 3479–3487.

Tervonen, T., I. Linkov, J.R. Figueira et al. 2009. Risk-based classification system of nanomaterials. *J. Nanopart. Res*. 11(4): 757–766.

The Royal Society & The Royal Academy of Engineering. 2004. *Nanoscience and Nanotechnologies: Opportunities and Uncertainties*. The Royal Society & The Royal Academy of Engineering, London.

Trujillo-Reyes, J., J. Peralta-Videa, S. Majumdar et al. 2014. Exposure studies of core–shell Fe/Fe_3O_4 and Cu/CuO nanoparticles to lettuce (*Lactuca sativa*) plants: Are they a potential physiological and nutritional hazard? *J. Hazard. Mater*. 267: 255–263.

Turktas, M., B. Inal, S. Okay et al. 2013. Nutrition metabolism plays an important role in the alternate bearing of the olive tree (*Olea europaea* L.). *PLoS One* 8: e59876.

Unrine, J.M., S.E. Hunyadi, O.V. Tsyusko et al. 2010. Evidence for bioavailability of Au nanoparticles from soil and biodistribution within earth worms (*Eisenia fetida*). *Environ. Sci. Technol.* 44(21): 8308–8313.

US EPA (US Environmental Protection Agency) Office of Inspector General. 2011. EPA needs to manage nanomaterial risks more effectively, Tech. Rep. 12-P-0162, US EPA, Washington, DC, 2011.

Verano-Braga, T., R. Miethling-Graff, K. Wojdyla et al. 2014. Insights into the cellular response triggered by silver nanoparticles using quantitative proteomics. *ACS Nano* 8: 2161–2175.

Walker, N.J. and J.R. Bucher. 2009. A 21st century paradigm for evaluating the health hazards of nanoscale materials? *Toxicol. Sci.* 110(2): 251–254.

Wang, S., J. Kurepa, and J.A. Smalle. 2011. Ultra-small TiO_2 nanoparticles disrupt microtubular networks in *Arabidopsis thaliana*. *Plant Cell. Environ*. 34(5): 811–1021.

Wang, Z.X.X., J. Zhao, X. Liu et al. 2012. Xylem- and phloem-based transport of CuO nanoparticles in maize (*Zea mays* L.). *Environ. Sci. Technol*. 46: 4434–4441.

Wei, C., Y. Zhang, J. Guo et al. 2010. Effects of silica nanoparticles on growth and photosynthetic pigment contents of *Scenedesmus obliquus*. *J. Environ. Sci.* 22(1): 155–160.

Wu, S.G.H.L., J. Head, D.R. Chen et al. 2012. Phytotoxicity of metal oxide nanoparticles is related to both dissolved metals ions and adsorption of particles on seed surfaces. *J. Petrol. Environ. Biotechnol.* 3: 126.

Yan, W., H.L. Lien, B.E. Koel et al. 2013. Iron nanoparticles for environmental clean-up: Recent developments and future outlook. *Env. Sci. Process Impact*. 15(1): 63–77.

Yang, L. and D.J. Watts. 2005. Particle surface characteristics may play an important role in phytotoxicity of alumina nanoparticles. *Toxicol. Lett.* 158: 122–132.

Yuan, H., S. Hu, P. Huang et al. 2011. Single walled carbon nanotubes exhibit dual-phase regulation to exposed *Arabidopsis* mesophyll cells. *Nanoscale Res. Lett*. 6: 44.

Zarafshar, M., M. Akbarinia, H. Askari et al. 2015. Toxicity Assessment of SiO_2 Nanoparticles to Pear Seedlings. *Int. J. Nanosci. Nanotechnol*. 11(1): 13–22.

Zhang, W.X. and C.B. Wang. 1997. Synthesizing nanoscale iron particles for rapid and complete dechlorination of TCE and PCBs. *Environ. Sci. Technol.* 31(7): 2154–2156.

Zhang, W.X. 2003. Nanoscale iron particles for environmental remediation: An overview. *J. Nanopart. Res.* 5(3–4): 323–332.

Zhao, L., Y. Sun, J.A. Hernandez-Viezcas et al. 2013. Influence of CeO_2 and ZnO nanoparticles on cucumber physiological markers and bioaccumulation of Ce and Zn: A life cycle study. *J. Agric. Food Chem*. 61: 11945–11951.

Zhou, D.J.S., L. Li, Y. Wang et al. 2011. Quantifying the adsorption and uptake of CuO nanoparticles by wheat root based on chemical extractions. *J. Environ. Sci*. 23: 1852–1857.

Zhu, H., J. Han, J.Q. Xiao et al. 2008. Uptake, translocation, and accumulation of manufactured iron oxide nanoparticles by pumpkin plants. *J. Environ. Monit.* 10(6): 713–717.

13

Phytochemicals and Their Functionalized Nanoparticles as Quorum Sensing Inhibitor and Chemotherapeutic Agent

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13.1 Introduction

Quorum sensing (QS) is a density-dependent bacterial intercellular communication and phenotype regulation associated with biofilm formation.[1] It was first described in 1970 when a gram-negative marine bacterium *Vibrio fischeri* was seen to luminesce in a cell density-dependent manner.[2] Since the first description of biofilms by Antonie van Leeuwenhoek, great advances have been made by the scientists/researchers. Ongoing studies have greatly increased our knowledge of the genetic and physiological bases of biofilm formation and their structure for a wide range of bacteria. Various bacterial activities can influence the structure and attributes of biofilms, including cell growth or death, nutrient acquisition, waste product accumulation, motility mechanisms, and exopolysaccharide synthesis.[3,4] Biofilm formations and QS are the interconnected and central feature of bacterial social life. In other words, QS is a cell density-dependent expression of species in bacteria mediated by hormonelike compounds called autoinducers (AIs). Biofilms consist of water, bacterial cells, and a wide range of self-generated extracellular polymeric substances referred to as the matrix. Microbial biofilms affect the world economy at the level of billions of dollars with regard to equipment damage, product contamination, energy losses, and infections. This is partially due to their ability, via various mechanisms, to develop antibiotic resistant and more virulent strains, making eradication more difficult.[5]

Several processes responsible for the successful establishment of microbial infection are mediated by QS. The inhibition of QS is therefore being considered as a new target for antimicrobial chemotherapy. Research efforts to block QS in bacteria and disrupt biofilms have enabled the identification of bioactive molecules that are also produced by plants. Dietary phytochemicals are secondary metabolites in plants known to have several health benefits including antimicrobial activity. However, their ability to inhibit QS has become a new scope in the field of phytomedicine and that could be generating an additional source of revenue for farmers. This chapter focuses on the importance of phytochemicals and their functionalized nanoparticles (NPs) in the field of biofilm treatment and their mechanisms of action as QS inhibitors (QSIs), which display potential for treating microbial infections and may enhance the safety of the food supply.

13.2 Quorum Sensing

At high cell densities, bacteria (gram-positive and gram-negative), fungi and archaea produce cell–cell communication or QS signal (Figure 13.1; Table 13.1), which, in turn, enhances the QS response and has led to these signals being named "autoinducers." These signaling molecules (AIs) and their receptors have been broadly divided into three major classes: (a) N-acyl homoserine lactones (AHLs), which vary in the length and oxidation state of the acyl side chain and produced by gram-negative bacteria for intraspecies communication; (b) oligopeptides or autoinducing peptides (AIPs), consisting of 5–34 amino acid residues, are generally used by gram-positive bacteria; (c) autoinducer-2 (AI-2) and a ribose derivative

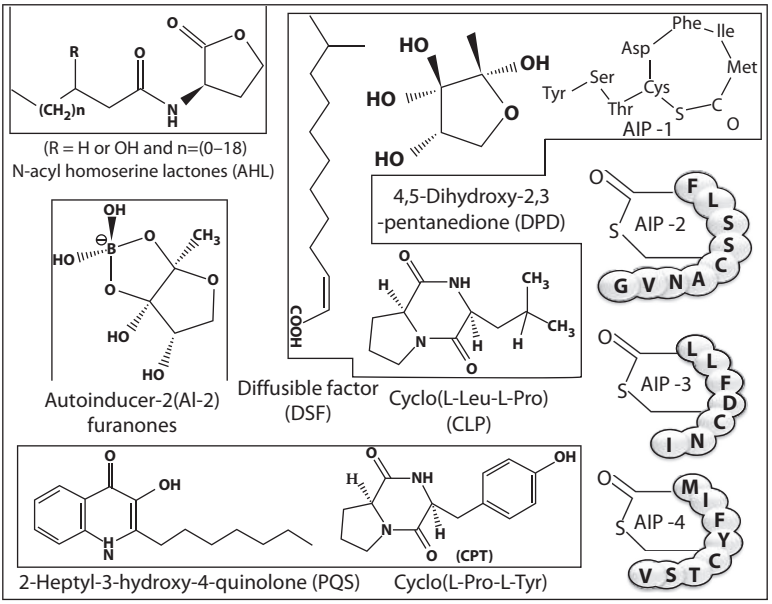


FIGURE 13.1 Structures of bacterial QS signaling molecules.

TABLE 13.1

Quorum Sensing Signaling Molecules of Microorganisms

AI	Representative Microorganism	Associated Phenomena
AHLs (intraspecies)	Gram-negative bacteria: *V. fischeri*, *P. aeruginosa*, *C. violaceum*, *A. hydrophila* Gram-positive bacteria: *Exiguobacterium* sp. MPO	Exopolysaccharide production, virulence, biofilm, pigmentation, swarming and bioluminescence
AI-2 (interspecies)	*V. harveyi*, *E. coli*, *Y. pestis*	Bioluminescence, biofilm formation, motility, virulence factor
AI-3 (interspecies)	EHEC	Virulence
PQS (intraspecies)	*P. aeruginosa*	Antibiotic production, virulence, and biofilm formation
DSF family (intraspecies)	*X. campestris*; *Xanthomonas* spp., and *Burkholderia cenocepacia*	Virulence, biofilm and antibiotic tolerance, endoglucanase production
DPD (interspecies)	Sa. enteric serovar typhimurium	Virulence factor
CLP and CPT (intraspecies)	*P. putida WCS358*, *P. aeruginosa*	Cross activates QS biosensors
AIPs (1–4) (intraspecies)	Gram-positive bacteria: *S. aureus* gram-negative bacteria: *Thermotoga maritime*	Virulence, biofilm, sporulation, exopolysaccharide production, cross-signaling between strains and species

(4,5-dihydroxy-2,3-pentanedione) employed by both gram-positive and gram-negative bacteria for interspecies communication.[6,7]

AHLs interact and activate a transcriptional regulator (e.g., LuxR in *V. fisheri*, the species in which the system was first described) that, in turn, activates the expression of target genes (e.g., LuxAB is responsible for bioluminescence in *V. fisheri*).[8] AIPs can either (a) activate, at the bacterial surface, a two-component system (histidine kinase and response regulator) that, via a phosphorylation cascade, subsequently induces the expression of target genes (e.g., the well-documented Agr system in *Staphylococcus aureus*)[9] or (b) be imported back into bacteria via the oligopeptide transport system and interact with an Response-Regulatory Aspartate (RAP) phosphatase or a transcriptional regulator that modulates the expression of target genes (e.g., the short hydrophobic peptides interacting with Rgg regulators in streptococci).[10] AI-2 signals are hypothesized to be used for interspecies communication. They are considered to be universal signaling molecules and are widespread among gram-negative and gram-positive bacteria. The AI-2s result from a detoxification step in the methyl cycle and are produced via a pathway involving the LuxS enzyme, which converts ribose-homocysteine into homocysteine and 4,5-dihydroxy-2,3-pentadione, an unstable compound that cyclizes into the furanones. AI-2s activate the regulation pathway that leads to the modulation of target gene expression,[11] whereas the autoinducer-3 (AI-3) molecule and its associated signaling pathway have been described in gram-negative bacteria and well documented in enterohemorrhagic *Escherichia coli* as well as in *Salmonella*, both responsible of food contaminations.[12] Gram-negative bacteria employ AHL, 3-hydroxy-2-heptyl-4-quinolone, fatty acid methyl esters, long-chain fatty acids, and a group of interconvertible furanones derived from 4,5-Dihydroxy-2,3-pentanedione (DPD) called AI-2. The ribose derivative AI-2 is

TABLE 13.2

Principal Biofilm-Forming Microorganism Cause Bacterial Infections

Microorganisms Involved	Type of Infection
S. epidermidis, *S. aureus*, *P. aeruginosa*	Device-related infection
Uropathogenic *E. coli*, *Klebsiella pneumoniae*	Chronic urinary tract infection
Streptococcus sanguinis, *Streptococcus mutans*, *S. mitis*, *S. salivarius*, and gram-negative anaerobic bacteria	Periodontitis
Nontypeable *Haemophilus influenzae*	Chronic bronchitis
P. aeruginosa	Cystic fibrosis pneumonia
S. aureus, *P. aeruginosa*	Chronic wounds
P. aeruginosa, *S. aureus*	Chronic rhinosinusitis
Streptococcus pneumonia, *Helicobacter influenzae*, *Moraxella catarrhalis*	Chronic otitis media

also produced by gram-positive bacteria, but these organisms generally prefer linear, modified, or cyclic peptide AIP made by *Staphylococcus* sp.[13]

Most gram-negative bacteria use AHLs to regulate QS-mediated behaviors, while gram-positive bacteria prefer linear, modified, or cyclic peptides such as the AIP to control QS phenotypes. The QS systems regulate the coordination of population behavior to enhance nutrient availability, collective defense against other antagonizing organisms, or community escape from adverse conditions. Since the discovery of QS regulation in bacteria, numerous such systems have been described. These QS systems regulate diverse functions in both gram-negative and gram-positive bacteria, which include biofilm formation, virulence factor, bioluminescence, motility patterns, exopolysaccharide production, antifungal or antibiotic production, endoglucanase production, pigmentation, competence, plasmid conjugal transfer, cross-signaling between strains, and species.[14] AHLs are the most common signal used by gram-negative bacteria for cell–cell communication. However, other small molecule signals have been associated with QS in gram-negative bacteria, including the boronate ester AI-2 used by *V. harveyi* and quinolone molecules (e.g., the *Pseudomonas quinolone* signal [PQS]) used by the opportunistic pathogen *Pseudomonas aeruginosa*. Gram-positive bacteria, such as *S. aureus*, use ~5–15-mer oligopeptides for QS (i.e., AIPs).[15] Table 13.2 summarizes the principal biofilm-forming microorganism and their chronic infections.[16]

13.3 Quorum Sensing and Quorum Quenching Strategies

The best-studied QS/biofouling systems in gram-negative bacteria produce diffusible AHL ligands via an inducer synthase (or LuxI-type protein), and the ligand is sensed by its cognate cytoplasmic receptor (or LuxR-type protein). AHL-based QS system functions through three key components: (a) AHL signal molecules, (b) AHL synthase protein for synthesis of AHL signals, and (c) a regulatory protein which responds to surrounding concentration of the AHL signal (Figure 13.2).[17] This process initiated with the synthesis and release of AHL signals into the surrounding environment which accumulates in a cell population density-dependent manner. When the concentration of AHL signals reaches at a higher level, the QS cells starts responding,

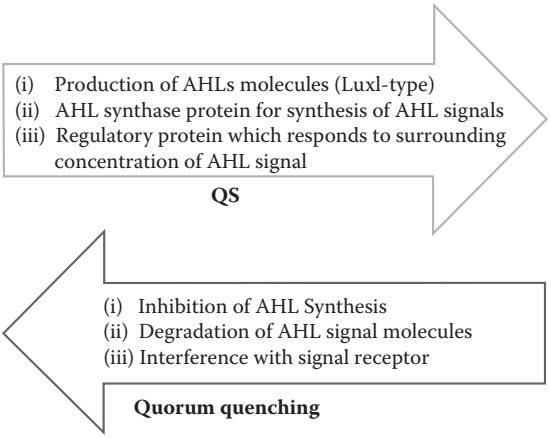


FIGURE 13.2 General strategies for QS and quorum quenching.

allowing them to regulate the production of secondary metabolites and control the expression of QS genes.[18]

The rapid growth of bacterial resistance underscores an urgent need for the development of new antibacterial strategies, and QS has considerably attracted recent attention as a new target. The inhibition of QS/biofouling combating in gram-negative bacteria is performed by the interception of any one of these three major components within the autoinduction circuit should lead to a bacterial "communication breakdown" (Figure 13.2). The inhibition of AHL synthesis by blocking of the LuxI-type protein appears to be a straightforward approach to the QS, as cell–cell signaling is clearly impossible without a signal. The second target is the AHL ligand itself. The degradation of the small molecular signal by AHL-acylase (AC) and AHL-lactonase that will prevent them from accumulating would be a complementary strategy for inhibiting its synthesis. It is worthwhile to note that several species, both prokaryotes and eukaryotes, are believed to degrade AHL signals in order to block the QS system of invading bacteria. The third and final target for the modulation of cell–cell communication in gram-negative bacteria is the receptor, or LuxR-type protein. These strategies can be applied to achieve quenching of AHL-mediated QS in gram-negative and AIP-mediated QS in gram-positive bacteria.[19–21] It opens up an exciting new strategy for antimicrobial chemotherapy and lead to the discovery of a new category of antibiotics, which can overcome the issues related to antimicrobial resistance.

13.4 Phytochemical-Based Quorum Sensing Inhibitors

The ideal QSI have been defined as chemically stable and highly effective, low molecular-mass molecules, which exhibit a high degree of specificity for the QS regulator without toxic side effects.[22] In the last few years, phytochemicals from the medicinal and dietary plants have long been used as new antipathogenic agents to treat microbial infections and have gained much interest as a source of anti-QS compounds for the treatment of biofilms.[23] Recently, dietary phytochemicals with a long history of medical use in humans are being investigated for the purpose of controlling biofilms, due

to their nontoxic nature.[24] Recently, several plant materials and their phytochemicals proven as QSIs have been reported (Figures 13.3 and 13.4; Table 13.3).

One such dietary phytochemical, curcumin, a major constituent of turmeric (*Curcuma longa* L.) rhizomes, was selected as therapeutic agents. Turmeric has traditionally been used as antiinflammatory, antimicrobial, and antifungal, but reports on its anti-QS properties are scarce.[24,25] Vasantha Packiavathy et al.[25] reported that turmeric inhibited the biofilm formation of uropathogens, such as *E. coli*, *P. aeruginosa* PAO1, *P. mirabilis*, and *S. marcescens*, possibly by interfering with their QS systems. The multifunctional activity of curcumin with promising antibiofilm activity could be used in the development of novel antipathogenic drugs to treat urinary infections.[25] The high prevalence of garlic within the human diet offers a distinct advantage over synthetically designed QSIs, as much of the information regarding pharmacokinetics and toxicity is already available. This, coupled with the potent QSI activity demonstrated *in vitro* and in model organisms, leads to garlic being the first QSI to be tested in humans. Garlic displayed high levels of QSI activity against *P. aeruginosa*; it was hypothesized that garlic may render *P. aeruginosa* less virulent and more susceptible to antibiotic treatment in humans.[26] Ajoene (allyl sulfide) was obtained as a major bioactive QSI compound isolated from garlic extract and capable of inhibiting LuxR-based QS networks in *P. aeruginosa* which lead to a significant decrease in the production of key *P. aeruginosa* virulence factors such as rhamnolipids. It was also shown to improve the efficacy of tobramycin to treat *P. aeruginosa* biofilm formation and increase bacterial mortality and clearing of the lungs of *P. aeruginosa* infected mice.[27] Abd-Alla and Bashandy[28] selected Pantolactone and myristic acid of onion bulb to explore their effects on growth and virulence factors of *P. aeruginosa*. The exogenous application of pantolactone and myristic acid significantly inhibited

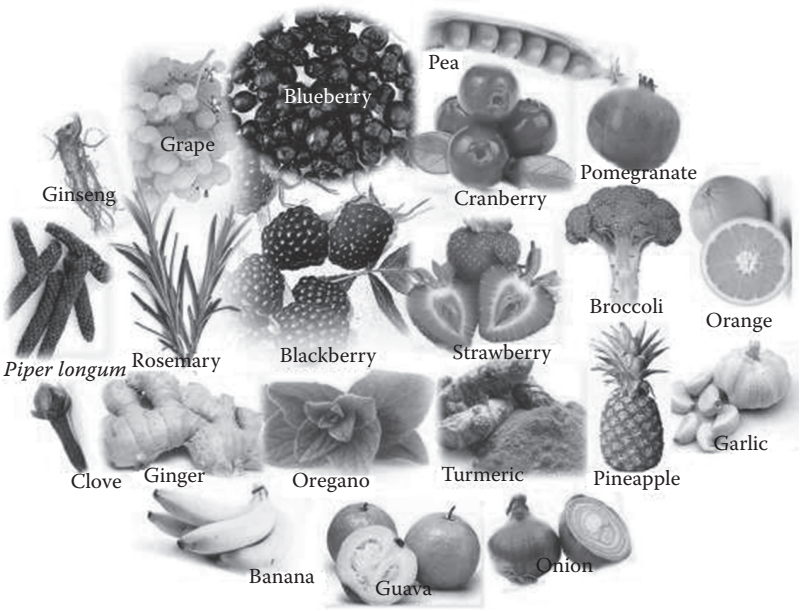


FIGURE 13.3 Fruits and vegetables proven as QSIs.

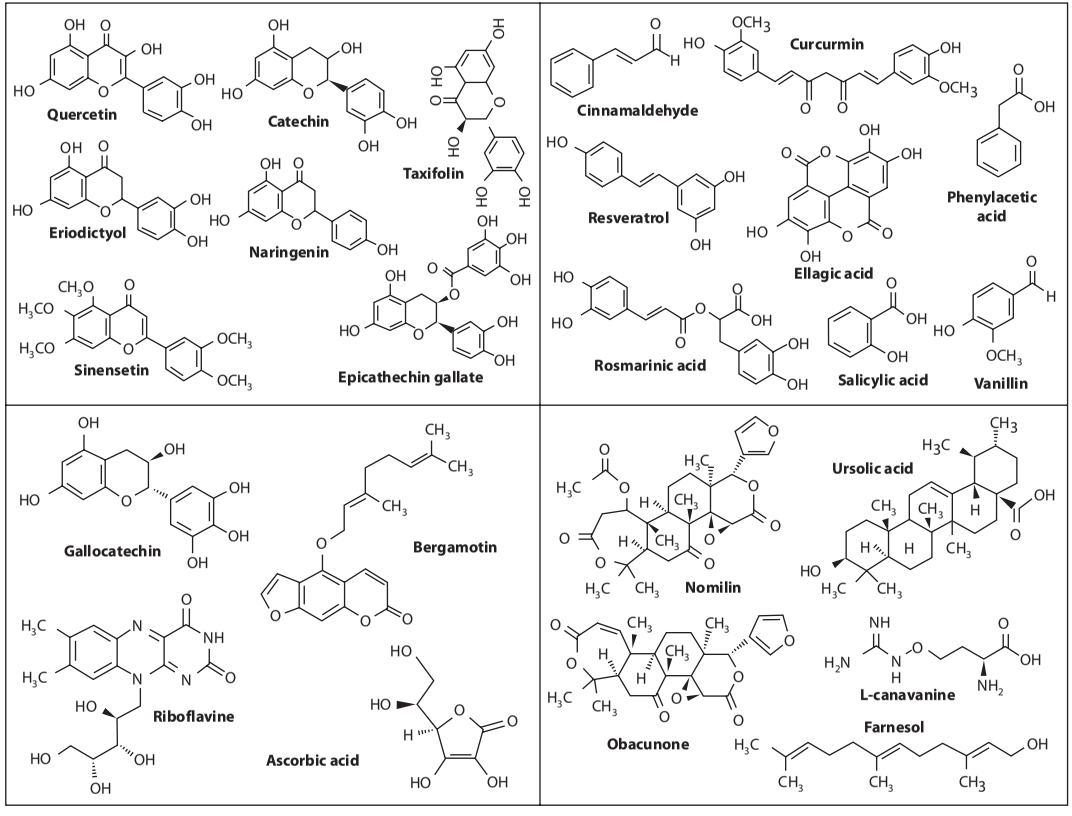


FIGURE 13.4 Important phytochemical constituents of major fruits and vegetables proven as QSIs.

TABLE 13.3

Phytochemicals as QS Inhibitors

Plant Source	Phytochemicals	Target Bacteria	QS Activity	References
Turmeric (*Curcuma longa*)	Curcumin	*E. coli*, *P. aeruginosa* PAO1, *Proteus mirabilis*, *and Serratia marcescens*	Inhibition of exopolysaccharide production, alginate production, swimming and swarming motility	Vasantha Packiavathy et al.[25]
Garlic (*Allium sativum*)	Ajoene	*P. aeruginosa*	Decrease in the production of rhamnolipids	Rasmussen et al.[26] Jakobsen et al.[27]
Onion (*Allium cepa*)	Pantolactone, myristic acid	*P. aeruginosa*	Inhibit pyocyanin production, protease, and lipase and polygalacturonase activity	Abd Alla and Bashandy[28]
Clove (*Syzygium aromaticum*)	–	*P. aeruginosa*	Decrease in lecA virulence factor, pyocyanin formation, and swarming motility	Krishnan et al.[29]
Ginger (*Zingiber officinale*)	Zingerone	*P. aeruginosa*	Decrease in swimming, swarming, and twitching	Lokender et al.[30]
Oregano (*Origanum vulgare*)	–	*E. coli* and *P. aeruginosa*	Inhibition of violacein pigment production and decrease swarming motility	Truchado et al.[32] Lokender et al.[31]
Blueberry, blackberry, strawberry, cranberry, grapes	Flavonoids, tannins, phenolic acids	*E. coli* and *P. aeruginosa*	Inhibition of violacein pigment production and decrease swarming motility	Truchado et al.[32] Lokender et al.[31]
Ginseng (*Panax notoginseng*)	–	*Chromobacterium violaceum* CV026 and *P. aeruginosa* PA01	Inhibition of violacein synthesis	Koh and Tham[33]
Piper longum	Alkaloids and terpenoids	*Chromobacterium violaceum*	Inhibition of violacein pigment	Zaki et al.[34]

(*Continued*)

TABLE 13.3 (CONTINUED)

Phytochemicals as QS Inhibitors

Plant Source	Phytochemicals	Target Bacteria	QS Activity	References
Rosemary (*Rosemarinus officinalis*)	Rosmarinic acid	*L. monocytogenes*	Inhibition of PVC adhesion and biofilm formation	Sandasi et al.[35]
Pea (*Pisum sativum*)	–	*S. liquefaciens* MG1 and *P. aeruginosa* PAO1	Inhibition of violacein production and swimming and swarming motility (AHL mimics)	Truchado et al.[32] Teplitski et al.[36]
Guava (*Psidium guajava*)	Quercetin and quercetin-3-o-arabinoside	*C. violaceum*, *P. aeruginosa*	Inhibition of violacein production	Vasavi et al.[37]
Pineapple (*Ananas comosus*) and banana (*Musa paradisiacal*)	–	*P. aeruginosa* PAOI, *C. violaceum* CV026	Inhibition of violacein and pyocyanin production	Musthafa et al.[38]
Broccoli (*Brassica oleracea*)	Sulforaphane, erucin	*E. coli* O157:H7	Inhibition of AI-2 production and swarming motility	Lee et al.[39]
Orange	Flavanones (naringin, neohesperidin, and hesperidin)	*Y. enterocolitica*	Inhibition of biofilm formation and swimming motility	Truchado et al.[40]
Pomegranate (*Punica granatum*)	–	*E. carotovora* and *Y. enterocolitica*	Degradation transformation and inhibition of AHL	Truchado et al.[41]
Citrus limon, *Citrus medica*, and *Citrus aurantium*	–	*Campylobacter jejuni*	Inhibition of swarming motility and biofilm formation	Castillo et al.[42]
Water lily (*Nymphaea tetragona*)	–	*C. violaceum* and *P. aeruginosa*	Reductions in pyocyanin production and LasA protease activity, swarming motility	Hossain et al.[43]
Rosa rugosa tea	Polyphenolic and flavonoids	*C. violaceum* 026, *E. coli* K-12, and *P. aeruginosa* PAO1	Inhibition of violacein production, swarming motility, and biofilm	Zhang et al.[44]

(*Continued*)

TABLE 13.3 (CONTINUED)

Phytochemicals as QS Inhibitors

Plant Source	Phytochemicals	Target Bacteria	QS Activity	References
Oak bark (*Quercus cortex*)	1,2,3-Benzenetriol and 4-propyl-1,3-benzenediol	*C. violaceum* CV026	Inhibition of violacein production	Deryabin et al.[45]
Cecropia pachystachya Trécul	Chlorogenic acid, isoorientin, orientin, isovitexin, vitexin, and rutin	*C. violaceum* ATCC 31532 and *E. coli* pSB403	Inhibition of violacein production, reduction in AHL production	Brango-Vanegas et al.[46]
Lavender oil (*Lavandula angustifolia*)	–	*E. coli* J53 R1	Membrane permeability and inhibition of bacterial QS	Yap et al.[47]
Vanilla (*Vanilla planifolia*)	Vanillin	*A. hydrophila*	Inhibition of C4-HSL, 3-oxo-C8-HSL and AHL molecules	Kappachery et al.[48] Ponnusamy et al.[49]
Cinnamon (*Cinnamomum verum* J. Presl)	Cinnamaldehyde	*P. aeruginosa* and *Vibrio* spp.	Inhibition of AI-2 production and decrease in DNA-binding ability of LuxR	Kalia[50]
–	Quercetin	*C. violaceum* CV026, *Y. enterocolitica*, *P. aeruginosa.*	Reduction in violacein exopolysaccharide and alginate production; motility	Gopu et al.[51]
	Ursolic acid	*E. coli*	Inhibition of biofilm formation	Ren et al.[53]
	Ascorbic acid	*C. perfringens*	Inhibition of AI-2 activity	Novak and Fratamico[54]
	Phenylacetic acid	*P. aeruginosa*	Inhibition of pyocianin production, protease, and swimming motility	Musthafa et al.[55]
–	Farnesol	*P. aeruginosa*	Inhibition of pyocyanin production and PQS levels	Cugini et al.[56]
Grapefruit	Nomilin and obacunone	*E. coli* O157:H7	Inhibition of biofilm formation	Vikram et al.[57]
Oregano oil	Carvacrol	*S. enterica* subsp. Typhimurium DT104 and *S. aureus* 0074	Inhibition of violacein production and chitinase activity	Burt et al.[58]

pyocyanin production and protease, lipase, and polygalacturonase activities in the bacteria. The inhibition of virulence factors without reduction in bacterial growth may be providing strong support that these chemicals are general QSIs than an antibacterial effect.[28]

Clove extracts (*Syzygium aromaticum*) showed the inhibition of QS activity in the *Chromobacterium violaceum* CVO26, *E. coli* pSB401, and *E. coli* pSB1075 biosensors. In fact, most of the studies used biosensors to demonstrate the anti-QS activity of these extracts. In *P. aeruginosa* PAO1, clove extract also decreased the gene expression of lecA virulence factor, pyocyanin formation, and swarming motility.[29] The results of the present study revealed the anti-QS activity of zingerone targeting ligand–receptor interaction, hence proposing zingerone as a suitable antivirulent drug candidate against *P. aeruginosa* infections. Zingerone, besides decreasing swimming, swarming, and twitching phenotypes of *P. aeruginosa* PAO1, reduced biofilm-forming capacity and production of virulence factors, including rhamnolipid, elastase, protease, pyocyanin, and cell-free and cell-bound hemolysin ($p < 0.001$) indicating antivirulent property attributing toward the attenuation of virulence of *P. aeruginosa*. Zingerone showed a good comparative docking score to respective AI molecules which was even higher than that of vanillin, a proven anti-QS phytochemical.[30] Vattem et al.[31] found that oregano extract inhibited violacein pigment production (*C. violaceum*) and decreased swarming motility of *E. coli* O157:H7 (49%) and *P. aeruginosa* PAO1 (19%).[32]

The biological activities of berries are partially attributed to their high content of phytochemicals such as flavonoids (anthocyanins, flavonols, and flavanols), tannins (proanthocyanidins, ellagitannins, and gallotannins), stilbenoids (e.g., resveratrol), phenolic acids (hydroxybenzoic and hydroxycinnamic acid derivatives), and lignans. Different berries (cranberry, wild blueberry, raspberry, blackberry, and strawberry) and grape extract showed QSI activity as AHL signal inhibitors in *C. violaceum*, but they also significantly reduced swarming motility in *E. coli* and *P. aeruginosa* PAO1.[31,32] It could be hypothesized that their activity as QSI could also be attributed to their phytochemical content of the berries.

Scientific research on complementary therapies such as traditional Chinese medicine has examined its antibacterial properties. It is used for the treatment of dizziness, vertigo, nosebleed, injuries, fractures, heart problems; it stops bleeding and reduces swelling and pain. Koh and Tham[33] screened 10 herbal medicines, including flower and root extracts of *Panax notoginseng* against two biomonitor strains, *C. violaceum* CV026, and *P. aeruginosa* PA01. Interestingly, extracts from different parts of *Panax notoginseng*, i.e., roots and flowers, showed slightly different effects on the reporter bacteria CV026 and PA01. Roots appeared to have only QSI activity, whereas flowers seemed to have both antimicrobial (AM) and QSI properties. This research confirmed that ginseng may be a rich source of compounds to combat pathogenic bacteria and reduce the development of antibiotic resistance.[33] Zaki et al.[34] investigated the effects of *Piper longum* fruit extract as anti-QS activity by the *C. violaceum* assay using the agar cup diffusion method. It showed moderate anti-QS activity of 6–9 mm radius.[34]

Rosemary extract reducing the AHL activity mediated the inhibition of violacein production by 40% and decreased swarming motility of *E. coli* O157:H7 and *P. aeruginosa*.[32] It also showed decreased *Listeria monocytogenes* cell attachment to polyvinyl chloride, biofilm growth, development, and metabolic activity.[35] Teplitski et al.[36] concluded that seed and seedling exudates seem to be very active as QSI.

For instance, seed from pea was identified as QSI against different biosensor strains. According to this, pea seeds and seedlings and their extracts and exudates were effective in inhibiting violacein production (*C. violaceum*) and swimming and swarming motility in *Serratia liquefaciens* MG1 and *P. aeruginosa* PAO1, respectively. It may be due the presence of AHL mimic molecules able to disrupt the QS system in biosensor bacteria.[31,36]

Guava or *Psidium guajava* L., which has been traditionally used as a medicinal plant, was explored for anti-QS activity using a biosensor bioassay with *C. violaceum* CV026. Vasavi et al.[37] reported that quercetin and quercetin-3-*O*-arabinoside inhibited violacein production in *C. violaceum* 12472 at 50 and 100 µg/mL and inhibited pyocyanin production, proteolytic and elastolytic activities, swarming motility, and biofilm formation in *P. aeruginosa* PAO1. Results of this study provide a scope for further research to exploit these active molecules as anti-QS agents.[37] Musthafa et al.[38] demonstrated anti-QS activity of pineapple and banana extracts against *C. violaceum* in a dose-dependent manner. The aqueous extract of pineapple and banana was able to inhibit the AHL system in *C. violaceum* CV026 as well as in *P. aeruginosa* PAO1, affecting violacein and pyocyanin pigment production, staphylolytic protease, elastase production, and biofilm formation, respectively.[38]

Lee et al.[39] demonstrated that broccoli extract was able to regulate virulence in *E. coli* O157:H7 by modulating the transcription of virulence genes. The broccoli extract was also able to protect a model organism (*Caenorhabditis elegans*) against the pathogenic attack by the *E. coli* O157:H7, and based on that, it has been proposed as an agent to attenuate bacterial virulence *in vivo*. Broccoli extract reduced not only AI-2 synthesis, but also swimming and swarming motility in *E. coli* O157:H7 in a dose-dependent manner.[39] On the other hand, orange extract, rich in flavanones (naringin, neohesperidin, and hesperidin), showed the capacity to reduce the levels of 3-oxo-C6-HHL and C6-HHL excreted to extracellular media and biofilm formation by *Yersinia enterocolitica*. These phenotypic responses were associated with the induction of two important QS regulatory genes, *yen*R and *flh*DC, which are involved in the synthesis and regulation of AHLs and motility in *Y. enterocolitica*, respectively.[40] Truchado et al.[40] demonstrated the anti-QS capacity of pomegranate extract using the biosensor strain *C. violaceum* by the reduction of AHL production partially due to the degradation–transformation of AHLs. Additionally, the ability of pomegranate to inhibit the AHL system of pathogenic bacteria such as *Y. enterocolitica* and *Erwinia carotovora* has been evidenced.[41]

Castillo et al.[42] investigated the ability of citrus extracts to inhibit the activity of AI-2 molecules that mediate QS in *Campylobacter jejuni* and the effects of these extracts on motility, biofilm formation, and expression of *fla*A-B. Cultures of *C. jejuni* were exposed to extracts of *Citrus limon*, *Citrus medica*, and *Citrus aurantium* peels at various concentrations. It showed that treatment with *Citrus limon* or *Citrus medica* peel extract reduced swarm motility by 44–59%, whereas treatment with *Citrus aurantium* extract reduced swarm motility by 35–40%. Biofilm formation was reduced by 60–75% by these extracts, and three citrus extracts decreased AI-2 activity by about 90%, and at 75% of minimal bactericidal concentration (MBC), significantly ($p \leq 0.05$) reduced expression of *fla*A-B.[42]

Hossain et al.[43] verified the inhibitory effects of 50% methanolic extract of *Nymphaea tetragona* on QS-controlled virulence factors of bacteria. The antibacterial activity of this extract was evaluated against *C. violaceum* and *P. aeruginosa*. There

were remarkable reductions in pyocyanin production and LasA protease activity in the overnight culture supernatant of *P. aeruginosa* supplemented with *Nymphaea tetragona* extract when compared with that of the untreated control. It was observed that the swarming motility of *P. aeruginosa* was inhibited by ≥70%, and no toxic effect was observed in *in vitro* and *in vivo* cytotoxicity assays.[43] Zhang et al.[44] studied the antibiofilm and QS inhibitory potentials of *Rosa rugosa* tea extract, which is rich in polyphenols (87.52%) and flavonoids (61.03%). It inhibited QS-controlled violacein production in *C. violaceum* 026 with an 87.56% reduction without significantly affecting its growth. It also exhibited inhibition in swarming motility (84.90% and 78.03%) and biofilm formation (67.02% and 72.90%) of *E. coli* K-12 and *P. aeruginosa* PAO1 in a concentration-dependent manner, respectively. These findings strongly suggest that RTP potentially could be developed as a new QSI and/or antibiofilm agent to enhance the shelf life and increase food safety.[44] *Quercus cortex* (oak bark) has been used in European folk medicine since medieval times for the treatment of diarrhea, stomatitis, pharyngitis, and skin inflammations. Its antimicrobial activity is a well-known therapeutic property of oak bark, and its novel anti-QS ability has also been recently described. Deryabin and Tolmacheva[45] examined the bioactive compounds of *Quercus cortex* extract and compared their direct antibacterial and regulatory anti-QS effects against *C. violaceum* CV026. The *Quercus cortex* extract showed weak antibacterial and prominent anti-QS activities due to the presence of aromatic hydrophobic compounds. The semiquantitative agar diffusion assay showed the violacein inhibition (235 ± 19 mm^2; 132%) without any observable changes in growth inhibition zones and recommended for the therapeutic treatment of bacterial infection.[45]

The *Cecropia* genus is widely distributed in Latin America, including at least 60 species, and some of them are commonly used in traditional medicine for the treatment of several diseases. Brango-Vanegas et al.[46] found that the aqueous extract of *Cecropia pachystachya* Trécul leaves is a promising source of substances with anti-QS activity. The phytochemicals of *Cecropia pachystachya* leaves showed a potent activity as QSIs, using *C. violaceum* (ATCC 31532) and *E. coli* (pSB403) as biosensors. Additionally, they did not show growth inhibition properties against the gram-positive and gram-negative bacteria tested, as well as against the yeast *Saccharomyces cerevisiae*. Thus, the extract and the compounds have a potential to be used in the development of antipathogenic drugs or for antifouling coatings.[46] Yap et al.[47] investigated the mode of action of the single lavender essential oil and its combination with piperacillin on antimicrobial activity against multidrug-resistant *E. coli* J53 R1. A complete killing of bacteria was observed when lavender oil was combined with piperacillin due to the disruption the bacterial cell membrane. In addition, the lavender oil has the potential to reverse bacterial resistance to piperacillin in *E. coli* J53 R1, and it may operate via two mechanisms: alteration of outer membrane permeability and inhibition of bacterial QS.[47]

Vanillin is a well-known food flavoring agent, chemically known as a phenolic aldehyde (4-hydroxy-3-methoxybenzaldehyde), which is the primary component of the extract of the vanilla bean (*Vanilla planifolia*). This compound has been recognized as a potential QSI compound for *A. hydrophila* biofilms without affecting cell growth in different membranes, including microfiltration, ultrafiltration, and reverse osmosis membranes.[48] Because it interferes with AHL by restricting the availability of AHL to cells and thus prevents the initiation of the QS mechanism for biofilm formation. It also showed significant inhibition in short-chain (C4-HSL and 3-oxo-C8-HSL)

and long-chain AHL molecules.[49] In other hand, cinnamaldehyde, an organic compound, given by *Cinnamomum verum* J. Presl (cinnamon), and its derivatives affect a wide range of QS-regulated activities such as biofilm formation in *P. aeruginosa* and AI-2-mediated QS in different *Vibrio* spp. by decreasing the DNA-binding ability of LuxR, resulting in several marked phenotypic changes, including reduced virulence and increased susceptibility to stress.[50]

Flavonoids have been the focus of research for their roles as antioxidant, anti-inflammatory, and anticancer agents. Quercetin is a typical flavonoid ubiquitously used in dietary supplements known for its antioxidant property. It is present in fruits, vegetables, nuts, and grains, which are nontoxic to humans and are readily available. Gopu et al.[51] studied the inhibitory activity of quercetin and its antibiofilm property against food-borne pathogens using a biosensor strain. Quercetin (80 μg/mL) showed the significant reduction in QS-dependent phenotypes such as violacein production, biofilm formation, exopolysaccharide production, motility, and alginate production in a concentration-dependent manner. The synergistic activity of conventional antibiotics with quercetin enhanced the susceptibility of all tested pathogens.[51] Keeping in view of these health benefits, flavonoids such as naringenin, kaempferol, quercetin, and apigenin were evaluated for their QSI activities. All these flavonoids inhibited AI-2-mediated bioluminescence in *V. harveyi BB886*. Quercetin and naringenin were found to inhibit biofilm formation by *V. harveyi* BB120 and *E. coli* O157:H7. In addition, naringenin altered the expression of genes for type 3 secretory system in *V. harveyi*.[52]

In this study, Ren et al.[53] identified ursolic acid as a new biofilm inhibitors, and it was able to decrease biofilm formation in *E. coli*, *V. harveyi*, and *P. aeruginosa* PAO1. The analyses of the gene expression profile by DNA microarrays of *E. coli* treated with ursolic acid showed an upregulation of genes implicated in chemotaxis, mobility, and heat shock response functions as well as a downregulation of genes involved in cysteine synthesis and sulfur metabolism.[53] On the other hand, ascorbic acid, a natural QS analogue, decreased the concentration of AI-2 and caused a reduction on sporulation and enterotoxin production in the anaerobic food-borne pathogen *Clostridium perfringens*.[54] Phenylacetic acid is an organic compound which can be predominantly found in fruits and known as phytohormone The capacity of this compound to interfere with QS has been recently reported due to its capacity for reducing the production of AHL-dependent factors in *P. aeruginosa* PAO1 (pyocyanin, exopolysaccharide, and protease and elastase production).[55]

Farnesol is a sesquiterpene commonly found in citrus fruits. However, anti-QS activity of farnesol has been mostly studied because this compound is also produced by the fungus *Candida albicans* and sufficient to stimulate phenazine production in LasR(−) laboratory strains and clinical isolates. Cugini et al.[56] showed that *P. aeruginosa* LasR mutants, which lack the master QS system regulator, regain the ability to produce QS-regulated phenazines when cultured with *Candida albicans*. *P. aeruginosa* ΔlasR mutants are defective in the production of the PQS due to their inability to properly induce pqsH, which encodes the enzyme necessary for the last step in PQS biosynthesis. LasR mutants frequently arise in the lungs of patients chronically infected with *P. aeruginosa*. The finding that *Candida albicans*, farnesol, or reactive oxygen species stimulate virulence factor production in LasR strains provides new insight into the virulence potential of these strains.[56] Nomilin and obacunone are one of the major constituents of grapefruit and citrus

fruits. They are characterized by a high degree of oxygenation and the presence of a furan ring, similar to that present in the AI molecules (AI-1 and AI-2) from *V. harveyi* and enterohemorrhagic *E. coli* (EHEC) cell–cell signaling and biofilm formation by modulating the response regulator LuxO and type three secretion systems, respectively.[57] Carvacrol is a monoterpenoid phenol and one of the major antimicrobial components of oregano oil. Burt et al.[58] reported that carvacrol was able to inhibit the formation of biofilms of *C. violaceum* ATCC 12472, *Salmonella enterica* subsp. typhimurium DT104, and *S. aureus* 0074, while it showed no effect on the formation of *P. aeruginosa* biofilms. Submicrobial influenced corrosion (MIC) concentrations of carvacrol reduced the expression of *cvil* (a gene coding for the N-acyl-L-homoserine lactone synthase), production of violacein (pigmentation), and chitinase activity (both regulated by QS) at concentrations coinciding with the inhibiting effect of carvacrol on biofilm formation. These results indicate that the activity of carvacrol in the inhibition of biofilm formation may be related to the disruption of QS.[58]

13.5 Phytochemical-Functionalized NPs as QSI

The alarming spread of bacterial resistance to traditional antibiotics has warranted the study of alternative antimicrobial agents. QS is integral to bacterial survival and therefore provides a unique target for antimicrobial therapy (Table 13.4). Ali et al.[59] has reported a simple and rapid microwave-assisted method of green synthesis of silver NPs using an aqueous leaf extract of *Eucalyptus globulus* and their antibacterial and antibiofilm potential. The data revealed 82 ± 3% and 81 ± 5% inhibition of biofilms formed by *S. aureus* and *P. aeruginosa*, respectively, at 30μg/mL after 24 hours of treatment due to cellular damage. Both *microbes* are known to be potent biofilm producers, which makes their clinical management difficult.[59]

Recently, Singh et al.[60] reported the QSI activity of *Usnea longissima* extract-modified gold NPs against *Streptococcus mutans. Usnea longissima*, a medicinal lichen is ethnobotanically known for tooth cleaning and the treatment of infectious diseases. They confirmed that polyphenols and usnic acid of *Usnea longissima* efficiently synthesize the gold NPs having spherical shape and size in the range of 5–23 nm. The treatment of phytochemical-modified gold NPs was found to inhibit the secretion of *Streptococcus mutans* virulence factors, including acid production, ATPase, enolase, lactate dehydrogenase, protease, total exopolysaccharide content, and glucosidase. The phytochemical-modified gold NPs inhibiting the biofilm formation in a concentration-dependent manner and showed enhanced antibiofilm and antimicrobial activity to its conventional antibiotics.[60] Silver NPs (AgNPs) have been developed as antibiofilm agent for the treatment of infections associated with the use of mechanical ventilations, such as endotracheal intubation. Meanwhile, curcumin, a phenolic plant extract, has displayed natural antibiofilm properties through the inhibition of bacterial QS systems. Loo et al.[61] reported that the combination therapy of curcumin NPs and AgNPs at 100 μg/mL disrupted 50% of established *P. aeruginosa* and *S. aureus* biofilms. Interestingly, at this concentration, no inhibitory activity on *P. aeruginosa* biofilm was observed using either curcumin NPs or AgNPs alone. However, further increase in the concentration of curcumin NPs and AgNPs failed to effectively eliminate the biofilms. These formulations could be administered either

TABLE 13.4

Phytochemicals Functionalized NPs as QSI

Plant Source/Phytochemicals	NPs	Target Bacteria	QS Activity	Reference
Eucalyptus globulus	Ag	*Pseudomonas aeruginosa, Staphylococcus aureus*	Inhibition of biofilms due to cellular damage	Ali et al.[59]
Usnea longissima	Au	*Streptococcus mutans*	Inhibition of acid production, ATPase, enolase, lactate dehydrogenase, protease, total exopolysaccharide content, and glucosidase	Singh et al.[60]
Curcumin	Ag	*P. aeruginosa* and *S. aureus*	Inhibition of bacterial quorum sensing systems through attenuation of exopolysaccharide, alginate, motility behaviors	Loo et al.[61]
Turbinaria conoides	Ag	*E. coli, Salmonella* sp., *S. liquefaciens*, and *A. hydrophila*	–	Vijayan et al.[62]
Psidium guajava	Ag	*E. coli* and *S. aureus*	Alteration of cell membrane permeability and interaction with macromolecules such as proteins and DNA	Gupta et al.[63]
Plumbago zeylanica	Ag, Au and Ag–Au	*Acinetobacter baumannii, S. aureus,* and *E. coli*	Enhanced penetrability of NPs within the bacterial biofilms	Salunke et al.[64]
Gum arabic	Ag	*P. aeruginosa*	Inhibition of exopolysaccharide synthesis and penetrability of NPs within the bacterial biofilms	Ansari et al.[65]

directly as solution to produce rapid alleviation in bacterial infections or as a coating on endotracheal tubes to achieve prolonged, sustained antibacterial effect.[61]

Silver and gold NPs were synthesized using an aqueous extract of the seaweed *Turbinaria conoides*, and their antibiofilm activity against marine biofilm forming bacteria was reported by Vijayan et al.[62] They showed that spherical and triangular nanostructures of the AgNPs of the size ranging from 2 to 17 nm are efficient in controlling the bacterial biofilm formation; however, AuNPs of 2–19 nm size did not show any remarkable antibiofilm activity. The maximum zone of inhibition was recorded against *E. coli* (17.6 ± 0.42 mm), followed by *Salmonella* sp., *S. liquefaciens*, and *A. hydrophila*. The macrotube dilution method inferred the MIC (20–40 L/mL) and MBC (40–60 L/mL) of Ag NPs. The confocal laser scanning microscopic (CLSM) images clearly showed the weak adherence and disintegrating biofilm formation of marine biofilm bacterial strains treated with AgNPs. The Artemia cytotoxicity assay recorded the LC_{50} value of 88.914 ± 5.04 L/mL. Thus, the present study proved the efficiency of Ag NPs as a potent antimicrofouling agent and became the future perspective for the possible usage in biofouling-related issues in the aquaculture installations and other marine systems.[62] Gupta et al.[63] describes a single-step green synthesis of extracellular AgNPs through an *in situ* bioreduction of aqueous solutions of $AgNO_3$ using *Psidium guajava* L. The phytosynthesized and ~60 nm-sized AgNPs showed effective antibiofilm activity at a minimum inhibitory concentration (250 µM) against *E. coli* and *S. aureus*, with 96% and 90% in a dose-dependent manner using crystal violet assay. However, the plant extract and $AgNO_3$ showed relatively lower antibiofilm activity. The results suggest AgNPs as a promising candidate for the design of effective antimicrobial and antibiofilm agents in the biomedical field.[63] Salunke et al.[64] demonstrated the synthesis of *Plumbago zeylanica* extract-modified 60 nm monodispersed Ag nanospheres, 20–30 nm Au nanospheres adhering to form Au nanotriangles, and about 90 nm hexagonal blunt-ended Ag–AuNPs. These NPs showed antimicrobial and antibiofilm activity against *E. coli*, *A. baumannii*, *S. aureus*, and a mixed culture of *A. baumannii* and *S. aureus*. AgNPs inhibited biofilm in the range of 96–99% and Ag–AuNPs from 93% to 98% in single-culture biofilms.

AuNPs also showed biofilm inhibition, with the highest of 98% in *S. aureus*.[64] Ansari et al.[65] demonstrate the antibiofilm efficacy of gum arabic capped AgNPs against the multidrug-resistant (MDR) biofilm forming *P. aeruginosa* using the Congo red agar, tube method, and tissue culture plate assays. The results exhibited the modified AgNP concentration dependent on the inhibition of bacterial growth and exopolysaccharides matrix of the biofilm colonizers on the surface of plastic catheters. The treatment of catheters with modified 7 nm of AgNPs at 50 µg/mL has resulted in 95% inhibition of bacterial colonization and suggested its applications, as an effective antimicrobial agent for protecting the surfaces of medical implants against pathogen colonization.[65]

13.6 Chemically Modified NPs as QSI

Recently, Mu et al.[66] developed a facile approach to overcome the chronic infections related to biofilm and their inherent resistance to both antimicrobial agents and host defenses through phosphatidylcholine-decorated AuNPs loaded with gentamicin (Table 13.5). The antibiofilm efficacy of the 180 nm-sized particles was evaluated against both gram-positive and gram-negative microorganisms. It showed that

TABLE 13.5

Chemically Modified NPs as QS Inhibitors

Chemicals	NPs/Metals	Target Bacteria	QS Activity/Biofilm Inhibition	Ref.
Phosphatidylcholine and gentamicin	Au	*P. aeruginosa, S. aureus, E. coli*, and *L. monocytogenes*	Inhibition of bacterial QS systems	Mu et al.[66]
One-pot sol–gel method	AgCl–TiO_2	*C. violaceum*	Absence of signalling molecule, oxo-octanoyl homoserine lactone	Naik et al.[67]
Ethylene glycol and polyvinylpyrrol idone	Ag nanowires	*P. aeruginosa*	Inhibition of bacterial QS systems	Wagh Nee Jagtap et al.[68]
Sigma Aldrich	CuO and Fe_2O_3	*S. aureus*, *E. coli*	Toxicity of CuO >Fe_2O_3 NPs, high potential of absorption, adsorption, penetration and availability of NPs resulting damage of the envelope of the microorganism	Agarwala et al.[69]
Sodium citrate and $NaBH_4$	Bi	*Streptococcus mutans*	Reduce cell growth	Hernandez-Delgadillo et al.[70]
–	Bi–thiol	*P. aeruginosa*	Disturb bacterial membrane integrity and to prevent biofilm formation	Halwani et al.[71]
–	Mesoporous Si	*P. aeruginosa*	Immobilizing and stabilizing an AC, disrupting bacterial cell-to-cell communication, in the form of cross-linked enzymes, effective prevention of enzyme leaching	Lee et al.[72]
	V_2O_5 nanowires	*S. aureus*, *E. coli*	HOBr interferes with the QS system of bacteria, preventing adhesion of the bacteria and biofilm formation	Natalio et al.[73]
	ZnO	*P. aeruginosa*	Control of pyocyanin and production and biofilm formation	Lee et al.[74]

(*Continued*)

TABLE 13.5 (CONTINUED)

Chemically Modified NPs as QS Inhibitors

Chemicals	NPs/Metals	Target Bacteria	QS Activity/Biofilm Inhibition	Ref.
	ZnO	*S. sobrinus*	Generate H_2O_2, inhibiting the active transport and metabolism of sugars as well as disrupting enzyme systems	Sevinc et al.[75]
–	Ni^{2+} and Cd^{2+}	*Burkholderia multivorans*	Inhibition (at the transcriptional level) of acyl-homoserine lactone quorum sensing	Vega et al.[76]
β-CD	SiO_2	*V. fischeri*	Block signaling molecules (acylhomoserine lactones), penetrate biofilms and eliminate infections	Miller et al.[77]

gentamicin-modified AuNPs were more effective in the eradication of preformed biofilm built by *P. aeruginosa*, *S. aureus*, *E. coli*, and *Listeria monocytogenes*. In addition, modified NPs had good biocompatibility and elicited a superior intracellular killing capability against multiple pathogenic bacteria in infected macrophages. The strategy may be useful to develop new therapeutics for treating chronic and stubborn infections related to biofilm and intracellular bacteria.[66] Anti-QS materials such as AgCl–TiO_2 are proposed as efficient models for controlling food spoilage. The AgCl–TiO_2 incorporated in food packaging materials could play an important role in food preservation and ensure the safety of food by prolonging their shelf life. The anti-QS activity of AgCl–TiO_2 was evaluated using the bacterial model *C. violaceum*. Silver present in AgCl–TiO_2 significantly reduced violacein production in a concentration-dependent manner, indicating the inhibition of QS. Anti-QS activity was confirmed by the absence of signaling molecule oxo-octanoyl homoserine lactone during growth in the presence of AgCl–TiO_2. TiO_2 acted as a good supporting matrix facilitating controled release of silver with prolonged residual activity and adds a further note on its anti-QS activity and its potential use in food packaging industry.[67] Wagh Nee Jagtap et al.[68] synthesized silver nanowires (200–250 nm in diameter and up to 3–4 μm in length) by using ethylene glycol as a reducing agent and polyvinylpyrrolidone as a capping agent under reflux reaction seedless process. It showed that silver nanowires have no antibacterial activity against test organisms, *Bacillus subtilis* NCIM 2063 and *E. coli* NCIM 2931. However, it showed a promising property of a QS-mediated inhibition of biofilm in *P. aeruginosa* NCIM 2948 and violacein synthesis in *C. violaceum* ATCC 12472, which is hitherto unattempted, by polyol approach.[68]

The effectiveness of the CuO and Fe_2O_3 as antibacterial agents against MDR biofilm-forming bacteria was evaluated by Agarwala et al.[69] They also experimented for antibiofilm and time kill assay. The CuO displayed maximum antibacterial activity with zone of inhibition of (22 ± 1) mm against methicillin-resistant *S. aureus*

(MRSA) followed by *E. coli* (18 ± 1) mm. The Fe_2O_3 showed the zone of inhibition against MRSA of (14 ± 1) mm followed by *E. coli* (12 ± 1) mm. The CuO proved to be more toxic than Fe_2O_3 NPs, showing significantly high antibacterial activity and found to possess dose-dependent antibiofilm properties.[69] The results of the study by Hernandez-Delgadillo et al.[70] showed that stable colloidal bismuth NPs had 69% antimicrobial activity against *Streptococcus mutans* growth and achieved complete inhibition of biofilm formation. These results are similar to those obtained with chlorhexidine, the most commonly used oral antiseptic agents. The minimal inhibitory concentration of bismuth NPs that interfered with *Streptococcus mutans* growth was 0.5 mM. These results suggest that Bi (0) NPs could be an interesting antimicrobial agent to be incorporated into an oral antiseptic preparation.[70] Recurrent pulmonary infection and inflammation are major risk factors for high morbidity and mortality in patients with cystic fibrosis (CF). As such, frequent antibiotic use and drug-resistant bacterial strains are main concerns in individuals with CF. Bacterial virulence and resistance are influenced by unique CF airway fluid lining and *P. aeruginosa* QS and biofilm formation. Halwani et al.[71] have developed a novel liposome formulation consisting of bismuth–thiol and tobramycin (LipoBiEDT–TOB) that is nontoxic and highly effective against planktonic bacteria. In this study, we examined the effect of LipoBiEDT–TOB on QS molecule AHL secretion by *P. aeruginosa* isolates in the presence of *Agrobacterium tumefaciens* reporter strain (A136). LipoBiEDT–TOB activity against biofilm-forming *P. aeruginosa* was compared to free tobramycin using the Calgary Biofilm Device. Our data indicate that LipoBiEDT–TOB prevents AHL production at low tobramycin concentration (as low as 0.012 mg/L) and stops biofilm-forming *P. aeruginosa* growth at 64 mg/L. The formulation is stable in different biological environments (biofilm, sputum, and bronchoalveolar lavage) and is able to penetrate CF sputum. Taken together, coencapsulation of bismuth–thiol metal with tobramycin in liposome improves its antimicrobial activities *in vitro*.[71]

Highly effective antifouling was achieved by immobilizing and stabilizing an AC, disrupting bacterial cell-to-cell communication, in the form of cross-linked enzymes in magnetically separable mesoporous silica. This so-called quorum-quenching AC was adsorbed into spherical mesoporous silica (S-MPS) with magnetic NPs (Mag-S-MPS) and further cross-linked for the preparation of nanoscale enzyme reactors of AC in Mag-SMPS (NER-AC/Mag-S-MPS). Lee et al.[72] showed that the NER-AC effectively stabilized the AC activity under rigorous shaking at 200 revolutions per minute for 1 month, while free and adsorbed AC lost more than 90% of their initial activities in the same condition within 1 and 10 days, respectively. When applied to the membrane filtration for advanced water treatment, NER-AC efficiently alleviated the biofilm maturation of *P. aeruginosa* PAO1 on the membrane surface, thereby enhancing the filtration performance by preventing membrane fouling. Highly stable and magnetically separable NER-AC, as an effective and sustainable antifouling material, has a great potential to be used in the membrane filtration for water reclamation.[72] Antifouling coatings that do not release biocides, such as silicone elastomers, have been considered enviromentally benign. Here, Natalio et al.[73] showed that vanadium pentoxide (V_2O_5) nanowires with an average length of 300 nm and a width of 20 nm act like naturally occurring vanadium haloperoxidases to prevent marine biofouling. In the presence of bromide ions and hydrogen peroxide, the nanowires catalyze the oxidation of bromide ions to

hypobromous acid (HOBr). Singlet molecular oxygen ($^1O_2$) is formed, and this exerts strong antibacterial activity, which prevents marine biofouling without being toxic to marine biota. V_2O_5 nanowires have the potential to be an alternative approach to conventional antibiofouling agents.[73]

The opportunistic pathogen *P. aeruginosa* produces a variety of virulence factors, and biofilms of this bacterium are much more resistant to antibiotics than planktonic cells. Lee et al.[74] investigated that zinc ions and ZnO NPs were found to markedly inhibit biofilm formation and the production of pyocyanin, PQS, pyochelin, and hemolytic activity of *P. aeruginosa* without affecting the growth of planktonic cells. Transcriptome analyses showed that ZnO NPs induce the zinc cation efflux pump czc operon and several important transcriptional regulators (porin gene opdT and type III repressor ptrA), but repress the pyocyanin-related phz operon, which explains observed phenotypic changes. A mutant study showed that the effects of ZnO NPs on the control of pyocyanin production and biofilm formation require the czc regulator CzcR. In addition, ZnO NPs markedly increased the cellular hydrophilicity of *P. aeruginosa* cells. Our results support that ZnO NPs are potential antivirulence materials against recalcitrant *P. aeruginosa* infections and possibly other important pathogens.[74] The resin-based dental composites, commonly used in restorations that result in more plaque accumulation than other materials, have been sought for several decades. Bacterial biofilm growth contributes to secondary caries and failure of resin-based dental composites. Sevinc and Hanley[75] demonstrated that zinc oxide NPs (ZnO-NPs) blended at 10% (w/w) fraction into dental composites display antimicrobial activity and reduce the growth of *Streptococcus sobrinus*/bacterial biofilms by roughly 80% for a single-species model dental biofilm. The minimum inhibitory concentration of ZnO-NPs suspended in *S. sobrinus* planktonic culture was 50 μg/mL. The insolubility of the ZnO-NPs prevented the diffusion of a sufficient amount of Zn^{2+} to the surrounding environment to impart an observable antibiotic effect, rendering the NPs ineffective for noncontact biofilm inhibition. Furthermore, media containing components eluted from the composite resins did not exhibit any antibacterial behavior at periods up to 1 month, implying that there was an insufficient release of Zn^{2+} from ZnO-NPs to activate this mechanism.[75]

MIC and biofouling are multibillion-dollar challenges typically associated with bacterial biofilms. Using microtiter plate and quantitative reverse transcriptase polymerase chain reaction assays, Vega et al.[76] show that submillimolar concentrations of nickel (Ni^{2+}) and cadmium (Cd^{2+}) inhibit biofilm formation by the bacterium *Burkholderia multivorans* through the inhibition (at the transcriptional level) of acyl-homoserine lactone QS. These results advance the understanding of environmental factors influencing the establishment and subsequent development of biofilms, as this is the first study linking heavy metals with QS and biofilm inhibition and may guide strategies to mitigate MIC and biofouling of infrastructure surfaces.[76] In this study, Miller et al.[77] demonstrated that β cyclodextrin (CD)-functionalized silica NPs (SiO_2 NPs) can be used to enhance the role of quenching agents *in vitro*, and β-CD is able to bind homoserine lactones (HSLs) and downregulate bacterial QS genes. They found that the quenching ability of β-CD was much greater on functionalized 50 nm SiO_2 NPs and provided a scaffold and critical stepping-stone for more pointed developments in antimicrobial therapy, especially with regard to QS a target that will reduce resistance pressures imposed by traditional antibiotics.[77]

13.7 Conclusions

One of the most promising strategies for the development of next-generation antimicrobials and food products is to target bacterial communication or QS. QS is a chemical signal-based communication method through which bacteria can adopt community-like behaviors implicated in bacterial pathogenicity and food spoilage. Biofilm or QSIs from natural products such as fruits and vegetables are promising sources that can potentially inhibit QS and biofilm formation. Here, with the advent of nanotechnology, these results confirm that phytochemical-functionalized smaller NPs are generally more effective against microorganisms than larger particles are, as the antibacterial activity appears to depend on the total contact surface area and biofilm penetration. Finally, phytochemicals and their functionalized NPs as QSIs open up an exciting new strategy for antimicrobial chemotherapy and lead to the discovery of a new category of antibiotics, which can overcome the issues related to antimicrobial resistance.

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REFERENCES

1. Sauer, K.; Camper, A.K.; Ehrlich G.D. et al. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *Journal of Bacteriology* 2002, 184, 1140–1154.
2. Nealson, K.H.; Platt, T.; Hastings, J.W. Cellular control of the synthesis and activity of the bacterial luminescent system. *Journal of Bacteriology* 1970, 104, 313–322.
3. Karatan, E; Duncan, T.R.; Watnick, P.I. NspS, a predicted polyamine sensor, mediates activation of *Vibrio cholerae* biofilm formation by norspermidine. *Journal of Bacteriology* 2005, 187, 7434–7443.
4. Haussler, S; Fuqua, C. Biofilms 2012: New discoveries and significant wrinkles in a dynamic field. *Journal of Bacteriology* 2013, 195, 2947–2958.
5. Yang, L.; Liu, Y.; Wu, H.; Song, Z.; Høiby, N.; Molin, S.; Givskov, M. Combating biofilms. *FEMS Immunology and Medical Microbiology* 2012, 65(2), 146–57.
6. Lade, H.; Paul, D.; Kweon, J.H. Quorum quenching mediated approaches for control of membrane biofouling. *International Journal of Biological Sciences* 2014, 10(5), 550–565.
7. Tang, K.; Zhang, X.H. Quorum quenching agents: Resources for antivirulence therapy. *Marine Drugs* 2014, 12, 3245–3282.
8. Rul, F.; Monnet, V. How microbes communicate in food: A review of signaling molecules and their impact on food quality. *Current Opinion in Food Science* 2015, 2, 100–105.
9. Novick, R.P.; Geisinger, E. Quorum sensing in staphylococci. *Annual Review of Genetics* 2008, 42, 541–564.
10. Monnet, V.; Juillard, V.; Gardan, R. Peptide conversations in gram-positive bacteria. *Critical Reviews in Microbiology* 2014, 8, 1–13.

11. Bai, A.J.; Rai, VR. Bacterial quorum sensing and food industry. *Comprehensive Reviews in Food Science and Food Safety* 2011, 10, 184–194.
12. Walters, M.; Sperandio, V. Quorum sensing in *E. coli* and *Salmonella*. *International Journal of Medical Microbiology* 2006, 296, 125–131.
13. Yarwood, J.M.; Bartels, D.J.; Volper, E.M. et al. Quorum sensing in *Staphylococcus aureus* biofilms. *Journal of Bacteriology* 2004, 186, 1838–1850.
14. Williams, P. Quorum sensing, communication and cross-kingdom signalling in the bacterial world. *Microbiology* 2007, 153, 3923–3938.
15. Geske, G.D.; O'Neill, J.C.; Blackwell, H.E. Expanding dialogues: From natural autoinducers to non-natural analogues that modulate quorum sensing in Gram-negative bacteria. *Chemical Society Reviews* 2008, 37, 1432–1447.
16. Hall-Stoodley, L.; Stoodley, P. Evolving concepts in biofilm infections. *Cellular Microbiology* 2009, 11, 1034–1043.
17. Shrout, J.D.; Nerenberg, R. Monitoring bacterial twitter: Does quorum sensing determine the behavior of water and wastewater treatment biofilms? *Environmental Science & Technology* 2012, 46, 1995–2005.
18. Hentzer, M.; Givskov, M.; Eberl, L. Quorum sensing in biofilms: Gossip in slime city. In: Ghannoum, M.; O'Toole, G. A., ed. *Microbial Biofilms*: ASM Press, Washington, DC; 2004: 118–140.
19. Parveen, N.; Cornell, K.A. Methylthioadenosine/S-adenosylhomocysteine nucleosidase, a critical enzyme for bacterial metabolism. *Molecular Microbiology* 2011, 79, 7–20.
20. Lin, Y.H.; Xu, J.L.; Hu, J.Y. et al. Acyl-homoserine lactone acylase from Ralstonia strain XJ12B represents a novel and potent class of quorum-quenching enzymes. *Molecular Microbiology* 2003, 47, 849–860.
21. Koch, B.; Liljefors, T.; Persson, T. et al. The LuxR receptor: The sites of interaction with quorum-sensing signals and inhibitors. *Microbiology* 2005, 151, 3589–3602.
22. Rasmussen, T.B.; Givskov, M. Quorum sensing inhibitors: A bargain of effects. *Microbiology* 2006, 152, 895–904.
23. Gibot, S. Fighting the enemy properly. *Critical Care Medicine* 2004, 32, 1223–1224.
24. Rudrappa, T.; Bais, H.P. *Curcumin*, a known phenolic from *Curcuma longa*, attenuates the virulence of *Pseudomonas aeruginosa* PAO1 in whole plant and animal pathogenicity models. *Journal of Agricultural and Food Chemistry* 2008, 56, 1955–1962.
25. Vasantha Packiavathy, I.A.S.; Priya, S.; Pandian, S.K.; Ravi, A.V. Inhibition of biofilm development of uropathogens by curcumin—An anti-quorum sensing agent from *Curcuma longa*. *Food Chemistry* 2014, 148, 453–460.
26. Rasmussen, T.B.; Bjarnsholt, T.; Skindersoe, M.E.; Hentzer, M.; Kristoffersen, P.; Kote, M.; Nielsen, J.; Eberl, L.; Givskov, M. Screening for quorum-sensing inhibitors (QSI) by use of a novel genetic system, the QSI selector. *Journal of Bacteriology* 2005, 187, 1799–1814.
27. Jakobsen, T.H.; van Gennip, M.; Phipps, R.K. et al. Ajoene, a sulfur-rich molecule from garlic, inhibits genes controlled by quorum sensing. *Antimicrobial Agents and Chemotherapy* 2012, 56, 2314–2325.
28. Abd-Alla, M.H.; Bashandy, S.R. Production of quorum sensing inhibitors in growing onion bulbs infected with *Pseudomonas aeruginosa* E (HQ324110). *ISRN Microbiology* 2012, 161890.
29. Krishnan, T.; Yin, W.F.; Chan, K.G. Inhibition of quorum sensing-controlled virulence factor production in *Pseudomonas aeruginosa* PAO1 by Ayurveda spice clove (*Syzygium aromaticum*) bud extract. *Sensors* 2012, 12(4), 4016–4030.

30. Lokender, K.; Sanjay, C.; Rajnish, K.; Manoj, K.; Kusum, H. Zingerone silences quorum sensing and attenuates virulence of *Pseudomonas aeruginosa*. *Fitoterapia* 2015, 102, 84–95.
31. Vattem, D.A.; Mihalik, K.; Crixell, S.H.; McLean, R.J.C. Dietary phytochemicals as quorum sensing inhibitors. *Fitoterapia* 2007, 78, 302–310.
32. Truchado, P.; Larrosa, M.; Castro-Ibáñez, I.; Tomás-Barberán, F.A.; Allende, A. Plant food extracts and phytochemicals: Their role as quorum sensing inhibitors. *Trends in Food Science & Technology* 2015, 43(2), 189–204.
33. Koh, K.H.; Tham, F.Y. Screening of traditional Chinese medicinal plants for quorum-sensing inhibitors activity. *Journal of Microbiology, Immunology and Infection* 2011, 44, 144–148.
34. Zaki, A.A.; Shaaban, M.I.; Hashish, N.E.; Amer, M.A.; Lahloub, M.F. Assessment of anti-quorum sensing activity for some ornamental and medicinal plants native to Egypt. *Scientia Pharmaceutica* 2013, 81, 251–258.
35. Sandasi, M.; Leonard, C.M.; Viljoen, A.M. The *in vitro* antibiofilm activity of selected culinary herbs and medicinal plants against *Listeria monocytogenes*. *Letters in Applied Microbiology* 2010, 50(1), 30–35.
36. Teplitski, M.; Robinson, J.B.; Bauer, W.D. Plants secrete substances that mimic bacterial N-acyl homoserine lactone signal activities and affect population density-dependent behaviors in associated bacteria. *Molecular Plant Microbe. Interactions* 2000, 13(6), 637–648.
37. Vasavi, H.S.; Arun, A.B.; Rekha, P.D. Anti-quorum sensing activity of *Psidium guajava* L. flavonoids against *Chromobacterium violaceum* and *Pseudomonas aeruginosa* PAO1. *Microbiology and Immunology* 2014, 58, 286–93.
38. Musthafa, K.S.; Ravi, A.V.; Annapoorani, A.; Packiavathy, I.S.; Pandian, S.K. Evaluation of anti-quorum-sensing activity of edible plants and fruits through inhibition of the N-acyl-homoserine lactone system in *Chromobacterium violaceum* and *Pseudomonas aeruginosa*. *Chemotherapy* 2010, 56(4), 333–339.
39. Lee, K.M.; Lim, J.; Nam, S. et al. Inhibitory effects of broccoli extract on *Escherichia coli* O157:H7 quorum sensing and *in vivo* virulence. *FEMS Microbiology Letters* 2011, 321(1), 67–74.
40. Truchado, P.; Giménez-Bastida, J.A.; Larrosa, M. et al. Inhibition of quorum sensing (QS) in *Yersinia enterocolitica* by an orange extract rich in glycosylated flavanones. *Journal of Agricultural and Food Chemistry* 2012, 60(36), 8885–8894.
41. Truchado, P.; Gil, A.; Tomás-Barberán, F.A.; Larrosa, M.; Allende, A. Food phytochemicals act as quorum sensing inhibitors reducing production and/or degrading autoinducers of *Yersinia enterocolitica* and *Erwinia carotovor*a. *Food Control* 2012, 24(1), 78–75.
42. Castillo, S.; Heredia, N.; Arechiga-Carvajal, E.; García, S. Citrus extracts as inhibitors of quorum sensing, biofilm formation and motility of *Campylobacter jejuni*. *Food Biotechnology* 2014, 28, 106–122.
43. Hossain, M.D.; Lee, S.J.; Park, J.Y.; Reza, M.A.; Kim, T.H.; Lee, K.J.; Suh, J.W.; Park, S.C. Modulation of quorum sensing-controlled virulence factors by *Nymphaea tetragona* (water lily) extract. *Journal of Ethnopharmacology* 2015, 174, 482–491.
44. Zhang, J.; Rui, X.; Wang, L.; Guan, Y.; Sun, X.; Dong, M. Polyphenolic extract from *Rosa rugosa* tea inhibits bacterial quorum sensing and biofilm formation. *Food Control* 2014, 42, 125–131.
45. Deryabin, D.J.; Tolmacheva, A.A. Antibacterial and anti-quorum sensing molecular composition derived from *Quercus cortex* (Oak bark) extract. *Molecules* 2015, 20, 17093–17108.

46. Brango-Vanegas, J.; Costa, G.M.; Ortmann, C.F.; Schenkel, E.P.; Reginatto, F.H.; Ramos, F.A.; Arévalo-Ferro, C.; Castellanos, L. Glycosylflavonoids from *Cecropia pachystachya* Trécul are quorum sensing inhibitors. *Phytomedicine* 2014, 21(5), 670–675.
47. Yap, O.S.X.; Krishnan, T.; Yiap, B.C.; Hu, C.P.; Chan, K.G.; Lim, S.H.E. Membrane disruption and anti-quorum sensing effects of synergistic interaction between *Lavandula angustifolia* (lavender oil) in combination with antibiotic against plasmid-conferred multi-drug resistant *Escherichia coli*. *Journal of Applied Microbiology* 2014, 116(5), 1119–1128.
48. Kappachery, S.; Paul, D.; Yoon, J.; Kweon, J.H. Vanillin, a potential agent to prevent biofouling of reverse osmosis membrane. *Biofouling* 2010, 26(6), 667–672.
49. Ponnusamy, K.; Paul, D.; Kweon, J.H. Inhibition of quorum sensing mechanism and Aeromonas hydrophila biofilm formation by vanillin. *Environmental Engineering Science* 2009, 26(8), 1359–1363.
50. Kalia, V.C. Quorum sensing inhibitors: An overview. *Biotechnology Advances* 2013, 31(2), 224–245.
51. Gopu, V.; Meena, C.K.; Shetty, P.H. Quercetin influences quorum sensing in food borne bacteria: *In-vitro* and *in-silico* evidence. *PLoS ONE* 2015, 10(8), e0134684.
52. Vikram, A.; Jesudhasan, P.R.; Jayaprakasha, G.K.; Pillai, S.D.; Patil, B.S. Citrus limonoid interfere with *Vibrio harveyi* cell-cell signaling and biofilm formation by modulating the response regulator LuxO. *Microbiology* 2011, 157, 99–110.
53. Ren, D.; Zuo, R.; González–Barrios, A.F.; Bedzyk, L.A.; Eldridge, G.R.; Pasmore, M.E. Differential gene expression for investigation of *Escherichia coli* biofilm inhibition by plant extract ursolic acid. *Applied Environmental Microbiology* 2005, 71(7), 4022–4034.
54. Novak, J.S.; Fratamico, P.M. Evaluation of ascorbic acid as a quorum-sensing analogue to control growth, sporulation, and enterotoxin production in *Clostridium perfringens*. *Journal of Food Science* 2004, 69(3), 72–78.
55. Musthafa, K.S.; Sivamaruthi, B.S.; Pandian, S.K.; Ravi, A.V. Quorum sensing inhibition in *Pseudomonas aeruginosa* PAO1 by antagonistic compound phenylacetic acid. *Current Microbiology* 2012, 65(5), 475–480.
56. Cugini, C.; Morales, D.K.; Hogan, D.A. *Candida albicans*-produced farnesol stimulates *Pseudomonas quinolone* signal production in LasR-defective *Pseudomonas aeruginosa* strains. *Microbiology* 2010, 156(10), 3096–3107.
57. Vikram, A.; Jesudhasan, P.R.; Jayaprakasha, G.K.; Pillai, S.D.; Patil, B.S. Citrus limonoids interfere with *Vibrio harveyi* cell–cell signalling and biofilm formation by modulating the response regulator LuxO. *Microbiology* 2011, 157(1), 99–110.
58. Burt, S.A.; Ojo-Fakunle, V.T.A.; Woertman, J.; Veldhuizen, E.J.A. The natural antimicrobial carvacrol inhibits quorum sensing in *Chromobacterium violaceum* and reduces bacterial biofilm formation at sub-lethal concentrations. *PLoS ONE* 2014, 9(4): e93414.
59. Ali, K.; Ahmed, B.; Dwivedi, S.; Saquib, Q.; AlKhedhairy, A.A.; Musarrat, J. Microwave accelerated green synthesis of stable silver nanoparticles with *Eucalyptus globulus* leaf extract and their antibacterial and antibiofilm activity on clinical isolates. *PLoS ONE* 2015, 10(7), e0131178.
60. Singh, B.N.; Prateeksha; Pandey, G.; Jadaun, V. et al. Development and characterization of a novel Swarna-based herbo-metallic colloidal nano-formulation—Inhibitor of *Streptococcus mutans* quorum sensing. *RSC Advances* 2015, 5, 5809–5822.

61. Loo, C.Y.; Rohanizadeh, R.; Young, P.M.; Traini, D.; Cavaliere, R.; Whitchurch, C.B.; Lee, W.H. Combination of silver nanoparticles and curcumin nanoparticles for enhanced anti-biofilm activities. *Journal of Agricultural and Food Chemistry* 2016, 64(12), 2513–2522.
62. Vijayan, S.R.; Santhiyagu, P.; Singamuthu, M.; Ahila, N.K.; Jayaraman, R.; Ethiraj, K. Synthesis and characterization of silver and gold nanoparticles using aqueous extract of seaweed, *Turbinaria conoides*, and their antimicrofouling activity. *The Scientific World Journal* 2014, 2014, Article ID 938272.
63. Gupta, K.; Hazarika, S.N.; Saikia, D.; Namsa, N.D.; Mandal, M. One step green synthesis and its anti-microbial and anti-biofilm properties of *Psidium guajava* L. leaf extract-mediated silver nanoparticles. *Materials Letters* 2014, 125, 67–70.
64. Salunke, G.R.; Ghosh, S.; Santosh Kumar, R.J. et al. Rapid efficient synthesis and characterization of silver, gold, and bimetallic nanoparticles from the medicinal plant *Plumbago zeylanica* and their application in biofilm control. *International Journal of Nanomedicine* 2014, 9, 2635–2653.
65. Ansari, M.A.; Khan, H.M.; Khan, A.A.; Cameotra, S.S.; Saquib, Q.; Musarrat, J. Gum arabic capped-silver nanoparticles inhibit biofilm formation by multi-drug resistant strains of *Pseudomonas aeruginosa*. *Journal of Basic Microbiology* 2014, 54, 1–12.
66. Mu, H.; Tang, J.; Liu, Q.; Sun, C.; Wang, T; Duan, J. Potent antibacterial nanoparticles against biofilm and intracellular bacteria. *Scientific Reports* 2016, 6, 18877.
67. Naik, K.; Kowshik, M. Anti-quorum sensing activity of AgCl-TiO_2 nanoparticles with potential use as active food packaging material. *Journal of Applied Microbiology* 2014, 117, 972–983.
68. Wagh Nee Jagtap, M.S.; Patil, R.H.; Thombre, D.K.; Kulkarni, M.V.; Gade, W.N.; Kale, B.B. Evaluation of anti-quorum sensing activity of silver nanowires. *Applied Microbiology and Biotechnology* 2013, 97, 3593–3601.
69. Agarwala, M.; Choudhury, B.; Yadav, R.N.S. Comparative study of antibiofilm activity of copper oxide and iron oxide nanoparticles against multidrug resistant biofilm forming uropathogens. *Indian Journal of Microbiology* 2014, 54(3), 365–368.
70. Hernandez-Delgadillo, R.; Velasco-Arias, D.; Diaz, D.; Arevalo-Niño, K.; Garza-Enriquez, M.; De la Garza Ramos, M.A.; Cabral-Romero, C. Zerovalent bismuth nanoparticles inhibit *Streptococcus mutans* growth and formation of biofilm. *International Journal of Nanomedicine* 2012, 7, 2109–2113.
71. Halwani, M.; Hebert, S.; Suntres, Z.E.; Lafrenie, R.M.; Azghani, A.O.; Omri, A.O. Bismuth–thiol incorporation enhances biological activities of liposomal tobramycin against bacterial biofilm and quorum sensing molecules production by *Pseudomonas aeruginosa*. *International Journal of Pharmaceutics* 2009, 373, 141–146.
72. Lee, B., Yeon, K.M.; Shim, J.; Kim, S.R.; Lee, C.H.; Lee, J.; Kim, J. Effective antifouling using quorum-quenching acylase stabilized in magnetically-separable mesoporous silica. *Biomacromolecules* 15, 2014, 1153–1159.
73. Natalio, F.; Andre, R.; Hartog, A.F.; Stoll, B.; Jochum, K.P.; Wever, R; Tremel, W. Vanadium pentoxide nanoparticles mimic vanadium haloperoxidases and thwart biofilm formation. *Nature Nanotechnology* 2012, 7, 530–535.
74. Lee, J.H.; Kim, Y.G.; Cho, M.H.; Lee, J. ZnO Nanoparticles inhibit *Pseudomonas aeruginosa* biofilm formation and virulence factor production. *Microbiological Research* 2014, 169(12) 888–896.
75. Sevinc, B.A.; Hanley, L. Antibacterial activity of dental composites containing zinc oxide nanoparticles. *Journal of Biomedical Materials Research B: Applied Biomaterials* 2010, 94B(1), 22–31.

76. Vega, L.M.; Mathieu, J.; Yang, Y.; Pyle, B.H.; McLean, R.J.C.; Alvarez, P.J.J. Nickel and cadmium ions inhibit quorum sensing and biofilm formation without affecting viability in *Burkholderia multivorans*. *International Biodeterioration & Biodegradation* 2014, 91, 82–87.
77. Miller, K.P.; Wang, L.; Chen, Y.P.; Pellechia, P.J.; Benicewicz, B.C.; Decho, A.W. Engineering nanoparticles to silence bacterial communication. *Frontiers in Microbiology* 2015, 6, 189.



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Nanotoxicological Evaluation in Freshwater Organisms

Lindsey C. Felix and Greg G. Goss

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14.1 Introduction

Introduced over a decade ago, nanotoxicology is an emerging multidisciplinary branch of toxicology that evaluates the safety of engineered nanomaterials by assessing their effects on living organisms (Oberdörster, Oberdörster, and Oberdörster 2005). Gallud and Fadeel (2015) suggested that nanomaterials should be defined as "materials that can interact with biological systems at the nanoscale." Although a "one-size-fits-all" definition of a nanomaterial is still lacking (Gallud and Fadeel 2015), the European Commission's recommended regulatory definition is a step forward (see Section 14.8) (European Commission 2011). For the purposes of this chapter, an engineered nanomaterial is defined as a synthetic material having at least one dimension between one and one hundred billionths of a meter, which can include a nanoparticle that has three nanoscale dimensions, as well as nanotubes and nanocrystals that have one dimension extending 100 nm or more (International Organization for Standardization 2015); the term *nanomaterial* will be consistently used throughout this chapter for clarity. Due to their small size, nanomaterials exhibit unique colloidal properties and functions that differ from their bulk counterparts (Schultz et al. 2014). For example, nanomaterials have a high surface area-to-volume ratio and, hence, have more exposed atoms on their surfaces compared to bulk materials (Pietroiusti, Magrini, and Campagnolo 2014). Therefore, potential nanomaterial effects may not be predicted by conventional toxicological approaches (Shvedova, Pietroiusti, and Kagan 2016). Furthermore, the volume of nanoproducts and consequent nanowastes are increasing at a much faster rate than information can be generated by nanotoxicologists and analyzed by regulators (Reinsborough and Sullivan 2011).

14.2 Transport, Transformation, and Fate of Nanomaterials

Despite recent advancements in the field of nanotoxicological assessment (Ong et al. 2014a; Petersen et al. 2015; Selck et al. 2016), there remains a gap in knowledge regarding nanobiointeractions, particularly the behavior, bioavailability, effects, and fate of nanomaterials in the environment (Schultz et al. 2014). Given that there are over 1800 nanotechnology-based consumer products currently available on the market (Vance et al. 2015), these and other nano-enabled or nano-enhanced applications will inevitably end up in the environment and interact with living organisms at some point during their product life cycle (Mitrano et al. 2015). It is noteworthy that nonengineered nanomaterials derived from sources including dust storms, forest fires, and volcanic eruptions are naturally found in the environment (Grillo, Rosa, and Fraceto 2015). However, these naturogenic nanomaterials are beyond the scope of this chapter and will not be discussed further. Rather, we shall begin by discussing the potential transport, transformation, and fate of engineered nanomaterials in the environment.

14.2.1 Environmental

The increased production of manufactured nanomaterials means that they will come in contact with the natural environment through various routes including the use, recycling, and/or disposal of nanotechnology-based consumer products, discharge of

effluent from wastewater treatment and industrial manufacturing plants, controlled release of nanoformulations for environmental remediation efforts (Dhar Dwivedi et al. 2015), and/or accidental spills associated with production or transport (Gottschalk and Nowack 2011). Nanoadditives in food, personal care, and other household products that are flushed as feces or urine, washed off skin, or poured down the drain enter wastewater treatment plants and subsequently the environment as treated effluent liberated into surface waters or as incinerated wastes, landfill solids, or biosolids derived from sewage sludge applied to agricultural land (Weir et al. 2012). Wastewater treatment plant processes may effectively remove carbon-based nanomaterials from effluent, whereas metal and metal oxide nanomaterials may persist and be discharged into freshwater environments (Gottschalk and Nowack 2011). Limited information is available regarding the behavior of nanomaterials in incinerated wastes, and data regarding nanomaterials in landfill leachate are scant (Bolyard, Reinhart, and Santra 2013). Certain nanomaterials such as zerovalent iron are typically injected directly into the subsurface environment to remediate groundwater contaminated with chlorinated solvents (Gottschalk and Nowack 2011). The release of nanomaterials in aerosol sprays is instantaneous when applied, whereas nanomaterial escape from emulsions or suspensions is expected within hours (Gottschalk and Nowack 2011). The immediate release of nanomaterials embedded in plastic or polymer matrices is improbable; however, the products themselves may be used outside or end up in landfills and degrade or be transformed upon exposure to environmental conditions (Mitrano et al. 2015). Exterior paint containing nanomaterials, for example, can wash off facades by rainwater, enter storm water runoff, and subsequently be transported to aquatic environments (Gottschalk and Nowack 2011). Most nanomaterials released from disposed products are present in matrix-bound form, but some can be freed by mechanical abrasion and material aging and exist as individual nanomaterials in the environment (Gottschalk and Nowack 2011).

Upon release into the environment, nanomaterial-containing products may be exposed to natural sunlight and degrade due to thermal and photooxidative processes (Gottschalk and Nowack 2011). Other abiotic factors including ionic strength, natural organic matter (NOM), and pH are known to facilitate the transformation of nanomaterials in aquatic environments (Dhar Dwivedi et al. 2015). Biotic interactions with animals, plants, fungi, and microorganisms can also affect nanomaterial transformation (Dhar Dwivedi et al. 2015). The behavior of nanomaterials in the aqueous phase may also be influenced by rainfall or other weather events that change the hydrology of NOM, suspended particles, and salinity of natural waters (Handy et al. 2008; Mitrano et al. 2015). Furthermore, some nanomaterials may settle in the water column and interact with benthic organisms while those that remain suspended will be transported considerable distances in the environment (Baalousha et al. 2008). Currently, analytical methods and techniques that detect and quantitatively monitor manufactured nanomaterials in different environmental compartments are lacking; therefore, reliable data regarding realistic environmental concentrations of nanomaterials are scarce (Gottschalk and Nowack 2011). Arguably, most nanotoxicity tests are not designed to assess the potential hazards of nanomaterials using environmentally relevant concentrations but are aimed instead at elucidating the mechanisms underlying nanomaterial toxicity (Krug 2014). Although actual environmental concentrations and production/use quantities of nanomaterials are largely unknown, modeled or predicted estimates have been reported elsewhere (Cowie et al. 2014; Gottschalk

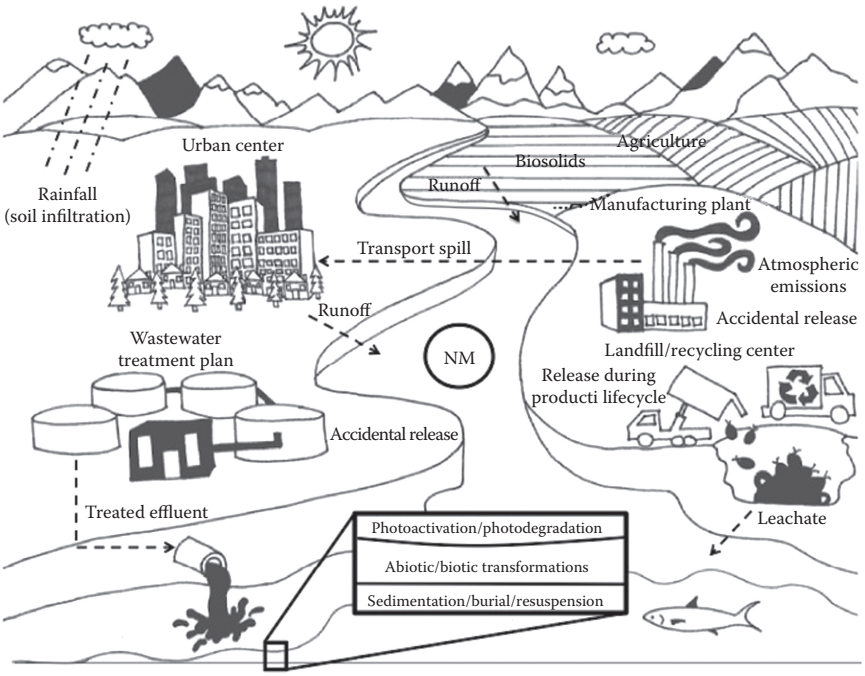


FIGURE 14.1 Illustrative overview of the probabilistic sources, transport routes, and key processes that determine the fate and behavior of engineered nanomaterials in freshwater environments.

et al. 2009, 2010; Gottschalk, Sun, and Nowack 2013; Piccinno et al. 2012). It is clear that aquatic organisms will likely be or have been exposed to engineered nanomaterials, potentially resulting in negative impacts on ecosystem health. Some of the aforementioned sources, transport routes, and key processes that determine the fate and behavior of engineered nanomaterials in freshwater environments are depicted in Figure 14.1.

14.2.1.1 Recommendations for Future Econanotoxicological Assessments

Our recommendations are not ranked based on order of importance but are rather intended to provide guidance for future experimental design and execution, as well as data reporting to improve the accuracy of nanotoxicity assessments. While this chapter highlights the progress related to nanotoxicological evaluation, further research is needed to ensure the safe use and disposal of nanomaterials. Several knowledge gaps regarding the potential hazard of water-dispersed nanomaterials need to be addressed in order for risk assessors to accurately predict their potential impact on freshwater organisms. To meet these needs, we recommend that nanotoxicologists move away from controlled mechanistic-oriented laboratory studies toward those that explore more complex, environmentally realistic matrices and exposure scenarios. Future nanotoxicology studies should attempt to address the potential effects resulting from a combination of chronic exposure and modeled or predicted environmental concentrations.

According to Amara's (1972) law, "we tend to overestimate the effect of a technology in the short run and underestimate the effect in the long run." Therefore, it is important to study lower concentrations over longer time periods because the physicochemical characteristics and toxic potential of nanomaterials may change as they age or transform in the environment (Mitrano et al. 2015). Although nanotoxicologists often employ high (i.e., mg/L) nanomaterial concentrations indicative of environmental spills in their studies (Felix et al. 2013, 2016), predicted environmental concentrations in surface water and wastewater treatment plant effluent are much lower (i.e., in the ng/L to µg/L range) (Maurer-Jones et al. 2013). Until reliable real-time detection techniques and monitoring instruments become available to accurately quantify engineered nanomaterials in the environment, nanotoxicologists should examine nanomaterial concentrations below and above modeled or predicted environmental concentrations to facilitate risk assessments (Oberdörster et al. 2005). Similarly, the thorough characterization of nanomaterial suspensions before, during, and after the experimental period is recommended. Moreover, natural waters obtained from local sources that contain NOM should be used instead of synthetic or pristine media to disperse nanomaterials for aquatic nanotoxicity studies. Synthetic or pristine media are often made fresh, yet nanomaterials can age in the environment, and thus, their physiochemical characteristics including dissolution can change upon aging (Mudunkotuwa, Pettibone, and Grassian 2012). The role of natural (e.g., NOM) and human-made (e.g., poly[acrylic acid]) surface coatings in determining nanomaterial phototoxicity also warrants further study. Nanomaterials including cerium dioxide, iron oxide, single-walled carbon nanotubes, titanium dioxide, and zinc oxide exhibit photocatalytic properties (Alarifi et al. 2014; Dahle and Arai 2015; Felix et al. 2017; Ma, Williams, and Diamond 2013; Valdiglesias et al. 2016). The polymer-coated and uncoated forms of these nanomaterials are available and could thus be used as models to close this gap in knowledge. Given the insufficient amount of information provided in the literature, reports that address ultraviolet (UV)-mediated toxicity of functionalized nanomaterials under solar radiation are needed (Ma, Williams, and Diamond 2013). Finally, nanotoxicologists that seek to represent realistic environmental conditions should consider using flow-through rather than static exposures, and all researchers must perform numerous controls (e.g., ionic metal, bulk material, and nanomaterial-assay interference) (see Section 14.4) as appropriate for their scientific hypotheses, objectives, and questions. Our recommendations have been summarized in Figure 14.2.

14.2.2 Biological

Toxicokinetics is a term used to describe how a substance (e.g., a nanomaterial) enters the body (i.e., absorption) and its fate afterward (i.e., distribution, metabolism, and excretion) (Shi et al. 2013). Direct interaction and interference with biological systems are expected because the sizes of nanomaterials and cellular machinery are comparable (Shvedova, Pietroiusti, and Kagan 2016). Nanomaterials are too big to be taken up by ion or other transporters, and although passive diffusion cannot be ruled out as a possible uptake mechanism for certain lipophilic nanomaterials (Handy et al. 2008), most are likely internalized by cells via active processes (Faille et al. 2012; Firdessa, Oelschlaeger, and Moll 2014; Kuhn et al. 2014). In the following subsections, we discuss the toxicokinetics of nanomaterial transportation, the interactions between nanomaterials and living systems, and the fate of nanomaterials both *in vitro*

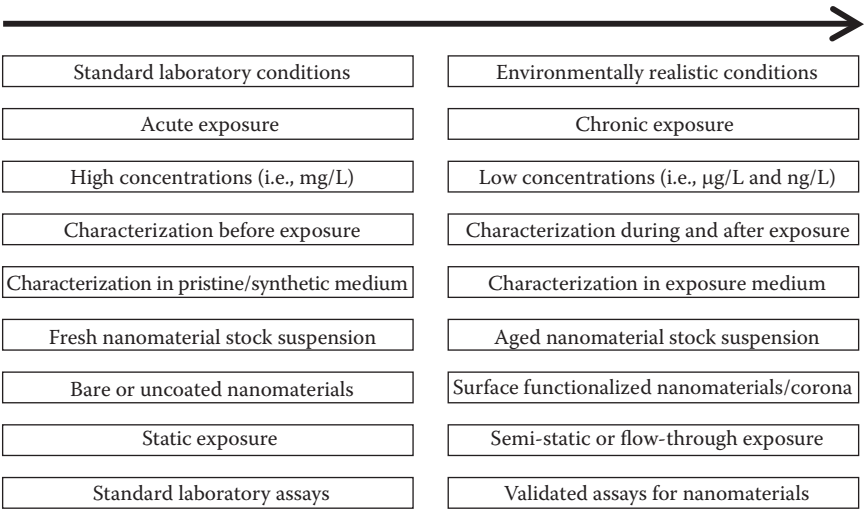


FIGURE 14.2 Summary of recommendations for future econanotoxicological assessments. Arrow represents the shift in research focus.

and *in vivo*. Specifically, we will introduce potential cellular transport pathways for nanomaterials (absorption) and their fate inside cells; describe how pathways and processes for nanomaterial uptake are studied; and finally, explore the distribution, metabolism, and excretion of nanomaterials. Although there are many fundamental similarities among mammals and fish (see Section 14.7), differences between these vertebrates can vary considerably, and some mammalian-specific traits cannot be assessed using fish model systems (Castaño and Gómez-Lechón 2005). Moreover, distribution, metabolism and excretion processes in fish have been well-described elsewhere (Tierney et al. 2013) and are thus not discussed in great detail.

14.2.2.1 Absorption and Uptake

The term *absorption* describes how nanomaterials translocate from the external environment or initial site of exposure to the internal milieu of a biological system (Li et al. 2010). Nanomaterials enter the human body either directly from injection (e.g., subcutaneous and intramuscular) or indirectly via pulmonary (i.e., inhaled into the lungs), oral (i.e., consumption of contaminated food or drink), and/or dermal (i.e., interaction between nanomaterials and skin) exposure (Li et al. 2010). In fish, the rate of xenobiotic (i.e., substances foreign to the body) absorption is increased by the fact that the gill has different epithelial tissue characteristics including a much larger surface area per gram animal, a shorter diffusion distance, and high relative rates of blood flow (Tierney et al. 2013). Two important processes are necessary for absorption to occur at the cellular level: a nanomaterial must first adsorb onto or come in close spatial proximity to the cell surface and, secondly, the nanomaterial has to be internalized into the cell (Landfester and Ostafin 2008) after moving across the apical membrane.

Xenobiotics utilize several mechanisms to traverse epithelial cells including passive diffusion and active transport (Tierney et al. 2013). Endocytosis is an energy-dependent

process by which cells internalize nutrients and signaling molecules allowing them to acquire energy and talk to each other, respectively (Oh and Park 2014), and is the major route of nanomaterial translocation across cell membranes (Behra et al. 2013). Endocytosis has been thoroughly characterized in mammalian cells (Engqvist-Goldstein and Drubin 2003) and is generally classified into phagocytosis or cell eating of large particles and pinocytosis or cell drinking of fluids, solutes, and suspensions containing small particles (Kou et al. 2013; Khalil et al. 2006). Mammalian phagocytic cells including dendritic cells, macrophages, monocytes, neutrophils, and mast cells utilize phagocytosis to internalize materials >0.5 μm in size including bacteria, yeast, and dead cells (Khalil et al. 2006; Oh and Park 2014). Using a rat basophilic leukemia-2H3 mast cell line, Ede et al. (2015) reported a strong association between fluorescein isothiocyanate-functionalized rosette nanotubes and immunoglobulin E receptors (FcεRI) located on the cell membrane surface and confirmed the internalization of these carbon-based nanomaterials. Pinocytosis occurs in all types of cells (Khalil et al. 2006) and functions to absorb biological fluids from the extracellular environment (Oh and Park 2014). Pinocytosis is typically divided into four morphologically distinct pathways including clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CavME), macropinocytosis (MP), and clathrin- and caveolae-independent endocytosis (Kou et al. 2013); the latter pathway is outside the range of this chapter and will therefore not be discussed further. CME, CavME, and MP routes are distinguished by the composition of the vesicular coat (if present), size of the formed vesicle, proteins involved, fate of the internalized ligand, and cell type (Khalil et al. 2006; Kou et al. 2013). Conversely, the intracellular fate of a nanomaterial depends on the endocytotic pathway selected for transport into the cell (Kou et al. 2013).

Researchers typically use a combination of inhibitors and fluorescent biomarkers (i.e., molecular probes) to determine whether the corresponding pathway plays a role in the cellular internalization of nanomaterials and to study the fate of nanomaterials within a cell, respectively (Kou et al. 2013). Given that endocytosis is an energy-dependent process, low temperature and ATPase inhibitors (e.g., sodium azide) can be used to distinguish nonendocytotic pathways (Kou et al. 2013). The inhibitors chlorpromazine (CPZ), genistein, and wortmannin (WN) are commonly employed to block CME, CavME, and MP pathways, respectively (Mercer and Helenius 2009; Nabi and Le 2003; Zhang and Monteiro-Riviere 2009), to confirm whether the corresponding pathway was exploited by nanomaterials to enter cells. However, these pharmacological inhibitors may induce nonspecific effects, and efficiencies vary across different cell types (Dutta and Donaldson 2013). For example, CPZ can inhibit both CME and clathrin-independent endocytosis in some cells leading to unintended consequences (Dutta and Donaldson 2013). Therefore, fluorescent markers have been routinely used alongside endocytosis inhibitors to confirm the internalization pathway involved and to provide more convincing evidence (Kou et al. 2013). Transferrin, cholera toxin B subunit, and dextran are known to be internalized through CME, CavME, and MP pathways, respectively (Liu et al. 2010; Patel and Insel 2009; Santos, Teixeira, and Rodrigues 2000), and are thus often used as markers of these pathways (Kou et al. 2013) when conjugated to fluorescent molecules (i.e., fluorophores). However, these markers may use different pathways upon entering a cell lacking specific endocytotic machinery (Kou et al. 2013) such as caveolin-/caveolae-deficient HepG2 cells (Fujimoto et al. 2000). The cellular uptake of fluorescently labelled nanomaterials

can be visualized by two- and three-dimensional computer-rendered confocal microscope imaging (Kou et al. 2013) and quantified by flow cytometry (Firdessa, Oelschlaeger, and Moll 2014). Organelle dyes including nonfixable LysoTracker can be used to detect the colocalization of fluorescently labelled nanomaterials in live cells using confocal microscopy (Van der Velden, Bertoncello, and McQualter 2013). Although evidence supporting nanomaterial uptake by cells is mounting (Firdessa, Oelschlaeger, and Moll 2014; Kuhn et al. 2014; Monti et al. 2015), it is debatable whether cellular internalization is a prerequisite for toxicity (Li et al. 2015a).

14.2.2.1.1 Recommendations for Future Cellular Uptake Methods

A common misconception among researchers is that an endocytosis inhibitor possesses an absolute specificity toward the pathway of interest (Ivanov 2008). Since many endocytosis inhibitors are known to induce nonspecific effects and undesirable modes of action (Dutta and Donaldson 2013), we recommend using more than one type of drug with the same desired function to verify the results of future mechanistic-oriented studies. For example, chloroquine, monensin, or dynasore could be used alongside CPZ to inhibit CME (Dutta and Donaldson 2013). However, dynasore may affect the actin cytoskeleton (Dutta and Donaldson 2013), and the reorganization of these filaments may disrupt uptake processes, leading to an erroneous interpretation of results (dos Santos et al. 2011). Therefore, a cytoskeletal stain (e.g., rhodamine phalloidin) should be used in conjunction with the inhibitor dynasore to assess possible structural damage. Among the different methods of CavME inhibition, filipin and nystatin produce the fewest nonspecific effects (Ivanov 2008). These antibiotics disrupt the structure and function of cholesterol-rich membrane domains, and decreasing the concentration of the drug can minimize potential side effects such as membrane permeabilization (Ivanov 2008). Although phosphoinositide 3-kinase inhibitors such as WN are widely employed to inhibit MP, they can effectively block the uptake of ligands involved in CME and CavME pathways (Ivanov 2008). We also recommend determining whether some combination of endocytosis inhibitors completely abolish nanomaterial uptake to gain knowledge of their cross-reactivity and specificity. Finally, the proton-sponge hypothesis describes how nanomaterials could potentially escape from lysosomes (see Section 14.3.1) (Boussif et al. 1995). Therefore, the fate of nanomaterials within other intracellular compartments including the endoplasmic reticulum, Golgi apparatus, and mitochondria should be subsequently investigated using molecular probes such as BODIPY™ FL Glibenclamide, CellLight™ Golgi-GFP, and MitoTracker™ Green FM, respectively.

14.2.2.2 Distribution, Metabolism, and Excretion

Distribution, metabolism (biotransformation), and excretion of nanomaterials in fish is poorly understood and information about these mechanisms is almost entirely lacking (Handy et al. 2008). Although knowledge of the body distribution of nanomaterials in fish is hindered by labor-intensive and time-consuming techniques (e.g., electron microscopy) for the measurement of nanomaterials in tissues, fluorescently labelled nanomaterials are increasingly being utilized in toxicokinetic studies to fill these gaps (Handy et al. 2008). Once a brachial epithelial cell internalizes individual or small groups of nanomaterials (Farkas et al. 2011), the next step in biological uptake is the transcellular transport through the cell to the blood (Handy et al. 2008). Following

absorption, nanomaterial distribution or translocation mainly occurs via the fish's circulatory system. A hydrophilic nanomaterial may have sufficient water solubility to be dissolved and transported in the aqueous plasma of the blood, whereas a more non-polar hydrophobic/lipophilic nanomaterial may associate with other components of the blood including cholesterol, triglycerides, and plasma proteins such as albumin (Tierney et al. 2013). Depending on their physicochemical properties (e.g., surface charge, size, and water solubility), nanomaterials may be stored in different organs with consequential bioaccumulation (Wang et al. 2013). The gill, gut, kidney, and liver are considered to be the primary target organs for nanomaterial toxicity in fish (Handy et al. 2008). Nanomaterials in liquid phase could directly interact with gill lamellae (Handy et al. 2008) because a fish must have a continuous flow of water passing over its gills. In addition, nanomaterial contaminated water drunk by freshwater fish could gain access to the gut mucosa and subsequently the liver and kidneys (Handy et al. 2008).

Biotransformation (i.e., the enzymatic conversion of a parent xenobiotic to its metabolite) predominantly occurs in the liver of fish and, to a lesser extent, in the gastrointestinal tract, gill, (Tierney et al. 2013) and kidneys (Bury, Schnell, and Hogstrand 2014). The main purpose of biotransformation is to enhance water solubility of the xenobiotic for excretion by introducing hydrophilic functional groups (e.g., -COOH, -OH, and/or -SH) to the parent molecule during phase I reactions or through conjugation of an endogenous molecule (e.g., glutathione [GSH], glucuronic acid, and/or sulfate) with the original compound or its phase I metabolite during phase II reactions (Tierney et al. 2013). Assuming that nanomaterials reach the liver and that metabolic processes affect nanomaterials, the dissolution of metal oxide nanomaterials (see Section 14.3.2) may occur while carbon-based nanomaterials could be oxidized or reduced by phase I enzymes (e.g., cytochrome P450 monooxygenases) leading to phase II conjugation reactions (e.g., acetylation, glucuronidation, and methylation) on the surface or surface coating of the nanomaterial (Handy et al. 2008; Tierney et al. 2013). The nanomaterial may not even be metabolized if it is small enough or if it already has a hydrophilic surface (Handy et al. 2008). Furthermore, biotransformation by cytochrome P450 monooxygenases might not result in detoxification and/or excretion (Tierney et al. 2013).

Eggs, milt, mucous, and scales are considered minor excretory routes in fish (Tierney et al. 2013). Nanomaterials and other substances transported from the blood to the kidneys and liver are primarily excreted into the surrounding water through urine and feces, respectively (Handy et al. 2008). Although elimination through the gills is largely determined by the hydrophobicity of the compound, fish rely on biotransformation processes to excrete water-soluble metabolites via the kidney and liver under most conditions. It remains unknown whether vesicles within gill epithelial cells are trafficked from the basolateral membrane to the apical membrane, and it is unlikely that nanomaterials larger than a few nanometers would be able to traverse the glomerular filtration barrier (Handy et al. 2008). Given that the gill is a major site for metabolic waste excretion (Bury, Schnell, and Hogstrand 2014), the cellular exocytosis of nanomaterials from cells cannot be ignored and warrants further investigation. Apart from branchial or renal clearance, hepatobiliary excretion into the bile is expected to be the most likely mechanism involved in the removal of nanomaterials or their metabolites from a fish (Handy et al. 2008). Clearance from hepatocytes involves the fusion of secretory vesicles at the plasma membrane, and given that these vesicles are ~200 nm in size, exocytosis of nanomaterials or their metabolites via the biliary system is the most plausible route (Hampton et al. 1988; Handy et al. 2008).

Improved knowledge of the mechanisms by which nanomaterials enter and exit cells could lead to new insights into toxicokinetics in fish and other freshwater organisms.

14.3 Factors Modulating Nanomaterial Toxicity

The characteristics of both the nanomaterial and environment are important in determining toxicity. Over the last decade, nanotoxicologists have gained a better understanding of the physical and chemical characteristics of nanomaterials and have identified a suite of properties that modulate nanomaterial toxicity to aquatic organisms. Several factors affect cellular import mechanisms, intracellular trafficking, nanobiointeractions, and toxicity of nanomaterials; the most important determinants are predestined by the nanomaterial itself or its surrounding environment (Firdessa, Oelschlaeger, and Moll 2014; Nel et al. 2009). Some interrelated factors intrinsic to the nanomaterials themselves (e.g., size, agglomeration, charge, dissolution, and surface functionalization) and extrinsic environmental factors including NOM and UV light that modulate nanomaterial toxicity are discussed in the following subsections.

14.3.1 Size, Agglomeration, and Surface Charge

Monitoring the sizes of nanomaterials at the beginning, middle, and end of the experimental period is essential to better predict their distribution, bioavailability, and fate in aquatic environments (Schultz et al. 2014). Moreover, the size of a nanomaterial largely determines its interaction with biological systems (Jiang, Oberdörster, and Biswas 2009). Many studies have suggested that size matters when it comes to nanomaterial internalization by cells or organisms (Shang, Nienhaus, and Nienhaus 2014), and nanomaterials above a certain threshold size may not be able to traverse biological membranes, such as the protective chorion surrounding embryonic fish (Rawson et al. 2000). The size of the formed cellular vesicle (i.e., clathrin-coated vesicle, caveosome, or macropinosome) that engulfs a nanomaterial depends on the selected endocytosis pathway (Kou et al. 2013). Large particles (≤5 μm) are likely internalized via MP, whereas smaller particles (≤150 nm) may be taken up via CME or CavME pathways (Kou et al. 2013). However, nanomaterial size does not solely govern cellular uptake, and some researchers suggest that agglomeration and surface charge also play important roles (Kühnel et al. 2009; Sokolova et al. 2013). According to the Derjaguin–Landau–Verwey–Overbeek theory, particles tend to agglomerate or flocculate when attractive forces (e.g., van der Waals) dominate repulsive forces (e.g., electrostatic), which destabilize the suspension (Derjaguin and Landau 1941; Verwey and Overbeek 1948). Agglomeration reduces both the specific surface area of the nanomaterial available for nanobiointeractions and the stability of the nanomaterial suspension (i.e., the likelihood that nanomaterials will remain suspended in an aqueous medium) (Schultz et al. 2014). Large nanomaterial agglomerates that form in aqueous solutions will likely settle and concentrate at the bottom of the exposure vessel where adherent cells, benthic organisms, and embryos are located (Schultz et al. 2014). Various abiotic (e.g., solution pH, ionic strength, and divalent ions) and biotic (e.g., NOM) factors influence nanomaterial agglomeration kinetics (Ghosh et al. 2010; Jiang, Oberdörster, and Biswas 2009). Agglomeration is suppressed when

solution pH is far from the point of zero charge because repulsive electrostatic forces overpower attractive van der Waals forces (Jiang, Oberdörster, and Biswas 2009). The electrostatic double layer surrounding a nanomaterial expands, and its surface charge increases in low ionic strength medium (Jiang, Oberdörster, and Biswas 2009). Divalent ions (e.g., calcium and magnesium) in natural waters are also known to induce nanomaterial agglomeration (Handy et al. 2008).

Cationic nanomaterials show a high affinity for negatively charged cytomembranes on the surface of cells due to electrostatic interactions (Wilhelm et al. 2003), resulting in rapid entry (Kou et al. 2013). Once taken up, cationic polymer nanomaterials may be released from lysosomes and become localized in the perinuclear region due to the proton-sponge effect (Boussif et al. 1995). This hypothesis suggests that polycations become protonated in acidic cellular vesicles which causes an influx of chloride ions and water leading to lysosomal swelling and rupture, thus providing an escape route for internalized cargo (Boussif et al. 1995). Cationic polymer nanomaterials and lipids have therefore been utilized as carriers in gene delivery applications to enhance the release of DNA into the cytosol prior to lysosomal degradation, thus allowing the gene to reach the nucleus (Khalil et al. 2006). At physiological pH, hydrophobic interactions and hydrogen bonds may facilitate the binding of nanomaterials with no charge to cell membranes (Vandamme and Brobeck 2005), whereas hydrophilic polymer coatings can prevent interactions between charge-neutral nanomaterials and cells leading to less adsorption (Kou et al. 2013). The large anionic domains of the cell surface should repel negatively charged nanomaterials (Patil et al. 2007); however, anionic nanomaterials have been proposed to cluster at positive sites (e.g., on proteins) favoring the adsorption of other nanomaterials of like charge to the plasma membrane (Wilhelm et al. 2003). Anionic poly(acrylic acid) polymers tend to agglomerate in acidic media and repel each other at neutral pH; the latter repulsive interaction results in less mucoadhesion compared to cationic dendrimers (Vandamme and Brobeck 2005).

14.3.2 Dissolution

High dissolution rates or release of ionic metal over time would be expected for metallic nanomaterials as a result of their large surface area per unit mass (Miao et al. 2010). The increased flux of metal and metal oxide nanomaterials in consumer products has raised concern as to whether the release of these nanomaterials and their accompanying dissolved metal ions will negatively impact the aquatic environment (Schultz et al. 2014). It has been well-documented that most heavy metals including cadmium, lead, nickel, and zinc are toxic to aquatic organisms (Govind and Madhuri 2014), and sublethal exposures are known to adversely affect an organism's endocrine system (Georgescu et al. 2011), growth (Farkas, Salánki, and Specziár 2002), and reproduction (Ebrahimi and Taherianfard 2011). Many nanotoxicological studies have identified the dissolution of metallic nanomaterials and the subsequent shedding of free metal ions as the primary mode of toxicity to freshwater and marine organisms (Franklin et al. 2007; Miao et al. 2010; Schultz et al. 2012) while other reports suggest that observed toxicities in fish were not exclusively attributed to dissolved metal species (Bai et al. 2010; Hao et al. 2013). Since aqueous exposure medium contains both metal-based nanomaterials and dissolved metal ions, observed toxicity is usually

the result of a combination of both components (Ma, Williams, and Diamond 2013). Humic or fulvic acids, nanomaterial agglomerates, small molecules, and surface coatings can all potentially impede dissolution rates by minimizing access of water to nanomaterial surfaces (Liu and Hurt 2010; Merdzan et al. 2014), thereby abrogating toxic effects. Conversely, high temperature and low pH both increase dissolution rates of metal nanomaterial colloids (Liu and Hurt 2010). For example, the maximum total metal ion concentration was shown to increase (by nearly 100 times) when the pH of a metal oxide nanomaterial suspension was lowered (i.e., adjusted from pH 9 to 7) (Miao et al. 2010). Given that suspension characteristics change as nanomaterials agglomerate, bind to molecules, and translocate across biological membranes, analytical techniques such as dialysis and ultracentrifugation are commonly employed to measure changes in total nanomaterial dissolution over time (Petersen et al. 2015; Schultz et al. 2014).

14.3.3 Surface Functionalization

The development of functionalized nanomaterials is central to the advancement of nanotechnology (Subbiah, Veerapandian, and Yun 2010). Various chemical methods have been employed to synthesize functionalized nanomaterials for specific use in many fields including agriculture and biomedical sciences (Liu and Lal 2015; Subbiah, Veerapandian, and Yun 2010; Xu et al. 2014). Functionalization is a technique utilized by chemists and material scientists to conjugate (i.e., physically attach or adsorb) chemical moieties such as -COOH groups or polymers onto the surfaces of nanomaterials to tailor their properties to fit specific applications (Meng, Fu, and Lu 2009; Priestly, Bartholomaeus, and Drew 2013; Subbiah, Veerapandian, and Yun 2010). Functional groups can be used to tune the hydrophobicity or hydrophilicity of the nanomaterial surface (Leung et al. 2011). Hydrophobic nanomaterials have a higher affinity for the phospholipid bilayer than hydrophilic nanomaterials and are thus expected to be efficiently internalized by a cell (Kou et al. 2013). After systematic administration *in vivo*, functionalized nanomaterials are more efficiently transported throughout the body compared to their nonfunctionalized counterparts, which, for example, has implications for targeted delivery of therapeutic agents (Subbiah, Veerapandian, and Yun 2010). Furthermore, hydrophilic polymers can suppress the interaction between the nanomaterial core and the cell surface (Kou et al. 2013). Wrapping polymers around carbon-based nanomaterials reduces their agglomeration tendencies by diminishing the attractive van der Waals forces between individual materials (Star et al. 2001), rendering them compatible with other matrices (Subbiah, Veerapandian, and Yun 2010). The dispersibility of certain carbon-based nanomaterials can be improved through the covalent attachment of functional groups or the noncovalent adsorption of polymers onto their surfaces (Zhao and Liu 2012). Some polymer coatings function to minimize or prevent the dissolution of free metal ions released from metal nanomaterials via chelation (Yang et al. 2012) while others can envelop nanomaterials with UV absorbing capabilities and repress substrate availability for free radical production without affecting their desired photocatalytic properties (Smijs and Pavel 2011). Figure 14.3 illustrates the effect of polymer coating, NOM or ecocorona, and biomolecules on nanomaterial properties. Another form of functionalization involves polymer encapsulation rather than physical adsorption onto the nanomaterial surface (Subbiah, Veerapandian, and Yun 2010). Polymeric

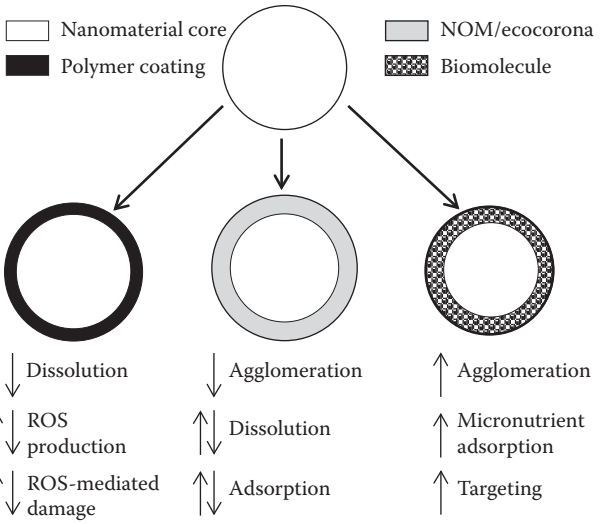


FIGURE 14.3 Physical, biophysical, and biological effects of polymer coating, NOM or ecocorona, and biomolecules on nanomaterial properties.

nanomaterials composed of biocompatible polymers have been extensively studied for use as nanosized carriers of various formulations (e.g., drugs, fungicides, and insecticides) in controlled delivery applications while metallic nanomaterials are tunable and can serve as vehicles for tracers or as contrast agents in medical imaging applications (Liu and Lal 2015; Subbiah, Veerapandian, and Yun 2010; Xu et al. 2014). The chemical functionalization of nanomaterials is not only expected to broaden the scope of potential applications but may also be used to modify nanomaterial toxicity. Therefore, each functionalized nanomaterial should be carefully examined for undesirable changes in toxicity.

14.3.4 Natural Organic Matter

Ubiquitous in natural waters, NOM is a complex and heterogeneous matrix (Dhar Dwivedi et al. 2015) formed during the microbial decomposition of plants and animals (Grillo, Rosa, and Fraceto 2015). The biopolymers and degraded products (i.e., humic and fulvic acids) that make up NOM vary in composition and molecular weight and are negatively charged due to carboxylic and phenolic functional groups (Dhar Dwivedi et al. 2015). It is well known that NOM can form an ecocorona around the nanomaterial surface that functions to minimize or prevent agglomeration through repulsive electrostatic and/or steric interactions (Ghosh et al. 2010) (Figure 14.3). This ecocorona can affect nanomaterial bioavailability to and biopersistence in aquatic organisms and thus modulates nanomaterial toxicity (Byrne et al. 2013; Westmeier, Stauber, and Docter 2016). Van Hoecke et al. (2011) observed that NOM adsorbed to the surfaces of metal oxide nanomaterials and suggested that their toxicity to algae (*Pseudokirchneriella subcapitata*) was reduced when NOM was added due to decreased bioavailability. In the same manner, NOM can adsorb to a metal oxide nanomaterial and inhibit its dissolution by blocking the interaction between

the nanomaterial surface and water molecules (Ma, Williams, and Diamond 2013) (Figure 14.3). Interestingly, Ong et al. (2017) demonstrated that humic acid both increased zinc oxide nanomaterial dissolution by complexing Zn^{2+} and ameliorated nanomaterial-induced developmental toxicity in zebrafish (*Danio rerio*).

14.3.5 Ultraviolet Light

Stratospheric ozone provides an important protective shield against the harmful components of solar radiation including UVC (200–280 nm) and UVB (280–320 nm) wavelengths shorter than 290 nm (Charron et al. 2000); UVA (320–400 nm) wavelengths pass through the ozone layer (Prasad 2012). The inevitable release of manufactured nanomaterials into the environment has raised concern as to whether nonionizing UV radiation will mediate deleterious health effects. Most nanotoxicity tests performed in laboratories under controlled conditions are considered effects- or mechanistic-oriented studies (Mitrano et al. 2015; Schultz et al. 2014). However, certain nanomaterials may be inert under ambient fluorescent laboratory lighting and become toxic due to UV-induced transformations in the environment (Wiesner et al. 2009). Fluorescent lamps that illuminate laboratories emit negligible or no UV radiation (Safari et al. 2015), yet certain nanomaterials are wide bandgap semiconductors (Li et al. 2015b). When a semiconductor absorbs enough energy (i.e., photon energy must be at or above the bandgap energy of the material) from sources such as natural sunlight, a valence electron is bumped up to the conduction band resulting in a free electron and a positively charged area or hole (Ma, Brennan, and Diamond 2012). This electron–hole pair reacts with dissolved oxygen and water in an aqueous environment to efficiently produce reactive oxygen species (ROS) (Ma, Brennan, and Diamond 2012) including hydrogen peroxide (H_2O_2), hydroxyl radicals (OH•), and superoxide anions $\left(O_2^-\right)$ (Cerutti 1985). These ROS contain unpaired valence electrons that make them highly reactive and capable of inducing severe cellular damage (Dahle and Arai 2015). Moreover, certain nanoscale semiconductors can convert harmful organic contaminants into carbon dioxide and water due to their photocatalytic properties (Iqbal et al. 2016; Mahlambi, Ngila, and Mamba 2015). The photoreactivity of metal oxides with a cerium, iron, titanium, or zinc core (see Sections 14.6.1 through 14.6.4) (Khan, Adil, and Al-Mayouf 2015; Valdiglesias et al. 2016) are directly related to their UV-induced toxicities and may thus be the primary mechanism of toxicity under environmentally realistic conditions (Ma, Williams, and Diamond 2013). Although endogenously produced ROS play a central role in normal cellular metabolic processes (Weydert and Cullen 2009), high intracellular concentrations of ROS generated by nanoscale semiconductors that undergo oxidation may overwhelm the antioxidant defense system resulting in irreversible damage to carbohydrates, membrane lipids, nucleic acids, and proteins (Riley 1994). Therefore, an enzymatic antioxidant system has evolved to ameliorate ROS-induced adverse effects (Riley 1994). The vital roles of defense enzymes including catalase (Cat), glutathione peroxidase (GPx), and superoxide dismutase (Sod), as well as antioxidants such as GSH in the mitigation of oxidative stress on DNA, lipids, and proteins, have been extensively reviewed (Gutteridge 1995; Kelly et al. 1998; Sies 2007). Figure 14.4 shows a diagrammatic representation of the relationship among the antioxidant defense system, ROS, and oxidative damage. Improved knowledge of the unique properties of nanosized bandgap semiconductors will enhance our understanding of their potential phototoxic effects and

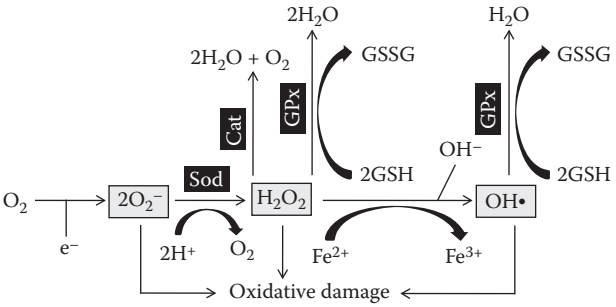


FIGURE 14.4 Diagram showing the interrelationship between antioxidant defense enzymes (*black*), ROS (*gray*), and oxidative damage. Free radicals including O_2^- are catalyzed to H_2O_2 by Sod. H_2O_2 are converted into water through the action of Cat or GPx. H_2O_2 not detoxified by these processes can be further reduced to OH• through the Fenton reaction and then converted into water, using GSH as a reductant. All ROS are capable of inducing oxidative damage to DNA, lipids, and proteins. (Felix, L. C. et al., *Environmental Science: Nano*, 4, 658–669, 2017. Reproduced by permission of The Royal Society of Chemistry.)

facilitate proper risk assessment (Schultz et al. 2014). Failure to consider this mode of action could severely underestimate the potential risk associated with the release of photoreactive nanomaterials into the aquatic environment.

14.4 Metrics of Nanotoxicity Testing and Associated Challenges

The combination of hazard and exposure is the major determinant of risk (Pitkänen, Kangas, and Vartiainen 2014). Toxicity testing plays a fundamental role in hazard evaluation (Warheit and Donner 2010); however, knowledge of environmental exposure is limited (Gottschalk and Nowack 2011). Accurate and efficient toxicity testing is necessary to determine whether nanomaterials should fall under regulations mandated for traditional bulk chemicals (Schultz et al. 2014). Conventional *in vivo* and *in vitro* toxicity tests for traditional chemicals are commonly employed due to their familiarity and interpretability (Balbus et al. 2007). However, these traditional testing methods may not be suitable for nanomaterials and could lead to an inaccurate assessment of toxicity and thus risk (Ong et al. 2014a; Schultz et al. 2014). Moreover, it is highly debated whether conventional methods originally designed for soluble chemicals are appropriate for nondispersible colloidal materials like most nanomaterials (Schultz et al. 2014).

To accurately assess the potential hazard associated with engineered nanomaterials, reliable toxicity screening methods are needed to determine how their physicochemical properties relate to differential biological or ecological responses (Oberdörster et al. 2005). The characterization of nanomaterial properties should be as comprehensive as possible to ensure that results are accurate, reliable, reproducible, and interpreted correctly for the establishment of regulations related to nanomaterial safety (Jiang, Oberdörster, and Biswas 2009). The measurement of every possible nanomaterial characteristic is not feasible (Jiang, Oberdörster, and Biswas 2009). Therefore, a list of physicochemical properties that must be addressed in nanotoxicological

studies including size, surface charge, material chemistry, and photocatalytic activity (Rasmussen et al. 2016) has been identified by the Organisation for Economic Co-operation and Development (OECD) Chemicals Committee Working Party on Manufactured Nanomaterials (WPMN). The noted physicochemical properties have been discussed in detail earlier (see Section 14.3). We emphasize that these and other nanomaterial properties should be characterized in the exposure medium (Schultz et al. 2014).

Standard reference materials prove useful for comparisons among nanomaterials and between instruments and laboratories, for instrument calibration and validation, and for nanotoxicity test controls. Analytical techniques including atomic force spectroscopy, dynamic light scattering (DLS), field flow fractionation, nanoparticle tracking analysis, and transmission electron microscopy (TEM) are commonly used to characterize nanomaterial properties. DLS is a well-established, noninvasive analytical technique that is often employed to measure the size, size distribution, and surface charge of nanomaterial agglomerates in a dispersed medium (Boluk and Danumah 2014); however, this characterization method has certain disadvantages. For example, data obtained from DLS do bias toward large particles that scatter more light compared to small particles (Murdock et al. 2008), and this technique may not provide meaningful size distribution measurements for high aspect ratio or cylindrically shaped nanomaterials (Rasmussen et al. 2016). Therefore, data acquired by DLS should be validated using standard reference materials and interpreted with caution. In addition, polymeric nanomaterials cannot be observed by TEM imaging. In lieu of TEM and DLS, nanotoxicologists may find analytical ultracentrifugation useful for the characterization of small (1–10 nm) polymeric nanomaterials (Diaz, Peyrot, and Wilkinson 2015).

Due to their small size and large surface area-to-volume ratio, nanomaterials can adsorb and effectively sequester soluble molecules including carbohydrates, growth factors, hormones, and nutrients from biological media that may be essential to an organism's survival (Brunner et al. 2010; Schultz et al. 2014). Biomolecules and small soluble molecules including salts/ions, serum proteins, and vitamins present in cell culture medium will inevitably interact with nanomaterials forming a biomolecular corona that cloaks the nanomaterial surface and can change its agglomeration kinetics (Figure 14.3), making the characterization of nanomaterials in physiological matrices difficult (Baolog et al. 2015; Monopoli et al. 2012; Schultz et al. 2014). Furthermore, complex biological fluid might also induce nanomaterial agglomeration, and certain nanomaterials can adsorb to and thus deplete essential micronutrients (e.g., folic acid) from these multicomponent aqueous matrices, potentially leading to reduced cell viability (Guo et al. 2008; Kühnel et al. 2009).

High-throughput *in vitro* assays are widely used by toxicologists to quantitatively determine the biochemical and physiological responses of organisms to xenobiotics (Shukla et al. 2010). Although researchers have begun to realize that nanomaterials can interfere with standard toxicity assays, the majority of published nanotoxicology literature fails to report or account for potential nanomaterial-assay interference (Ong et al. 2014a; Petersen et al. 2015). Intrinsic optical absorbance or fluorescence of nanomaterials, adsorption of nanomaterials to assay components, and high nanomaterial concentrations increase the likelihood of nanomaterial-assay interference, which can lead to false-positive results, and an inaccurate assessment of toxicity will ensue (Ong et al. 2014b; Schultz et al. 2014). Due to their quantum properties, nanoscale

semiconductors typically absorb or fluoresce at wavelengths in the UV region, which may overlap with the wavelength monitored in the assay (Kroll et al. 2012). As mentioned earlier, nanomaterials can adsorb to molecules, and this interaction may hinder the proper transformation of indicator molecules used in classic endpoint assays (Wörle-Knirsch, Pulskamp, and Krug 2006). The final nanomaterial concentration present in a biological sample may not be lowered by repeated washes, centrifugations, or filtrations that attempt to remove noninternalized nanomaterials adsorbed onto tissue and cell surfaces; therefore, controlling for nanomaterial interference with assay measurements is essential (Ong et al. 2014a). Fluorescence and absorbance measurements of the nanomaterials themselves coupled with the assessment of biochemical assay results before and after nanomaterial addition to kit reagents are some of the steps nanotoxicologists need to take to avoid nanomaterial-assay interference (Ong et al. 2014a).

Nanotoxicologists have begun to collaborate with cell biologists, chemists, engineers, material scientists, and physicists to explore the role of different physicochemical properties in determining nanomaterial toxicity, and by introducing appropriate controls into their experimental design (e.g., bulk material, different nanomaterial sizes, ionic metal, and surface coatings without core material), researchers are now more equipped conceptually and methodologically to accurately assess and interpret their results (Schultz et al. 2014). A number of informative literature reviews have been published in recent years that present a series of recommendations aimed at alleviating some of the challenges associated with nanotoxicity testing (Baun et al. 2008; Ong et al. 2014a; Petersen et al. 2015; Schultz et al. 2014; Taurozzi, Hackley, and Wiesner 2011). Taken together, a variety of factors must be considered by nanotoxicologists to make accurate toxicological assessments. As a result, regulators who seek to minimize risk will be able to extrapolate useful information from these well-designed and executed studies to better predict nanomaterial toxicity.

14.5 Test Guidelines for Evaluation and Interpretation of Nanotoxicological Effects

The regulation of nanomaterials is still in its infancy (Ngarize, Makuch, and Pereira 2012). At present, there are few nanomaterial-specific regulations, and many of these involve reporting requirements for any substantial new use of the base material (Canadian Environmental Protection Act 2015). Generally, most nanomaterials are regulated in the same manner as their corresponding bulk counterparts (Pitkänen, Kangas, and Vartiainen 2014). Moreover, hazard and risk assessment of nanomaterials remains at the development stage (Pitkänen, Kangas, and Vartiainen 2014), and advancement requires the standardization of toxicity tests to identify deleterious human health and environmental effects associated with exposure to nanomaterials and other new substances (Hund-Rinke et al. 2016). Although no nanomaterial-specific standards or comprehensive, explicit, and systematic evidence-based guideline for the risk assessment of nanomaterials currently exists (Peixe et al. 2015; Pitkänen, Kangas, and Vartiainen 2014), a couple of guidance documents are available (OECD 2012a; SCENIHR 2009). Several OECD test guidelines (TGs) and guidance documents for traditional chemicals can be generally applied to nanomaterials; however, some need to be adapted or newly developed to address endpoints more suited for nanomaterial testing (Rasmussen et al. 2016). Recently, Hund-Rinke et al. (2016)

proposed nanomaterial-specific modifications for several ecotoxicity-related OECD TGs including acute toxicity measurements using the crustacean *Daphnia magna* (TG 202) and developmental toxicity assays with zebrafish (TG 210) based on testing of silver and titanium dioxide nanomaterials. One nanomaterial-specific OECD TG is publically available, which provides direction for sample preparation and dosimetry for nanomaterial testing (OECD 2012b). The intent of the latter TG was not to provide detailed method descriptions for individual toxicity tests. Furthermore, nanomaterial-specific issues including appropriate characterization methods (Rasmussen et al. 2016), biomolecule and small molecule interactions (Nel et al. 2009), and assay interference (Ong et al. 2014a) are not covered in present TGs or guidance documents. Unconventional approaches to nanotoxicity testing are essential to accurately assess the potential hazard of nanomaterials and to safely use them in real-world applications.

14.6 Toxicological Assessment of Nanomaterials

Nanotoxicologists need to have a keen, fundamental understanding of biology, chemistry, and physics to make accurate and meaningful toxicological assessments (Schultz et al. 2014). Of the reported 1827 nanotechnology-based consumer products currently available on the market, 279 products were identified to contain either metal oxide or carbon-based nanomaterials (Vance et al. 2015), which are the most widely studied among nanotoxicologists. Five of the major OECD priority nanomaterials (OECD 2010), cerium dioxide, iron oxide, single-walled carbon nanotubes, titanium dioxide, and zinc oxide, as well as one novel nonpriority nanomaterial, cellulose nanocrystals, are examined separately in this chapter. In the following subsections, we discuss the applications and products that incorporate each of the aforementioned nonfunctionalized nanomaterials, the unique physicochemical properties that modulate their toxicity, and summarize recent research illustrating their toxic effects on aquatic freshwater organisms including algae, invertebrates, and vertebrates. Given that published research related to nanomaterial effects on marine organisms is scant and sufficient toxicological information is lacking, we will not summarize marine nanomaterial toxicology in this chapter. Studies of nanomaterial toxicity on human health using mammalian model systems are also not the subject of this chapter and have thus been excluded. The intention here is not to provide an exhaustive review of the nanotoxicological literature, but rather to draw attention to the wide diversity of nanomaterials and their effects across multiple levels of biological organization.

14.6.1 Cerium Oxide Nanomaterials

Cerium (IV) oxide (also known as ceria, cerium oxide, or cerium dioxide) nanomaterials have received much attention in recent years due to their use as fuel-borne catalysts and glass additives and use in ceramic coating and fuel cell applications (Dahle and Arai 2015). These nanomaterials are commonly used in catalytic converters of motor vehicles to reduce harmful nitrogen oxide emissions and convert carbon monoxide to carbon dioxide by releasing or absorbing oxygen in the exhaust system of an internal combustion engine (Dahle and Arai 2015). Abiotic factors including ionic

strength, NOM, and pH have been shown to affect cerium dioxide nanomaterial toxicity and their stability in natural waters (Van Hoecke et al. 2011). Using concentrations at and well above predicted environmental concentrations (0.0005–80 mg/L), Taylor et al. (2016) demonstrated that ceria nanomaterials were internalized into intracellular vesicles of the unicellular green alga *Chlamydomonas reinhardtii* but did not affect their growth at any exposure concentration tested. Manier et al. (2013) reported that nonaged cerium dioxide nanomaterials inhibited the growth of green microalga (*Pseudokirchneriella subcapitata*; 72 h half maximal effective concentration [EC_{50}] = 5.6 mg/L) and suggested that both direct physical effects and indirect membrane damage due to oxidative stress mediated the inhibitory mode of action. After exposing adult zebrafish to cerium dioxide nanomaterials (0.5 mg/L) via the water column (as opposed to oral dose by incorporation into feed pellets) for 7 days, Johnston et al. (2010) noted significant uptake of cerium in the livers of exposed fish. It should be noted that cerium dioxide is a bandgap semiconductor, and to date, only one study (Zhang et al. 2018) investigating the effects of natural sunlight containing UV light on cerium oxide-mediated toxicity has been published.

14.6.2 Iron Oxide Nanomaterials

Due to their unique biochemical, catalytic, and magnetic properties, iron oxide nanomaterials such as hematite (α-Fe_2O_3) are often employed in the biomedical field as carriers in targeted drug delivery applications, as contrast agents for magnetic resonance imaging (approved by the U.S. Food and Drug Administration in 1996) and for induced hyperthermia cancer treatments (Valdiglesias et al. 2016). Iron oxide nanomaterials are typically composed of a ferri (Fe^{3+}) or ferro (Fe^{2+}) crystalline core and a surface coating that stabilizes the material, prevents agglomeration, and enhances biocompatibility (Valdiglesias et al. 2016). Despite the rapidly growing number of clinical applications and mammalian toxicology studies involving iron oxide nanomaterials (Valdiglesias et al. 2016), information regarding their potential toxicity to aquatic organisms is limited. Zhu, Tian, and Cai (2012) associated iron oxide nanomaterial-mediated toxicity in zebrafish with both metal ion release and ROS generation. The latter study showed that iron oxide nanomaterials (≥10 mg/L) induced lethality, hatching delay, and malformations including tissue ulceration, pericardial edema, and tail curvature (Zhu, Tian, and Cai 2012). Using TEM and scanning transmission X-ray microscopy, Kwon, Nho, and Yoon (2014) observed several incidences of malformation (e.g., irregularly shaped microvilli, protrusion of epithelial cells, and dilatation of intracellular spaces) in the digestive tract of α-Fe_2O_3 and magnetite (Fe_3O_4) nanomaterial-exposed *Daphnia magna* after 48 h.

14.6.3 Titanium Dioxide Nanomaterials

Nanotitanium dioxide is a white pigment commonly added to a variety of food and personal care products including candies, chewing gums, deodorants, powdered donuts, shampoos and conditioners, shaving cremes, sunscreens, and toothpastes (Weir et al. 2012). Titanium dioxide nanomaterials are wide bandgap semiconductors with photocatalytic properties and have thus been extensively studied for the purposes of environmental remediation (Hoffmann et al. 1995; Ijadpanah-Saravy et al. 2014;

Lee and Choi 2002), particularly air purification, bacterial disinfection, and water treatment (Pelaez et al. 2012). The photocatalytic properties of titanium dioxide nanomaterials have also been exploited for self-cleaning and antifogging applications (Shi et al. 2013). Two major crystal structures of titanium dioxide, anatase and rutile, define their surface reactivity (Watanabe et al. 1999). The bandgap energies of anatase (3.2 eV/$\lambda \leq 387$ nm) and rutile (3.0 eV/$\lambda \leq 413$ nm) (Lin et al. 2006) crystalline forms of titanium dioxide nanomaterials that govern their photoreactivity may also induce or predict phototoxicity (Ma, Brennan, and Diamond 2012). The photoxicity of titanium dioxide nanomaterials to *Daphnia magna* and Japanese medaka (*Oryzias latipes*) has been shown to increase by several orders of magnitude in the presence of near full-spectrum artificial sunlight containing UV wavelengths known to activate titanium dioxide (Ma, Brennan, and Diamond 2012). Alterations in thiobarbituric acid reactive substances and total GSH levels were reported during a 14-day exposure of titanium dioxide nanomaterials (≤1 mg/L) to trout (Federici, Shaw, and Handy 2007). After a 14-day semistatic exposure to 1 mg/L titanium dioxide nanomaterials, mature adult female zebrafish produced significantly fewer viable embryos by the end of a 21-day postexposure recovery period (Ramsden et al., 2013). While few nanotoxicological studies have investigated whether the photoreactivity of titanium dioxide nanomaterials may increase the risk associated with their release into the environment (Bar-Ilan et al. 2012, 2013; Ma, Brennan, and Diamond 2012), even fewer have explored the role of surface coatings on titanium dioxide nanomaterial phototoxicity (Pan et al. 2009; Yin, Casey, and McCall 2010). Furthermore, only one study reported the *in vivo* phototoxicity of polymer-coated titanium dioxide nanomaterials (Felix et al. 2017).

14.6.4 Zinc Oxide Nanomaterials

Nanozinc oxide is a dispersible metal oxide nanomaterial currently used in a wide variety of commercial and consumer products including batteries, ceramics, electronics, glasses, industrial coatings, paints, and personal care products, namely, cosmetics and sunscreens. Prevalence in these applications is due to their unique antimicrobial, catalytic, optoelectronic, reflective, and UV absorption properties (Ma, Williams, and Diamond 2013; Schultz et al. 2014). Analogous to titanium dioxide nanomaterials, zinc oxide nanomaterials are also photocatalysts that generate ROS when photon energy from UV irradiation is equal to or greater than their bandgap energy (3.37 eV/$\lambda \leq 368$ nm); which is integral to their effectiveness in environmental remediation of pollutants and in water disinfection (Ma, Williams, and Diamond 2013; Masoumbaigi et al. 2015). The formation of ROS by zinc oxide nanomaterials and their dissolution to free zinc ions are considered to be the two primary modes of action for toxicity (Ma, Williams, and Diamond 2013). Many nanotoxicological studies have shown that uncoated zinc oxide nanomaterials are highly toxic (in the low mg/L range) to a variety of aquatic organisms (Aruoja et al. 2009; Blinova et al. 2010; Zhu et al. 2008). Zhao et al. (2013) reported a strong correlation ($R^2 = 0.90$) between ROS production by zinc oxide nanomaterials and DNA damage in 144-h postfertilization zebrafish larvae and clearly demonstrated that exposure to both zinc oxide nanomaterials (≤100 mg/L) and dissolved zinc ions altered the activities of the aforementioned antioxidant defense-related enzymes (see Section 14.3.5). Both Aruoja et al. (2009) and Franklin et al. (2007) indicated that the toxicity of zinc

oxide nanomaterials to the freshwater microalgae (*Pseudokirchneriella subcapitata*) was solely attributed to ionic metal release. Furthermore, zinc oxide nanomaterials (10 and 100 mg/L) have been shown to completely inhibit the hatching of zebrafish embryos, and this effect was most likely caused by the interaction between nanomaterials and the zebrafish hatching enzyme (see Section 14.7) (Ong et al. 2014b), thus compromising enzyme activity (Lin et al. 2011).

14.6.5 Single-Walled Carbon Nanotubes

First discovered by Iijima and Ichihashi (1993), carbon nanotubes have been described as elongated cylindrical-shaped structures with unique chemical, electronic, and physical properties (Liang and Chen 2010) that make them suitable for a broad range of applications in the fields of architecture, electronics, and optics (Zhao and Liu 2012). Carbon nanotubes are poorly dispersible in water and will likely exist at the air/water interface, depending on the degree of physical mixing that occurs in the water column (Handy et al. 2008). The rolling of a single layer of graphene forms single-walled carbon nanotubes, whereas multi-walled carbon nanotubes consist of multiple graphene layers (Zhao and Liu 2012). Although an exact mechanism of single-walled carbon nanotube toxicity has yet to be elucidated, nanotoxicologists have proposed that noted toxicity was attributed to both direct physical irritation and indirect occlusion of biological tissues such as gills (Smith, Shaw, and Handy 2007), ROS production, lipid peroxidation (Alarifi et al. 2014), and/or trace metal impurities that originate from production processes (e.g., bimetallic catalyst addition, centrifugation, isolation, and sonication) (Cheng, Flahaut, and Cheng 2007; Colvin 2003; Felix et al. 2016). Embryonic zebrafish embryos are protected from single-walled carbon nanotube agglomerates because most are too large to pass through the pore canals of the chorion (see Section 14.7) (Cheng, Flahaut, and Cheng 2007). Carboxylated single-walled carbon nanotube dispersions have been shown to generate ROS including OH•, singlet oxygen, and O_2^- in the presence of artificial sunlight (Chen and Jafvert 2010). However, amorphous carbon and metallic catalyst impurities may have contributed to ROS generation by these colloidal dispersions (Chen and Jafvert 2010).

14.6.6 Cellulose Nanocrystals

Biocompatible and biodegradable cellulosic nanomaterials are produced at low cost from many different renewable resources including algae, bacteria, and plants (Roman 2015). Due to their high water absorption capacity, mechanical strength, and stiffness, cellulose-based nanomaterials are becoming increasingly attractive for use as drug delivery vehicles, in polymer nanocomposites, and for wound dressings (Roman 2015). Although several excellent reviews covering the use of cellulose nanocrystals in biomedical applications and their toxic effects on human health are available (Jorfi and Foster 2015; Lin and Dufresne 2014; Roman 2015; Roman et al. 2009), to our knowledge, only two comprehensive toxicological assessment involving aquatic organisms exists (Felix et al. 2016; Kovacs et al. 2010). Kovacs et al. (2010) evaluated the effects of different concentrations (≤6000 mg/L) of cellulose nanocrystals on rainbow trout (*Oncorhynchus mykiss*) hepatocytes and nine aquatic species (*Ceriodaphnia dubia*, *Danio rerio*, *Daphnia magna*, *Hydra attenuate*, *Oncorhynchus*

mykiss, *Pimephales promelas*, *Pseudokirchneriella subcapitata*, *Thamnocephalus platyurus*, and *Vibrio fischeri*) using multiple endpoints (cytotoxicity, development, growth, lethality, morphology, reproduction, etc.) for up to 10 days and found the material to have low toxicity. However, simple toxicity screening rather than mechanism appeared to be the major focus of this study. Felix et al. (2016) reported reduced viability of 1G8 channel catfish (*Ictalurus punctatus*) cells following a 48-h exposure to 100 and 200 mg/L carboxylated cellulose nanocrystals and suggested that noted effects were likely attributed to nanomaterial adsorption to essential micronutrients. Given that the same exposure conditions had no effect on the expression patterns of *hypoxia-inducible factor 1 alpha subunit b*, *alpha-tocopherol transport protein*, *glutamate-cysteine ligase catalytic subunit*, and *heat shock protein 70 kDa* zebrafish genes, the underlying molecular mechanism(s) of cellulose nanocrystal toxicity remains to be elucidated (Felix et al. 2016).

14.7 Aquatic Freshwater Animal Models

In addition to the freshwater species mentioned earlier, frogs (*Rana catesbeiana*) (Hammond, Carew, and Helbing 2013), mussels (*Elliptio complanata*) (Gagné et al. 2013), and snails (*Lymnaea stagnalis*) (Croteau et al. 2014) have also been employed by nanotoxicologists in their testing methods; all of which likely have different sensitivities. Song et al. (2015) compared species sensitivities among juvenile rainbow trout, fathead minnow, and zebrafish during a 96 h semistatic exposure to spherical 50 nm copper nanomaterials and demonstrated that zebrafish were the most sensitive while rainbow trout were the least sensitive species. Incubation temperature, intrinsic physiology, and body weight were speculated to account for sensitivity differences between these salmonid and cyprinid fish (Song et al. 2015).

Numerous characteristics make fish an excellent experimental model for aquatic toxicology-, developmental biology-, and environmental science-related studies (Powers 1989). Developing fish are highly sensitive and vulnerable to toxicants and may provide advanced warning of their potential hazard upon release into aquatic environments (Powers 1989). Vardy et al. (2013) reported that white sturgeon (*Acipenser transmontanus*) yolk-sac larvae (8 days posthatch) were more sensitive to waterborne copper exposure than rainbow trout and fathead minnows at the same developmental stage. Many fish species can be easily raised and bred under laboratory conditions at a much lower cost compared to amphibian, avian, mammalian, and reptilian animal models (Powers 1989). Mammals and fish share many developmental pathways, organ systems, and physiological mechanisms (Crollius and Weissenbach 2005), making cross-species comparisons possible. For example, the basic features of gill and gut epithelium that line the external surfaces and internal body cavities of a fish are similar to the lung, kidney, and gut of other vertebrates (e.g., rodents) (Handy et al. 2008). In recent years, there has been a movement toward using cultured fish cells in toxicity testing for cost, ethical, and logistic reasons and to minimize the sheer number of animals needed for research and toxicology studies (Bury, Schnell, and Hogstrand 2014). Although fish models cannot replace traditional mammalian systems for answering certain scientific questions, they may provide a more acceptable alternative for nanotoxicity testing (Powers 1989). *In vivo* assays should augment *in vitro* tests to better understand the biological effects of nanomaterials and

to complement or validate research findings (Oberdörster et al. 2005). Zebrafish and fish gill cells were selected as examples of *in vivo* and *in vitro* aquatic animal models, respectively, and we discuss these widely used experimental systems in detail below.

Wild-type zebrafish are commonly used in nanotoxicology studies due to their well-understood biology and acceptance by regulatory authorities as a model aquatic organism (Ma, Williams, and Diamond 2013). The zebrafish is a member of the family Cyprinidae and is native to India and Pakistan. Zebrafish offer several advantages for toxicity testing over other mammalian model systems (e.g., primates, rabbits, and rodents) including economical husbandry requirements, high fecundity, low cost, short breeding cycle, small size, transparent embryos (Dai et al. 2014; Hill et al. 2005), and considerable genome sequence homology to humans (Howe et al. 2013). However, experiments using nonnative (to Canada) zebrafish require a large sample size, making statistical analysis cumbersome (Dai et al. 2014).

The zebrafish chorion, mainly composed of glycoproteins, acts as a physical and chemical barrier to shield developing zebrafish from noxious external environmental conditions (Bonsignorio et al. 1996) and is the first biological membrane to interact with nanomaterials upon exposure (Cheng, Flahaut, and Cheng 2007). The chorion has two distinct membranes, the outer chorion dotted with protuberances and the inner vitelline membrane containing numerous pore canals (Bai et al. 2010) that are permeable to certain nanomaterials (Felix et al. 2013). Alternatively, nanomaterials could adsorb to the surface of the chorion (Lin et al. 2011) and block pore canals, thereby creating hypoxic conditions for the embryo (Cheng, Flahaut, and Cheng 2007). Lee et al. (2007) demonstrated that some nanomaterials passively diffused into and out of the embryo through pore canals, while others got stuck, aggregated with incoming particles and consequently clogged the pores of the chorion. Lin et al. (2011) reported an increase in metal ion concentration inside the chorion following 1 and 24 h exposures to different metal oxide nanomaterials, and results suggested that uptake was time-dependent. At time of hatch (48–72 h) (Kimmel et al. 1995), a zinc metalloprotease is released from a hatching gland located on the anterior surface of the yolk sac that functions to partially digest the inner vitelline membrane of the chorion (Sano et al. 2008). Contractile movements of the zebrafish can then rupture the softened chorion allowing the animal to emerge (Sano et al. 2008).

Direct contact between water and respiratory epithelia of fish allows for rapid and efficient uptake of xenobiotics (Tierney et al. 2013). Fish gill cells are increasingly being utilized in nanotoxicological testing and effects assessment (Farkas et al. 2011; Kühnel et al. 2009; Stott et al. 2015). The fish gill is a complex, multifunctional tissue made up of a variety of different cell types including pavement, mitochondrial-rich, and mucous cells and is the major site of acid–base regulation, gas exchange, nitrogenous waste excretion, and osmoregulation (Bury, Schnell, and Hogstrand 2014; Evans, Piermarini, and Choe 2005). Given their large surface area, thin epithelial barrier (Farkas et al. 2011), and need for continuous water flow to function, the gills are one of the primary target organs and uptake sites of aqueous contaminates such as waterborne nanomaterials (Bury, Schnell, and Hogstrand 2014; Handy et al. 2008). Certain nanomaterials are expected to adsorb onto gill surfaces and be taken up by epithelial cells via endocytosis rather than by membrane transporters or by passive diffusion through cell membranes (Handy et al. 2008).

Permanent gill cell lines (i.e., cells passaged multiple times *in vitro*) are often used in nanotoxicological studies due to their freshwater tolerance (Stott et al. 2015), ease of manipulation, high reproducibility, low maintenance, and amenability to culture in both complete and simplified media (Kühnel et al. 2009; Lee et al. 2009). For example, the rainbow trout gill epithelial cell line, RTgill-W1, proliferates at room temperature in the absence of 5% carbon dioxide, thus eliminating the need for an expensive carbon dioxide incubator typically set at 37°C for culturing mammalian cells. Furthermore, certain gill preparations and culturing techniques using permeable membrane inserts generate polarized epithelial cells bearing tight junctions that resemble *in vivo* scenarios (Kelly et al. 2000). Cultured gill epithelium derived from rainbow trout consist of different cell types including mucous-secreting goblet-like cells and mitochondria-rich cells (Lee et al. 2009), and these cells contain intracellular structures including nuclei, mitochondria, lysosomes, and intermediate filaments (Bols et al. 1994).

14.8 Outlook

The interrelated fields of nanotechnology and nanotoxicology have progressed over the past decade. The OECD Chemicals Committee WPMN was established in 2006 to advise on issues related to human health and environmental safety of nanomaterials and to ensure that hazard, exposure, and risk assessments are of the highest quality. In 2009, the WPMN determined whether OECD TGs, originally developed for more conventional chemicals, were appropriate for nanomaterial testing (OECD 2009). In 2011, the European Commission recommended that nanomaterials be defined as "a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1–100 nm" (European Commission 2011). Although this was an important step and many believe that a strict definition is needed to ensure that nanotechnology is safely integrated into society, some argue that nanomaterials should not be defined (Maynard 2011). The mineral Libby vermiculite, for example, contains harmful asbestiform fibers, but for many years, it was left unregulated because it did not fit the definition of asbestos (Maynard 2011). In 2013, the European Commission announced new cosmetics regulations that require engineered nanomaterials and their physicochemical characteristics to be listed on product labels (European Commission 2013). In 2014, a nanomaterial-specific OECD guidance document for aquatic and sediment toxicity tests was developed during a groundbreaking workshop; the key findings and recommendations that arose from this workshop have been published (Petersen et al. 2015). A few guidelines and guidance documents related to nanotoxicity testing have emerged in recent years (OECD 2012a; OECD 2012b; SCENIHR 2009) and more are expected to become publically available in the near future (Rasmussen et al. 2016). Nanotoxicologists have begun to collaborate with scientists and engineers from all disciplines, modify their approaches to nanotoxicity testing, and think beyond conventional toxicology paradigms. We have seen more concerted and coordinated efforts that aim to close the gap between the production of nanotechnology-based products and toxicity testing of base nanomaterials to ensure the safe and responsible development of nanoscience. Nanotoxicology is now a key component in research

and development processes rather than a posthoc determination once materials have entered commerce, and this connection will ensure human health and environmental sustainability over the upcoming decades. Overall, improved knowledge and understanding of the factors that modulate nanomaterial toxicity and the underlying mechanisms by which nanomaterials induce toxic effects will facilitate accurate assessments by researchers and thus allow risk assessors to better predict the potential impact of nanomaterials when unintentionally or purposefully released into freshwater environments.

REFERENCES

Alarifi, S., D. Ali, A. Verma, F. N. Almajhdi, and A. A. Al-Qahtani. 2014. Single-walled carbon nanotubes induce cytotoxicity and DNA damage via reactive oxygen species in human hepatocarcinoma cells. *In Vitro Cell Dev Biol Anim* 50:714–22.

Amara, R. 1972. A framework for national science policy analysis. *IEEE Trans Syst Man Cybern* SMC-2 (1):3–8.

Aruoja, V., H. C. Dubourguier, K. Kasemets, and A. Kahru. 2009. Toxicity of nanoparticles of CuO, ZnO and TiO_2 to microalgae *Pseudokirchneriella subcapitata*. *Sci Total Environ* 407:1461–68.

Baalousha, M., A. Manciulea, S. Cumberland, K. Kendell, and J. R. Lead. 2008. Aggregation and surface properties of iron oxide nanoparticles: Influence of pH and natural organic matter. *Environ Toxicol Chem* 27 (9):1875–82.

Bai, W., Z. Zhang, W. Tian et al. 2010. Toxicity of zinc oxide nanoparticles to zebrafish embryo: A physicochemical study of toxicity mechanism. *J Nanopart Res* 12:1645–54.

Balbus, J. M., A. D. Maynard, V. L. Colvin et al. 2007. Meeting report: Hazard assessment for nanoparticles—Report from an interdisciplinary workshop. *Environ Health Perspect* 115 (11):1654–59.

Baolog, S., L. Rodriguez-Lorenzo, C. A. Monnier et al. 2015. Characterizing nanoparticles in complex biological media and physiological fluids with depolarized dynamic light scattering. *Nanoscale* 7:5991–7.

Bar-Ilan, O., C. C. Chuang, D. J. Schwahn et al. 2013. TiO_2 nanoparticle exposure and illumination during zebrafish development: Mortality at parts per billion concentrations. *Environ Sci Technol* 47:4726–33.

Bar-Ilan, O., K. M. Louis, S. P. Yang et al. 2012. Titanium dioxide nanoparticles produce phototoxicity in the developing zebrafish. *Nanotoxicology* 6 (6):670–9.

Baun, A., N. B. Hartmann, K. Grieger, and K. O. Kusk. 2008. Ecotoxicity of engineered nanoparticles to aquatic invertebrates: A brief review and recommendations for future toxicity testing. *Ecotoxicology* 17:387–95.

Behra, R., L. Sigg, M. J. D. Clift et al. 2013. Bioavailability of silver nanoparticles and ions: From a chemical and biochemical perspective. *J R Soc Interface* 10:20130396.

Blinova, I., A. Ivask, M. Heinlaan, M. Mortimer, and A. Kahru. 2010. Ecotoxicity of nanoparticles of CuO and ZnO in natural water. *Environl Pollut* 158:41–7.

Bols, N. C., A. Barlian, M. Chirino-Trejo, S. J. Caldwell, P. Goegan, and L. L. E. J. Lee. 1994. Development of a cell line from primary cultures of rainbow trout, *Oncorhynchus mykiss* (Walbaum), gills. *J Fish Dis* 17:601–11.

Boluk, Y. and C. Danumah. 2014. Analysis of cellulose nanocrystal rod lengths by dynamic light scattering and electron microscopy. *J Nanopart Res* 16:2174.

Bolyard, S. C., D. R. Reinhart, and S. Santra. 2013. Behavior of engineered nanoparticles in landfill leachate. *Environ Sci Technol* 14:8114–22.

Bonsignorio, D., L. Perego, L. Del Gaicco, and F. Cotelli. 1996. Structure and macromolecular composition of the zebrafish egg chorion. *Zygote* 4:101–8.

Boussif, O., F. Lezoualc'h, M. A. Zanta et al. 1995. A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: Polyethylenimine. *Proc Natl Acad Sci* 92:7297–301.

Brunner, D., J. Frank, H. Appl, H. Schöffl, W. Pfaller, and G. Gstraunthaler. 2010. Serum-free cell culture: The serum-free media interactive online database. *ALTEX* 27:53–62.

Bury, N. R., S. Schnell, and C. Hogstrand. 2014. Gill cell culture systems as models for aquatic environmental monitoring. *J Exp Biol* 217:639–50.

Byrne, H., A. Ahluwalia, D. Boraschi, B. Fadeel, and P. Gehr. 2013. The bionanointerface in predicting nanoparticle fate and behaviour in living organisms: Towards grouping and categorising nanomaterials and ensuring nanosafety by design. *BioNanoMaterials* 14:195–216.

Castaño, A. and , M. J. Gómez-Lechón. 2005. Comparison of basal cytoxicity data between mammalian and fish cell lines: A literature survey. *Toxicol In Vitro* 19 (5):695–705.

Canadian Environmental Protection Act. 2015. CEPA 1999 section 71 notice with respect to certain nanomaterials in Canadian commerce. *Can Gaz Part I* 149 (30):1939–2136.

Cerutti, P. A. 1985. Prooxidant states and tumor promotion. *Science* 227 (4685):375–81.

Charron, R. A., J. C. Fenwick, D. R. S. Lean, and T. W. Moon. 2000. Ultraviolet-B radiation effects on antioxidant status and survival in the zebrafish, *Brachydanio rerio. Photochem Photobiol* 72 (3):327–33.

Chen, C. Y. and C. T. Jafvert. 2010. Photoreactivity of carboxylated single-walled carbon nanotubes in sunlight: Reactive oxygen species production in water. *Environ Sci Technol* 44:6674–9.

Cheng, J., E. Flahaut, and S. H. Cheng. 2007. Effect of carbon nanotubes on developing zebrafish (*Danio rerio*) embryos. *Environ Toxicol Chem* 26 (4):708–16.

Colvin, V. L. 2003. The potential environmental impact of engineered nanomaterials. *Nat Biotechnol* 21 (10):1166–70.

Cowie, J., E. M. Bilek, T. H. Wegner, and J. A. Shatkin. 2014. Market projections of cellulose nanomaterial-enabled products—Part 2: Volume estimates. *Tappi J* 13 (6):57–69.

Crollius, H. R. and J. Weissenbach. 2005. Fish genomics and biology. *Genome Res* 15:1675–82.

Croteau, M. N., S. K. Misra, S. N. Luoma, and E. Valsami-Jones. 2014. Bioaccumulation and toxicity of CuO nanoparticles by a freshwater invertebrate after waterborne and dietborne exposures. *Environ Sci Technol* 48:10929–37.

Dahle, J. T. and Y. Arai. 2015. Environmental geochemistry of cerium: Applications and toxicology of cerium oxide nanoparticles. *Int J Environ Res Public Health* 12:1253–78.

Dai, Y. J., Y. F. Jia, N. Chen et al. 2014. Zebrafish as a model system to study toxicology. *Environ Toxicol Chem* 33 (1):11–7.

Derjaguin, B. and L. Landau. 1941. Theory of the stability of strongly charged lyophobic sols and of the adhesion of strongly charged particles in solutions of electrolytes. *Acta Physicochim URSS* 14:633–62.

Dhar Dwivedi, A., S. P. Dubey, M. Sillanpää, Y. N. Kwon, C. Lee, and R. S. Varma. 2015. Fate of engineered nanoparticles: Implications in the environment. *Coord Chem Rev* 287:64–78.

Diaz, L., C. Peyrot, and K. J. Wilkinson. 2015. Characterization of polymeric nanomaterials using analytical ultracentrifugation. *Environ Sci Technol* 49:7302–7309.

dos Santos, T., J. Varela, I. Lynch, A. Salvati, and K. A. Dawson. 2011. Effects of transport inhibitors on the cellular uptake of carboxylated polystyrene nanoparticles in different cell lines. *PLoS ONE* 6 (9):e24438.

Dutta, D. and J. G. Donaldson. 2013. Search for inhibitors of endocytosis. *Cell Logis* 2 (4):203–8.

Ebrahimi, M. and M. Taherianfard. 2011. The effects of heavy metals exposure on reproductive systems of cyprinid fish from Kor River. *Iran J Fish Sci* 10 (1):13–24.

Ede, J. D., V. A. Ortega, D. Boyle et al. 2015. Rosette nanotubes alter IgE-mediated degranulation in the rat basophilic leukemia (RBL)-2H3 cell line. *Toxicol Sci* 148 (1):108–120.

Engqvist-Goldstein, Å. E. Y. and D. G. Drubin. 2003. Actin assembly and endocytosis: From yeast to mammals. *Annu Rev Cell Dev Biol* 19:287–332.

European Commission. 2013. Commission implementing decision on guidelines on annex I to regulation (EC) no 1223/2009 of the European Parliament and of the council on cosmetic products. Official Journal of the European Union, L 83–105.

European Commission. 2011. Commission recommendation of 18 October 2011 on the definition of nanomaterial (text with EEA relevance). Official Journal of the European Union, L. 275:38–40.

Evans, D. H., P. M. Piermarini, and K. P. Choe. 2005. The multifunctional fish gill: Dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol Rev* 85:97–177.

Faille, D., F. El-Assaad, A. J. Mitchell et al. 2012. Endocytosis and intracellular processing of platelet microparticles by brain endothelial cells. *J Cell Mol Med* 16 (8):1731–8.

Farkas, A., J. Salánki, and A. Specziár. 2002. Relation between growth and the heavy metal concentration in organs of ream *Abramis brama* L. populating lake Balaton. *Arch Environ Contam Toxicol* 43:236–43.

Farkas, J., P. Christian, J. A. Gallego-Urrea et al. 2011. Uptake and effects of manufactured silver nanoparticles in rainbow trout (*Oncorhynchus mykiss*) gill cells. *Aquat Toxicol* 117–25.

Federici, G., B. J. Shaw, and R. D. Handy. 2007. Toxicity of titanium dioxide nanoparticles to rainbow trout (*Oncorhynchus mykiss*): Gill injury, oxidative stress, and other physiological effects. *Aquat Toxicol* 84:415–30.

Felix, L. C., E. J. Folkerts, Y. He, and G. G. Goss. 2017. Poly (acrylic acid)-coated titanium dioxide nanoparticle and ultraviolet light co-exposure has minimal effect on developing zebrafish (*Danio Rerio*). *Environ Sci Nano* 4:658–669.

Felix, L. C., J. D. Ede, D. A. Snell et al. 2016. Physicochemical properties of functionalized carbon-based nanomaterials and their toxicity to fishes. *Carbon* 104:78–89.

Felix, L. C., V. A. Ortega, J. D. Ede, and G. G. Goss. 2013. Physicochemical characteristics of polymer-coated metal-oxide nanoparticles and their toxicological effects on zebrafish (*Danio Rerio*) development. *Environ Sci Technol* 47 (12):6589–96.

Firdessa, R., T. A. Oelschlaeger, and H. Moll. 2014. Identification of multiple cellular uptake pathways of polystyrene nanoparticles and factors affecting the uptake: Relevance for drug delivery systems. *Eur J Cell Biol* 93:323–37.

Franklin, N. M., N. J. Rogers, S. C. Apte, G. E. Batley, G. E. Gadd, and P. S. Casey. 2007. Comparative toxicity of nanoparticulate ZnO, bulk ZnO, and $ZnCl_2$ to a freshwater microalga (*Pseudokirchneriella subcapitata*): The importance of particle solubility. *Environ Sci Technol* 41:8484–90.

Fujimoto, T., H. Kogo, R. Nomura, and T. Une. 2000. Isoforms of caveolin-1 and caveolar structure. *J Cell Sci* 113:3509–17.

Gagné, F., P. Turcotte, J. Auclair, and C. Gagnon. 2013. The effects of zinc oxide nanoparticles on the metallome in freshwater messels. *Comp Biochem Physiol C* 158:22–8.

Gallud, A. and B. Fadeel. 2015. Keeping it small: Towards a molecular definition of nanotoxicology. *Eur J Nanomed* 7 (3):143–51.

Georgescu, B., C. Georgescu, S. Dărăban, A. Bouaru, and S. Paşcalau. 2011. Heavy metals acting as endocrine disrupters. *Sci Pap Anim Sci Biotechnol* 42 (2):89–93.

Ghosh, S., H. Mashayekhi, P. Bhowmik, and B. Xing. 2010. Colloidal stability of Al_2O_3 nanoparticles as affected by coating of structurally different humic acids. *Langmuir* 26 (2):873–9.

Gottschalk, F. and B. Nowack. 2011. The release of engineered nanomaterials to the environment. *J Environ Monit* 13:1145–55.

Gottschalk, F., T. Sonderer, R. W. Scholz, and B. Nowack. 2009. Modeled environmental concentrations of engineered nanomaterials (TiO_2, ZnO, Ag, CNT, fullerenes) for different regions. *Environ Sci Technol* 43:9216–22.

Gottschalk, F., T. Sonderer, R. W. Scholz, and B. Nowack. 2010. Possibilities and limitations of modeling environmental exposure to engineered nanomaterials by probabilistic material flow analysis. *Environ Toxicol Chem* 29 (5):1036–48.

Gottschalk, F., T. Sun, and B. Nowack. 2013. Environmental concentrations of engineered nanomaterials: Review of modeling and analytical studies. *Environ Pollut* 181:287–300.

Govind, P. and S. Madhuri. 2014. Heavy metals causing toxicity in animals and fishes. *Research J Anim Vet Fish Sci* 2 (2):17–23.

Grillo, R., A. H. Rosa, and L. F. Fraceto. 2015. Engineered nanoparticles and organic matter: A review of the state-of-the-art. *Chemosphere* 119:608–19.

Guo, L., A. Von Dem Bussche, M. Buechner, A. Yan, A. B. Kane, and R. H. Hurt. 2008. Adsorption of essential micronutrients by carbon nanotubes and the implications for nanotoxicity testing. *Small* 4 (6):721–27.

Gutteridge, J. M. C. 1995. Lipid peroxidation and antioxidants as biomarkers of tissue damage. *Clin Chem* 41 (12):1819–28.

Hammond, S. A., A. C. Carew, and C. C. Helbing. 2013. Evaluation of the effects of titanium dioxide nanoparticles on cultured *Rana catesbeiana* tailfin tissue. *Front genet* 4 (251):1–8.

Hampton, J. A., R. C. Lantz, P. J. Goldblatt, D. J. Lauren, and D. E. Hinton. 1988. Functional units in rainbow trout (*Salmo gairdneri*, Richardson) liver: II. The biliary system. *Anat Rec* 221:619–34.

Handy, R. D., T. B. Henry, T. M. Scown, B. D. Johnston, C. R. Tyler. 2008. Manufactured nanoparticles: Their uptake and effects on fish—A mechanistic analysis. *Ecotoxicology* 17:396–409.

Hao, L., L. Chen, J. Hao, and N. Zhong. 2013. Bioaccumulation and sub-acute toxicity of zinc oxide nanoparticles in juvenile carp (*Cyprinus carpio*): A comparative study with its bulkcounterparts. *Ecotoxicol Environ Saf* 91:52–60.

Hill, A. J., H. Teraoka, W. Heideman, and R. E. Peterson. 2005. Zebrafish as a model vertebrate for investigating chemical toxicity. *Toxicol Sci* 86 (1):6–19.

Hoffmann, M. R., S. T. Martin, W. Choi, and D. W. Bahnemann. 1995. Environmental applications of semiconductor photocatalysis. *Chem Rev* 95:69–96.

Howe, K., M. D. Clark, C. F. Torroja et al. 2013. The zebrafish reference genome sequence and its relationship to the human genome. *Nature* 496:498–503.

Hund-Rinke, K., A. Baun, D. Cupi et al. 2016. Regulatory ecotoxicity testing of nanomaterials—Proposed modifications of OECD test guidelines based on laboratory experience with silver and titanium dioxide nanoparticles. *Nanotoxicology* 10 (3):1442–47.

Iijima, S. and T. Ichihashi. 1993. Single-shell carbon nanotubes of 1-nm diameter. *Nature* 363:603–605.

Ijadpanah-Saravy, H., M. Safari, A. Darban-Khodadadi, and A. Razaei. 2014. Synthesis of titanium dioxide nanoparticles for photocatalytic degradation of cyanide in wastewater. *Anal Lett* 47 (10):1772–82.

International Organization for Standardization. 2015. Nanotechnologies—Vocabulary—Part 2: Nano-objects, ISO/TS 80004-2:2015(en). https://www.iso.org/obp/ui/#iso:std:iso:ts:80004:-2:ed-1:v1:en (accessed April 4, 2016).

Iqbal, M., T. K. Purkait, G. G. Goss, J. R. Bolton, M. G. El-Din, and J. G. C. Veinot. 2016. Application of engineered Si nanoparticles in light-induced advanced oxidation remediation of a water-borne model contaminant. *ACS Nano* 10 (5):5405–12.

Ivanov, A. I. 2008. Pharmacological inhibition of endocytosis pathways: Is it specific enough to be useful? In: Ivanov, A.I. (Ed.), *Exocytosis and Endocytosis*. Human Press, New Jersey, pp. 15–33.

Jiang, J., G. Oberdörster, and P. Biswas. 2009. Characterization of size, surface charge, and agglomeration state of nanoparticle dispersions for toxicological studies. *J Nanopart Res* 11:77–89.

Johnston, B. D., T. M. Scown, J. Moger et al. 2010. Bioavailability of nanoscale metal oxides TiO_2, CeO_2, and ZnO to fish. *Environ Sci Tech* 44:1144–51.

Jorfi, M. and E. J. Foster. 2015. Recent advances in nanocellulose for biomedical applications. *J Appl Polym Sci* 132:41719.

Kelly, S. A., C. M. Havrilla, T. D. Brady, K. H. Abramp, and E. D. Levin. 1998. Oxidative stress in toxicology: Established mammalian and ererging piscine model systems. *Environ Health Perspect* 106 (7):375–84.

Kelly, S. P., M. Fletcher, P. Part, and C. M. Wood. 2000. Procedures for the preparation and culture of "reconstructed" rainbow trout branchial epithelia. *Methods Cell Sci* 22:153–63.

Khalil, I. A., K. Kogure, H. Akita, and H. Harashima. 2006. Uptake pathways and subsequent intracellular trafficking in nonviral gene delivery. *Pharmacol Rev* 58:32–45.

Khan, M. M., S. F. Adil, and A. Al-Mayouf. 2015. Metal oxides as photocatalysts. *J Saudi Chem Soc* 19:462–4.

Kimmel, C. B., W. W. Ballard, S. R. Kimmel, B. Ullmann, and T. F. Schilling. 1995. Stages of embryonic development of the zebrafish. *Dev Dynam* 203:253–310.

Kou, L., J. Sun, Y. Zhai, and Z. He. 2013. The endocytosis and intracellular fate of nanomedicines: Implication for rational design. *Asian J Pharm Sci* 8:1–10.

Kovacs, T., V. Naish, B. O'Connor et al. 2010. An ecotoxicological characterization of nanocrystalline cellulose (NCC). *Nanotoxicology* 4 (3):255–70.

Kroll, A., M. H. Pillukat, D. Hahn, and J. Schnekenburger. 2012. Interference of engineered nanoparticles with *in vitro* toxicity assays. *Arch Toxicol* 86:1123–36.

Krug, H. F. 2014. Nanosafety research—Are we on the right track? *Angew Chem Int Ed* 53:12304–19.

Kuhn, D. A., D. Vanhecke, B. Michen et al. 2014. Different endocytotic uptake mechanisms for nanoparticles in epithelial cells and macrophages. *Beilstein J Nanotechnol* 5:1625–36.

Kühnel, D., W. Busch, T. Meißner et al. 2009. Agglomeration of tungsten carbide nanoparticles in exposure medium does not prevent uptake and toxicity toward a rainbow trout gill cell line. *Aquat Toxicol* 93:91–9.

Kwon, D., H. W. Nho, and T. H. Yoon. 2014. X-ray and electron microscopy studies on the biodistribution and biomodification of iron oxide nanoparticles in *Daphnia magna*. *Colloids Surf B Biointerfaces* 122:384–9.

Landfester, K. and A. Ostafin. 2008. Enhancing effectiveness of nanoparticles and nanoreactor in human (stem) cells—Understanding and influencing the uptake of nanostructured materials in (stem) cells. In *Nanoreactor Engineering for Life Sciences and Medicine*, eds Landfester, K. and A. Ostafin, 251–262. Norwood, MA: Artech House.

Lee, H. and W. Choi. 2002. Photocatalytic oxidation of arsenite in TiO_2 suspension: Kinetics and mechanisms. *Environ Sci Technol* 36 (17):3872–8.

Lee, K. J., P. D. Nallathamby, L. M. Browning, C. J. Osgood, and X. H. N. Xu. 2007. *In vivo* imaging of transport and biocompatibility of single silver nanoparticles in early development of zebrafish embryos. *ACS Nano* 1 (2):133–143.

Lee, L. E. J., V. R. Dayeh, K. Schirmer, N. C. Bols. 2009. Applications and potential uses of fish gill cell lines: Examples with RTgill-W1. *In Vitro Cell Dev Biol Anim* 45:127–34.

Leung, A. C. W., S. Hrapovic, E. Lam et al. 2011. Characteristics and properties of carboxylated cellulose nanocrystals prepared from a novel one-step procedure. *Small* 7 (3):302–5.

Li, M., K. T. Al-Jamal, K. Kostarelos, and J. Reineke. 2010. Physiologically based pharmacokinetic modeling of nanoparticles. *ACS Nano* 4 (11):6303–17.

Li, S., R. J. Erickson, L. K. Wallis, S. A. Diamond, and D. J. Hoff. 2015b. Modeling TiO_2 nanoparticle phototoxicity: The importance of chemical concentration, ultraviolet radiation intensity, and time. *Environ Pollut* 205:327–32.

Li, X., K. Schirmer, L. Bernard, L. Sigg, S. Pillai, and R. Behra. 2015a. Silver nanoparticle toxicity and association with the alga *Euglena gracilis*. *Environ Sci Nano* 2:594–602. Liang, F. and B. Chen. 2010. A review on biomedical applications of single-walled carbon nanotubes. *Current Med Chem* 17 (1):10–24.

Lin, H., C. P. Huang, W. Li, C. Ni, S. Ismat Shah, and Y. H. Tseng. 2006. Size dependency of nanocrystalline TiO_2 on its optical property and photocatalytic reactivity exemplified by 2-chlorophenol. *Appl Catal B* 68:1–11.

Lin, N. and A. Dufresne. 2014. Nanocellulose in biomedicine: Current status and future prospect. *Eur Polym J* 59:302–25.

Lin, S., Y. Zhao, T. Xia et al. 2011. High content screening in zebrafish speeds up hazard ranking of transition metal oxide nanoparticles. *ACS Nano* 5 (9):7284–95.

Liu, A. P., F. Aguet, G. Danuser, and S. L. Schmid. 2010. Local clustering of transferrin receptors promotes clathrin-coated pit initiation. *J Cell Biol* 191 (7):1381–93.

Liu, J. and R. H. Hurt. 2010. Ion release kinetics and particle persistence in aqueous nanosilver colloids. *Environ Sci Technol* 44:2169–75.

Liu, R. and R. Lal. 2015. Potentials of engineered nanoparticles as fertilizers for increasing agronomic productions. *Sci Total Environ* 514:131–9.

Ma, H., A. Brennan, and S. A. Diamond. 2012. Phototoxicity of TiO_2 nanoparticles under solar radiation to two aquatic species: *Daphnia magna* and Japanese medaka. *Environ Toxicol Chem* 31 (7):1621–9.

Ma, H., P. L. Williams, and S. A. Diamond. 2013. Ecotoxicity of manufactured ZnO nanoparticles—A review. *Environ Pollut* 172:76–85.

Mahlambi, M. M., C. J. Ngila, and B. B. Mamba. 2015. Recent developments in environmental photocatalytic degradation of organic pollutants: The case of titanium dioxide nanoparticles—A review. *J Nanomat* 2015 (790173):1–29.

Manier, N., A. Bado-Nilles, P. Delalain, O. Aguerre-Chariol, and P. Pandard. 2013. Ecotoxicity of non-aged and aged CeO_2 nanomaterials towards freshwater microalgae. *Environ Pollut* 180:63–70.

Masoumbaigi, H., A. Rezaee, H. Hosseini, and S. Hashemi. 2015. Water disinfection by zinc oxide nanoparticle prepared with solution combustion method. *Desalin Water Treat* 56 (9):2376–81.

Maurer-Jones, M. A., I. L. Gunsolus, C. J. Murphy, and C. L. Haynes. 2013. Toxicity of engineered nanoparticles in the environment. *Anal Chem* 85 (6):3036–49.

Maynard, A. D. 2011. Don't define nanomaterials. *Nature* 475:31.

Meng, L., C. Fu, and Q. Lu. 2009. Advanced technology for functionalization of carbon nanotubes. *Prog Nat Sci* 19:801–10.

Mercer, J. and A. Helenius. 2009. Virus entry by macropinocytosis. *Nat Cell Biol* 11 (5):510–20.

Merdzan, V., R. F. Domingos, C. E. Monteiro, M. Hadioui, and K. J. Wilkinson. 2014. The effects of different coatings on zinc oxide nanoparticles and their influence on dissolution and bioaccumulation by the green alga, *C. reinhardtii*. *Sci Total Environ* 488–489:316–24.

Miao, A. J., X. Y. Zhang, Z. Luo et al. 2010. Zinc oxide-engineered nanoparticles: Dissolution and toxicity to marine phytoplankton. *Environ Toxicol Chem* 29 (12):2814–22.

Mitrano, D. M., S. Motellier, S. Clavaguera, and B. Nowack. 2015. Review of nanomaterial aging and transformations through the life cycle of nano-enhanced products. *Environ Int* 77:132–47.

Monopoli, M. P., C. Åberg, A. Salvati, and K. A. Dawson. 2012. Biomolecular coronas provide the biological identity of nanosized materials. *Nat Nanotechnol* 7:779–86.

Monti, D. M., D. Guarnieri, G. Napolitano, R. Piccoli, P. Netti, S. Fusco, and A. Arciello. 2015. Biocompatibility, uptake and endocytosis pathways of polystyrene nanoparticles in primary human renal epithelial cells. *J Biotechnol* 193:3–10.

Mudunkotuwa, I. A., J. M. Pettibone, and V. H. Grassian. 2012. Environmental implications of nanoparticle ageing in the processing and fate of copper-based nanomaterials. *Environ Sci Technol* 46:7001–10.

Murdock, R. C., L. Braydich-Stolle, A. M. Schrand, J. J. Schlager, and S. M. Hussain. 2008. Characterization of nanomaterial dispersion in solution prior to *in vitro* exposure using dynamic light scattering technique. *Toxicol Sci* 101 (2):239–53.

Nabi, I. R. and P. U. Le. 2003. Caveolae/raft-dependent endocytosis. *J Cell Biol* 161 (4):673–77.

Nel, A. E., L. Mädler, D. Velegol et al. 2009. Understanding biophysicochemical interactions at the nano-bio interface. *Nat Mater* 8:543–57.

Ngarize, S., K. E. Makuch, and R. Pereira. 2012. The regulation of nanotechnologies. In *Environmental and Energy Law*, eds Makuch, K. E. and R. Pereira, 439–464. Hoboken, NJ: John Wiley & Sons.

Oberdörster, G., A. Maynard, K. Donaldson et al. 2005. Principles for characterizing the potential human health effects from exposure to nanomaterials: Elements of a screening strategy. *Part Fibre Toxicol* 2 (8):1–35.

Oberdörster, G., E. Oberdörster, and J. Oberdörster. 2005. Nanotoxicology: An emerging discipline evolving from studies of ultrafine particles. *Environ Health Perspect* 113 (7):823–39.

OECD (Organisation for Economic Co-operation and Development). 2012a. Important issues on risk assessment of manufactured nanomaterials. Series on the Safety of Manufactured Nanomaterials No. 33—ENV/JM/MONO(2012)8. http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2012)8&doclanguage=en (accessed November 6, 2016).

OECD. 2012b. Guidance on sample preparation and dosimetry for the safety testing of manufactured nanomaterials. Series on the Safety of Manufactured Nanomaterials No. 36—ENV/JM/MONO(2012)40. http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2012)40&doclanguage=en (accessed November 6, 2016).

OECD. 2010. List of manufactured nanomaterials and list of endpoints for phase one of the sponsorship programme for the testing of manufactured nanomaterials. Series on the Safety of Manufactured Nanomaterials No. 27—ENV/JM/MONO(2010)46. http://www.oecd.org/officialdocuments/displaydocumentpdf/?cote=env/jm/mono(2010)46&doclanguage=en (accessed November 6, 2016).

OECD. 2009. Preliminary review of OECD test guidelines for their applicability to manufactured nanomaterials. Series on the Safety of Manufactured Nanomaterials—ENV/JM/MONO(2009)21. http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2009)21&doclanguage=en (accessed November 6, 2016).

Oh, N. and J. H. Park. 2014. Endocytosis and exocytosis of nanoparticles in mammalian cells. *Int J Nanomed* 9:51–63.

Ong, K. J., L. C. Felix, D. Boyle et al. 2017. Humic acid ameliorates nanoparticle-induced developmental toxicity in zebrafish. *Environ Sci Nano* 2017 (4):127–37.

Ong, K. J., T. J. MacCormack, R. J. Clark et al. 2014a. Widespread nanoparticle-assay interference: Implications for nanotoxicity testing. *PLoS ONE* 9 (3):e90650.

Ong, K. J., X. Zhao, M. E. Thistle et al. 2014b. Mechanistic insights into the effect of nanoparticles on zebrafish hatch. *Nanotoxicology* 8 (2):295–304.

Pan, Z., W. Lee, L. Slutsky, R. A. F. Clark, N. Pernodet, and M. H. Rafailovich. 2009. Adverse effects of titanium dioxide nanoparticles on human dermal fibroblasts and how to protect cells. *Small* 5 (4):511–20.

Patel, H. H. and P. A. Insel. 2009. Lipid rafts and caveolae and their role in compartmentation of redox signaling. *Antioxid Redox Signal* 11 (6).1357–72.

Patil, S., A. Sandberg, E. Heckert, W. Self, and S. Seal. 2007. Protein adsorption and cellular uptake of cerium oxide nanoparticles as a function of zeta potential. *Biomaterials* 28:4600–7.

Peixe, T. S., E. de Souza Nascimento, K. L. Schofield, A. S. A. Arcuri, and R. P. Bulcão. 2015. Nanotoxicology and exposure in the occupational setting. *Occup Diseases and Environmental Medicine* 03:35–48.

Pelaez, M., N. T. Nolan, S. C. Pillai et al. 2012. A review on the visible light active titanium dioxide photocatalysts for environmental applications. *Appl Catal B* 125:331–49.

Petersen, E. J., S. A. Diamond, A. J. Kennedy et al. 2015. Adapting OECD aquatic toxicity tests for use with manufactured nanomaterials: Key issues and consensus recommendations. *Environ Sci Technol* 49:9532–47.

Petersen, E. J., T. B. Henry, J. Zhao et al. 2014. Identification and avoidance of potential artifacts and misinterpretations in nanomaterial ecotoxicity measurements. *Environ Sci Technol* 48:4226–46.

Piccinno, F., F. Gottschalk, S. Seeger, and B. Nowack. 2012. Industrial production quantities and uses of ten engineered nanomaterials in Europe and the world. *J Nanopart Res* 14:1109.

Pietroiusti, A., A. Magrini, and L. Campagnolo. 2014. Mechanisms of nanomaterial toxicity. In *Health and Environmental Safety of Nanomaterials: Polymer Nanocomposites and Other Materials Containing Nanoparticles*, eds Njuguna, J., K. Pielichowski, and H. Zhu, 28–43. Amsterdam: Elsevier.

Pitkänen, M., H. Kangas, and J. Vartiainen. 2014. Toxicity and heath issues. In *Handbook of Green Materials: Processing Technologies, Properties and Applications*, eds Oksman, K., A. P. Mathew, A. Bismarck, O. Rojas, and M. Sain, 1–1200. Singapore: World Scientific.

Powers, D. A. 1989. Fish as model systems. *Science* 246:352–358.

Prasad, K. N. 2012. Health risks of nonionizing radiation and their prevention and mitigation. In *Radiation Injury Prevention and Mitigation*, 1–312. Boca Raton, FL: CRC Press.

Priestly, B. G., A. Bartholomaeus, R. Drew. 2014. Hazards of food contact material: Nanotechnologies and nanomaterials. In *Reference Module in Food, from Encyclopedia of Food Safety*, 444–448. Cambridge, MA: Academic Press.

Rasmussen, K., M. González, P. Kearns, J. R. Sintes, F. Rossi, and P. Sayre. 2016. Review of achievements of the OECD Working Party on Manufactured Nanomaterials' Testing and Assessment Programme. From exploratory testing to test guidelines. *Regul Toxicol Pharmacol* 74:147–160.

Rawson, D. M., T. Zhang, D. Kalicharan, and W. L. Jongebloed. 2000. Field emission scanning electron microscopy and transmission electron microscopy studies of the chorion, plasma membrane and syncytial layers of the gastrula-stage embryo of the zebrafish *Brachydanio rerio*: A consideration of the structural and functional relationships with respect to cryoprotectant penetration. *Aquacult Res* 31 (3):325–36.

Reinsborough, M., and G. Sullivan. 2011. The regulation of nano-particles under the European biocidal products directive: Challenges for effective civil society participation. *European J Law Technol* 2 (3):1–19.

Riley, P. A. 1994. Free radicals in biology: Oxidative stress and the effects of ionizing radiation. *Int J Radiat Biol* 65 (1):27–33.

Roman, M. 2015. Toxicity of cellulose nanocrystals: A review. *Ind Biotechnol* 11 (1):25–33.

Roman, M., S. Dong, A. Hirani, and Y. W. Lee. 2009. Cellulose nanocrystals for drug delivery. In *ACS Symposium Series*, 81–91. Washington, DC: American Chemical Society.

Safari, S., S. E. Dehkordy, M. Kazemi, H. Dehghan, and B. Mahaki. 2015. Ultraviolet radiation emissions and illuminance in different brands of compact fluorescent lamps. *Int J Photoenergy* 2015 (504674):1–6.

Sano, K., K. Inohaya, M. Kawaguchi, N. Yoshizaki, I. Iuchi, and S. Yasumasu. 2008. Purification and characterization of zebrafish hatching enzyme—An evolutionary aspect of the mechanism of egg envelope digestion. *FEBS J* 275:5934–46.

Santos, M., J. Teixeira, and A. Rodrigues. 2000. Production of dextransucrase, dextran and fructose from sucrose using *Leuconostoc mesenteroides* NRRL B512(f). *Biochem Eng J* 4:177–88.

Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR). 2009. Risk Assessment of Products of Nanotechnologies. European Commission, 1–71. http://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_o_023.pdf (accessed April 15, 2016).

Schultz, A. G., D. Boyle, D. Chamot et al. 2014. Aquatic toxicity of manufactured nanomaterials: Challenges and recommendations for future toxicity testing. *Environ Chem* 11:207–26.

Schultz, A. G., K. J. Ong, T. MacCormack, G. Ma, J. G. C. Veinot, and G. G. Goss. 2012. Silver nanoparticles inhibit sodium uptake in juvenile rainbow trout (*Oncorhynchus mykiss*). *Environ Sci Technol* 46:10295–301.

Selck, H., R. D. Handy, T. F. Fernandes, S. J. Klaine, and E. J. Petersen. 2016. Nanomaterials in the aquatic environment: A European Union–United States perspective on the status of ecotoxicity testing, research priorities, and challenges ahead. *Environ Toxicol Chem* 35 (5):1055–67.

Shang, L., K. Nienhaus, and G. U. Nienhaus. 2014. Engineered nanoparticles interacting with cells: Size matters. *J Nanobiotechnol* 12 (5):1–11.

Shi, H., R. Magaye, V. Catstranova, and J. Zhao. 2013. Titanium dioxide nanoparticles: A review of current toxicological data. *Part Fibre Toxicol* 10:1–33.

Shukla, S. J., R. Huang, C. P. Austin, and M. Xia. 2010. The future of toxicity testing: A focus on *in vitro* methods using a quantitative high-throughput screening platform. *Drug Discov Today* 15 (23–24):997–1007.

Shvedova, A., A. Pietroiusti, and V. Kagan. 2016. Nanotoxicology ten years later: Lights and shadows. *Toxicol Appl Pharmacol* 299:1–2.

Sies, H. 2007. Biological redox systems and oxidative stress. *Cell Mol Life Sci* 64:2181–88.

Smijs, T. G. and S. Pavel. 2011. Titanium dioxide and zinc oxide nanoparticles in sunscreens: Focus on their safety and effectiveness. *Nanotechnol Sci Appl* 4:95–112.

Smith, C. J., B. J. Shaw, and R. D. Handy. 2007. Toxicity of single walled carbon nanotubes to rainbow trout, (*Oncorhynchus mykiss*): Respiratory toxicity, organ pathologies, and other physiological effects. *Aquat Toxicol* 82:94–109.

Sokolova, V., D. Kozlova, T. Knuschke, J. Buer, A. M. Westendorf, and M. Epple. 2013. Mechanism of the uptake of cationic and anionic calcium phosphate nanoparticles by cells. *Acta Biomaterialia* 9:7527–35.

Song, L., M. G. Vijver, W. J. G. M. Peijnenburg, T. S. Galloway, and C. R. Tyler. 2015. A comparative analysis on the in vivo toxicity of copper nanoparticles in three species of freshwater fish. *Chemosphere* 139:181–9.

Star, A., J. F. Stoddart, D. Steuerman et al. 2001. Preparation and properties of polymer-wrapped single-walled carbon nanotubes. *Angew Chem Int Ed* 40 (9):1721–25.

Stott, L. C., S. Schnell, C. Hogstrand, S. F. Owen, and N. R. Bury. 2015. A primary fish gill cell culture model to assess pharmaceutical uptake and efflux: Evidence for passive and facilitated transport. *Aquat Toxicol* 159:127–37.

Subbiah, R., M. Veerapandian, and K. S. Yun. 2010. Nanoparticles: Functionalization and multifunctional applications in biomedical sciences. *Curr Med Chem* 17:4559–77.

Taurozzi, J. S., V. A. Hackley, and M. R. Wiesner. 2011. Ultrasonic dispersion of nanoparticles for environmental, health and safety assessment—Issues and recommendations. *Nanotoxicology* 5 (4):711–29.

Taylor, N. S., R. Merrifield, T. D. Williams, K. J. Chipman, J. R. Lead, and M. R. Viant. 2016. Molecular toxicity of cerium oxide nanoparticles to the freshwater alga *Chlamydomonas reinhardtii* is associated with supra-environmental exposure concentrations. *Nanotoxicology* 10 (1):32–41.

Tierney, K. B., C. J. Kennedy, F. Gobas, M. Gledhill, and M. Selkela. 2013. Organic Contaminants and Fish. In *Fish Physiology: Organic Chemical Toxicology of Fishes*, eds Tierney, K. B., A. P. Farrell, and C. J. Brauner, 1–52. Cambridge, MA: Academic Press.

Valdiglesias, V., N. Fernández-Bertólez, G. Kiliç et al. 2016. Are iron oxide nanoparticles safe? Current knowledge and future perspectives. *J Trace Elem Med Biol* 1–11.

Van der Velden, J. L., I. Bertoncello, and J. L. McQualter. 2013. LysoTracker is a marker of differentiated alveolar type II cells. *Respir Res* 14 (123):1–7.

Van Hoecke, K., K. A. C. De Schamphelaere, P. Van der Meeren, G. Smagghe, and C. R. Janssen. 2011. Aggregation and ecotoxicity of CeO_2 nanoparticles in synthetic and natural waters with variable pH, organic matter concentration and ionic strength. *Environ Pollut* 159:970–76.

Vance, M. E., T. Kuiken, E. P. Vejerano et al. 2015. Nanotechnology in the real world: Redeveloping the nanomaterial consumer products inventory. *Beilstein J Nanotechnol* 6:1769–80.

Vandamme, T. F. and L. Brobeck. 2005. Poly (amidoamine) dendrimers as ophthalmic vehicles for ocular delivery of pilocarpine nitrate and tropicamide. *J Control Release* 102:23–38.

Vardy, D. W., J. Oellers, J. A. Doering, J. P. Giesy, and M. Hecker. 2013. Sensitivity of early life stages of white sturgeon, rainbow trout, and fathead minnow to copper. *Ecotoxicology* 22:139–47.

Verwey, E. J. W. and J. T. G. Overbeek. 1948. *Theory of the Stability of Lyophobic Colloids*, 1–108. Amsterdam: Elsevier.

Wang, B., X. He, Z. Zhang, Y. Zhao, and W. Feng. 2013. Metabolism of nanomaterials *in vivo*: Blood circulation and organ clearance. *Acc Chem Res* 46 (3):761–69.

Warheit, D. B. and M. Donner. 2010. Rationale of genotoxicity testing of nanomaterials: Regulatory requirements and appropriateness of available OECD test guidelines. *Nanotoxicology* 44, 409–13.

Watanabe, T., A. Nakajima, R. H. Wang et al. 1999. Photocatalytic activity and photoinduced hydrophilicity of titanium dioxide coated glass. *Thin Solid Films* 351 (1–2):260–63.

Weir, A., P. Westerhoff, L. Fabricius, K. Hristovski, and N. von Goetz. 2012. Titanium dioxide nanoparticles in food and personal care products. *Environ Sci Technol* 46 (4):2242–50.

Westmeier, D., R. H. Stauber, and D. Docter. 2016. The concept of bio-corona in modulating the toxicity of engineered nanomaterials (ENM). *Toxicol Appl Pharmacol* 299, 53–7.

Weydert, C. J. and J. J. Cullen. 2009. Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. *Nat Protoc* 5:51–66.

Wiesner, M. R., G. V. Lowry, K. L. Jones et al. 2009. Decreasing uncertainties in assessing envrironmental exposure, risk, and ecological implications of nanomaterials. *Environ Sci Technol* 43 (17):6458–62.

Wilhelm, C., C. Billotey, J. Roger, J. N. Pons, J. C. Bacri, and F. Gazeau. 2003. Intracellular uptake of anionic superparamagnetic nanoparticles as a function of their surface coating. *Biomaterials* 24 (6):1001–11.

Wörle-Knirsch, J. M., K. Pulskamp, and H. F. Krug. 2006. Oops they did it again! Carbon nanotubes hoax scientists in viability assays. *Nano Lett* 6 (6):1261–68.

Xu, L., L. D. Cao, F. M. Li, X. J. Wang, and Q. L. Huang. 2014. Utilization of chitosan-lactide copolymer nanoparticles as controlled release pesticide carrier for pyraclostrobin against colletotrichum gossypii southw. *J Dispers Sci Technol* 35 (4):544–50.

Yang, X., A. P. Gondikas, S. M. Marinakos et al. 2012. Mechanism of silver nanoparticle toxicity is dependent on dissolved silver and surface coating in *Caenorhabditis elegans*. *Environ Sci Technol* 46 (2):1119–27.

Yin, H., P. S. Casey, and M. J. McCall. 2010. Surface modification of ZnO nanoparticles and their cytotoxicity. *J Nanosci Nanotechnol* 10 (11):7565–70.

Zhang, L. W. and N. A. Monteiro-Riviere. 2009. Mechanisms of quantum dot nanoparticle cellular uptake. *Toxicol Sci* 110 (1):138–55.

Zhang, Y., T. A. Blewett, A. L. Val, and G. G. Goss. 2018. UV-induced toxicity of cerium oxide nanoparticles (CeO_2 NPs) and the protective properties of natural organic matter (NOM) from the Rio Negro Amazon River. *Environ Sci Nano* 5 (2):476–86.

Zhao, X. and R. Liu. 2012. Recent progress and perspectives on the toxicity of carbon nanotubes at organism, organ, cell, and biomacromolecule levels. *Environ Int* 40:244–55.

Zhao, X., S. Wang, Y. Wu, H. You, and L. Lv. 2013. Acute ZnO nanoparticles exposure induces developmental toxicity, oxidative stress and DNA damage in embryo-larval zebrafish. *Aquat Toxicol* 136–137:49–59.

Zhu, X., S. Tian, and Z. Cai. 2012. Toxicity assessment of iron oxide nanoparticles in zebrafish (*Danio rerio*) early life stages. *PLoS ONE* 7 (9):e46286.

Zhu, X., L. Zhu, Z. Duan, R. Qi, Y. Li, and Y. Lang. 2008. Comparative toxicity of several metal oxide nanoparticle aqueous suspensions to zebrafish (*Danio rerio*) early developmental stage. *J Environ Sci Heath A* 43 (3):278–84.

15

Guidelines and Protocols for Nanotoxicity Evaluation

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15.1 Introduction

Nanomaterials, although small in size, possess a large surface area per unit volume. The novel physical characteristics of nanomaterials can result in their ability to drastically have different chemical and biological properties compared to the same material in bulk form. The unique chemical and biological properties of nanomaterials make

them useful in many products for humans, including some in industry, agriculture, business, medicine, clothing, cosmetics, and food [Nasser et al., 2016]. The development of an environmental health and safety risk management system for nanoscale particle types requires a base set of hazard data. The accurate determination of health and environmental risks of nanomaterials is a function of the integration of hazard and exposure datasets. Many challenges are faced to assess such risks, which emerge due to uncertainty of interactions of nanoparticles with human and environment. In order to reduce these uncertainties, sound scientific data on hazard and exposure have to be generated by means of relevant frameworks and tools [Hristozov et al., 2016]. Recently, a nanoparticle risk assessment strategy was promulgated, and the components are described in a document entitled "Nanorisk Framework" (http://www.nanoriskframework.com). Further into the chapter, important guidelines for nanotoxicity evaluation are discussed. These guidelines are applied to varied models of nanotoxicity such as *in vitro* and *in vivo* plant and animal systems. Along with the guidelines, various methods to assess the toxicity caused due to nanomaterials are also covered in a systematic manner.

15.2 Guidelines for Nanotoxicity Evaluation

Guidelines over the years are very important and comprehensive for the nanotoxicity evaluation based on all the principles and protocols. Sampling for *in vitro* and *in vivo* studies is divided into plant and animal models and is based on number of test procedures. Toxicity assessments are grounded on the following test guidelines.

15.2.1 Organisation for Economic Co-Operation and Development

The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental organization in which representatives of 34 industrialized countries in North and South America, Europe, and Asia Pacific region, as well as the European Commission, meet to coordinate and harmonize policies, discuss issues of mutual concern, and work together to respond to international problems. More than 200 committees and working groups work on OECD program, and all the OECD programs and workshops are carried out by all members of the committees. All the panels, committees, and working groups are served by the OECD Secretariat, located in Paris, France, which is organized into directorates and divisions. There are many divisions including environment, health, and safety which publish free-of-charge documents in 11 different series: Testing and Assessment, Good Laboratory Practice and Compliance Monitoring, Pesticides, Biocides, Risk Management, Harmonization of Regulatory Oversight in Biotechnology, Safety of Novel Foods and Feeds, Chemical Accidents, Pollutant Release and Transfer Registers, Emission Scenario Documents; and Safety of Manufactured Nanomaterials. Most of the information about the Environment, Health and Safety Programme (EHS) and EHS publications are available on OECD's World Wide Web site (http://www.oecd.org/chemicalsafety/).

Several genetic toxicology tests have been validated for chemicals according to the OECD Test Guidelines. The relevance of these assays for nanoparticle material remains to be determined. In an attempt to overcome this issue, the OECD has established current projects designed to evaluate the relevance and reproducibility of

safety hazard tests for representative nanomaterial, including genotoxicity and phytotoxicity assays. The implementation of well-accepted genotoxicity assay for testing nanomaterial remains a controversial issue. This is because many of these genotoxicity tests were designed for screening general nanoparticle chemicals and might not be suitable for the screening of nanomaterial. In a recent inhalation study, evaluating micronucleus formation in rat erythrocytes following exposures to engineered amorphous nanosilica particles was conducted. It seems clear that the development of standardized approaches will be necessary in order to determine whether exposure to specific nanoparticle types is associated with genotoxicity events. The appropriateness of available genotoxicity test systems for nanomaterial requires confirmation and standardization. Accordingly, it seems reasonable to conclude that any specific regulatory testing requirements for nanoparticles would be premature [Warheit and Donner, 2010].

15.2.1.1 OECD Guidelines for Testing of Chemicals

OECD guidelines for the Testing of Chemicals are periodically reviewed in the light of scientific progress or changing assessment practices. In 1985, Bruce proposed an up-and-down procedure (UDP) for the determination of acute toxicity of chemicals [Bruce, 1985]. There exist several variations of up-and-down experimental design for estimating a lethal dose 50 (LD50). This guideline is based on the procedure by Bruce as adopted by the American Society for Testing and Materials (ASTM) in 1987 [ASTM, 1987] and revised in 1990. A study comparing the results obtained with the UDP, the conventional LD50 test, and the fixed dose procedure was published in 1995 [Lipnick et al., 1995]. The rationale of genotoxicity testing of nanomaterial relies on regulatory requirements and appropriateness of available OECD Test Guidelines. OECD guidelines for testing of chemicals for genotoxicity assay have been periodically reviewed both *in vivo* and *in vitro*. The Comet assay, such as *in vivo* alkaline comet (single cell gel electrophoresis) assay, is used for the detection of DNA strand breaks in cells or nuclei isolated from multiple tissues of animals and rodents on exposure to genotoxic materials. There are several reviews on comet assay, and many of the suggestions have been published by various expert groups [Kirkland and Speit, 2008; Brendler-Schwaab et al., 2005; Burlinson et al., 2007; Burlinson, 2012; Smith et al., 2008; Hartmann et al., 2003; McKelvey-Martin et al., 1993; Tice et al., 2000; Singh et al., 1988; Rothfuss et al., 2010]. The comet assay can detect single- and double-stranded breaks and cross-links [Vasquez, 2012]. However, the proof of evidence shows that they cannot be reliably detected when applying standard experimental conditions [OECD, 2014a, 2014b], and therefore, it is not appropriate to use the standard assay to detect cross-linking agents.

There are scientific peer review panels such as Japanese Center for the Validation of Alternative Methods (JaCVAM) to evaluate the validation status of the reactive oxygen species (ROS) assay and established international standard norm according to OECD guidelines [OECD, 2005a]. The ROS assay is a test method proposed as a component of an integrated photo-safety testing protocol to evaluate whether test substances such as pharmaceuticals have the potential to cause phototoxicity. Many decisions and suggestions have been given by the committee, which initially met in February and again in August 2013 in Tokyo, Japan. Finally, the panel considered the reports of two international validation studies and a proposed ROS assay protocol at

their initial meeting. The ROS assay protocol was subsequently reviewed and updated according to the suggestions of the panel, and the validation study reports was revised by the Validation Management Team.

Following are the points which the panel concluded: Negative results in the ROS assay would not require further testing in animals or other models, while positive, weakly positive, and inconclusive results would proceed to the next level of testing in an *in vitro* test system such as the 3T3 phototoxicity assay [OECD, 2005b]. The ROS assay could potentially provide significant savings in time and cost and reduced animal use for photo-safety assessments. Furthermore, incorporating the ROS assay into a photo-safety testing strategy is expected to significantly reduce the overall number of substances that would require additional testing in the *in vitro* 3T3 phototoxicity assay and subsequent testing in animals. The panel considered the test method protocols used for the two validation studies and key aspects of a proposed standardized ROS assay protocol. The proposed ROS assay protocol was sufficiently detailed to allow for users to successfully perform the procedure. The protocol included adequate and appropriate analysis and classification criteria. Solar simulators used should be equipped with an appropriate temperature control unit or fan since ROS production can be influenced by temperature. The list of proficiency chemicals provided in the test method protocol for laboratories to use to demonstrate ability to perform the assay was appropriate. Each lab should develop historical positive and negative control value acceptance ranges that can be used to determine the acceptability of an individual test.

15.2.1.2 OECD Guidelines for Dosage

Dosages in OECD guidelines are fixed, since classical mass dose trend applied in conventional toxicological research may not be sufficient in nanotoxicology testing due to the extensively large surface area in comparison with large particles. Large doses will amplify toxicity in nanomaterial; hence, dosage will have to take into account the size of the nanoparticles. Other parameters to be considered are the number, surface coatings on particle, degree of aggregation, surface charges, and particle synthesis. Thus, nanoparticle should be well characterized to reflect these parameters so that proper dosage could be fixed. Dosage also reflects real-world conditions. High nanoparticle doses administered as bolus do not reflect real situations where exposure may take hours, days, or weeks. Studies showed that *Allium cepa* used for *in vitro* toxicity assay was exposed to nanoparticle suspension for about 4 hours [Kumari et al., 2009]. Generally, rodents, mice, and rat are used as the first animal model followed by larger models such as dogs, pigs, or nonhuman primates. Published literature has shown that the time of exposure of nanoparticles in animals could be from hours to days.

The JaCVAM recently established the formal validation of *in vivo* comet assay in rodents, in collaboration with the European Centre for the Validation of Alternative Methods (ECVAM) and the Interagency Coordinating Committee on the Validation of Alternative Methods [OECD, 2014a, 2014b]. The present test guideline by the panel includes the recommended use and limitations of the comet assay and is based on the final protocol used in the validation study. The laboratory should have competence to obtain single cell or nuclei suspensions of sufficient quality from each target tissue. The quality of the preparations will be firstly identified in the laboratory, and finally, results of group of experiments should be statistically presented based on

mean and median. All data should be validated according to JaCVAM based on mean and median [OECD, 2014a, 2014b]. There are not enough data at this time to make recommendations about optimum or acceptable ranges for other tissues. All the test reports should provide appropriate review of the performance of the comet assay in these tissues in relation to the published literature.

A micronucleus test is a test used in the toxicological screening for potential genotoxic compounds. The assay is now recognized as one of the most successful and reliable assays for genotoxic carcinogens, i.e., carcinogens that act by causing genetic damage and are the OECD guideline for the testing of chemicals. The presence of micronuclei in cultured human cells has been reported as early as the 1960s [Elston, 1963] and 1970s [Countryman and Heddle, 1976]. The *in vitro* micronucleus test (IVMNT) is the best quantitative assay of chromosome damage by the development of the cytokinesis-block technique that eliminated the confounding effects on micronuclei expression by the cytostatic effects caused by poor culture conditions, treatment effects, cell senescence, and variability in mitogen response in the lymphocyte test system [Fenech and Morley, 1985; Fenech and Morley, 1986]. In the cytokinesis-block micronucleus assay, the scoring of micronuclei discriminates between once-divided cells that are accumulated and recognized by their binucleated appearance and mononucleated cells that did not divide during the *in vitro* given the culturing period. Most of the genotoxicity testing accuracy is based on recommended guidelines of OECD guideline 478 [OECD, 2010]. The IVMNT has become an attractive tool for genotoxicity testing because of its capacity to detect not only clastogenic and aneugenic process but also some epigenetic effects and its simplicity of scoring, accuracy, and wide applicability in different cell types. Initially, the recommendations for this guideline came from two workshops (International Workshops on Genotoxicity Testing), which proposed an internationally harmonized protocol designed for both human primary lymphocytes and cell lines [Kirsch-Volders et al., 2000, 2003]. The validation of the methodology was achieved by the ECVAM, based on the retrospective examination of the existing data published on the IVMNT [Lorge et al., 2006; Oliver et al., 2006; Aardema et al., 2006; Wakata et al., 2006; Clare et al., 2006] using the modular approach for validation [Hartung et al., 2004]. ECVAM confirmed that the IVMNT is reliable, reproducible, transferable, and predictive [Corvi et al., 2008] and was endorsed by the ECVAM Scientific Advisory Committee [ECVAM, 2010; ESAC, 2010]. The final step before acceptance by the OECD consisted of an interlaboratory exercise to evaluate different measures of cytotoxicity/cytostasis that can be applied when the *in vitro* micronucleus (IVMN) is performed in the absence of cytochalasin B (reviewed in ECVAM [2010]).

The use of the IVMNT within a battery of tests will be defined by the various regulatory bodies responsible for developing such test strategies. The advantages of the IVMNT are well defined [Bonassi et al., 2010; Decordier et al., 2010; Elhajouji et al., 2010]. It is a multitarget genotoxic end point predictive for cancer [Bonassi et al., 2010] and amenable for automation [Decordier et al., 2010] and allows good extrapolation for potential limits of exposure or thresholds [Elhajouji et al., 2010]. In addition, micronuclei can be scored and easily measured in a variety of systems, *in vitro* and *in vivo* [Hayashi et al., 2007]. The implementation of IVMN assays in the battery of tests for hazard and risk assessment of potential mutagens/carcinogens is therefore fully justified.

The chemicals and dose of the chemicals should be selected, for example, from the JaCVAM validation study [OECD, 2014a, 2014b] or from other published data, if appropriate, with justification, and demonstrate clear positive responses in the tissues of interest. The ability to detect weak effects of known mutagens, e.g., ethyl methanesulfonate at low doses, should also be demonstrated, for example, by establishing dose–response relationships with appropriate numbers and spacing of doses.

15.2.1.3 OECD Guideline for Exposure Route of the Nanoparticles to the System

"OECD activities on the safety evaluation and risk assessment of manufactured nanomaterials," by Mar Gonzalez, from the OECD's Working Party on Manufactured Nanomaterials (WPMN) Secretariat, who provided a presentation entitled "OECD WPMN Work on the Safety of Manufactured Nanomaterials." First, she introduced the work of the OECD's division of the EHS on chemical safety. The objectives of this division are to protect humans and the environment, to increase efficiency in the management of chemicals, and to avoid nontariff trade barriers. The aims of the division are (1) harmonization, (2) coordination, and (3) outreach to stakeholders and nonmember countries (global harmonization). Second, she described how the activities of the WPMN are organized. She also introduced the OECD database on research into the safety of manufactured nanomaterials (http://www.oecd.org/env/nanosafety/database).

The OECD WPMN initiated a series of expert meetings to assess the applicability of the OECD test guidelines (used for regulatory testing of chemicals) to nanomaterial. A preliminary report was published in 2009 [OECD, 2009]. The current OECD Test Guidelines [OECD, 2012] series address agreed end points used for chemical safety assessment but were not specifically developed for addressing nanomaterial. It was expected that by reviewing the new findings from the OECD Sponsorship Programme for the Testing of Manufactured Nanomaterials (hereafter Testing Programme), experts will be in a better position for evaluating the applicability of current OECD Test Guidelines to nanomaterial. If test guidelines were not applicable, there is a need to identify and update current ones or develop new test guidelines for those agreed end points that are relevant for safety and regulatory decision-making.

The Institute of Food Technologists, International Life Sciences Institute (ILSI), and OECD have made progress with testing methodology, risk assessment and communication/education, environmental issues, and nanotechnologies. Nanowaste is a topic that OECD has recently started to investigate. This topic has wide implications for policy making; therefore, more research and collaboration at international level are needed. Multiagency collaborations at the international level (OECD) Food and Agricultural Organization (FAO)/Nutrition and Consumer Protection Division (AGN) and OECD have been closely associating on the issue of nanotechnology. It is recommended that AGN (food safety) should be involved more in the mechanism of the Inter-organization Program for the Sound Management of Chemicals (IOMC) so that the FAO activities related to nanotechnology can be recognized by the members of the IOMC. The IOMC members met in Nairobi in September 2012, and the FAO representative presented the work at the AGN.

TABLE 15.1

End Points for the Evaluation of Toxicity

Animal Model	Plant Model
Body weight and food/water consumption	Microscopic examination
Hematology	Root/shoot length
Clinical biochemistry	Biochemical analysis
Pathology	Effect on DNA and protein
Gross necropsy	ROS mechanism to check on DNA content and lipid peroxidation
Histopathology	Electron microscopy for biological sample
ROS mechanism	–
Electron microscopy for biological samples	–

Traditionally, test items are given orally, especially the ones developed for health and medical purposes. However, delivery can be via respiratory, intravenous, intraperitoneal, or dermal systems. Currently, similar routes are employed for nanoparticle testing. Studies showed that the absorption is much dependent on the physical properties of the nanoparticle as well as its interactions with cellular proteins. The rate of absorption again varies with surface properties.

According to OECD guidelines, there are some end points (Table 15.1) for observation after the administration of the doses of chemicals to check the changes in the test system. Studies have also shown that there are some end points for the plant and animal models after the administration of the nanoparticles to check the nanotoxicity [Kumari et al., 2009].

15.2.1.4 OECD Guidelines for Observation Period

The observation period should be 28 days. Animals in a satellite group scheduled for follow-up observations should be kept for at least 14 days without treatment to detect delayed occurrence or persistence of or recovery from toxic effects. General clinical observations should be made at least once a day, preferably at the same time(s) each day and considering the peak period of anticipated effects after dosing. The health condition of the animals should be recorded. At least twice daily, all animals should be observed for morbidity and mortality. Once before the first exposure (to allow for within-subject comparisons), and at least once a week thereafter, detailed clinical observations should be made in all animals. These observations should be made outside the home cage in a standard arena and preferably at the same time of day on each occasion.

The animals should be carefully observed and carefully recorded based on each day observation. All the signs and changes should be noted, such as changes in skin, fur, eyes, and mucous membranes and occurrence of secretions and excretions and autonomic activity (e.g., lacrimation, piloerection, pupil size, and unusual respiratory pattern). Changes in gait, posture, and response to handling as well as the presence of clonic or tonic movements, stereotypy (e.g., excessive grooming, and repetitive circling) or bizarre behavior (e.g., self-mutilation and walking backward) should also be recorded [Dixon, 1965]. The observation should be carried out for 90 days.

15.2.1.4.1 Body Weight and Food/Water Consumption

All animals should be weighed at least once a week. Measurements of food consumption should be made at least weekly. If the test substance is administered via drinking water, water consumption should also be measured at least weekly.

15.2.1.4.2 Hematology

The following hematological examinations should be made at the end of the test period: hematocrit, hemoglobin concentrations, erythrocyte count, reticulocytes, total and differential leukocyte counts, platelet count, and a measure of blood clotting time/potential. Blood sample should be periodically taken for hematology.

15.2.1.4.3 Clinical Biochemistry

Clinical biochemistry determinations to investigate major toxic effects in tissues and, specifically, effects on kidney and liver should be performed on blood samples obtained from all animals prior to or as part of the procedure for euthanasia of the animals (apart from those found moribund and/or euthanized prior to the termination of the study). Investigations of plasma or serum shall include sodium, potassium, glucose, total cholesterol, urea, creatinine, total protein and albumin, at least two enzymes indicative of hepatocellular effects (such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, γ-glutamyl trans-peptidase, and glutamate dehydrogenase), and bile acids. Measurements of additional enzymes (of hepatic or other origin) and bilirubin may provide useful information under certain circumstances.

15.2.1.4.4 Pathology

Gross necropsy: All animals in the study shall be subjected to a full, detailed gross necropsy, which includes the careful examination of the external surface of the body; all orifices; and the cranial, thoracic, and abdominal cavities and their contents. The liver, kidneys, adrenals, testes, epididymides, prostate and seminal vesicles with coagulating glands as a whole, thymus, spleen, brain, and heart of all animals are also examined.

15.2.1.4.5 Histopathology

Full histopathology should be carried out on the preserved organs and tissues of all animals in the control and high-dose groups. These examinations should be extended to animals of all other dosage groups, if treatment-related changes are observed in the high dose group.

15.2.1.4.6 Common Mechanism for Cytotoxicity

Cytotoxicity due to nanoparticle interaction is based on shape, size, and surface area of the particle. Nanoparticles react at areas where larger particles cannot enter, such as, in some cases, they enter the nucleus, or in *in vivo* studies, nanoparticles can transfer across the placental barrier from pregnant mice to pups [Gu et al., 2009]. Due to the high surface area-over-volume ratio of nanoparticles, they react with cellular components. The subcellular structure such as endosomes can react with nanoparticles by producing free ions, which show high toxicity and can locally exceed the LD50.

15.2.2 World Health Organization/Food and Agricultural Organization

Many countries have identified the potential use of nanotechnology in the food and agriculture sectors and are significantly investing in its application considering the food safety implications of the technology. According to FAO and the World Health Organization (WHO), issues pertaining to food safety, new issues, and technologies continue to emerge; hence, FAO/WHO convened a joint meeting on nanotechnologies in food and agriculture. The FAO/WHO analysis report showed state of the art on the initiatives and activities relevant to risk assessment. Dr A. Chiodini, scientist, and his team introduced their work on the development of the practical guidance for the safety assessment of engineered nanomaterials in food, to conduct a practical, systematic, and robust safety assessment of engineered nanomaterials. This activity was supported by the ILSI, Europe Novel Foods, and Nanotechnology Task Force. This guidance suggests that the four-step risk assessment paradigm of FAO/WHO is also applicable to engineered nanomaterials.

The risk assessment of engineered nanomaterials should be conducted according to the following steps: Step 1: identification of engineered nanomaterials (e.g., from a known bulk material? Does an appropriate comparator exist?) Step 2: physicochemical characterization of engineered nanomaterials (e.g., solubility and agglomeration/aggregation). Step 3: decision tree to determine the necessity of the reevaluation of an existing risk assessment or tiered risk assessment, depending on the resolvability under gastric conditions and solubility of the engineered nanomaterials. Step 4: a tiered approach, consisting of tier 1, screening for potential hazards of ENM, and tier 2, determination of the effects of the engineered nanomaterials that is different from those of a comparator. Step 5: exposure assessment and risk characterization, i.e., consideration of the engineered nanomaterials in the food matrix.

Dr. Chiodini explained some of the imported nanorelated activities that focus on specific areas related to nanomaterial and nanotechnology, and the process is elaborately explained in the "Workshop on Outlook and Challenges of Nanotechnologies for Food Packaging." According to FAO, animal health has both direct and indirect implications for food safety and eventually human health. Thereby, nanotechnology has real benefits for the global public. According to FAO/Plant Production and Protection Division (AGP) protocol related to nanotoxicity explained similarly in plant health sector, nanopesticides may have implications for food safety and the environment. It is difficult for national authorities to differentiate between nanopesticides and nonnanopesticides because there is no specification for nanopesticide formulation due to minor differences in their efficacy. In addition, performing environmental risk assessment for developing countries is a challenge as there are no internationally harmonized standards/criteria for nanopesticides. Occupational health is another issue in the area of pesticide use. There is an international guideline on the safe use of wastewater with the collaboration of FAO, WHO, and United Nations (UN) Environment Programme.

15.2.3 United Nations

UN Guidelines for Consumer Protection (UNGCP) are a valuable set of principles for setting out the main characteristics of effective consumer protection legislation,

enforcement institutions, and redress systems. They assist interested member states in formulating and enforcing domestic and regional laws, rules, and regulations that are suitable to their own economic, social, and environmental circumstances. They also promote international enforcement cooperation among member states and encourage the sharing of experiences in consumer protection. The guidelines were first adopted by the General Assembly in resolution 39/248 of April 16, 1985, later expanded by the Economic and Social Council in resolution E/1999/INF/2/Add.2 of July 26, 1999, and recently revised by the General Assembly in resolution 70/186 of December 22, 2015.

The UN Conference on Trade and Development (UNCTAD), established in 1964 as a permanent intergovernmental body, promotes the guidelines and encourages interested member states to create awareness on the many ways in which member states, businesses, and civil society can promote consumer protection in the provision of public and private goods and services. The intergovernmental group of experts on consumer protection law and policy has been established to monitor the implementation of the guidelines, provide a forum for consultations, produce research and studies, provide technical assistance, undertake voluntary peer reviews, and periodically update the UNGCP. The UNGCP was first adopted by consumers in 1985. The guidelines follow a long campaign by consumer associations in many countries. The UNGCP were significantly expanded in 1999, followed by a number of meetings on rules and protocols related to the guidelines. In July 2012, the first meeting decided that UNCTAD should begin a consultation process on the revision of UNGCP. Likewise, a second meeting held in July 2013 discussed the implementation report.

15.2.4 International Organization for Standardization

Standardization in the field of nanotechnology includes discussion on the size, shape, and the surface characterization of nanomaterial. It includes understanding and control of the matter that are processed at the nanoscale level. It also deals with the properties of nanoscale materials, which differ from the properties of materials in bulk. International standardization protocols deal with not just measurement standards, but according to the International Organization for Standardization (ISO), in order to ensure consistency, repeatability, and accuracy, there must be standards of practice (e.g., procedure and guidelines) and standard for verification (i.e., reference materials). ISO is an active committee publishing more than 40 documents, which are in various stages of development. They have a tremendous impact on the global advancement of nanotechnology standardization in critical areas such as nanotechnology vocabulary, appropriate measurement techniques for characterization of nanoobjects and nanomaterial, and health and safety practices for those individuals working with nanoobjects or nanomaterial.

ISO Technical Committee 229, along with four working groups, work independently or jointly depending upon the potential overlap of subject areas, focusing on the development of international standards for nanotechnologies according to the following scopes: working group 1, terminology and nomenclature; working group 2, measurement and characterization; working group 3, health, safety, and environment; and working group 4, materials specifications. Nanotechnology, which refers to the manipulation and control of matter in the nanoscale (approximately 1–100 nm), is revolutionizing virtually all industry sectors, from information technology to medicine to clean energy production.

15.2.5 Other Guidelines

ASTM International's Committee E56 (Nanotechnology) (formerly known as the American Society for Testing Chemicals and Materials) is an international standards organization that develops and publishes voluntary consensus technical standards for a wide range of materials, products, systems, and services. Hence, ASTM is also one of the best guidelines to follow for toxicity assessment.

The American National Standards Institute (ANSI) is the standard panel on nanotechnology and has recently started the ANSI–Nanotechnology Standards Panel (NSP) newsletter with the objective of getting information about standards related to developments. The panel is a private nonprofit organization that oversees the development of standards for products, services, processes, systems, and personnel in the United States. The organization also coordinates US standards with international standards so that American products can be used worldwide. The Interagency Committee on Standards Policy, also known as the ICSP, provides advice and recommendations to the secretary of Commerce and other executive branch agencies on matters related to standards policy.

15.3 Protocols for Nanotoxicology Evaluation

15.3.1 Cytotoxicity Evaluation

15.3.1.1 In Vitro Methods

15.3.1.1.1 Trypan Blue Exclusion

Trypan blue is a dye which is taken up only by dead cells (Figure 15.1). Its International Union of Pure and Applied Chemistry name is (3*Z*,3′*Z*)-3,3′-[(3,3′-dimethylbiphenyl-4,4′-diyl)di(1*Z*)hydrazine-2-yl-1-ylidene]bis(5-amino-4-oxo-3,4-dihydronaphthalene-2,7-disulfonic acid). This dye is also called diamine blue/Niagara blue. Trypan blue exclusion method is used to determine cell viability.

It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue or eosin whereas dead cells do not (Figure 15.2). The cytoplasm of live cell appears clear whereas that of a dead cell appears blue in color [Strober, 1997]. Since live cells are excluded from the staining, this staining method

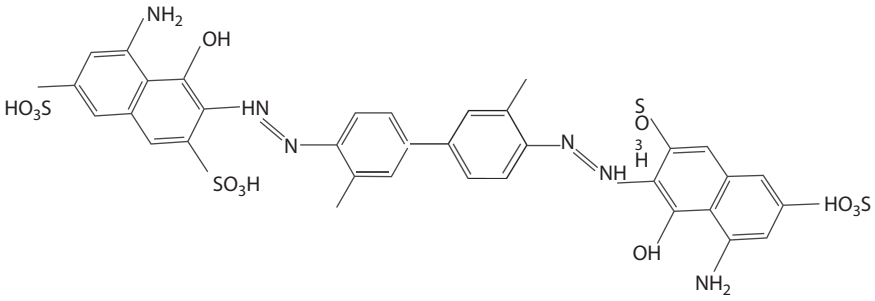


FIGURE 15.1 Molecular structure of trypan blue.

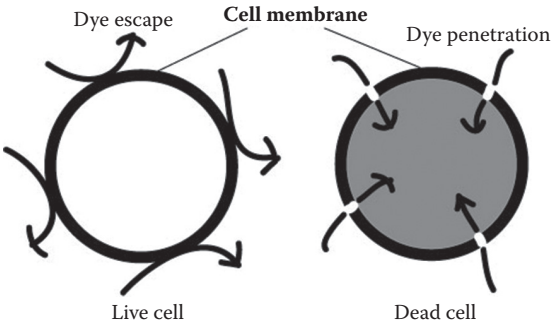


FIGURE 15.2 Principle of trypan blue.

is also referred to as dye exclusion method. This method is extended to cells which are exposed to nanoparticles. Cell cytotoxicity is determined after exposure to the nanoparticle dispersion. The cells should be exposed to nanoparticles for about 7 days followed by incubation for 24 hours. The incubated cells after 24 hours of incubation are ready for treatment with different concentrations of magnetic nanoparticle–polyethyleneimine. The following formulas may be used to determine the volume of medium to be added to the cell stock to achieve the desired cell density (Table 15.2). The desired cell density varies for suspension assays but is most commonly taken to be 1×10^6 cells mL^{-1}.

$$\text{Total volume needed} = \text{viable cell yield/desired cell density}$$

$$\text{Volume to add to cell stock} = \text{total volume needed} - \text{current volume}$$

Cell viability can be determined using the following formula:

$$\text{Viability} = \text{live cell count/total cell count}$$

TABLE 15.2

Desired Cell Densities for Different Species

	6-Well	12-Well	24-Well	48-Well	96-Well
Species	**Cell Density (10^6 Cells mL^{-1})**				
Human, rat, dog	0.9–1.1	0.8–1.0	0.7–0.9	0.6–0.8	0.5–0.7
Monkey	1.1–1.3	1.0–1.2	0.9–1.1	0.8–1.0	0.7–0.9
Mouse	0.5–0.7	0.4–0.6	0.3–0.5	0.2–0.4	0.1–0.3

Source: Triangle Research Labs, Research Triangle Park, NC, http://www.triangleresearchlabs.com.

The percentage of viable cells is calculated as

$$\text{Viable cells (\%)} = (\text{total number of viable cells per milliliter of aliquot}) / (\text{total number of cells per milliliter of aliquot}) \times 100.$$

The formula for total viable cell yield is

$$\text{Viable cell yield} = \text{viable cell count/quadrants counted} \times \text{dilution factor} \times \text{hemocytometer factor} \times \text{current volume (mL)}.$$

The disadvantage with dye exclusion is that cell viability is mainly determined by the cell membrane integrity. Sometimes, even when cell membrane integrity is abnormal, the cells may repair themselves and become viable. Conversely, when the membrane integrity is maintained, cell viability may be compromised. Since this method is solely dependent on the uptake of the dye, a small amount of the dye taken up due to cell injury goes unnoticed [Strober, 1997]. Another limitation is the time; cells have to be visualized and analyzed within 5 min of staining to avoid errors. As the time prolongs, the number of stained cells increases as the dye competes in penetrating even the live cells [Altman et al., 1993].

15.3.1.1.2 Microculture Tetrazolium Assay

Microculture tetrazolium assays are used to understand both cell activation and cell damage. The assay is based on the reduction of tetrazolium salt (INT) to colored formazan (Figure 15.3) [Goodwin et al., 1995]. INT is a yellow dye, which is reduced to purple-colored formazan crystals by the activity of mitochondrial succinate dehydrogenase enzyme in viable cells [Ezhilarasi et al., 2016]. Leading examples of INTs are 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazolyl)-3-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS), 2,3-*bis*-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide, and 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium [Ginouves et al., 2014]. An extra step of solubilization is required only in the case of MTT, whereas in other assays, the direct solubilization of INT to formazan can be achieved. Hence, error caused by using MTT INT is comparatively more. Among the preceding salts, MTS has shown better results [Scudiero et al., 1988; Goodwin et al., 1995].

The advanced *in vitro* detection of cytotoxicity induced by nanoparticles could be determined by microculture tetrazolium assay (Figure 15.4). The cell line after

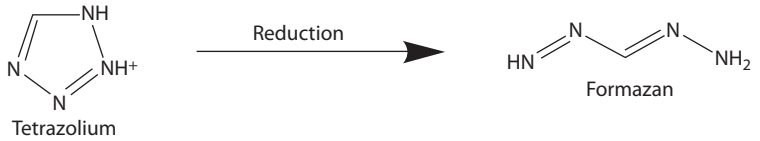


FIGURE 15.3 Reduction of tetrazolium to formazan.

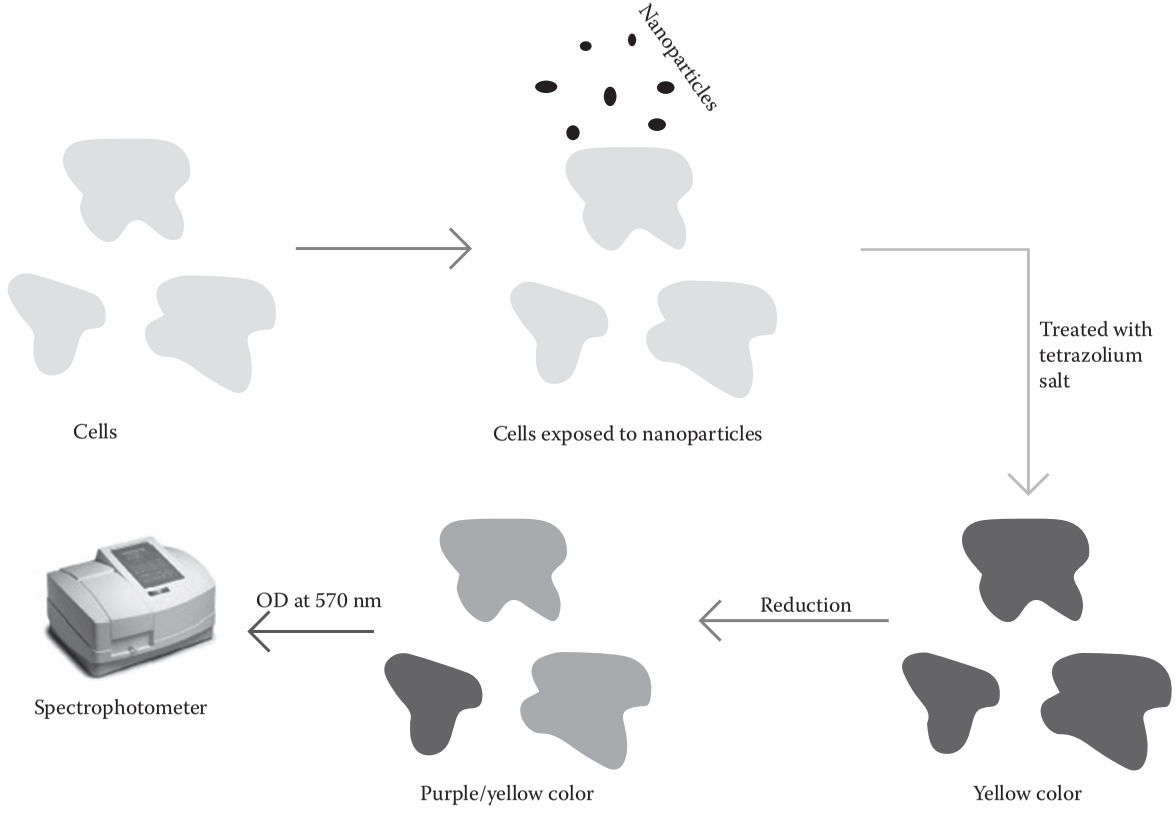


FIGURE 15.4 Microculture tetrazolium assay.

exposure to nanoparticle is ready to check for the inhibitory effect of nanoparticles on growth and proliferation of cells. The cell lines should be exposed to thiazolyl blue tetrazolium bromide, followed by the uptake of thiazolyl blue tetrazolium bromide by viable cells [Mosmann, 1983]. Cells should be plated on to 96-well plates (Orange Scientific, Brussels, Belgium) at a density of 1.5×10^4 cells 100 μL^{-1} per well. After incubation at 37°C for 24 hours, the medium should be replaced with either control medium or medium containing specialized concentration of Gd_2O_3 for 2, 8, and 24 hours. The concentration of Gd_2O_3 for cell culture treatment should be done at concentration rates that are as follows: 0.1, 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, and 2.5 mM. The zero concentration is used as negative control. The treated cells are kept in 50 µL of MTT solution (0.5 mg ml^{-1}) in each well followed by incubation for about 3 hours at 37°C. After incubation, the cells are solubilized in precipitated formazan with 100 µL dimethyl sulfoxide (DMSO); the cells are subjected for measurement of optical densitometry at a wavelength of 570 nm.

The inhibition rate (IR) was evaluated using the following equation:

$$\text{IR}(\%) = 1 - \text{ODexp/ODcon} \times 100,$$

where ODexp and ODcon are the optical densitometries of treated and untreated cells, respectively.

The viability rate of Gd_2O_3 was evaluated using the following equation [Derfus et al., 2004]:

$$\text{Viability}(\%) = 100 - \text{IR}(\%).$$

15.3.1.1.3 LDH Leakage Assay

Studying the effect of cell inhibition or cell death has become very important in biological research. Lactate dehydrogenase (LDH) is a soluble cytoplasmic enzyme, which is leaked out into the extracellular space from the plasma membrane of damaged cells. Initially, LDH produced reduces nicotinamide adenine dinucleotide (NADH) when it catalyzes the oxidation of lactate to pyruvate. In the next step, tetrazolium converts into a colored formazan using newly synthesized NADH in presence of an electron acceptor (Figure 15.5) [Chan et al., 2013]. LDH assay could be performed for the detection of cytotoxicity using a commercially available kit (Roche Applied Science). The serum LDH used for assay should be kept at 20°C [Braydich-Stolle et al., 2005]. The cell lines should be exposed to nanoparticle suspension for about 2 hours followed by transferring of the supernatant into an optically clear 96-well flat-bottomed microtiter plate. The concentration of the nanoparticle suspension should be selected based on the experiment. The test samples should be kept with negative control (zero concentration). To determine LDH activity, 100 µL of reaction mixture should be added to each well followed by incubation for 30 min at 15–25°C with a control group of 0.1% DMSO. The spectrophotometer should be calibrated to zero absorbance using culture medium without cells. The relative LDH leakage (%) related to control wells containing cell culture medium without nanoparticles or phosphate-buffered saline (PBS) as a vehicle should be calculated by

$$\text{LDH leakage}(\%) = [\text{A}]\ \text{test}/[\text{A}]\ \text{control} \times 100,$$

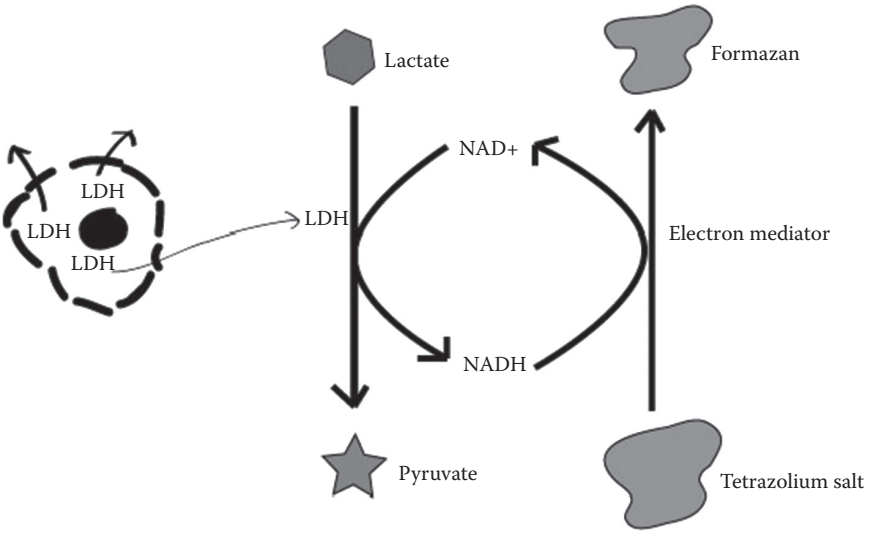


FIGURE 15.5 LDH assay.

where [A] test is the absorbance of the test sample and [A] control is the absorbance of the control sample. The absorbance of the samples should be measured at 490 nm using an enzyme-linked immunosorbent assay (ELISA) reader. Released LDH in culture supernatants results in the conversion of a INT into a red formazan product. The amount of color formed is proportional to the number of lysed cells.

$$\% \text{ Cytotoxicity} = (\text{corrected reading from test well} - \text{corrected reading from untreated well}) / (\text{corrected maximum LDH release control} - \text{corrected reading from untreated well}) \times 100$$

Cytotoxicity is expressed relative to the basal LDH release by untreated control cells followed by the study of cell viability after incubation with nanoparticle suspension with trypan blue. The cells should be exposed to 0.5 and 2.5 mM trypan blue, and dyed cells ready for observation should be taken for counting in a Burker chamber. One set of samples should be washed after 2 hours of incubation, and another set of samples should be kept unwashed. The cell samples should be then kept in culture for 8 days followed by viability directly monitored after 2 and 24 hours of incubation [Tracy et al., 2000].

NOTE: Precipitation can be seen in the LDH dye assay solution upon thawing since the solution is saturated. Once the solution is warmed to 37°C, the product will partially dissolve, and the solution can be decanted for any remaining precipitation. The precipitated solution has no effect on the results of the experiment.

15.3.1.2 In Vivo Methods

15.3.1.2.1 Lactate Dehydrogenase Assay

The protocol for the LDH assay for cytotoxicity assay is discussed earlier in Section 15.3.1.1.3. The same protocol could be used for the *in vivo* cytotoxicity assay.

15.3.1.2.2 Apoptosis Assay

Apoptosis is a process of programmed cell death that ensures the normal development and homeostasis of an organism. It is necessary for immunity and is implicated in neurodegenerative diseases, cancer, and aging. The loss of plasma membrane asymmetry is one of the earliest features of apoptosis. P53 is a vital protein for apoptosis; more than half of all cancers have inactive P53. When activated by either DNA damage or chromosome abnormalities, P53 can halt the cell cycle and initiate DNA repair. If repair is successful, the cell cycle is restarted ensuring cellular and genomic stability. If repair is not an option because the damage is too large, then P53 can facilitate apoptosis leading to the death and elimination of the abnormal cell (Figure 15.6). There are

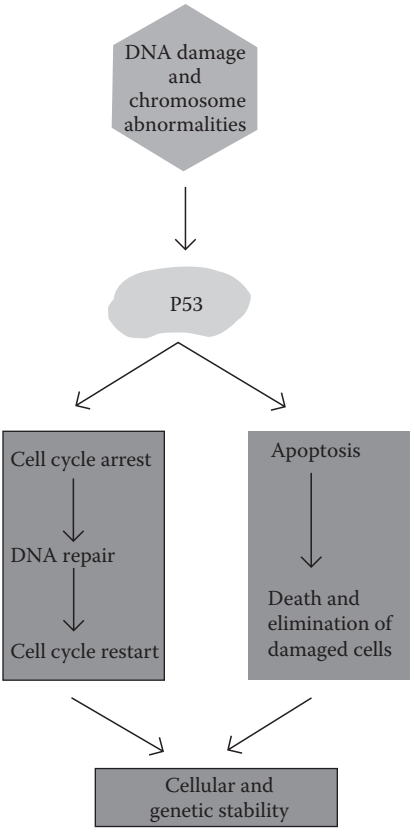


FIGURE 15.6 Apoptosis mechanism.

several means of studying apoptosis; some of them include antibodies, ELISAs, active proteins, and activity assays. There are also many commercially available kits which are used to study the apoptosis such as terminal deoxynucleotidyl transferase dUTP nick end labeling assay, mitochondrial assay, annexin V kits, and ApoStat apoptosis detection kit. Cell death cannot be fully defined by a single parameter. Hence, several approaches are used to study apoptosis. Some of them are apoptotic assays using nucleic acids stains, apoptosis assays which detect DNA strand breaks, apoptosis assays which detect membrane asymmetry, apoptosis assays based on protease activity, apoptosis assays using mitochondrial stains, and assays for autophagy.

In this test, the cells (approximately 2.5×10^5) should be grown on a coverslip, 20 × 20 mm, in tube culture of 10 cm^2 surface area containing 3 mL of culture medium. After washing cells with PBS, the coverslip is removed from the culture tube and fixed in Carnoy's fixative for 5 min. The coverslip is then rapidly immersed in a series of plates containing decreasing concentrations of ethanol (95–25%), followed by washing with McIlvaine's buffer for 5 min, staining with acridine orange (0.01%, 5 min), and again washing with buffer. The apoptotic cells should be analyzed by the pattern of the fragmentation of nuclear DNA after staining with acridine orange. The cells should be treated with concentrations of rutin after 24 hours of acclimation period. After 24 hours of drug exposure, the cells should be harvested as described by Rovozzo and Burke [1973] and Tsuboy et al. [2010]. The experiments are performed in triplicates and 1500 cells are analyzed per treatment.

15.3.2 Genotoxicity Evaluation

Genotoxicity is a deleterious action on cell genetic material affecting its integrity. The substances which cause genotoxicity are known to be potential carcinogens or mutagens. Genetic toxicity testing is routinely performed to identify potential genotoxic carcinogens and germ cell mutagens. *In vitro* genotoxicity test batteries recommended by regulatory agencies to detect genotoxic carcinogens include at least two or three test procedures, such as bacterial reverse mutation test (Ames test), mammalian cell chromosome damage test, and mammalian cell mutation assay [Kirkland et al., 2005]. Strategies for assessing the safety of nanoparticles have been proposed [Warheit and Donner, 2010]. Several initiatives, for example, OECD, WPMN, and Nanogenotox Joint Action have been convened, resulting in the establishment of standardized testing methods to determine the genotoxicity of nanoparticles.

There are many companies and industries which follow certain best guidelines for genetic toxicological assays. One such example of a company is the Bio Reliance. Bio Reliance is a global leader in genetic toxicity evaluation services. For a good product development, Bio Reliance has always given importance and end-to-end solution for genotoxicity. For safety and hazard assessment of their products, Bio Reliance has always given their best assays, testing paradigms, and expert advices. They have individual toxicological assays as well as tailor their program according to individual needs. They follow international guidelines such as those from the Food and Drug Administration, the Environmental Protection Agency, OECD, the European Union, the Japanese Ministry of Health and Welfare, and the Japanese Ministry of Agriculture, Forestry, and Fisheries along with good laboratory practice. They apply the most current battery of tests available for the detection of genotoxic potential. Genotoxicity assays support the interpretation of other toxicology results, provide

support for regulatory decisions, alert producers and users of potential hazard, and help with drug discovery decisions.

Testing categories are screening assays, regulatory submission assays, tier II (follow-up mechanistic assays), and reach testing services.

15.3.2.1 In Vitro Methods

15.3.2.1.1 Ames Test

Ames test is a widely used method to analyze whether the chemical or substance nanoparticle can cause mutations in the DNA of the test organism. It is the key evaluation test for genetic toxicity for the detection of toxic, mutagenic, clastogenic, and morphological transforming activity in a number of well-characterized test systems. It can assess the mutagenic potential of chemical compounds and serves as a quick and convenient method for their estimation compared to the standard carcinogen assays on mice and rats, which are time consuming and expensive [Mortelmans and Zeiger 2000]. A positive test indicates that the chemical or nanoparticle is mutagenic and may therefore act as carcinogen because cancer is often linked to mutation. Ames test is known as the most accurate and commonly used procedure to detect genotoxic carcinogens, which cause two classes of gene mutation, base pair substitution, and small frameshift [Ames et al., 1973]. It is a very quick and convenient assay to estimate the carcinogenic potential of a compound [Charnley, 2002]. It is an essential test within the current battery of assays required for genotoxicity evaluation. According to UK expert advisory Committee on Mutagenicity, Ames test is recommended for the mutagenicity test. Although this test has proven to be invaluable in the safety testing of chemical substances, it has been less commonly used with nanoparticles [Doak et al., 2012; OECD, 2014a, 2014b]. One interesting result of Ames test is that there is no threshold concentration for mutagenesis as the dose–response curve using varying concentrations of chemical is almost always linear. However, some organisms can tolerate the low level of mutagens due to protective mechanisms such as DNA repair, and threshold may exist for certain chemical mutagens. Ames test is often used as one of the initial screens for potential drugs to weed out possible carcinogens, and it is one of the eight tests required under the Pesticide Act (United States) and one of six tests required under the Toxic Substances Control Act (United States).

Ames test was usually done on agar plates, but in recent times, due to technology advancements, an alternative method known as fluctuation method has been developed. The concept remains the same as the agar based method, but here, a pH indicator is added which indicates the level of mutagenesis by the change in color from yellow to purple on the microtiter plate.

15.3.2.1.2 Comet Assay

Comet assay also known as single-cell gel electrophoresis assay is a sensitive and rapid technique for detecting DNA damage. The term *comet* refers to the pattern of DNA migration through the electrophoresis gel, which often resembles a comet. This is one of the techniques used as chemoprevention by evaluating genotoxicity for the existence of cancer.

Swedish researchers Ostling and Johansson [1984] developed this technique, which was modified late in 1988, as the alkaline comet assay [Singh et al., 1988]. Most of

the time, comet assay application has been to study animal eukaryotes, but nowadays, there have been reports of successful application in the study of plant cells as well. The extent of DNA liberated from the head of the comet is directly proportional to the amount of DNA damage. The comet assay can be used to detect DNA damage caused by double-strand breaks, single-strand breaks, alkali labile sites, oxidative base damage, and DNA cross-linking with DNA or protein. The comet assay is also used to monitor DNA repair in living cells [Collins, 2004]. The principle involves the encapsulation of cells in a low-melting-point (LMP) agarose suspension, lysis of the cells in neutral or alkaline conditions, and electrophoresis of the suspended lysed cells. DNA migration through electrophoretic gel resembles a comet and hence termed as *comet assay*. After staining with a DNA-specific fluorescent dye such as ethidium bromide or propidium iodide, the gel is read for the amount of fluorescence in head and tail and length of tail. The head of the comet is composed of intact DNA, while the tail consists of damaged or broken pieces of DNA (Figure 15.7) [Collins, 2004]. Comet assay is carried out according to the protocol by Tice et al. [2000]. Approximately 5×10^5 cells should be seeded per experimental culture flask followed by 24 hours of treatment of hepatoma tissue culture cells with the DNA damage-inducing agent or with rutin. Cells should be trypsinized (500 mL of trypsin–ethylenediaminetetraacetic acid [EDTA], 0.025%; 37°C); cell suspension should be centrifuged (5 min, 1000 revolutions per minute [rpm]); and cell pellet should be resuspended in 500 ml of culture medium. Next, 120 mL of LMP agarose (0.5%) should be added to the tube and mixed with the cells. This mixture deposition should be done on pregelatinized slides (normal melting point agarose: 1.5%), and the slides should be placed in lysis solution for at least 1 hour. After denaturation (2 min) and alkaline electrophoresis (25 V, 300 mA, 20 min), the slides are neutralized and fixed and should be kept for refrigeration followed by analysis. Slides should be stained with ethidium bromide and visually inspected using a fluorescence microscope (excitation filter of 420–49 nm and emission filter of 52 nm) at ×400 magnification.

Comets are classified as follows: Class 0: nucleoids that do not show a tail. Class 1: nucleoids with tail less than the diameter of the nucleoid. Class 2: nucleoids with tail size between one and two times the diameter of the nucleoid. Class 3: nucleoids with tail more than two times greater than the diameter of the nucleoid. The experiments should be carried out in triplicates, where 300 cells are analyzed per treatment. Apoptotic cells that showed the nucleoid completely fragmented should not be counted. The total score calculation is done by summing the resultant values of the multiplication of the total cells observed in each lesion class by the class value (0–3). Cell viability should be determined along with the comet assay for each treatment. After the treatment period and harvesting the cells by trypsinization, 20 mL of the cell suspension should be mixed with 20 mL of trypan blue (Gibco) in a 1.5 mL microtube. The cells are ready for counting in a Neubauer chamber using a light microscope, where the viable cells were unstained and nonviable cells appeared blue. Cell viability is taken to be the percentage of viable cells examined.

15.3.2.2 In Vivo Methods

15.3.2.2.1 Micronucleus Test with Cytokinesis Block (MNCtB)

Potential genotoxic compounds can be tested using micronucleus test. This assay is very successful and reliable in assessing genotoxic carcinogens, i.e., carcinogens that

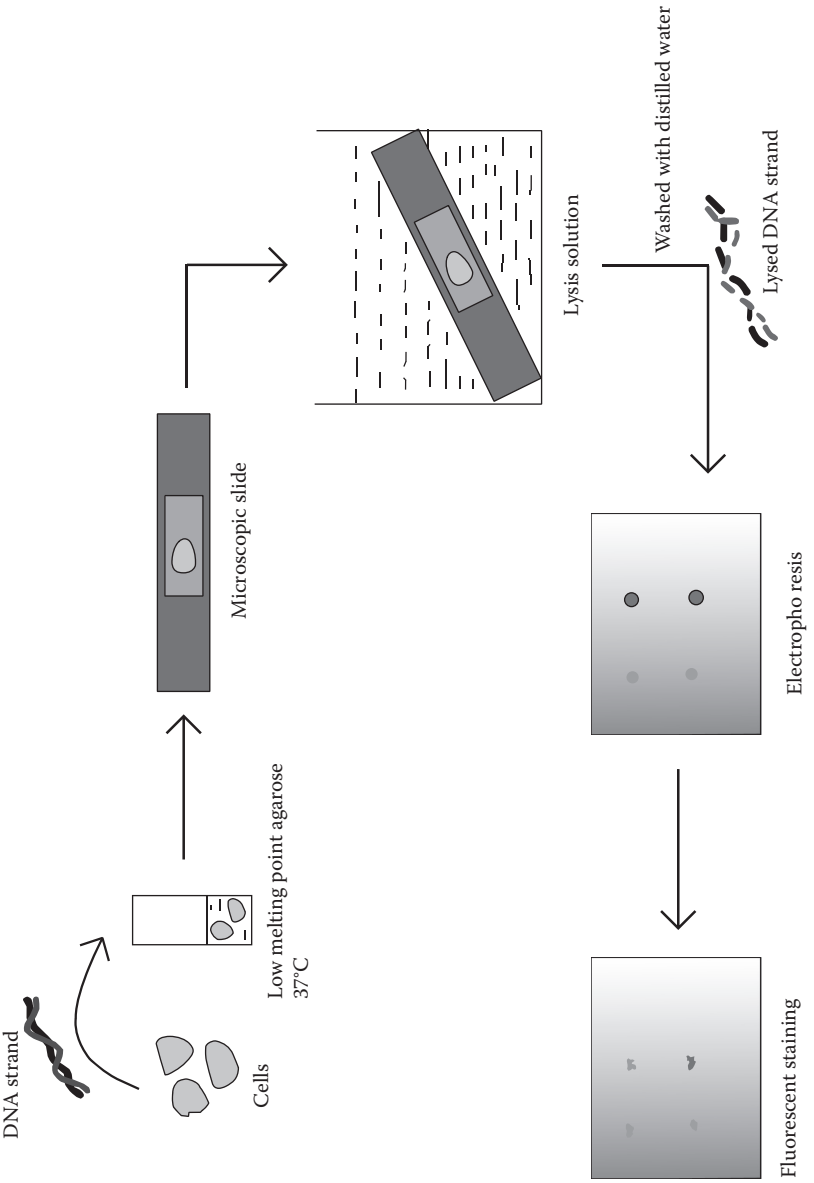


FIGURE 15.7 Comet assay.

act by causing genetic damage, and they are the OECD guideline for the testing of chemicals. The presence of micronuclei in cultured human cells has been reported as early as the 1960s [Elston, 1963] and 1970s [Countryman and Heddle, 1976]. The IVMNT has evolved into a robust quantitative assay of chromosome damage by the development of the cytokinesis-block technique that eliminated the confounding effects on micronuclei expression by the cytostatic effects caused by poor culture conditions, treatment effects, cell senescence, and variability in mitogen response in the lymphocyte test system (Figure 15.8) [Fenech and Morley, 1985; Fench and Morley, 1986].

The cells with micronucles formation after the treatment with the nanoparticles should be treated with five concentrations of rutin for about 24 hours. The treatment with rutin and benzopyrene gives better results for the evaluation of antigenotoxicity. Approximately 5×10^5 cells per culture flask should be seeded in an experimental flask. In this assay, after 24 hours of treatment with rutin and benzopyrene, it is followed by 26 hours of incubation after the addition of 3 mg mL^{-1} cytochalasin-B to obtain binucleated cells. The cells are harvested according to the protocol of Oliveira et al. [2002]. Briefly, the cells should be trypsinized (500 mL of 0.025% trypsin–EDTA, at 37°C), centrifuged (5 min, 1000 rpm), hypotonic-treated (1% sodium citrate) and fixed (3:1 methanol/acetic acid). The experiments should be performed

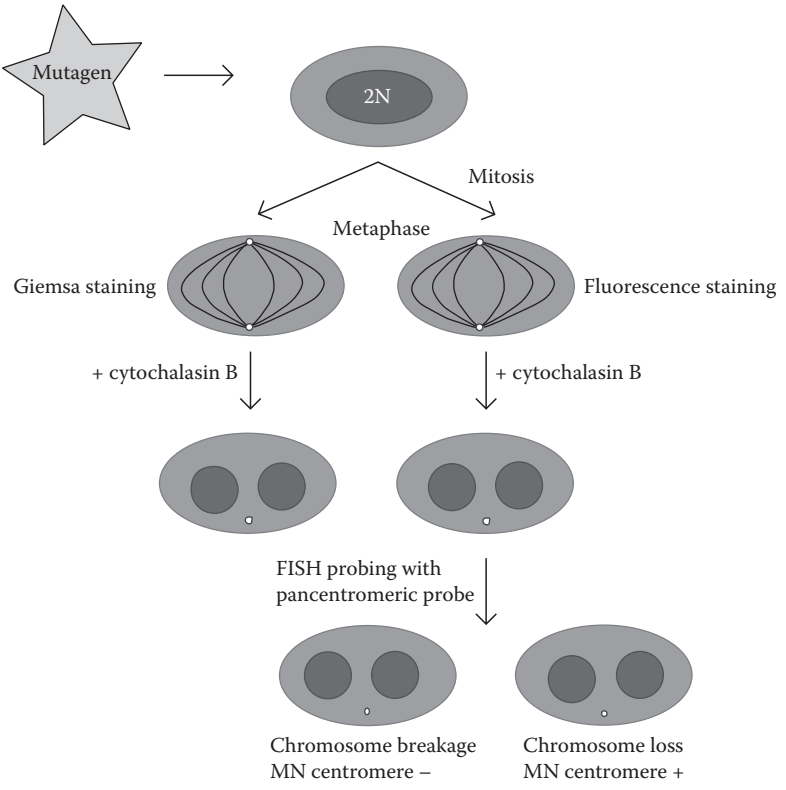


FIGURE 15.8 Micronucleus test.

in triplicates, where approximately 6000 binucleated cells should be analyzed per treatment for the frequency of micronuclei and 1500 cells for determination of the nuclear division index. The criteria for the selection of binucleated cells, identification of micronuclei, and calculation of the nuclear division index followed the description by Fenech [2000].

The percent reduction in DNA damage by the genotoxic agent was calculated using the following formula:

$$\text{Reduction}\,(\%) = \text{A} - \text{B}/\text{A} - \text{C} \times 100.$$

A is the mean number of cells with micronuclei observed with DNA damage-inducing agent (benzopyrene); B is the number of cells with micronuclei observed in the antigenotoxicity treatment (rutin + benzopyrene); and C is the number of cells with micronuclei of the control. The criteria for selecting binucleated cells to score are the following: main nuclei that are separate and of approximately equal size, main nuclei that touch and even overlap as long as nuclear boundaries are able to distinguished, and main nuclei that are linked by nucleoplasmic bridges.

15.3.2.2.2 Biochemical Assays

Biochemical assays analyze the substances found in living organisms and the chemical reactions underlying the life processes. For a biochemical assay, the biomolecule selected should be in its pure form. Once a pure biomolecule is obtained, it may be employed for a specific purpose such as an enzymatic reaction, used as a therapeutic agent, or in an industrial process. Biochemical assay is basic profiling for the DNA quantification and protein profiling in both treated and negative sample (treatment with zero concentration of the nanoparticles). The total protein concentration is analyzed according to the protocol by Lowry et al. [1951]. And the DNA isolation and quantification is done according to Hoisington et al. [1994].

15.3.2.2.3 ROS Mechanism

ROS are chemically reactive chemical species containing oxygen. These are peroxides, superoxide, hydroxyl radical, and singlet oxygen [Hayyan et al., 2016]. In terms of biological context, ROS are by-products of the natural metabolism of oxygen and have important role in cell signaling and homeostasis [Devasagayam et al., 2004]. The ROS level can increase during the time of environmental stress (e.g., ultraviolet or heat exposure and treatment with nanoparticle or chemicals). This may result in damage of the cells and cell structures. Collectively, this is also known as oxidative stress [Devassagayam et al., 2004; Sosa Torres et al., 2015]. Similarly, the molecules are also produced during the electron transport of mitochondrial aerobic respiration or by oxidoreductase enzymes and metal catalyzed oxidation. ROS is not only involved in the induction of cell death, but also regulates the expression of certain genes and is able to activate cell-signaling cascades (Figure 15.9) [Hancock et al., 2001]. ROS can be measured by various assays. ROS mechanism assay could be used for both *in vitro* and *in vivo* genotoxicity test. Some of the assays done to analyze the ROS mechanism are as follows:

15.3.2.2.3.1 Fluorescent and Chemiluminescent Assays The fluorescent and chemiluminescent assays are important assays for the analysis of the different ROS

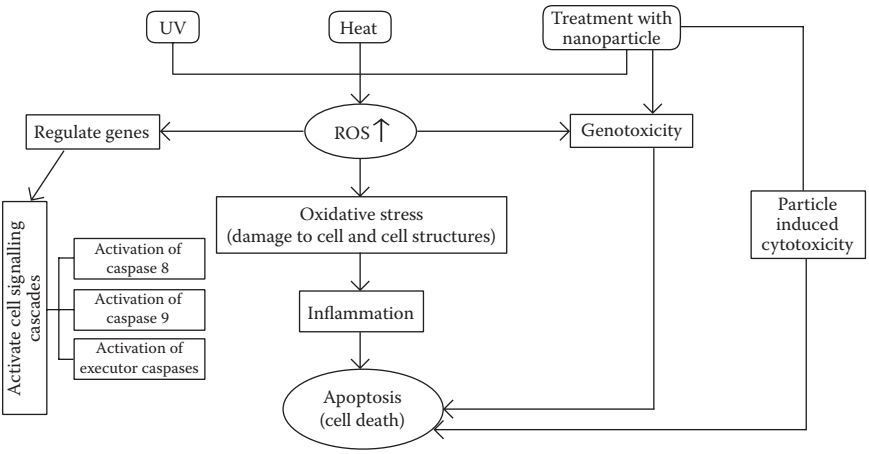


FIGURE 15.9 ROS mechanism induced through nanoparticles.

such as HO•. It is generally performed with fluorogenic or chemiluminogenic substances, which serve as hydrogen donors. The commonly used substrates for these types of assays are 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) [Johnston et al., 2010], homovanillic acid [Zhou et al., 1997], and AmplexRed [Halliwell and Whiteman 2004]. But a number of these fluorogenic substrates can only efficiently detect the cellular peroxides in the presence of a peroxidase (e.g., horseradish peroxidase [HRP]) or a transition metal [Gasser et al., 2012; Tarpey et al., 2004]. The HRP or transition metal could decompose the peroxides to radicals; after decomposition to radicals, it is able to react with the dye. However, the use of HRP in the assay can lead to incorrect results, because cellular compounds, such as thiols, can serve as a substrate for HRP and thus affect the fluorescence by either enhancing or quenching the signal [Ruch et al., 1983]. Some other fluorescent dyes are used to detect ROS species, e.g., H_2DCF. The active dye in the medium will react with oxidizing compounds, which will influence the measurement result [Tarpey et al., 2004]. Unlike this dye, many other fluorescent substrates are not highly specific, which can be an advantage in assays where different types of ROS are produced or in assays where a certain nanocompound induces the release of unknown ROS. Furthermore, DCF has a high fluorescence quantum yield and is hence detectable by fluorescence spectroscopy.

15.3.2.2.3.2 Nitric Oxide Assay Nitric oxide assay is useful for the detection of reactive molecules. In this assay, the reduced dye, dihydrorhodamine (DHR), is oxidized by ROS, by free radical oxidants derived from them, to its corresponding fluorescent product, rhodamine [Kohno, 2010]. This test also has its limitations. The oxidative conversion of DHR to rhodamine is mediated by an intermediate DHR radical, which is usually further oxidized by oxygen to yield a stable fluorescent product. The reaction of the intermediate DHR radical can cause an artificial enhancement of the fluorescent signal intensity (Figure 15.10) [Rothe and Valet, 1990]. The DHR conversion is not very specific because DHR reacts with NO•, as well as with superoxide and several other oxidants.

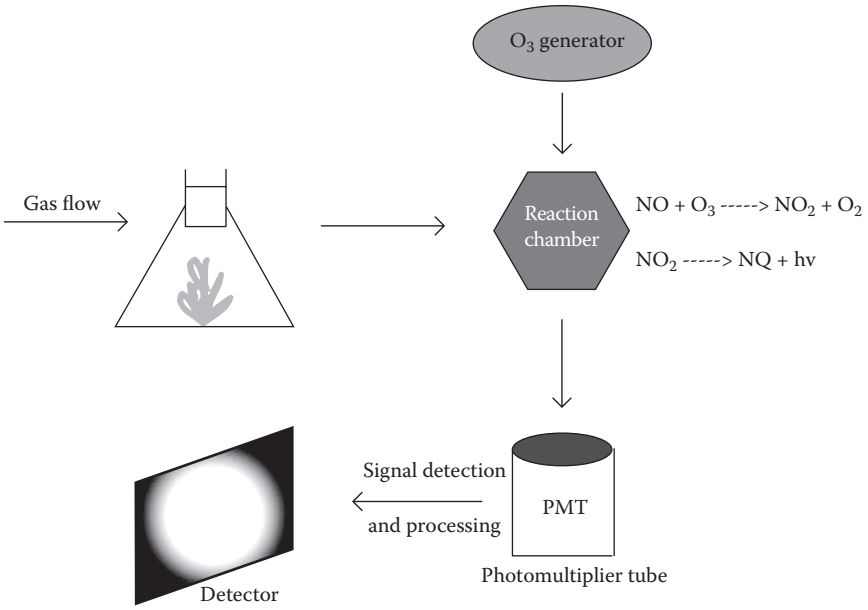


FIGURE 15.10 Nitric oxide assay.

5.3.2.2.3.3 Electron Spin Resonance Assay Electron spin resonance (ESR) assay, also known as the electron paramagnetic resonance technique, is the only technique that can give the measurement of free radicals, such as HO• and superoxide (O^{2-}). This assay could detect fairly unreactive radicals only. Reactive radicals do not accumulate to levels that are sufficiently high to allow detection [Tarpey et al., 2004]. Highly reactive radicals can be converted to relatively stable radicals by the spin-trapping technique [Herman et al., 2007]. A five-membered ring nitron derivative, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), is widely considered to be the most powerful spin-trapping reagent [Hartley et al., 1999]. ESR assay can then readily detect the more stable species. DMPO is commonly used for *in vitro* studies of living cells, but DMPO is cytotoxic at high concentrations, which is limiting its use [Hartley et al., 1999]. A few drawbacks are there for this assay such as free radicals can immediately react with a molecule other than the spin-trapping agent and the high cost of the equipment is another major drawback of this method [Van Acker et al., 1996].

15.3.2.2.3.4 Lipid Peroxidation Assay Lipid peroxidation is a ROS-mediated process by which polyunsaturated fatty acids undergo peroxidation, producing α,β-unsaturated aldehydes such as malondialdehyde [Hodges et al., 1999]. In contrast to other radical scavenging assays, the lipid peroxidation assay can evaluate test compounds in the presence of other antioxidants, iron, and radicals [Van Acker et al., 1996]. Lipid peroxidation is one of the most widely used indicators of free radical formation. The previously described assays (H_2DCF, nitric oxide assay, and ESR assay) directly measure the free radicals, whereas the lipid peroxidation assay does not measure released radicals; rather, it measures the amount of oxidized fatty acids, which correlates with the amount of released radicals. The measurement of

lipid peroxidation is generally done by detecting malondialdehyde generated from the decomposition of lipid peroxidation products. The big problem is that this method is not necessarily specific to malondialdehyde [Byrdwell and Neff, 2002]. Various compounds have a similar absorption maximum as malondialdehyde, which leads to an overestimation of the malondialdehyde values [Buerki-Thurnherr et al., 2013]. Progress in mass spectrometry has prompted the development of more accurate and sensitive methods for various lipid peroxidation products [Herman et al., 2007].

15.3.2.2.3.5 Glutathione Peroxidase Assay Glutathione is a three-amino acid peptide, active in a nonenzymatic oxidant defense mechanism, which serves to detoxify peroxides and to regenerate a number of important antioxidants [Ruch et al., 1983]. Different colorimetric and fluorescent-based assays are available for measuring the amount of reduced glutathione (GSH)/glutathione disulfide (GSSG) in *in vitro* cultures. GSH is regenerated from its oxidized form (GSSG) by the action of a NADPH-dependent reductase, which can influence the measurement results and the GSSG concentration might be underestimated.

15.3.2.3 Microscopic Analysis

15.3.2.3.1 Light Microscopy

Microscopy was created to satisfy our urge of seeing objects at higher magnification. Light microscopy needs light as a source to visualize the objects. Light microscopy can be used to study objects from cytogenetic techniques to histological and histochemical techniques [Fontanetti et al., 2010]. Light microscopic studies are used to analyze *in vitro* genotoxicity evaluation. They are used to study a number of aberrant cells per total cells scored in treated and untreated (samples treated with zero concentration) samples. The slides for observation could be prepared following the protocol by Fiskesjo [1997]. The staining procedure should be done by keeping cells in 1 M HCl for about 6 min followed by staining with 40–50% Safranin. Staining should be continued for about 5–6 min. Slides are ready for observation followed by analysis at 1000× magnification for cytological changes. The micronucleus index should be calculated following Tolbert et al. [1992]. It should be calculated as the number of dividing cells per number of 1000 observed cells. The number of aberrant cells should be noted per total cells scored at that concentration [Bakare et al., 2000].

15.3.2.3.2 Electron Microscopy for Biological Samples

Ultramorphological analyses can be done using electron microscopy; Scanning electron microscopy and transmission electron microscopy are such evaluations. Scanning electron microscopy proves the tridimensional view of the cell surface; Transmission electron microscopy provides the bidimensional view of the interior of the cell. An electron microscope has scanning coils that capture images produced by an electron beam tracing over the object. Electron microscopy analysis could be very much useful for treated and untreated samples for both *in vitro* and *in vivo* studies. The sample preparation for scanning electron microscopy and transmission electron microscopy analysis are as follows: 1 mL of treated cells should be fixed for 2 hours in 2% gluteraldehyde in 0.1 M cacodylate buffer, pH 7.2. The next step is to fix the cells in 1% osmium tetraoxide (12 hours) followed by dehydration in alcohol.

Later, the material should be embedded in Epox 812. Semithin and ultrathin sections from the middle fragment of the central longitudinal and cross section of the treated sample are selected. One part is stained with uranyl acetate and Reynold's reagent, and the other half should be left unstained for observation under electron microscope [Wierzbicka, 1998].

15.3.3 Importance and Significance

Nanotoxicology is one of the important areas of the research. It could be the area of biological research in past few years, which addresses the adverse effect of nanomaterials in biological systems. Despite the growing interest in nanoparticles and their effect on the body, standardized procedures have not yet been outlined for the evaluation of nanoparticle toxicity. To date, however, nanoparticle toxicity studies have mainly focused on *in vitro* examinations due to the ease in execution, control, and interpretation of the experiments compared with *in vivo* tests, yet there are very limited studies that have been done to evaluate the toxicity of nanoparticles on skin and other parts of the body. Hence, more consideration must be placed on improving the experimental procedures of nanoparticle toxicity assays in order to gain reliable data. Several critical issues should be addressed when designing an experiment to study the cytotoxicity of nanoparticles. Due to the increased production and use of nanoparticles in various fields, the unintended adverse effect of nanoparticles is an emerging and growing concern both academically and socially. Several studies have investigated the toxicity of nanoparticles based on various characteristics such as shape, size, surface chemistry, chemical composition, surface activity, and solubility.

The recommendations for the use of nanoparticles in various fields have emerged as a result of these initial toxicity studies. For example, the Royal Society recommended the conditional use of nanoparticles in cosmetics upon a favorable assessment by the European Commission safety advisory committee after identifying that nanoparticles are capable of penetrating the skin. However, a more rigorous evaluation of toxicity is needed in order to confidently regulate the safe use of all forms of nanoparticles. There should be improved technology for further studies on the use of nanoparticle and its cytotoxic studies, since this is the growing interest of nanoparticle research. There are many areas of application with nanomaterials, such as design of optical devices, catalysts, biosensors, imaging agents, drugs, and gene delivery. A large number of nanoparticles including gold nanoparticles, polymeric nanoparticles, quantum dots, and bioceramic-based nanoparticles are applied as carriers in the drug delivery systems. Dominant physicochemical properties of these materials such as small size and high surface-to-volume ratio lead to the improvement of their effectiveness as suitable carriers in drug and gene delivery. Extensive work on nanotoxicity should be conducted for drug delivery systems. Although nanoparticles find application in advanced research, their drawback is with the toxicity levels; hence, nanoparticle cytotoxicity should be confirmed on cell lines. It is important to understand nanoparticle–cell interactions in order to check cell viability, DNA, and protein profile. Lipid peroxidation and oxidative stress associated with cytotoxicity are also to be analyzed.

Guidelines and protocols for *in vitro* and *in vivo* nanotoxicities discussed in this chapter could be beneficial for industries, researchers, students, and society at large.

15.4 Conclusions

Despite the growing interest in nanoparticles and their innumerable applications, an emerging scientific and social concern is that standardized procedures have not yet been outlined for the evaluation of nanoparticle specific toxicity. To date, however, nanoparticle toxicity studies have mainly focused on *in vitro* examinations due to the ease in execution, control, and interpretation of the experiments compared with *in vivo* tests. Several studies have investigated the toxicity of nanoparticles based on various characteristics such as shape, size, surface chemistry, chemical composition, surface activity, and solubility. Recommendations for the use of nanoparticles in various fields have been formulated as a result of these initial toxicity studies. However, a more rigorous evaluation of toxicity is needed in order to confidently regulate the safe use of all forms of nanoparticles.

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REFERENCES

Altman, S. A., Randers, L., Rao, G. 1993. Comparison of trypan blue dye exclusion and fluorometric assays for mammalian cell viability determinations. *Biotechnol* 9:671–674.

Aardema, M. J., Snyder, R. D., Spicer, C. 2006. SFTG international collaborative study on *in vitro* micronucleus test III. Using CHO cells. *Mutat Res* 607:61–87.

Ames, B. N., Lee, F. D., Durston, W. E. 1973. An improved bacterial test system for the detection and classification of mutagens and carcinogens. *Proc Natl Acad Sci USA* 70:782–786.

ASTM (American Society for Testing and Materials). 1987. E 1163-87, standard test method for estimating acute oral toxicity in rats. ASTM Internation, West Conshohocken, PA.

Bakare, A. A., Mosuro, A. A., Osibanjo, O. 2000. Effect of simulated leachate on chromosomes and mitosis in roots of *Allium cepa* (L). *J Environ Biol* 21:263.

Bonassi, S., El-Zein, R., Barale, R., Fenech, M. 2010. Associations of micronucleus frequency with cancer risk. *Mutagenesis* 26:93–100.

Braydich-Stolle, L., Hussain, S., Schlager, J. 2005. *In vitro* cytotoxicity of nanoparticles in mammalian germline stem cells. *J Toxicol Sci* 8:412–419.

Bruce, R. D. 1985. An up-and-down procedure for acute toxicity testing. *Fundam Appl Tox* 5:151–157.

Brendler-Schwaab, S., Hartmann, A., Pfuhler, S., Speit, G. 2005. The *in vivo* comet assay: Use and status in genotoxicity testing. *Mutagenesis* 20:245–254.

Burlinson, B., Tice, R. R., Speit, G. et al. 2007. Fourth International Workgroup on Genotoxicity Testing: Result of the *in vivo* Comet assay workgroup. *Mutat. Res* 627:31–35.

Burlinson, B. 2012. The *in vitro* and *in vivo* comet assays. *Methods Mol Biol* 817:143–163.

Buerki-Thurnherr, T., Xiao, L., Diener, L. et al. 2013. *In vitro* mechanistic study towards a better understanding of ZnO nanoparticle toxicity. *Nanotoxicol* 7:402–416.

Byrdwell, W. C., Neff, W. E. 2002. Dual parallel electrospray ionization and atmospheric pressure chemical ionization mass spectrometry (MS), MS/MS and MS/MS/MS for the analysis of triacylglycerols and triacylglycerol oxidation products. *Rapid Commun Mass Spectrom* 16:300–319.

Countryman, P. I., Heddle, J. A. 1976. The production of MN from chromosome aberrations in irradiated cultures of human lymphocytes. *Mutat Res* 41:321–332.

Charnley, G. 2002. Ames test. *Encyclopedia of Public Health*. eNotes.com.

Chan, F. K., Moriwaki, K., De Rosa, M. J. 2013. Detection of necrosis by release of lactate dehydrogenase (LDH) activity. *Methods Mol Biol* 979:65–70.

Clare, M. G., Lorenzon, G., Akhurst, L. C. et al. SFTG international collaborative study on *in vitro* micronucleus test II: Using human lymphocytes. *Mutat Res* 607:37–60.

Corvi, R., Albertini, S., Hartung, T. et al. 2008. ECVAM retrospective validation of *in vitro* micronucleus test (MNT). *Mutagenesis* 23:271–283.

Collins, A. R. 2004. The comet assay for DNA damage and repair: Principles, applications and limitations. *Mol Biotechnol* 26:249–261.

Derfus, A., Chan, W., Bhatia, S. J. 2004. Probing the cytotoxicity of semiconductor quantum dots. *Nano Lett* 4:11–18.

Devasagayam, T., Tilak, J. C., Boloor, K. K., Sane Ketaki, S., Ghaskadbi Saroj, S., Lele, R. D. 2004. Free radicals and antioxidants in human health: Current status and future prospects. *J. Assoc Physicians India (JAPI)* 52:796.

Decordier, I., Papine, A., Vande Loock, K., Plas, G., Soussaline, F., Kirsch-Volders, M. 2010. Automated image analysis of MN by IMSTAR for biomonitoring. *Mutagenesis* 26:163–168.

Dixon, W. J. 1965. The up-and-down method for small samples. *J Am Stat Assoc* 60:967–978.

Doak, S. H., Manshian, B., Jenkins, G. J. S., Singh, N. 2012. *In vitro* genotoxicity testing strategy for nanomaterials and the adaptation of current OECD guidelines. *Mutat Res* 745:104–111.

ECVAM (European Centre for the Validation of Alternative Methods). Statement by the European Centre for the Validation of Alternative Methods (ECVAM) Scientific Advisory Committee (ESAC) on the Scientific Validity of the *In vitro* Micronucleus Test as an Alternative to the *In vitro* Chromosome Aberration Assay for Genotoxicity Testing. 2006. ESAC 25th meeting, November 16–17, 2006. http://ecvam.jrc.it /index.htm (date last accessed September 28, 2010).

ESAC (ECVAM Scientific Advisory Committee). ECVAM Scientific Advisory Committee (ESAC) Peer Review: Retrospective Validation of the *In Vitro* Micronucleus Test. 2006. Summary and Conclusions of the Peer Review Panel. European Centre for the Validation of Alternative Methods (ECVAM), Ispra, Italy, November 16–17, 2006. http://ecvam.jrc.it/index.htm (date last accessed September 28, 2010).

Elston, R. N. 1963. Nuclear budding and MN formation in human bone marrow, skin and fascia lata cells *in vitro* and in oral mucosa cells in *vivo*. *Acta Pathol Microbiol* 59:195–199.

Elhajouji, A., Cammerer, Z., Lukamowicz, M., Kirsch-Volders, M. 2010. Potential thresholds for genotoxic effects by MN scoring. *Mutagenesis* 26:199–204.

Ezhilarasi, A., Vijaya, J. J., Kaviyarasu, K., Maaza, M., Ayeshamariam, A., Kennedy, L. J. 2016. Green synthesis of NiO nanoparticles using *Moringa oleifera* extract and their biomedical applications: Cytotoxicity effect of nanoparticles against HT-29 cancer cells. *J Photochem Photobiol* 164:352–360.

Fenech, M., Morley, A. 1985. Solutions to the kinetic problem in the micronucleus assay. *Cytobios* 43:233–246.

Fenech, M., Morley, A. A. 1986. Cytokinesis-block micronucleus method in human lymphocytes: Effect of *in vivo* ageing and low dose X-irradiation. *Mutat Res* 161:193–198.

Fenech, M. 2000. The *in vitro* micronucleus technique. *Mutat Res Fundam Mol Mech Mutagenesis* 455:81–95.

Fiskesjo, G. 1997. Allium test for screening chemicals: Evaluation of cytologic parameters. In: *Plants for environmental studies*, Wang W., Gorsuch J. W., Hughes J. S., Eds. Boca Raton, FL: CRC Lewis Publishers; p. 308.

Fontanetti, C. S., Christofoletti, C. A., Pinheiro, T. G., Souza, T. S., Pedro-Escher, J. 2010. Microscopy as a tool in toxicological evaluation. In: *Microscopy: Science, technology, applications and education*, Méndez-Vilas, A. and Díaz, J., Eds. Badajoz: Formatex, pp. 1002–1007.

Gasser, M., Wick, P., Clift, M. J. et al. 2012. Pulmonary surfactant coating of multi-walled carbon nanotubes (MWCNTs) influences their oxidative and pro-inflammatory potential *in vitro. Part Fibre Toxicol* 9:17.

Ginouves, M., Carme, B., Couppie, P., Prevot, G. 2014. Comparison of tetrazolium salt assays for evaluation of drug activity against *Leishmania* sp. *J Clin Microbiol* 56:2131–2138.

Goodwin, C. J., Holt, S. J., Downes, S., Marshall, N. J. 1995. Microculture tetrazolium assays: A comparison between two new tetrazolium salts, XTT and MTS. *J Immunol Methods* 179:95–103.

Gu, Y. J., Cheng, J., Lin, C. C., Lam, Y. M., Cheng, S. H., Wong, W. T. 2009. Nuclear penetration of surface functionalized gold nanoparticles. *Toxicol Appl Pharmocol* 237:196–204.

Halliwell, B., Whiteman, M. 2004. Measuring reactive species and oxidative damage *in vivo* and in cell culture: How should you do it and what do the results mean? *Br. J Pharmacol* 142:231–255.

Hancock, J. T., Desikan, R., Neill, S. J. 2001. Role of reactive oxygen species in cell signalling pathways. *Biochem Soc Trans* 29:345–350.

Hartley, D. P., Kolaja, K. L., Reichard, J., Petersen, D. R. 1999. 4-Hydroxynonenal and malondialdehyde hepatic protein adducts in rats treated with carbon tetrachloride: Immunochemical detection and lobular localization. *Toxicol Appl Pharmacol* 161:23–33.

Hayyan, M., Hashim, M. A., AlNashef, I. M. 2016. Superoxide ion: Generation and chemical implications. *Chem Rev* 116:3029–3085.

Hayashi, M., MacGregor, J. T., Gatehouse, D. G. et al. 2007. *In vivo* micronucleus assay working group, IWGT. *In vivo* erythrocyte micronucleus assay III: Validation and regulatory acceptance of automated scoring and the use of rat peripheral blood reticulocytes, with discussion of non-hematopoietic target cells and a single dose-level limit test. *Mutat Res* 627:10–30.

Hartung, T., Bremer, S., Casati, S. et al. 2004. A modular approach to the ECVAM principles on test validity. *Altern Lab Anim* 32:467–472.

Hartmann, A., Agurell, E., Beevers, C. et al. 2003. Recommendations for conducting the *in vivo* alkaline Comet assay. *Mutagenesis* 18:45–51.

Hodges, D. M., DeLong, J. M., Forney, C. F., Prange, R. K. 1999. Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. *Planta* 207:604–611.

Hoisington, D., Khairallah, M., Gonzales de leon, D. 1994. *Laboratory protocols: CIMMYT Applied Biotechnology Center*, 2nd (ed.). Texcoco: Centro Internacional de Mejoramiento de Maíz y Trigo.

Hristozov, D., Gottardo, S., Semenzin, E. et al. 2016. Frameworks and tools for risk assessment of manufactured nanomaterials. *Environ Int* 95:36–53.

Johnston, H. J., Hutchison, G. R., Christensen, F. M. et al. 2010. A critical review of the biological mechanisms underlying the *in vivo* and *in vitro* toxicity of carbon nanotubes: The contribution of physico-chemical characteristics. *Nanotoxicol* 4:207–246.

Kirkland, D., Aardema, M., Henderson, L., Müller, L. 2005. Evaluation of the ability of a battery of three *in vitro* genotoxicity tests to discriminate rodent carcinogens and non-carcinogens: I. Sensitivity, specificity and relative predictivity. *Mutat Res* 84:1–256.

Kirkland, D., Speit, G. 2008. Evaluation of the ability of a battery of three *in vitro* genotoxicity tests to discriminate rodent carcinogens and non-carcinogens: III. Appropriate follow-up testing *in vivo*. *Mutat Res* 654:114–132.

Kohno, M. 2010. Applications of electron spin resonance spectrometry for reactive oxygen species and reactive nitrogen species research. *J Clin Biochem Nutr* 47:1–11.

Kumari, M., Mukherjee, A., Chandrasekaran, N. 2009. Genotoxicity of silver nanoparticles. *Sci Total Environ* 407:5243–5246.

Kirsch-Volders, M., Sofuni, T., Aardema, M. et al. 2000. Report from the *in vitro* micronucleus assay working group. *Environ Mol Mutagen* 35:167–172.

Kirsch-Volders, M., Sofuni, T., Aardema, M. et al. 2003. Report from the *in vitro* micronucleus assay working group. *Mutat Res* 540:153–163.

Lorge, E., Thybaud, V., Aardema, M. J. et al. 2006. SFTG international collaborative study on *in vitro* micronucleus test I. General conditions and overall conclusions of the study. *Mutat Res* 607:13–36.

Lipnick, R. L., Cotruvo, J. A., Hill, R. N., Bruce, R. D., Stitzel, K. A., Walker, A. P. et al. 1995. Comparison of the up-and-down, conventional LD50 and fixed dose acute toxicity procedures. *Fd Chem Toxicol* 33:223–231.

Lowry, O. H., Rosbrough, N. J., Farr, A. L., Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:267–275.

McKelvey-Martin, V. J., Green, M. H., Schmezer, P., Pool-Zobel, B. L., De Méo, M. P., Collins, A. 1993. The single cell gel electrophoresis assay (comet assay): A European review. *Mutat Res* 288:47–63.

Mortelmans, K., Zeiger, E. 2000. The Ames Salmonella/microsome mutagenicity assay. *Mutat Res* 455:29–60.

Mosmann, T. J. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Immunol Methods* 65:55–63.

Nasser, F., Davis, A., Valsami-Jones, E., Lynch, I. 2016. Shape and charge of gold nanomaterials influence survivorship, oxidative stress and moulting of *Daphnia magna*. *Nanomaterials* 6:222.

Oliver, J., Meunier, J. R., Awogi, T. et al. 2006. SFTG international collaborative study on *in vitro* micronucleus test V. Using L5178Y cells. *Mutat Res* 607:125–152.

Ostling, O., Johanson, K. J. 1984. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochem Biophys Res Commun* 123:291–298.

Oliveira, D. C., de Lencastre, H. 2002. Multiplex PCR strategy for rapid identification of structural types and variants of the mec element in methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* 46:2155–2161.

OECD (Organisation for Economic Co-operation and Development). 2005a. Guidance Document No. 34: Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment. OECD, Paris. Available at: http://search.oecd.org/officialdocuments/displaydocumentpdf/?doclanguage=en&cote=env/jm/mono(2005)14.

OECD. 2005b. Test Guideline 432: *In Vitro* 3T3 NRU Phototoxicity Assay. OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects. OECD, Paris. Available at: http://www.oecd-ilibrary.org/environment/test-no-432-in-vitro-3t3-nru-phototoxicitytest_9789264071162-en.

OECD. 2009. Guidance Manual for the Testing of Manufactured Nanomaterials: OECD Sponsorship Programme: First Revision [ENV/JM/MONO(2009)20/REV].

OECD. 2010. *In Vitro* Mammalian Cell Micronucleus Test (MNvit). OECD Guideline for Testing of Chemicals No. 487 (draft). OECD, Paris. Available at: http://www.oecd.org/env/testguidelines (date last accessed September 28, 2010).

OECD. 2012. Important Issues on Risk Assessment of Manufactured Nanomaterials [ENV/JM/MONO (2012)8].

OECD. 2014a. Reports of the JaCVAM initiative international pre-validation and validation studies of the *in vivo* rodent alkaline comet assay for the detection of genotoxic carcinogens, Series on Testing and Assessment, Nos. 193 and 194, Paris: OECD (not yet available).

OECD. 2014b. Environment, Health and Safety Publications: Series on the Safety of Manufactured Nanomaterials No. 43 Genotoxicity of Manufactured Nanomaterials: Report of the OECD Expert Meeting, Paris.

Rothe, G., Valet, G. 1990. Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 2′,7′-dichlorofluorescin. *J Leukoc Biol* 441:440–448.

Rovozzo, G. C., Burke, C. N. 1973. *A manual of basic virological techniques*, pp. 165–177. Prentice-Hall, Englewood Cliffs, NJ.

Rothfuss, A., O'Donovan, M., De Boeck, M. et al. 2010. Collaborative study on fifteen compounds in the rat-liver Comet assay integrated into 2- and 4-week repeat-dose studies. *Mutat Res* 702:40–69.

Ruch, W., Cooper, P. H., Baggiolini, M. 1983. Assay of H2O2 production by macrophages and neutrophils with homovanillic acid and horse-radish peroxidase. *J Immunol Methods* 63:347–357.

Scudiero, D. A., Shoemaker, R. H., Paull, K. D. et al. 1988. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. *Cancer Res* 48:4827–4833.

Singh, N. P, McCoy M. T., Tice, R. R. et al. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* 175:184–91.

Smith, C. C., Adkins, D. J., Martin, E. A., O'Donovan, M. R. 2008. Recommendations for design of the rat Comet assay. *Mutagenesis* 23:233–240.

Sosa Torres, M. E., Saucedo-Vázquez, J. P., Kroneck, P. M. 2015. Chapter 1, Section 3 The dark side of dioxygen. In *Metal ions in life sciences series 15.* Springer. US. pp. 1–12.

Strober, W. 1997. Trypan blue exclusion test of cell viability. *Curr Protoc Immunol* 111:A3.B.1–A3.B.3.

Tarpey, M. M., Wink, D. A., Grisham, M. B. 2004. Methods for detection of reactive metabolites of oxygen and nitrogen: *In vitro* and *in vivo* considerations. *Am J Physiol Regul Integr Comp Physiol* 286:431–444.

Tice, R. R., Agurell, E., Anderson, D. et al. 2000. Single cell gel/comet assay: Guidelines for *in vitro* and *in vivo* genetic toxicology testing. *Environ Mol Mutagenesis* 35:206–221.

Tolbert, P. E., Shy, C. M., Allen, J. W. 1992. Micronuclei and other nuclear abnormalities in buccal smears, method and development. *Mutat Res* 271:69–71.

Tracy, F., Sandra, U., Hewett, J. 2000. A microtiter trypan blue absorbance assay for the quantitative determination of excitotoxic neuronal injury in cell culture. *J Neurosci Methods* 100:157–163.

Tsuboy, M. S., Marcarini, J. C., Luiz, R. C. et al. 2010. *In vitro* evaluation of the genotoxic activity and apoptosis induction of the extracts of roots and leaves from the medicinal plant *Coccoloba mollis* (Polygonaceae). *J Med Food 13*:503–508.

Van Acker, S. A., Van den Berg, D. J., Tromp, M. N. et al. 1996. Structural aspects of antioxidant activity of flavonoids. *Free Radic Biol Med* 20:331–342.

Vasquez, M. Z. 2012. Recommendations for safety testing with the *in vivo* comet assay. *Mutation Research* 747:142–156.

Wakata, A., Matsuoka, A., Yamakage, K. et al. 2006. SFTG international collaborative study on *in vitro* micronucleus test IV. Using CHL cells. *Mutat Res* 607:88–124.

Warheit, D. B., Donner, E. M. 2010. Rationale of genotoxicity testing of nanomaterials: Regulatory requirements and appropriateness of available OECD test guidelines. *Nanotoxicol* 4:409–413.

Wierzbicka, M. 1998. Lead in the apoplast of *Allium cepa* L. root tips—Ultrastructural studies. *Plant Science* 105–119.

Zhou, M., Diwu, Z., Panchuk-Voloshina, N., Haugland, R. P. 1997. A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: Applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. *Anal Biochem* 253:162–168.



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Regulations for Safety Assessment of Nanomaterials

Preetika Biswas and Ashutosh Yadav

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16.1 Introduction: The Need for Nanoparticle Regulation and Their Expanding World

In everyday scenarios, we come across the terms *nanotechnology* and *nanomaterials* in products we use, in technologies being developed, or in some science fiction movies. The question that naturally arises is what makes this field so intuitive. Why is that approximately every science or technology news we hear has the term nanotechnology associated with it?

In order to answer the question of "Why nanomaterials/nanoparticles?" we must begin with the fundamental definition of nanomaterials. While several definitions and terms exist, a widely accepted definition is the definition recommended by the European Commission (EC). It defines nanomaterials as the following (European Commission, 2017):

> "Nanomaterial" means a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm–100 nm.

It further defines the terms used as previously described:

1. *Particle* means a minute piece of matter with defined physical boundaries
2. *Agglomerate* means a collection of weakly bound particles or aggregates where the resulting external surface area is similar to the sum of the surface areas of the individual components
3. *Aggregate* means a particle comprising strongly bound or fused particles

It also warrants that fullerenes, graphene flakes, and single-walled carbon nanotubes with one or more external dimensions below 1 nm should be considered as nanomaterials.

In specific cases and where there are concerns about the health and safety of the environment as well as health, safety, or competitiveness, the number size distribution threshold of 50% (in the definition) may be replaced by a threshold between 1% and 50%.

To visualize how small these nanoparticles are, let us first think about a human hair. On a rough estimate, it is of the size of 80,000 nm. That makes these particles roughly 10,000 smaller than the width of a human hair. That is pretty small! These particles are a few hundred to a thousand atoms in size. Let us take an example of the human skin. The human skin has pores of different sizes. On an average, we can estimate them to be of 80 μm (80,0000 nm) in diameter. It is easy to guess that these nanoparticles can have an easy way to our body. But their entry into our body is not the main concern. The main concern is how these nanoparticles will affect us or how potent these effects are in short and long terms on our body and ultimately on the environment.

The nanoparticles are very small and hence tend to have a very high surface-to-volume ratio. This means that for a given volume of the surface, they will have an extremely large surface area. A larger surface area means that there is more surface available for any reaction. This is one of the primary reasons for the high reactivity of these nanoparticles. The topic of nontoxicity has already been discussed in earlier chapters in detail, and we will not go in detail on it.

This not the only concern that affects us. Nanoparticles have a unique property that makes them different from most of the other chemicals being manufactured; nanoparticles have a size- and shape-dependent property. It means that the property of a nanoparticle may change drastically if you change the size of the particles. Take an example of silver; in the bulk form, silver is silvery in color (Oldenburg, 2011) and typically nonreactive. In the nanoform, silver is used as an antibacterial agent due to

its high reactivity and has a black to green color. The exact color varies and depends on the size of the nanoparticle. What is wonderful is that a noble metal of the periodic table that is quite inert is used extensively for its high reactivity in the nanoscale. The interesting part does not end here. It was mentioned in the definition that a particle of the size of 1–100 nm is considered as a nanoparticle. The particle can have these small dimensions in one, two, or all the three directions (i.e., *x*, *y,* and *z*). This again creates a great deal of difference in the properties of the nanoparticles depending on the number of dimensions the particle is confined to the nanoscale. Hence, we have many parameters that can change the properties of the particles and where each and every change can render a particle with a new set of properties. This makes them difficult to be classified under the existing regulations that mostly deal with bulk or microparticle production.

As per Lux Research (2014), the global market of nanotechnology-based products increased by 40–45% in just 3 years (2010–2013), from a $339 billion industry in 2010 to $731 billion in 2012 to more than $1 trillion in 2013. They forecast that this giant will grow to a massive $4.4 trillion by 2018. This makes the point pretty clear that the magnanimity of the nanomaterial-based products is large and the quantity of materials being manufactured to satisfy this billion-dollar industry can have a significant impact, especially when we have very little information about them.

Like any other technology, nanotechnology has its share of pros and cons. It is necessary to understand them in order to proceed further with the use and development of such technologies. It is hard to deny the fact that most technologies have the potential to do harm than good. What may seem a short-term benefit can also have a long-term negative impact. The subject of the environmental impact of these nanoparticles has been debated especially after studies showed that the nanoparticles can have an adverse effect on laboratory animals. The data available are still less, and most of the studies do not take into account the long-term impact of these nanoparticles on the environment as the topic of nanomaterials is relatively new. The existing regulations do not cover nanoparticles specifically, but they come under the broad spectrum of their bulk counterparts. It is wiser to, therefore, to recognize a threat and address it in the beginning stage before it expands to an incorrigible problem. It is highly likely that if these regulations are not able to deal with the current nanomaterials, they would not be able to deal with the future nanomaterials.

Richard Feynman, a notable American physicist, in 1959 gave his famous lecture at the Caltech University, titled “There is plenty of room at the bottom.” This lecture marked the milestone in the history of nanotechnology where he talked about the manipulation of individual atoms (Nature Nanotechnology, 2009). It was the first time that the possibility of having to manipulate a few atoms was realized. His unconventional thoughts got strong support when the International Business Machines (IBM) Corporation developed the scanning tunneling microscope in 1981 where IBM wrote its logo on a cooled nickel substrate using only 35 xenon atoms (IBM, 2016). The support was furthered by the discovery of fullerenes, quantum dots, and carbon nanotubes.

It is common today for us to hear the term *nano* in one form or another—maybe in a water filter technique, in a cosmetic product, in paint technology, in a refrigerator that keeps the food fresh for long, in a new clothing that does not need to be washed for long, or in a new medicine that promises a better cure—the list may go on. The past decade has seen a steep growth in the number of products that have been utilizing

nanomaterials to promote a new and better generation of nanomaterials. It is hard to ignore them. We are surrounded by them, and it is hard to deny that nanomaterials will soon dominate the market and almost every sector will soon be using them on a large scale. The recent trends in the patents filed in the field of nanotechnology suggest the same. According to the annual report published by StatNano in March 2016, both the European Patent Office and the United States Patent and Trademark Office (USPTO) have seen a noticeable growth of patent applications in the field of nanotechnology from 2006 to 2015 (StatNano, 2016b). Figure 16.1 shows that the year 2010 saw a steep increase in the number of patents granted with nanotechnology by the USPTO. Since then, there has been a regular increase in the number of patents granted in this field. The growth not only suggests the increasing interest in nanomaterial-based products but also suggests the increasing interest that the industries are putting in this field.

The Nanotechnology Products Database of StatNano (2016a) provides vital information regarding the use of nanomaterials in the field. The information is categorized by the industry, companies, and countries they are produced in. It gives detailed information about each product, the technology, and the nanomaterial being used. This gives us detailed information about the 5113 products manufactured by 653 companies in 45 countries. The findings have been summarized in Figures 16.2 and 16.3. It is found that of all these products, the United States takes the major share with 1939 products being manufactured in the United States, followed by Switzerland, United Kingdom, Japan, Germany, Taiwan, China, South Korea, Canada, and Iran with 389, 379, 343, 293, 290, 255, 213, 170, and 93, respectively. Still, the details of the amount of nanomaterials used in the products are not clearly mentioned. There are very few companies that actually state the amount of nanomaterial being used. Also, there is very limited or no information about the form of the nanoparticles. It is seen that while construction accounts for only 13% of the total nanomaterial market, it uses the most number of nanomaterials. The industrial patents filed by these industries, government funding, and the scientific articles published in journals give us a valuable

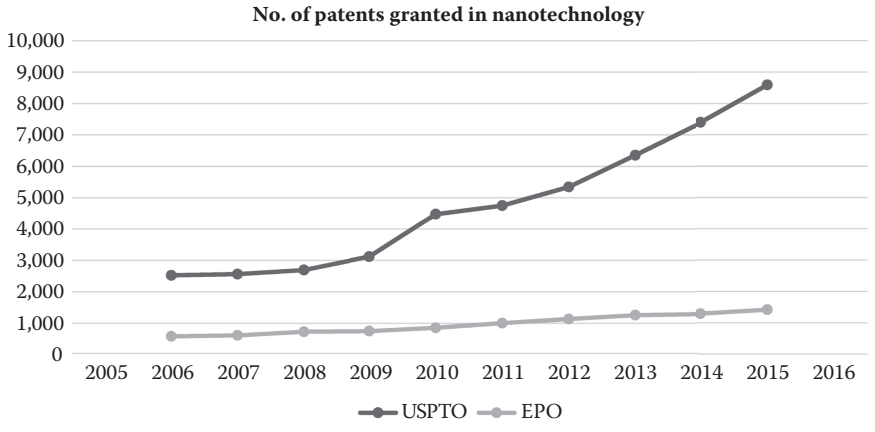


FIGURE 16.1 Patents granted in nanotechnology in the USPTO and the EPO. (From StatNano, *StatNano Annual Report 2015*, StatNano, Tehran, 2016.)

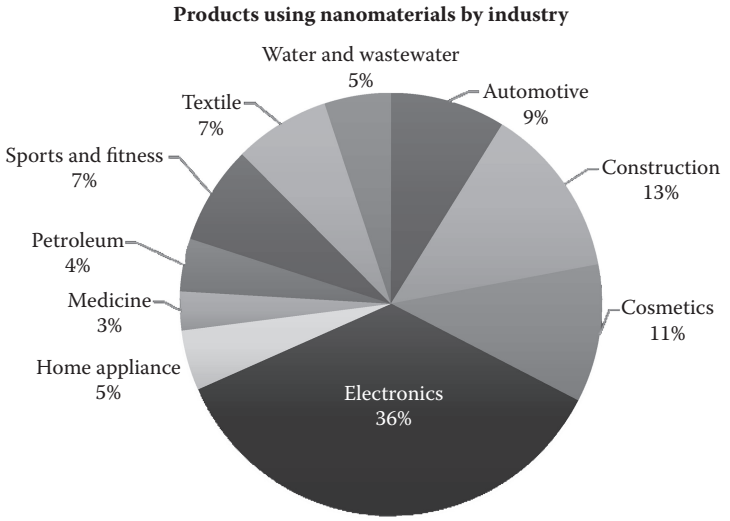
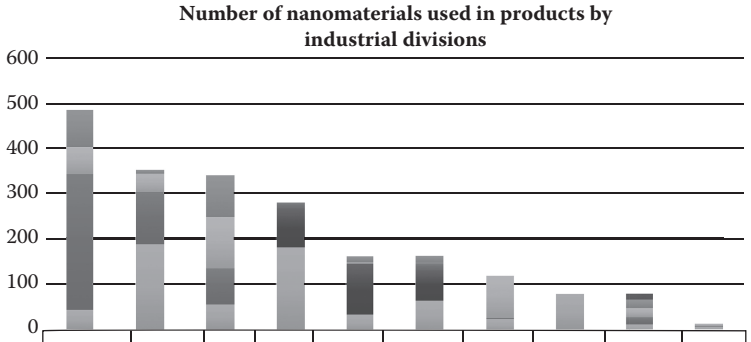


FIGURE 16.2 Five thousand one hundred thirteen products using nanomaterials distributed by industry. (From StatNano, Nanotechnology Products Database, StatNano, Tehran, 2016.)



	Construction	Cosmetics	Textile	Sports and Fitness	Petroleum	Automotive	Home Appliance	Electronics	Water and Wastewater	Medicine
Silicon dioxide	82	8	91		13	15			18	
Graphite			1							
Silver	59	41	112		3	1	92		20	5
Titanium dioxide	298	114	79	12		15	2		16	1
Tungsten di Sulphide				86	111	67				
Others	46	190	58	183	38	68	28	83	16	11

FIGURE 16.3 Nanomaterials being used by products by Industrial Divisions. (From StatNano, Nanotechnology Products Database, StatNano, Tehran, 2016.)

insight into the increasing interest in this field. The United States of America, which has been spending a great amount of money on the development of nanomaterials in the 1990s, finally launched the National Nanotechnology Initiative (NNI) in 2000. This was a turning point in the development of nanomaterials as, after this, almost all major countries either launched an NNI or strengthened the resources being put

in the field of nanomaterial development. The NNI of the United States has a budget of $1.4 billion for 2017. There is a race to dominate this new field of nanotechnology with many countries trying to exploit this novel field as soon as possible.

16.2 Problems in Regulating the Toxicity of Nanoparticles

Nanomaterials, right from their inception, have been a source of hope and fear equally. The great potential of nanomaterials is in conjunction with the risks they pose to the human health and the environment. These challenges have been associated with a series of uncertainties with regard to the development and commercial application of nanomaterials, hazards, exposure pathways, the direction and speed of technology change, and the suitability and effectiveness of existing frameworks. It is very important that the legislators and the advocates of nanotechnology understand to achieve a total balance of the risks with the novel applications. The main concerns regarding nanotechnology can be classified under the following labels (Falkner et.al., 2010).

16.2.1 Uncertainty Regarding Nanomaterial Risks

There is a profound lack of data on the toxicity and the exposure pathways for the available nanomaterials to access them property. Even for the available data, there is uncertainty about the testing methods, the reproducibility, and the authenticity of the data. This is the most important factor that impedes the regulation of nanomaterials in the commercial market. The government frameworks in different countries are trying their best to deal with this new class of materials, either with their existing frameworks or by developing new frameworks to incorporate nanotechnology.

16.2.2 Rapid Technology Change

The very development of nanotechnology and the commercial usage of the plethora of nanomaterials have shown an exponential growth. This has given the regulators very little time to emulate on the possible effects of nanomaterials on human health and the environment. Hence, there is still a vast knowledge gap for the existing nanomaterials. Until a few years back, the nanomaterials used were usually limited to a passive nature; i.e., they do not require any external stimulation to perform their activities. A good example is the use of nano titanium dioxide in cosmetics where it is used as a sunscreen to block the ultraviolet rays. The mere presence of nano titanium dioxide is sufficient and renders its function. With the integration of nanomaterials in other technologies such as biocognitive technologies, the nanomaterials are being used as active components. This means that the nanomaterials do active work and interact with external forces such as electric and magnetic fields to do their work. These nanomaterials are more complex, and their effects are more difficult to foresee. The regulators need to swiftly expand their knowledge database to cover multiple areas of scientific and engineering enquiry and develop flexible responses to a constantly changing technological environment. The development of nanomaterials does not stop with the active and passive components. Each of these sections has generations of product that enter the market as an improved version of the preceding parent product. The presence of these different nanomaterials combined with the different ways in

which a small change in the nanomaterial can affect its properties drastically. This is a major challenge in their effective regulation. The regulations need to be ready in the future to incorporate any known danger that nanomaterials may pose in the future.

16.2.3 Uncertainty of Commercialization Paths

The number of commercial products utilizing nanotechnology is increasing, and with this increases the number of ways in which a nanomaterial can cause damage. As the range of commercial applications expands, governments have to address potential risks of nanomaterials in diverse regulatory contexts covering different industries and commercial applications, potentially adding to the existing uncertainty about the regulatory coverage of nanomaterial risks.

16.2.4 Uncertainty Regarding the Suitability of Regulatory Frameworks

Legislators have been in debate whether the existing laws cover nanotechnology or not. US and EU agencies have suggested that the existing framework is good for the regulation of nanotechnology with some minor amendments. This is, however, a matter of debate as the conclusion is based on the small amount of information that is available and uncertainties cannot be ruled out given the broad range of applications of nanomaterials.

It is undeniable that there are many problems faced by the regulators of nanotechnology, and there is still much to be developed to offer a safe and fruitful usage of nanomaterials.

16.3 Nanoparticle Regulations across the World

All governments have the direct and indirect responsibility of protecting their citizens from any risk. With this increasing popularity of the nanomaterials, government organizations across the world were drawn toward the urgent need to exploit the vast untapped potential of nanotechnology using novel custom-designed nanomaterials as well as to tackle the problems that may arise with the development of these nanomaterials: the environmental hazards, the long-term impact on the human body, and the ways that these materials could harm. The setup of the NNI by the United States was an attempt in this direction. The Royal Society and the Royal Academy of Engineering (2004) in the United Kingdom published the article "Nanoscience and nanotechnologies: opportunities and uncertainties" (Dowling et. al., 2004). It was a landmark in drawing the attention of the scientists and the policymakers across the globe toward the urgent need to address the uncertainties about health and environmental effects of nanoparticles.

The major questions raised by many politicians, regulators, and members of the public have been the following:

1. Whether nanotechnology is safe
2. Whether existing regulation is adequate in the short and in the long term

3. What is and should be done to learn more about protection of the public
4. Workers from any potential environmental, health, and safety risks related to nanotechnology and nanomaterials

The first question has already been explained in detail. We will be exploring the rest of the points later in this chapter. Apart from the government regulations, there are many voluntary environmental programs run by the government (Berger, 2013), with the aim to bypass the lengthy political and legal considerations for setting up of a formal regulation. The governments ask the manufacturers of the nanomaterials to voluntarily submit the health and safety of nanomaterials they use or commercialize. In 2013, it was estimated that around 300 voluntary environment programs were running in the European Union (EU) and over 200 in the United States. As it is hard for governments to create any lengthy regulations themselves, this is being seen as a favorable approach to the regulation of nanomaterials. This chapter will mainly focus on the regulations being developed in Europe (mainly the EU) and the United States. Other countries such as Australia, New Zealand, Canada, and Japan are also developing their regulations in this field in conjunction with EU and the United States (Gorjiara and Baldock, 2014). The Latin American countries have their own collaboration for the regulation of nanotechnology.

16.3.1 Europe

The EC is the foremost authority in determining the regulations governing the European member states (MSs). It has several rules that relate to the regulations of nanomaterials in the environment. To regulate nanomaterials, the EU has also established several agencies that assist in the development and implementation of rules in different domains of application (Hansen, 2009; Maria et al., 2010). This section will deal with the regulations in different domains including chemicals, pharmaceuticals, and environment and waste management.

The EU defined the term nanomaterials in 2011 after a review by EC. This was followed by a regulatory review in 2012 (European Commission, 2012; Prinz, 2014) to understand nanomaterials better. The review provided an overview of nanomaterials in the market and the available information on hazard properties and assessed the regulatory options for the existing chemical regulation, including the possible amendments that could be incorporated in it. It identified nanotechnology as a key enabling technology with a global market of around 11 million tons, providing employment to around 300,000 to 400,000 people. As per the second review, the genre of materials attracting the most attention were nano titanium dioxide, nano zinc oxide, fullerenes, carbon nanotubes, and nanosilver. It goes beyond doubt that these recommendations have been highly instrumental in framing the current legislations. We will now proceed with discussing the major regulations that help in the safe regulation of nanomaterials in the market of the EU.

16.3.1.1 Registration, Evaluation, and Authorisation of Chemicals

The EU adopted the regulation of the Registration, Evaluation, and Authorisation of Chemicals (REACH) in 2007 (Regulation (EU) No 1907/2006), replacing the former

legislative framework for the chemical in the EU, to improve the protection of human health and the environment from the risks that can be posed by chemicals. It aims for a better and earlier identification of intrinsic properties of chemical substances. This is done by the four processes of REACH, namely, the registration, evaluation, authorization, and restriction of chemicals. It passes on the responsibility of providing proof for the safety of a chemical on the company. To comply to REACH, the manufacturers should identify, and manage risks linked to the substances they manufacture and market in the EU. The manufacturers and the marketers have to demonstrate to the European Chemicals Agency (ECHA) the safe usage of the substances and have to also communicate the risk management measures to the users (ECHA, 2012). If the risk is too large to be managed adequately, the authorities can restrict the use of substances in different manners with a long-term aim of substituting them with less dangerous ones. It also promotes alternative methods for the hazard assessment of substances in order to reduce the number of tests on animals. REACH applies not only to the chemical substances that are used in the industries but also to those used in the daily lives of people such as paints, polishes, and electrical appliances (ECHA, 2016). It ensures that the end user of any product is well informed and not harmed due to the chemical used in the manufacturing of the product and the user, after the life cycle or consumption of the product, and has adequate knowledge on the proper disposal of the residue. This also helps to encompass as many companies as possible across the EU. One of the main reasons to formulate REACH was that there were a large number of companies that had been manufacturing various chemicals in the EU before REACH, yet there was insufficient information available on hazards that these chemicals posed to the human health and environment. There was an urgent need to identify and implement the risk management techniques for the safety of the human health and the environment.

16.3.1.1.1 REACH and Nanomaterials

REACH, although it identifies the nanomaterials, does not mention them explicitly in its definition. It encompasses them in the broad category of chemical substances, and it defines a substance as "a chemical element and its compounds in the natural state or obtained by any manufacturing process, including any additive necessary to preserve its stability and any impurity deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition." In 2008, REACH published a review in which they clarified that "When an existing chemical substance, already placed on the market as bulk substance, is introduced on the market in a nanomaterial form (nanoform), the registration dossier will have to be updated to include specific properties of the nanoform of that substance. The additional information, including different classification and labelling of the nanoform and additional risk management measures, will need to be included in the registration dossier. The risk management measures and operational conditions will have to be communicated to the supply chain."

REACH provisions are complemented by an extensive Technical Guidance Document (TGD) that specifies how to implement the various provisions under reach. But, as both of them came into effect before the nanomaterials gained a fair share in the market, neither of these are fully adapted to face the questions that are raised by REACH.

The gaps that still remain in REACH for the regulation of nanomaterials have been highlighted specifically in a report published by the Center for International Environmental Law (CIEL) (CIEL, 2012), Geneva, Switzerland. They can be broadly classified into four categories:

1. Identification of nanomaterials
2. The phase-in status of nanomaterials
3. Tonnage threshold and nanomaterials
4. Risk assessment provisions

16.3.1.1.1.1 Identification of Nanomaterials Although the nanomaterials were later explicitly included in REACH, they are never defined in the scope of REACH, which means that there remain very limited opportunities to identify them to take into account their special characteristics. The limitation becomes even more severe when a bulk material having the same chemical composition already exists in the market (reason discussed in the next point). This often leads to an underestimation of their effects by the regulators in identifying and implementing regulatory provisions relevant to them and therefore produces a major problem in utilizing the full scope of REACH. This is quite contrary to the basic principle of REACH that states "no data, no market." Due to this limitation, the registrant has the final decision of determining whether the material falls under the category of nanomaterials or not.

Nanomaterials possess unique properties that are exhibited only at the nanoscale regime, and this differentiates them greatly from their bulk counterpart that shares the chemical composition or molecular structure. Typical properties such as solubility, rate of dissolution, dynamics of dispersion, melting point, conductivity, and physical absorption or magnetic properties can greatly vary for nanomaterials. The Second REACH Implementation Plans on Nanomaterials were focused on information required for characterizing and evaluating the nanomaterials. It proposed several modifications of the TGD and REACH for nanomaterials based on several physiochemical properties, toxicological properties, etc., and took into account their preparation, exposure, and quantification. However, for these regulations to be successfully adopted and implemented, the nanomaterials need to be identified first. The fifth version of the International Uniform Chemical Information Database allows the registrants to identify nanomaterials in registration dossiers. But as REACH does not specify nanomaterials, the final decision in determining whether a substance is a nanomaterial rests with the applicant.

16.3.1.1.1.2 The "Phase-In" Status of Nanomaterials Another major point to be noted is that REACH distinguishes the chemicals that were already there in the market when it encompassed nanomaterials under its hood, from the new chemicals that are seeking approval to enter into the market. REACH identifies the substances by their chemical composition alone. When two substances are of the same chemical composition, they are considered to be the same substances. As a consequence, if a substance exists both in the bulk and in the nanoform, and the bulk substance exists as a "phase-in substance," nanomaterials sharing the same chemical composition will automatically benefit from the phase-in status of the bulk material, regardless of its

new novel properties. Phase-in substances are the chemicals listed in the European Inventory of Existing Commercial Chemical Substances (EINECS) prior to the entry of REACH in nanomaterials on June 1, 2008. This can be deduced from the EINECS reporting rules and the Manual of Decisions, which state that "substances in nanoform which are in EINECS shall be regarded as existing substances." Irrespective of their production volume, phase-in substances benefit from delayed registration deadlines and, in case of a higher tonnage band (>10 tons), need to provide just the physiochemical properties instead of the toxicological and (eco-)toxicological information, provided that they were successfully preregistered by December 1, 2008. The deadline for the registration of these phase-in substances was spread in three phases with specific deadlines in 2010, 2013, and 2018.

The preregistration requires simple steps of submitting information on the identity of the chemical substance to the ECHA, including its EINECS number (if applicable), tonnage band, and any applicable identifier such as the Chemical Abstract Service (CAS) index number or International Union of Pure and Applied Chemistry name by the manufacturer or the importer. This failure to include additional identifiers for nanomaterials, such as material size, shows as one of the fundamental gaps in the ability of REACH to effectively regulate them. Due to this, the materials sharing the same chemical composition with the bulk phase-in substance were not registered until 2013 if they were manufactured or imported in quantities above 100 tons per year per registrant. Nanomaterials which are manufactured or imported in quantities of 1–100 tons per year will not be registered until 2018. As the result, there is still a long gap for these materials to effectively spread in the market and at the same time prolong the gap on knowledge of nanomaterials.

The reason the category of phase-in substances exists is the practical limitation of registering and evaluating thousands of substances that were already present in the EU market prior to the adoption of REACH. It relies on the basic principle that basic information of such substances already exists; therefore, phase-in substances are not prioritized under REACH unless it is warranted by specific hazardous characteristics (Article 57). This reason may not hold true for nanomaterials. Although they may share the same chemical composition, their physicochemical, toxicological, and ecotoxicological properties along with their uses and exposure patterns may differ greatly from their bulk counterpart. The toxicological and ecotoxicological properties are not always transferred from the bulk form to the nanoform. As a result, the availability of toxicological and ecotoxicological information on the bulk form does not justify the phase-in status granted to nanomaterials.

Studies have shown that most of the nanomaterials that are there in the market already have their parent substances as materials that are pretty common and preexisting in the bulk form. A study done by Hansen et al. (2008) found that all the nanomaterials existing in the market could be traced back to six parent substances and all these parent substances had a phase-in status. It showed that a vast majority of nanomaterials being used in the market are benefiting from the phase-in status by delaying registration deadlines. As a result, there is a vast majority of nanomaterials in the market with little or no information regarding their potential risks which is in direct contradiction with the "no data, no market" principle underlying REACH, thereby undermining the effectiveness and credibility of REACH as a primary regulatory tool to bridge the knowledge gap on nanomaterials.

16.3.1.1.1.3 Tonnage Threshold and Nanomaterials REACH determines the role of a material based on how many tons of the material is produced per year. The general rule is that the higher the volume of the material (tons), the more exposure there is, the higher the risk posed by the substance, and hence the more are the data required and the sooner is the registration required. They have therefore divided the regulatory mechanism into four tonnage bands: 1–10, 10–100, 100–1000, and 1000+ tons. The information required for registration increases with the tonnage band.

The registration of nanomaterials is only obligated when the materials manufactured or imported exceed quantities of 1 ton per year. The registration has to be submitted directly to the ECHA unless stated otherwise. The registration deadline and the information required vary depending on the hazard profile and the quantity manufactured or imported. While this might be true for the bulk materials, nanomaterials may challenge this assumption as there are several factors apart from the quantity of material that determine their activity such as the shape, size, or form.

A major problem faced by the usage of tonnage bands is that reliable information on the production volumes of nanomaterials is very scarce and is "hampered by the lack of definite inventory on the types and uses of nano substances." Nanomaterials are typically marketed in very small quantities as the amount in which they are present in the products is very small; hence, their production volume typically falls below 1 ton a year, which is below the threshold that warrants the application of REACH. This again violates the basic principle in Article 5 of REACH—"no data, no market." The German Advisory Council on the Environmental (SRU) suggests that a core data set should still have to be submitted if a nanomaterial is produced in quantities of less than 1 ton per year. Even if nanomaterials are produced in quantities greater than 1 ton per year, they still benefit from the phase-in status of their bulk counterparts. As per REACH, the information that the manufacturers and the importers of nanomaterials require to submit will still be limited to the physiochemical properties of the substance, excluding any toxicological and ecotoxicological information, which may be otherwise mandated. It will also not include the information that is required for substances of very high concern. A detailed chemical safety assessment, which contains detailed information on the human health as well as a physicochemical and environmental hazard assessment, is mandated only for substances that are manufactured or imported above 10 tons. As nanomaterials are usually produced in low volumes (under the 10-ton threshold), chemical safety assessment will be unavailable for the majority of nanomaterials.

16.3.1.1.1.4 Risk Assessment Provisions As discussed earlier, REACH provides rules and methodologies that were developed primarily for the bulk substances and were later extended to the nanomaterials. Nanomaterials have faced a major problem when their safety is considered: the lack of information. While it might be overwhelming to see the number of articles published each year on the usage of nanomaterials, there is very little information on their safety. There have been several commentators that have highlighted the urgent need to modify the current rules to address the risk posed by nanomaterials.

The Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), in particular, mentions that there are several areas where the risk assessment, as outlined in REACH (SCENIHR, 2007), and explained further in greater

detail in the ECHA 2011 guidance, may need modifications to better encompass the risks posed by nanomaterials. As an example, SCENIHR observed that the uptake distribution, clearance, and elimination of nanoparticles may differ from the chemical substances for which the TGDs were initially developed. The committee had doubts if the base set of standard tests and procedures that were developed to evaluate the effects of nanoparticles was adequate. Specifically, the traditional dose-dependent relationship, relying on mass as an indicator of exposure and toxicity, was found to be inappropriate by the committee. The committee also found the exposure assessment to be quite inaccurate and warranted further changes to reflect the physiochemical characteristics of nanomaterials and their potential to change throughout their life cycle. This problem remains even under the stringent requirements under the 1000-tonnage threshold. SCENIHR reported that "on the basis of current knowledge, the risk characterization of bulk materials as described in the technical guidance document cannot be directly extrapolated to nanomaterials." REACH does not distinguish between the bulk and the nanomaterial and treats them similarly. This means that the same regulation applies to both irrespective of the fact that the risk of the substance is not same at all the scales.

The reason given by industry in applying the same rules to the nanoparticles was that there is a profound lack of information on fate and effect of nanoparticles on the environment and human health and therefore it was infeasible to impose strict rules for their usage. The lack of data should not come as an escape hole to warrant the use of conventional rules and assessment methods. REACH, at this stage, cannot assess effectively the state of health and environmental risks of the nanomaterial primarily due to the time involved and the high cost of generating substantial meaningful data. Apart from being a serious concern for the environment, this also places ambiguity in the role of the manufacturers and importers who are often unsure of their complete responsibility in regulating the nanomaterials they market.

These concerns have also been highlighted in the Joint Research Centre's evaluation of registration dossiers containing nanomaterials that was published by EC in 2012. The report has 21 adaptations to REACH regulation to remove the ambiguity and to ensure that adequate registration information is provided in all dossiers related to nanomaterials. The Commission also launched comprehensive REACH Implementation Projects on Nanomaterials (RIPoN 1, 2, and 3) in 2009 to articulate key recommendations for the inclusion of nanomaterials in REACH without modifying the text of the regulation. RIPoN 1, in particular, focused on substance adaptation rules. The final report on this is available, but as it was not possible to reach consensus among the experts of recommendations in these reports, further work is therefore being carried out in collaboration with CARACAL, an expert group which advises EC and ECHA on questions related to REACH and Classification, Labeling, and Packaging regulation, before the recommendations can be forwarded to ECHA. RIPoN 2 and 3 focused on information requirements and chemical safety assessment, respectively. The corresponding reports were published in July 2011.

The CIEL reevaluated the status of inclusion of nanomaterials in REACH and published a position paper in 2015. The paper highlighted that although the RIPoN were published way back in 2011, there are still no nano-specific regulations included in REACH annexes, and the registration dossiers still do not contain information on nanoforms. This may be linked directly to the fact that nanomaterials with the same

chemical composition in the bulk are not considered to be a different substance but a derivative of the parent substance.

16.3.1.1.2 Attempts to Fill the Gaps of REACH

In May 2015, the EC presented a series of proposed amendments to the Competent Authorities Subgroup on Nanomaterials to ensure efficiency of nanomaterial registration. The major recommendations were as follows:

1. Manufacturers and importers of a nanoform of a substance have to state which forms of the nanosubstance they have included in their Chemical Safety Report (CSR). If they use the nanoform of a substance to demonstrate the safety of other forms of the substance, they should include it in their CSR along with the justification that their safety assessment includes all nanoforms of substance covered by the registration. (This amendment was proposed in Sections 0.1 and 0.3 of Annex I.) This means that if the manufacturer intends to use data from one nanoform of a substance to demonstrate the safety of some other nanoforms, he must provide scientific evidence to do the same and record it. The commission has proposed to add the following sentence to Annex 1 Section 0.1: "The chemical safety report shall also describe whether and which different nanoforms of substances manufactured and imported are included, including a statement [on] when and how information on one form is used to demonstrate safety of other forms. The requirements specific to nanoforms of a substance in this Annex are without prejudice to requirements applicable to other forms of that substance."
2. The commission recommends making amendments to the phase-in status of nanoforms of substance. The nanoforms of substance in the category of 1 to 10 tons are considered as phase-in materials and need to provide just their physiochemical properties as per Article 12 (a) and (b) unless they fall into the category of Annexure III which states that the ecotoxicological and toxicological information may be required if they are carcinogenic, mutagenic, or toxic for reproduction or are substances with diffusive or dispersive use, particularly those used in consumer mixtures or are incorporated in consumer articles. The proposed amendment wants to include the phase-in substances that have one or more nanoforms in Annexure III. Annex III deals with substances that are likely to cause harm to the health and the environment.
3. The registrant should describe the test material and the sample preparation method that was used. The commission recommends adding the following text to the introductory text of Annexes VII to X: "Without prejudice to the information submitted for other forms, any relevant physicochemical, toxicological and ecotoxicological information shall include characterization of the nanoform tested and test conditions. The same shall apply when information is provided by application of quantitative structure activity relationships (QSARs) or evidence obtained via other means than testing." Annex VII to X deal with chemicals that are produced in large quantities above 1 ton.

The commission intends to classify nanoforms and the non-nanoforms of a substance as different forms of the same substance and therefore recommends the amendment of Annex VI to require the characterization of nanoforms of a substance according to the following criteria:

a. Names or other identifiers of nanoforms of substance
b. An indication of the fraction of constituent particles that are in the range of 1–100 nm
c. Description of the treatment done for the surface functionalization
d. Details on the morphological characterization of the nanoforms such as the shape and aspect ratio with information on the type of assembly structure such as hollow or shell-like structures
e. Volume-specific or mass-specific surface area
f. Sufficient information on the references or analytical methods used for the identification of the elements, such that the methods can be reproduced

4. Human toxicology and ecotoxicology of nanomaterials are different from the bulk material. The commission, therefore, recommends the following changes under different categories in Annexes VII–XI. The changes recommended are as follows:
   a. *Acute toxicity*: The commission recommends the requirement of dermal or inhalation studies for nanoforms of substances produced above 1 ton instead to just an oral test in cases where they may be the more appropriate exposure route.
   b. *Degradation*: The stability of a bulk material is tested by physiochemical properties such as hydrolysis, but for nanoforms of a material, the same is not true. Other abiotic mechanisms such as ion degradation may be more useful. The commission proposes to replace the conventional testing mechanisms with mechanisms such as photocatalysis or interaction with other chemicals.
   c. *Bioaccumulation*: Bioaccumulation of materials in the tonnage band of 100–1000 tons is mandated and is determined by their solubility in fat. This test can we waived off if a material is not fat soluble. But some nanoforms of materials can be nonsoluble in fat. The commission recommends scrapping of this recommendation for nanoforms of substances.
   d. *Long-term toxicity*: Currently, the long-term toxicity of a material can be waived off it does not show toxicity in short-term tests. The commission proposes to do away with the waiver of long-term toxicity tests for nanoforms of materials based on lack of short-term toxicity evidence. It also proposes to include cardiovascular toxicity and respiratory sensation as an additional criterion when the toxicity of nanoforms of a substance is considered.
5. The downstream users are mandated to be responsible for the safety of the chemicals they use at their site by maintaining a CSR for any situation that the manufacturer or importer may not have included in his list of exposure

scenarios. The commission proposes to introduce an obligation for downstream users to keep available information on the physical state, concentration, concentration range, or quantities of nanoforms in mixtures and articles that they use in accordance with Annex III.

The commission in its best possible means has tried to address the concerns of the nanomaterials. The CIEL, however, recommends the following to seal the possible loopholes:

1. The reach regulation should state clearly in Annex I that the uses of the nanoforms of a substance have to be identified by the registrant in the CSR.
2. If the nanoforms of a substance show different characteristics than the bulk form, then it should be classified as a separate substance than as a derivative of the nonnanoform.
3. Along with the details of the sample preparation and methods, the registrant should justify the use of the method scientifically.
4. When the commission proposes the methods to characterize the nanoforms of a material, it fails to acknowledge important characterization information such as information on the particle size distribution outside the 1 and 100 nm range, the surface chemistry and surface charge, the formation of agglomerated/aggregated forms during use and (environmental) release, the stability of aggregates/agglomerates, and the possible "crystalline state" of the nanoform. Information that the commission recommends acquiring on surface treatment, coating, or functionalization is extremely limited, although this is considered to be one of the key parameters relevant for risk assessment of nanoforms.
5. Grouping of nanoforms of a material for hazard assessment relies on the assumption that the safety of data of one nanoform can be used to demonstrate the safety of another nanoform, but there are only few parameters that can affect the toxicity of a nanomaterial such as the form of a material, the shape, and surface characteristics. Therefore, it would not be very scientific to group several uncharacterized materials under a common banner; rather, the materials should be characterized individually and then grouped into different classes. REACH has been a strong attempt by the EU to regulate the use of chemicals in the European market, but unfortunately despite its revisions, it is not fully suited in its current state to regulate the nanomaterials in its market. The delayed registration times for the phase-in substances and the failure to consider the unique properties of nanomaterials are some major reasons for the same. The CIEL recommends the setup of new regulations that are carefully tailored along the chemical rules but more suited to the nanomaterials. The suitability and amendments of REACH are still a subject of debate.

16.3.1.2 Pharmaceutical Regulations

The broad application of nanotechnology often warrants a stringent control over the products that enter the market. The pharmaceutical sector is one such sector where

the utmost stringent measures are needed to govern all the nanomedicine products that enter the market. Nanotechnology with its entry into the domain of medicines has brought about some significant changes in the development of a new genre of medicines that can be taken with low doses and still be much more effective over the conventional doses of medicines. The nanomaterials offer comprehensive and novel solutions for diseases such as cancer, infections, autoimmune diseases, and infections. They offer increasingly novel advantages such as the following: The pharmaceutical ingredient can be targeted directly at the site of requirement, and its release at other places is shielded leading to a higher efficiency and a reduction of side effects.

Drugs with low solubility can be used, and their bioavailability can be increased. Nanomedicines due to their small sizes can reach their targets by crossing through small places in biological barriers such as that in the blood–brain barrier. They have the ability to use molecules that otherwise could not be used due to their high toxicity such as Mepact. There is a possibility to precisely engineer a medicine as per the demand. Along with their advantage, there is also a strong cautionary evidence for the long- and short-term toxicity of these products in healthcare and on the environment. This is backed by several published papers that have demonstrated this. The benefits and risks of the nanoproducts need to be sufficiently evaluated as there is still a big knowledge gap in the full understanding of the impact of these nanomaterials. To achieve this, an improved process or method for the characterization of the products is needed to achieve a deeper understanding of how minor changes in nanomaterials can affect their clinical profile. This will be helpful in developing tools for successful nomenclature and classification of these materials, aiding in their easier and efficient access to the market along with a complete control over life cycle monitoring.

The EU came into play in 2002, but the advent of nanomaterials in medicine has a history that even supersedes the history of nanomedicines in the United States. The first nanomedicines that were approved by the European Medicines Agency (EMA) for commercial use were in 1994. The EMA is the decentralized agency of the EU that oversees the approval of medicinal products that enter the European market. It evaluates and supervises the development of medicines for human and veterinary use by providing independent recommendations based on scientific evaluation of data (de la Ossa, 2015). To qualify as a nanomedicinal product, it should have the following properties:

1. Has purposely designed systems for clinical applications
2. At least one component in the nanoscale regime
3. As a result, has definable specific properties and characteristics that are
   a. Related to the specific nanotechnology application and characteristics for the intended use
   b. Associated with the expected clinical advantages of nanoengineering
4. Has met the definition of a medicinal product as per European legislation

However, the EMA is not the final deciding authority, and the approval of a drug is regulated by the national agencies or the EU. It continuously develops methods for evaluating the characterization and stability of nanosystems, functionalities of nanosystems, the biomarkers for nanofunctionalities, biodistribution and biopersistence of nanomaterials, and degradation of products for long-term safety, dose determination,

and other unique aspects associated with treatment procedures such as the impact of energy sources within and outside the clinical setting. It weighs the potential benefits of the products against the potential risks, utilizing the principle of benefit/risk analysis to give a verdict on the approval of the product in the European market.

The EU published the definition of a nanomaterial in 2011 defining it as a product with at least one dimension in the range of 1–100 nm. The EMA, however, uses a modified definition for nanotechnology that defines it as the use of tiny structures that are "less than 1000 nanometers across—that are designed to have specific properties." This enables them to cover a wider range of materials than what the definition of a nanomaterial by the EU would allow them to. It defines nanomedicine as "the application of nanotechnology in view of making a medical diagnosis or treating or preventing diseases. It exploits the improved and often novel physical, chemical and biological properties of materials at nanometer scale."

As per a study published by Noorlander et al. (2015), the EMA has approved 43 nanomedicinal products until 2013, approving at least one per year. The results are summarized in Figure 16.4. The EU granted access additionally to four products in Germany, Netherlands, France, and United Kingdom. Different studies often have come up with different numbers of nanomedicine products often due to unavailability of information about the structure of the active constituents. A great majority, 94%, of the approved nanomedicinal products were for therapeutic use, while 2% and 4% were for diagnostic and vaccines, respectively.

It is good to note that these first-generation medicines that were approved by EMA, including liposomal formulations, iron-based preparations, and drug nanocrystal technologies in oral dosage forms, have been established as safe and effective for many years. But it still raises the question whether the methods that were used to characterize these nanomedicines were suitable enough to gauge the toxicity of these nanomaterials (Mühlenach et al., 2015).

There are several regulatory issues that arise while evaluating the toxicity of the nanomedicine. The majority of them are as follows.

16.3.1.2.1 Difficulty in Identification of a Nanomedicinal Product

Several manufacturers have been marketing different information in the market that have contained the materials or drugs in the nanoscale regime. The contents of the medicines are often available, but information on the forms and the scale of the materials used is kept aloof from the user. This is often done to keep trade secrets that give a product an edge over the competing products. Thus, there is no way to verify if the manufacturer is using nanomaterials in its medicinal products. Another reason that the products obscure information of any containing nanomaterials is that if in future there is a stricter control over the nanomedicinal products or a negative public opinion, their products would be safe from any scrutiny. On the other hand, many manufacturers of medicines have now started marketing the products with the label of a nanocomponent that they may use as an active or a supporting ingredient. But the information on the form and in many cases the quantity of the nanomaterial being used is still obscured from public databases.

This, from the regulatory point of view, is a great disadvantage. Etheridge et al. (2013) attributed this to the lack of clear definition and the characterization of nanomedicines as a unique product class. There is no separate provision, as for now, in the EU regulations that give a special consideration to the nanomedicinal products.

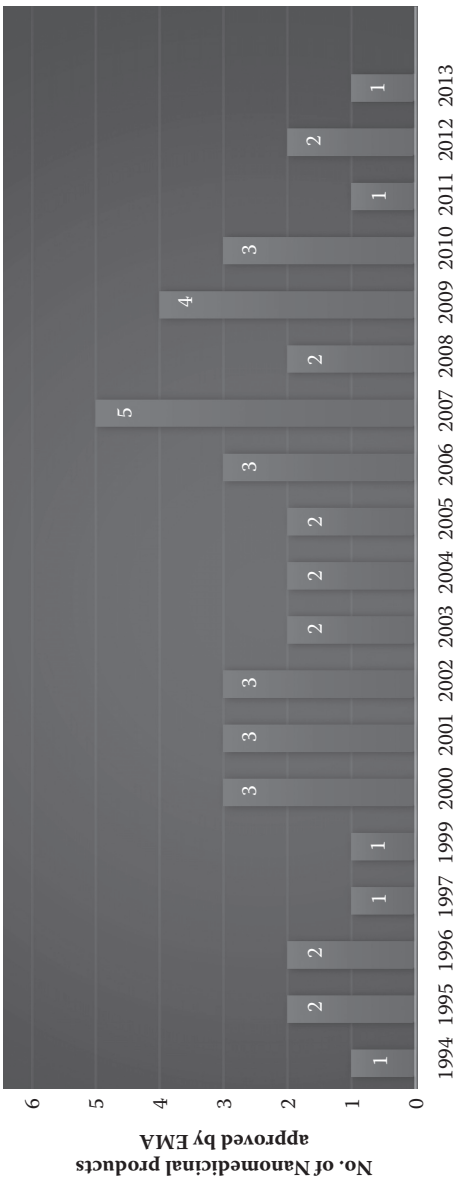


FIGURE 16.4 Number of nanomaterial-based products approved by the EMA by year. (From Noorlander, C.W. et al., *Nanomedicine (London)*, 10, 1–10, 2015.)

They are often treated as a new drug, such as any other non-nanomedicinal product (Gatof and Bowman, 2015). The mere definition of nanomedicines is not sufficient to identify a drug-using nanomaterial. The definition just tells what the EMA refers to as a nanomedicine and what products classify as products using nanotechnology under its banner. But the absence of its characterization as a unique product class marks the main problem. Even if the definition would cover all the characteristics of a nanomedicinal product, if there is no product classification, the manufacturer may choose to completely obscure the information on the form of the nanomaterial used. Etheridge et al. (2013) further noted in their study that the nanomedicines are playing an active role in the prevailing healthcare market, but their extent and penetration along with their economic value remain largely unknown and this is a worrying trend.

16.3.1.2.2 Difficulty in Characterizing a Nanomedicinal Product

It comes as no surprise that the toxicokinetic profile of these nanomedicines is much different from the other medicines and that they depend on the specific formulations of the products. Therefore, the characterization methods of the non-nanomedicinal products cannot be duplicated for the nanomedicinal counterparts. The nanomedicinal products have a higher surface-to-volume ratio that their size and form dictate the properties that they possess. For example, a nanorod and a nanoball of the same material will have different properties such as penetration across a biological barrier, and their reactivity would depend on their surface area and the specific coatings applied to the nanomaterial.

There is a need to develop characterization methods that uniquely identify the toxicity of a nanomaterial and that are not based on the standard testing methods used for determining the toxicity of non-nanomedicinal products. A strong emphasis has to be paid on the in vivo and in vitro stability of the nanomaterials and the physiochemical functionality of the biointerface, i.e., of the surface of the nanosystem. The toxicity of a product would depend not only on its form but also on the specific dose chosen for administration to the patient and the frequency of the dosage. Another important factor is the patient to whom the medicine is being administered. An old person may have a different reaction to a medicine than an adult. Some medicines may not be suitable for administration to small children. This can be an important factor in characterizing the toxicity of the product. Without a thorough consideration of all these factors, it is far likely that a robust system that can characterize the existing nanomaterials and is ready for the challenges of the future nanomedicines is far from development.

16.3.1.2.3 "Nanosimilars"

After the successful launch of several nanomedicines in the market, the "follow-on" medicines have begun to enter the market. These are the medicines that are derived from the parent nanoproduct. Such medicines are defined as "nanosimilars" or similar nanoproducts. They often claim a similar behavior to their parent product. The regulations of the EU make it easier for them to be classified under one band (see "REACH" section).

Ehmann et al. (2013) pointed out that "In order to demonstrate similarity there is a need for stepwise comparability studies to generate evidence substantiating the similar nature, in terms of quality, safety and efficacy, of the nanosimilar and the chosen reference (originator/innovator) nanomedicine. As nanomedicines differ significantly

in their complexity and nature, a case-by-case or product class specific approach for their evaluation might be necessary." A small change in their form, size, or surface coating can have a significant change in their properties, and hence a robust classification that takes into the many factors that contribute to the properties of the nanomaterials is still far from development. The EU follows a more class-based approach, and this makes it easier for nanomaterial-based drugs to penetrate the market. A case-by-case approach, though lengthy and cumbersome, is more suited to determine the toxicity of a nanomaterial assess its "fitness" to enter the market.

Also, the information about the process used to manufacture a drug is often kept at bay. The nanomaterial may be used as an active drug, surrounded by much larger molecules, or the nanomaterial may be used in the manufacturing process of a drug. Nanosimilars may have certain characteristics similar to their parent product, but their manufacturing process may differ. The change of a single component in the manufacturing process may lead to a change in the stability of the coating of the nanomedicine and which can be a vital factor in determining the toxicity of the nanomaterial. The nanomaterial may not demonstrate a significant change in its toxicity immediately, but over its lifetime, it may demonstrate a different bioaccumulation profile.

Ehmann et al. (2013) further stressed that "any drug developed to be comparable to the reference patented drug (brand, originator or innovator) product must demonstrate equivalence in terms of quality, safety and efficacy before a market authorization can be granted. Given the degree of complexity of many nanomedicine products, special scientific considerations may be needed to ensure this equivalence of performance." If a nanomedicinal product is authorized to enter the market as a derivate of an existing parent nanomedicinal product, it should undertake specific tests tailored to demonstrate that the drug is safe in terms of quality, safety, and efficiency.

16.3.1.2.4 "Next-Generation" Nanomedicines

The domain of nanotechnology is expanding every day in terms of its applicability and efficiency. There is an increasing trend to utilize the novel properties of the nanomaterials, especially for different therapeutic purposes (Hafner et. al., 2014). This has led to the creation of new complex hybrid structures that have increased uses as imaging agents, pharmaceutical drugs, or a complex combination of both. The main problem with their regulation is the lack of information. It is not necessary that the combination of two or more drugs may yield a linear result and that the result may possess the exact properties of the parent materials. The degree of complexity increases with the following:

1. Number of parent materials
2. Complexity of the parent materials
3. Modifications done to combine the parent materials to compose the hybrid structure
4. Any other modifications that may have been done to increase the efficiency

The relation may not be a linear, i.e., the complexity of the drug may not add up linearly, but a combination of changes may lead to the emergence of entirely new properties. These products produce a challenge of their own. The nanomedicinal products

of the first generation have their "nanosimilars" following on which still have not been identified clearly for their properties, and with the emergence of these next-generation nanomedicinal products, the challenges have increased. A further increase is expected as there is a significant increase in the use of nanomaterials for medicinal products to fulfill the growing demand for more efficient medicines.

Thus, there is a significant growing pressure on EMA to alone advise the EU on the nanomedicinal products that should enter the market. In 2006, the EMA created a cross-agency Nanomedicine Expert Group to assess the potential needs for regulatory requirements for the evaluation and assessment of nanomedicines. This was further expanded into the International Regulators Subgroup on Nanomedicines, an initiative which was jointly launched by EMA, Food and Drug Authority (FDA), United States, Ministry of Health Labor and Welfare, Japan, and Health Canada, Canada. The experts from this group organized the first international nanomedicines workshop in September 2010 in London.

16.3.1.3 Agriculture and Nanofood Regulations

Nanotechnology is combining with other technologies to create new products for the market. These products have increasing application in the food industry including food production, food storage, food transportation, traceability, and security. Nanotechnology is being employed in food safety where it is used as nanobiosensors and nanotracers. Nanotechnology is also being used to create food customized to the nutritional needs and tastes of the user (Prakash et al., 2013). In the past decade, these technologies were confined to the laboratory shelves but now they are gradually making their way into the market. Their use can be classified into four major categories (Amenta et al., 2015):

- Food products
  - Delivery
  - Formulation
  - Packaging
- Food particles
  - Nanoparticles
  - Nanoemulsions
  - Nanocomposites
- Food safety
  - Nanosensors
  - Nanotracers
- Food processing
  - Nanobiotechnology
  - Molecular synthesis

It is interesting to note that "nanofood" also occurs naturally and has been consumed safely for generations. During normal food processing, raw materials such as starch, protein, and fat undergo several structural changes at the nanoscale level.

Homogenized milk has droplets at the nanoscale level that may contain fat, proteins, or casein micelles. Nanotechnology has also been used in food packaging to make food safer and to prolong its shelf life. For example, polylactic acid has gained special attraction in the food packaging industry due to its biocompatibility, thermoplasticity, and good processability but suffers from a major drawback, just like any other pure polymer, of the low–medium gas barrier, thermal stability, and low solvent water resistance. This has been resolved by the usage of clay nanocomposites in low volumes (less than 5% by weight) that improve the polymer matrix in terms of mechanical, gas barriers, and optical properties and hence provide a barrier against water vapor transmission. Clay nanocomposites have also been combined with other polymers to yield better food packaging products for different requirements. Similarly, silver nanoparticles have been used in the food industry for a long time to enhance the quality of beverages by preventing any microbial growth. The list of the applications in the specific industry may go on endlessly. The main point to understand here is that nanomaterials are being used worldwide and there is a need for a central regulation that governs its uses and applications (Chellaram et. al., 2014; Gallocchio et al., 2015).

The European Food and Safety Authority (EFSA) is the main governing body that helps in the formation of the regulations related to nanomaterials in food in Europe. It is an organization that is funded by the EU but functions independently of the European legislative and executive institutions and EU MSs. It is responsible for risk assessment and communicates its scientific findings to the public. It provides independent scientific advice to the decision makers that regulate food safety: EC, other national food and safety bodies, or the European Parliament. It may also take up a task on itself if it identifies a risk where the safety of the environment or humans is at stake.

The EFSA has panels of scientific experts, selected through a vigorous procedure, that look over the following specific topics: Pesticides, Animal Feed, Animal Health and Welfare, Biological Hazards, Contaminants, Nutrition, Food Additives, Contact Food Materials, Genetically Modified Organisms, and Plant Health. There may be cases that are cross-disciplinary in nature and require the expertise of more than one panel. Such cases are governed by a Scientific Committee that consists of 10 chairs of the scientific panels and 6 additional experts. The scientific committee then sets up a working group that is tailored to address the specific risks to the question. The working group performs a detailed scientific work based on scientific evidence from data available and from literature and their expertise and then drafts an opinion and submits it to the scientific committee for discussion and possible adoption. The scientific committee then holds a public consultation on the opinion and, after a thorough revision of feedback and a possible adoption, forwards the final opinion (draft) to the original requester of the information.

Just like the pharmaceutical regulations, the final deciding authority that lays down the rules for the regulation of nanomaterials in food are the EC and the national food bodies. The term nanotechnology was not mentioned for long in the definition of EC. In October 2011, when EC released a definition of nanomaterials, it was not legally binding on all the monitoring bodies of the EU and the EFSA did not immediately adapt to it. It wrote a letter to the director general of the EU stating that it did not accept the definition of engineered nanomaterial (ENM) as the threshold of 50% stated by the EC was high especially for food and agriculture feed-related products (EFSA, 2012). It recommended the revision of the definition to lower the threshold of

1–50% wherever applicable for the environment, health, safety, or competitiveness. It also pointed out other problems with the definition that did not take into account the full range of applications of nanomaterials and their possible toxicity. It also stated that the only definition of a nanomaterial that was relevant to the food and feed applications was provided under the Food Information Regulation Article 2(2) (Regulation (EU) No. 1169/2011) that defined an ENM as follows (European Parliament and the Council of the European Union, 2011):

> any intentionally produced material that has one or more dimensions of the order of 100 nm or less or that is composed of discrete functional parts, either internally or at the surface, many of which have one or more dimensions of the order of 100 nm or less, including structures, agglomerates or aggregates, which may have a size above the order of 100 nm but retain properties that are characteristic of the nanoscale.
>
> Properties that are characteristic of the nanoscale include:
>
> (i) those related to the large specific surface area of the materials considered; and/or
> (ii) specific physico-chemical properties that are different from those of the non-nanoform of the same material.

This definition, although extensive, still did not include the threshold value for the nanomaterials. The EC plans to amend this definition. The process is still ongoing and is expected to take some time to come in action. The European Parliament in November 2014 voted to change its definition to adopt a 10% threshold of particles in the nanorange to be applied while considering the definition of nanomaterial in food. The Novel Food Regulation (EFSA, 2016) does not have its own definition of nanomaterial but refers to this definition of an "engineered nanomaterial." Any change in this definition of engineered nanomaterial would automatically apply to novel food.

It is also interesting to note that different sectors in the EFSA have a different definition of a nanomaterial. Example, the Biocidal Product Regulation adopted most of the aspects of the EC definition of nanomaterials. However, EFSA was not content with the fact that the EC definition did not mention a specific area (>60 m^2/cm^3), which as per the EC recommendation can also be used to define nanomaterials. It also does not take into consideration a possible reduction in the threshold value of 50% for nanomaterial products. Other regulations concerning agriculture, feed, or food do not contain a fixed definition of nanomaterial.

The use of different definitions, while helping to address the specific needs of the nanomaterial regulation based on the sector they were used, also creates a lot of confusion where a material may be present in its nanoform in the biocidal products. A nanomaterial may reach the stage of the final food, and while the biocidal regulation may regard it as a nanomaterial, the food regulation may not. These sector-specific definitions are still being discussed for a possible alignment. An important barrier in a uniform definition of nanomaterials is the life cycle stage at which a nanomaterial should be identified, as pointed out by the EFSA Scientific Committee (2011). A nanomaterial can change at any step in its life cycle in terms of size, agglomeration, or surface properties. A thorough development of the procedures previously mentioned is needed if the procedure is to be developed further to apply to a complex system such as a food chain.

The pesticides are regulated by the Plant Protection Product (PPP) Regulation (EC 1107/2009) (European Parliament and the Council of the European Union, 2009), which requires a premarket authorization of all pesticides at the EU level for the active substance and MS level for all PPPs. The active substances are dealt on a case-by-case basis which helps them to regulate ENMs even if they are not explicitly mentioned in the legislation. Environmental studies are undertaken on the active ingredient of the substance or a representative formulation. The authorization can only be granted to a product under plant protection product regulation if the active substance along with its safeners (added to eliminate or reduce phytotoxic effect) and synergists (added to enhance the activity of the active ingredient) has been approved (EFSA, 2011). Pesticides containing nanoforms or nanoencapsulations of approved active substances are considered as different pesticide products and require separate risk assessment and authorization. Kah and Hoffmann (2014) pointed out that the interaction between the active substances and the formulations also needs to be studied along with the long-term processes such as distribution and degradation.

The EC lays down a high level of protection for human health and consumer interest in relation to food by the use of Regulation (EC) No. 178/2002 (European Parliament and the Council of the European Union, 2002b), which explains the general principles and requirements of food law. Different regulations govern different sectors of food safety such as novel foods and novel food ingredients, food additives, feed additives, food supplements, vitamins, minerals, and food contact materials. Depending on the type of nanomaterial or the purpose of a food product, a food product may fall under the respective legislation. The EU has two major laws that govern the areas of food and food packaging regulations: EU General Food Law Regulation and EU Novel Food Regulation. The EU General Food Law Regulation is defined in Articles 5 to 10 and forms the horizontal framework that guides and regulates all food and food packaging. The EU Novel Food Regulation covers all nanomaterials under its scope. It first came into existence on May 15, 1997, and was then revised on November 25, 2015. The EC defines a novel food as "food that has not been consumed to any significant degree in the EU before May 1997. This can be newly developed, innovative food or food produced using new technologies and production processes as well as food traditionally eaten outside of the EU."

Engineered nanomaterials are defined in the new regulation (Regulation (EU) No. 1169/2011) that was revised on April 25, 2015. The EC clarified this inclusion in a press by stating that "the new Regulation specifies that engineered nanomaterials defined in the new legislation, require a Novel Food authorization before being used in foodstuffs. Their safety will be assessed by the European Food Safety Authority. The applicants must also demonstrate that most up-to-date test methods have been used for testing the engineered nanomaterials for which an authorization is sought for." Before the introduction of this regulation, the EFSA carries out a scientific risk assessment of a novel food only if an MS or the EU raises an objection to an intended market introduction of a novel food. The new regulation centralizes the procedure of risk assessment, and after its implementation in January 2018, all the risk assessment on the safety of novel food will be processed by EFSA on the request of the EU or an MS. The substances that are added to food to improve the taste or for any technological reasons are covered under the "Food Improvement Agent Package," which comprises a set of regulations that cover different aspects of food additives such as minerals, vitamins, and enzymes. An important point to note is that the nanoforms of

minerals and vitamins fall under the banner of Novel Food Regulation, due to their differences in production, nutritional value, and bioavailability when compared to their microscale counterparts. All the food additives, enzymes, and flavorings must undergo a premarket authorization. Feed additives, novel food, and food supplements must also do the same. Any change in the starting material (e.g., change in size) used in the production method of an additive must undergo a new scientific evaluation for its safety. All food additives that were permitted before 2009 are still under evaluation under the new guidelines. These include additives such as SiO_2 and nanosilver. For nanosilver, knowledge gaps have been identified and EFSA is reevaluating its efficiency as a safe food additive.

Food, before its consumption and during its production, processing, storage preparation, and serving, comes into contact with many materials and articles. Such materials are known as food contact materials (European Commission, 2016a). These materials are either intended to be brought into contact with food, are already in contact with food, or can reasonably be in contact with food or can transfer their constituents to food during their normal or foreseeable use. This includes direct or indirect contact. Some examples are containers for transporting food, machinery to process food, packaging materials, kitchenware, and tableware. Regulation (EC) No. 1935/2004 oversees this category of materials. The EU laws are general in nature and cover all food contact materials, and some apply to specific materials only. The MSs may impose additional laws if specific provisions do not exist in the EU rules. There are only two regulations that take nanomaterials into consideration: Regulation (EC) 450/2009 on "Active Intelligent Materials and Articles" and "Plastic Food Contact Materials" (European Commission, 2009). Regulation (EC) 450/2009 on Active Intelligent Materials and Articles is general with respect to nanomaterials and suggests a case-by-case risk assessment of nanomaterials used in packaging materials in intelligent packaging systems until more information is known. Intelligent packaging systems are systems that provide the user with information on the conditions of the food (European Commission, 2016a). They may be positioned on the outer surface of the package and may be separated from the food by a functional barrier. A functional barrier is a barrier within the food contact materials that prevent the migration of substances from behind the barrier into the food. A nonauthorized substance may be used behind the barrier provided that they fulfill a certain criterion and remain below a detection limit. But nanoparticles exhibit physical and chemical properties that are quite different from the bulk and hence a case-by-case assessment is required for them. The "Plastic Food Contact Materials" specifies nanomaterials more specifically. It states that substances in nanoforms can only be used if they are explicitly in Annex I of the regulation. Annex I has specified only one material as nanoparticle: titanium nitride as an additive or in production aid. Carbon black and amorphous silicon dioxide also fall under the category of nanomaterial, but they are not explicitly named but specified with size ranges below or around 100 nm. Substances that are released into the food using active food contact materials are considered to be actively added to food.

The EU also accounts for the safety of its consumers through a more transparent, traceable, and information regarding the use and possible exposure of nanomaterials on food, cosmetics, and biocides. Regulation (EU) No. 1169/2011 on the provision of food information to consumers entered into application on December 13, 2014, and mandates the information on all the ENMs used in the product to be labeled on the

product. All the ingredients used in food and biocides that are present in the nanoform have to be clearly indicated in the list of ingredients with the name of the ingredients followed by the word *nano* in brackets. Labeling of the products helps the consumers to make informed choices about the products. This may also overstrain the customer by having too much information on a single label but is a necessary step to ensure the safety of the customers. In addition to a label, there may be a separate register that records the possible advantages and side effects of the nanomaterial being used in the product.

16.3.1.4 Water Protection

The presence of clean air, water, and healthy soil is extremely important for the environment. Nanoparticles are continuously released in aquatic and terrestrial environments (CONTAM, 2016). The complete effect of the nanoparticles in these environments still suffers from the lack of adequate data. Humans and several terrestrial ecosystems rely on freshwater resources such as lakes and rivers for their sustenance. The nanoparticles that are present in the discharge have the potential to enter these complex ecosystems and cause short- or long-term damage that may be irreversible (Gottschalk et. al., 2012). Studies by Oberdörster et al.(2005) and Radomski et al. (2005) showed that the nanoparticles may undergo alterations inside a living organism. Silver nanoparticles at low concentrations were shown by Wijnhoven et al. (2009) to harm aquatic invertebrates and fish. There is also a high possibility of the nanoparticles getting bioaccumulated along the food chain. The European Environmental Bureau in its literature review found that "release of nanosized titanium dioxide into water could have detrimental effects on the overall ecosystem health, especially given that the concentration is large enough to have some detrimental effect on organisms and that it readily accumulates in drinking water."

The EU established the Water Framework Directive (WFD) in October 2000, with the aim of cleaning up and reducing dangerous chemicals (i.e., priority substances) in the European surface water and groundwater (Ganzleben and Hansen 2012a, 2012b; Hansen, 2012a). The WFD aims to protect surface waters, transitional waters, coastal waters, and ground waters. It seeks to promote long-term sustainable water use and prevent the further deterioration and to protect and enhance the status of aquatic ecosystems. The WFD also aims to protect and improve the aquatic environment through specific measures to reduce the discharges and emissions and promote losses of priority substances. In 2001, the Directive 2455//2011/EC established a list of 33 priority substances. The Directive 2008/105/EC replaced this old directive in 2008 and defined 33 priority substances along with 8 other substances that were previously regulated under earlier pieces of legislation of surface waters (Ganzleben and Hansen, 2012b). It also set up a set of environmental quality standards (ESQs) for these substances. The WFD has set up a goal of achieving a "good status" for all EU waters ideally by 2015 and latest by 2027. A good status, as per Annex V, is reached for a surface water body when it meets all the ESQs set for all priority substances and for the other pollutants defined in Annex I of the ESQs in their field of water policy (EQSD).

The WFD uses a river basin approach for water management. Under this measure, MSs have to develop river basin management plans that specify the measures to be taken to meet the environmental objectives. The geography and the hydrological formation of each river basin determine which MS will be responsible for establishing

the "river basin management plan." The MSs need to update the river basin management plan every 6 years. A summary report of the measures taken to address the pollution from priority substances is also warranted under the regulation. The MSs are also required to implement necessary measures with the "aim of progressively reducing pollution from priority substances and ceasing or phasing out emissions, discharges and losses of priority hazardous substances." Under the river basin management plan, the MSs are also obligated to include an analysis of the characteristics of the river basin (region, salinity, depth, etc.) and also provide a thorough review of the impact of human activity on the status of surface waters and on groundwater. They should also make an economic analysis with suggestions about the most cost-effective combination of measures in respect of water uses based on estimates of the potential costs of such measures. This analysis includes analysis on water use, involving relevant calculations of the recovery costs of the services that water brings as well as the volume, prices, and costs associated with these water services.

The WFD has two major terms to define the quality of water: "Good Ecological Status" and "Good Chemical Status." Good Ecological Status is defined in terms of the quality of the biological community, the hydrological characteristics, and the chemical characteristics, while Good Chemical Status is defined in terms of compliance with all the quality standards established for chemical substances at the EU level. The ecological conditions vary from place to place, and hence setting up a universal ecological standard that will be constant across all the EC is not possible. The term Good Chemical Status is more relevant to nanomaterials as it is defined in terms of compliance with all the quality standards established for chemical substances at the European level.

An important feature of WFD is the integration of groundwater and surface water management. Under WFD, surface water is evaluated in terms of Good Ecological Status and Good Chemical Status, but these are not followed for groundwater. Groundwater is assessed on the assumption that it should not be polluted at all and therefore any form of discharge in the groundwater is strictly prohibited. The Groundwater Directive sets up chemical quality standards for nitrates, pesticides, and biocidal products which need to be strictly adhered to. But these directives are advised not to serve as an acceptable level of pollution, and they serve as the minimum measures required to protect the groundwater. The MSs are also required to monitor indirect discharges to groundwater by keeping a watch on the chemical composition of groundwater.

The increased use of nanomaterials in several products makes it inevitable that they will be released into the EU waters whether directly or indirectly. Nanomaterials have multiple exposure routes through which they can contaminate the water sources such as the following:

- Nanomaterials used in environmental remediation of soil and groundwater
- Cleaning process of chambers used for nanomaterial production
- Spills from production, transport, and improper disposal of nanomaterials or products containing nanomaterials
- Improper treatment of wastewater containing nanomaterials
- Nanomaterials produced as a by-product of degradation of microsized materials such as beads
- Nanomaterials released during natural phenomena such as volcanic eruptions

WFD differentiates point source of pollution from a diffuse source. A point source of pollution may be wastewaters from industrial manufacturing facilities that produce nanomaterials either as a product or as a by-product of their production process. Point sources may also include wastewater from urban wastewater treatment facilities that include waste from cosmetics and sunscreens washed out of body and face of user, detergents, and nanotextiles from indoor and outdoor paints and from nanomaterials used to disinfect water and in wastewater treatment. Diffuse sources of nanomaterials include nanomaterials that leach into the groundwater and reach the surface water through landfills or through runoff of waste disposed improperly on the land near surface waters, runoffs of nanomaterials contacting pesticides from cultivated areas and sewage sludge, off-road spills of fuels, and lubricants that make their way into stormwater discharges and nanomaterials released into the atmosphere by automobiles and incinerators. Natural phenomena that release nanomaterials in water are also there, but they are beyond plausible control and do not fall under the category of an "engineered nanomaterial." There cannot be rules to regulate them.

The question of regulating a nanomaterial in EU waters has a major precursor to its application: the identification of a nanosubstance and the acknowledgement of its presence. The WFD mostly regulates a nanomaterial if it is in the list of its "priority substances." These are substances that have an EU-wide concern. The WFD requires the EC to review its list of priority substances every 4 years. As an overarching principle, the list of priority substances should include the following:

1. Hazardous substances agreed for the phase-out or for the cessation of discharges, emissions, and losses in international agreements
2. Hazardous substances which give rise to "an equivalent level of concern" as substances that are persistent, toxic, and liable to bioaccumulate (PTBs)

In order to determine the priority substances, the methodology has to take account for the following:

1. Evidence regarding the intrinsic hazard of the substance concerned and, in particular, its aquatic ecotoxicity and human toxicity via aquatic exposure routes
2. Evidence from monitoring of widespread environmental contamination
3. Other proven factors which may indicate the possibility of widespread environmental contamination, such as production, use volume, and use pattern of the substance concerned

The EC follows a Combined Monitoring-Based and Modeling-Based Priority Setting (COMMPS) approach while identifying a priority substance. It consists of a set of steps that help in narrowing down the substances that are a major concern across the EU. Unfortunately, each of these steps poses a major hurdle in recognizing nanomaterials a priority substance. The main reasons are the following:

1. There are various official substance lists and monitoring programs such as List I and II of Council Directive 76/464/EEC, OSPAR list of individual candidate substances, and HELCOM lists of priority substances that need

to be considered. A candidate for a priority substance has to be chosen from these lists, and no nanomaterial is listed so for on these lists.

2. The COMMPS requires exposure indices based on surface water monitoring data and modeling data for organic substances in the aquatic department. Exposure lists are also established for pollutants adsorbed by sediments and for metals based only on monitoring data. Surface water monitoring data are currently not available for any nanomaterial and the detection and monitoring of widespread environmental presence of nanomaterials in natural waters present some profound challenges.

   The concentrations of nanomaterials in the environment are shown to be low especially in regard to nanosilver and nanoparticles used in cosmetics and personal care products. The detection limits for most monitoring methods are not low enough to encompass nanomaterials under their banner.

3. Another requirement of COMMPS is the calculation of effect indices based on test data on direct and indirect effects on aquatic organisms as well as selected effects on humans (carcinogenicity, mutagenicity, reproductive and chronic effects from oral uptake) for organic pollutants and metals in the aquatic compartment and sediments. The method of grading involves scoring of direct and indirect effects of pollutants on the aquatic health, using methods such as Predicted Number Effect Concentrations. In such methods, chronic or acute data for the pollutant are extrapolated according to the methods laid down in the Technical Guidance Documents on Chemicals.

   Such data are rarely available for nanomaterials. Even the most tested nanomaterials such as C60 (Oberdörster, 2004) and carbon nanotubes do not possess complete data (Baun et. al., 2008). If the data are available, the reliability and interpretation of the ecotoxicity data are hindered by factors such as particle impurities, suspension preparation methods, the release of free metal ions, and particle aggregation.

The indirect effects of a nanomaterial are calculated using the octanol–water partitioning coefficient (Kow) which is used to measure the bioaccumulation potential. There is not much information available on the Kow values of most nanomaterials, and the available values cannot be used for nanomaterials. Furthermore, the Kow values may not be relevant for nanomaterials because they tend to be unstable and exhibit colloidal properties. Praetorius et al. (2014) suggested that the use of the Kow method may lead to significant errors in determining the fate of engineered nanoparticles and the associated risk assessment.

The EC is required to review the WFD every 4 years, and in the 2012 proposal, there was no mention of a nanomaterial as a priority substance. It is expected that ENMs may be listed as priority substances in the 2016 review. Along with this, the MSs are also required to arrange for long-term analysis of concentrations of these priority substances that tend to accumulate in sediments and/or in biodata. Monitoring plans need to be established within 6 years of the entry of WFD. As an example for surface waters ". . . such programmes shall cover among other things the ecological and chemical status and ecological potential." The frequency of monitoring should provide sufficient data for a reliable long-term trend analysis and should, as a guideline, take place every three years. Also, it is the duty of the MSs to take measures so

that the concentrations of the pollutants do not significantly increase in sediment and/or relevant biodata.

Analysis of nanomaterials in natural waters presents a challenge on its own. The first major challenge is developing methods with sufficiently low detection limits, the second being able to distinguish between natural and intentionally produced nanoparticles. The third challenge is to develop suitable methods that allow complete in situ analysis of samples in the natural environment and also a complete nanoparticle characterization. The last and the most important challenge is to understand that even the slightest change in physicochemical properties can make two nanomaterials of the same molecular form very different from each other in terms of characterization and environmental fate and behavior. Therefore, it is more suitable to individually monitor a nanomaterial. This is really challenging and is not expected to be overcome easily.

16.3.2 United States

The United States is one of the early users of nanotechnology due to its fast-paced product-oriented development. The development of biotechnology in the United States sparked debate about the security of biotechnology products in the market and the regulation that would govern them. This was closely followed by the debate on the nanotechnology, its safety, and the future of the products containing nanomaterials (Marchant et al., 2007; Beaudrie et al., 2013).

The US federal government established a "Coordinated Framework for Regulation of Biotechnology" in 1986 on the central idea that no new rules were needed to regulate biotechnology. Instead, the Coordinating Framework consisted of ten existing statutes administered by five different federal agencies. The product would be regulated by one or more statues based on the product type. An important aspect of the framework was that it did not regard the biotechnology products to be more dangerous than other types of products and it decided the regulation of the product based on its specific properties not by the process it was made. A reason for the success of biotechnology products in the United States than in other countries, especially in Europe, was the absence of any strong public opposition.

The US government has adopted a similar approach toward the development of nanotechnology. Europe and most other countries have developed rules that specifically aim to target nanotechnology-related products. The US government, on the other hand, has developed a much more flexible and adaptable approach toward the development of nanotechnology. The Risk Policy Report of 2006 stated that no new regulations are required to regulate nanotechnology. Existing statutes administered by various federal agencies can be applied based on the category the product falls into. The US Federal Government launched the NNI, which was equivalent to the Coordinated Framework for Regulation of Biotechnology. The NNI is the Government research and development (R&D) initiative involving 20 departments and independent agencies working together toward the shared vision of "a future in which the ability to understand and control matter at the nanoscale leads to a revolution in technology and industry that benefits society." It is coordinated by the White House Office of Science and Technology Policy's National Science and Technology Committee. An interagency coordination committee at the Nanoscale Science Engineering and Technology (NSET) committee has been established as a subcommittee of the Nano Science and Technology Council (NSTC). The NSTC coordinates the activities of

several federal agencies to form investment packages aimed at accomplishing multiple national goals. It was established in 1993 with the aim of establishing clear goals for the Federal Science and Technology investments in all its mission areas.

The NNI gives several considerable emphases to the ethical legal and social issues. The NNI has divided the plan to tackle challenges related to nanomaterials and nanotechnology under two banners: (a) environmental health and safety and (b) ethical legal and other social issues. A workgroup of the Nanomaterials Environmental Health Implications was established in 2004 under the NSET to address and coordinate environmental and health issues with nanotechnology.

Three major agencies under the US Federal Government have been mainly active in regulating the nanomaterials in the environment: (1) FDA, (2) US Environmental Protection Agency (EPA), and (3) National Institute of Occupational Safety and Health (NIOSH).

16.3.2.1 Food and Drug Administration

The FDA is responsible for protecting the public health by ensuring the safety, efficacy, and security of human and veterinary drugs, biological products, and medical devices and by ensuring the safety of US food supply (including food additives and dietary supplements), color additives, cosmetics, and products that emit radiation (US FDA, 2016b).

The FDA has been encountering several combinations of promise, risk, and uncertainty that accompany emerging technologies. The same has been true for nanotechnology, which has both the promise of a better future with the uncertainty of unfathomable risks. It conducts its functions using a variety of laws and regulations which establish the scientific premarket and postmarket oversight mechanisms applicable to a particular class of product. Substances used as food and color additives are always subjected to premarket authorization while for food ingredients that are generally recognized as safe, premarket authorization is not required (US FDA, 2016c). Materials can exhibit a wide range of properties at the nanoscale level and can have unique properties even if one of their dimensions is in the nanoscale. A universal definition of nanomaterials is not adopted by FDA as the definition may be very meaningful for one respect and too narrow or too broad for another. It, therefore, focuses on a broader prospectus whether a product in question contains nanomaterials or uses nanotechnology. In the application of nanotechnology or the use of nanomaterials, FDA, during its evaluation, focuses on the unique properties or behaviors that the product may exhibit. It does not categorize a product as benign or harmful based on the fact that it applies nanotechnology or uses nanomaterials or not. It rather judges the product based under existing statutory authorities, in accordance with the specific legal standards applicable to each type of product under its jurisdiction. The central idea is that the product willing to enter the market should not damage or have a minimal impact on the environment. In agreement with the Executive Order 13563 on improving regulation, as well as with the White House policy statement on emerging technologies and the application of nanotechnology, FDA supports innovation and the safe use of nanotechnology in FDA-regulated products under appropriate and balanced regulatory oversight.

It has released several guidance documents addressing the issues of nanotechnology. In June 2011, FDA issued a draft guideline for the industry titled "Considering Whether an FDA-Regulated Product Involves the Application of Nanotechnology" to present its views to the industry on nanotechnology. It was followed by three products of specific guidelines (US FDA, 2014, 2016a). The four final guidances were as follows.

16.3.2.1.1 Final Guidance for Industry: Considering Whether an FDA-Regulated Product Involves the Application of Nanotechnology

This is the overarching guidance for all FDA-regulated products, identifying the points to consider while determining whether a product uses nanotechnology. It states that while considering whether a product contains nanomaterials, FDA looks at the size of the material (around 1–100 nm), properties, or phenomena that are attributable to the external dimensions of the material, even if these dimensions are not in the nanoscale range but instead up to 1 μm. Structures such as agglomerates and aggregates along with coated, functionalized, or hierarchically assembled structures are of special interest in this context. The guidance also encourages the manufacturers to consult FDA in the early stages of product development to address the question about the regulatory status of products or address questions related to the safety, effectiveness, or other attributes of nanotechnology products.

16.3.2.1.2 Final Guidance for Industry: Safety of Nanomaterials in Cosmetic Products

It states that nanomaterials may exhibit new or altered physicochemical properties that may affect biological interactions, which may raise questions about the safety of the product containing nanomaterials. Any such unique properties or biological effects of nanomaterials should be identified and appropriately addressed during safety evaluations. It expresses the current thinking of the FDA on the safety assessment of nanomaterials when used in cosmetic products. It emphasizes on the strong need for a thorough nanomaterial characterization with respect to its physical and chemical properties including its toxicity profile, exposure routes, uptake, and absorption. The main highlights of the regulation are as follows:

1. Cosmetics and cosmetic ingredients (with the exception of color additives) are not subject to premarket approval; however, they must demonstrate to be safe for customers under labeled or customary conditions of use, and they must be properly labeled.
2. Cosmetics containing nanomaterials also follow under the umbrella of the regulations that govern cosmetics not containing nanomaterials. The marketer of a cosmetic is legally responsible for the safety of their products.
3. It recognizes that the framework currently in use for assessing the safety of cosmetics is appropriate for cosmetics containing nanomaterials. However, the data needs and testing methods should be evaluated in light of the properties, behaviors, and/or effects that may be exhibited by nanomaterials used in cosmetic products.

16.3.2.1.3 Final Guidance for Industry: Assessing the Effects of Significant Manufacturing Process Changes, Including Emerging Technologies, on the Safety and Regulatory Status of Food Ingredients and Food Contact Substances, Including Food Ingredients That Are Color Additives

This guidance informs manufacturers of the potential impact of a change in the manufacturing process, including those on using nanotechnology and on the safety and regulation status of food substances. It informs the manufacturers of the factors that they should consider while determining whether there is a significant change in the manufacturing of a food substance already in the market. The change is considered to be substantial if the manufacturing process does any of the following:

1. Affects the identity of the food substance
2. Affects the safety of use of the food substance
3. Affects the regulatory status of the use of food substance
4. Warrants a regulatory submission to FDA

It also recommends manufacturers to consult the FDA if there is a significant change in the manufacturing process for a food substance that is already in the market.

16.3.2.1.4 Final Guidance for Industry: Use of Nanomaterials in Food for Animals

This guidance highlights the approach of FDA toward the use of nanomaterials or the application of nanotechnology in food for animals. It defines the legal regulations for adding a substance to food for animals and recommends the submission of a Food Additive Petition (FAP) for a nanomaterial animal food ingredient. In the development of a FAP, the FDA recommends the following sections to be taken into consideration:

- Identity
- Manufacturing methods and controls
- Intended use, use level, and labeling
- Analytical methods
- Safety, evaluation, and proposed tolerances for the food additive
- Proposed regulation
- Environmental assessment

It also recommends the manufacturer to consult the FDA before the submission of FAP, in the early phases of development of nanomaterial animal food ingredient.

The products under FDA can fall under two categories: (a) products that require premarket approval (e.g., new drugs, biological products, certain devices, food and color additives) and (b) products that do not require premarket approval (e.g., cosmetics). In either case, the product has to meet the applicable safety standards and has to comply with other applicable requirements. Safety requirements can only be met on the basis of scientific evidence. The FDA nanotechnology task force helps in determining whether the currently available tests are suitable to support risk management

decisions. FDA invests significantly in the developing techniques for the characterization of nanomaterials, in vivo and in vitro modeling, and product-focused research. There are regions of special interest, for example, cosmetics that do not require a premarket approval, and therefore their characterization is of prime importance. The FDA also coordinated its activities with other agencies under the NNI.

16.3.2.1.5 Regulatory Strategies of FDA

As stated earlier, the FDA does not categorically judge products as benign or harmful by the application of nanotechnology or the use of nanomaterials. It follows an iterative, adaptive, and flexible approach. It is iterative by developing and delivering incremental components of a regulatory system such as guidance specific to product areas, each as warranted, when ready. It is adaptive in its approach as it provides a mechanism within the statutory constraints to change the rules or pathways for regulating a product if it finds new information from research or from regulating earlier products.

FDA has a different approach to regulating a product based on the class the product falls into. Food additives are considered safe when there is a reasonable certainty of no harm from their intended use. Drugs, by contrast, are evaluated on the basis not only of their risk profile but also of their predicted benefit. There are different legal standards to demonstrate how different contexts could lead to different regulatory outcomes, even if two products present the same level of risk.

Premarket review exists for certain class of products, such as new drugs, new animal drugs, biologics, food additives, color additives, certain human devices, and certain new dietary ingredients in dietary supplements, which requires the applicant to submit data and answer questions related to the safety, effectiveness (where applicable), or regulatory status of the product. The FDA is incorporating nanomaterials into its standard procedure of premarket approval. Individual premarket review procedures include attention to whether the use of nanomaterials suggests the need for additional data on safety or effectiveness, as applicable.

There are also classes of products that do not require premarket approval. In these cases, the ability of the FDA to regulate the product before it enters the market is severely limited. For these products, the FDA relies on publicly available or voluntarily submitted information, adverse event reporting (where applicable), and postmarket surveillance activities, to provide oversight. FDA also encourages manufacturers and marketers to consult them before they take their products to the market. Such consultation can help the FDA to advise companies, review safety information, and design any necessary postmarketing safety oversight.

The responsibility for the safety of a product, whether it requires premarket approval or not, falls on the manufacturer and the marketer of the product. The industry remains responsible for ensuring that its products meet all applicable legal requirements, including safety standards. All industries are required to work with current information in product development and continue to monitor products once marketed. Along with the option of consultation, the FDA also assists in public meetings to advance regulatory science, to identify product-specific data needs, and to seek input on specific issues. It coordinates its activities with domestic and international agencies to provide for better regulation through scientific understanding and uses of any possible threat posed by a substance. The FDA also continuously monitors products containing nanomaterials in the market and takes action when needed to protect the customer.

16.3.2.2 US Environmental Protection Agency

The US Environmental Protection Agency (US EPA) is the lead agency in the United States for addressing the concerns of the potential environmental risks from nanotechnology exposure. The EPA initially had the focus on supporting research and building internal expertise on environmental applications and implications of nanotechnology.

Particularly in the last decade, with more than 1300 known commercial products in the US market containing nanomaterials, concerns about the potential environmental and health risks have considerably risen. There is increased use of nanomaterials in products such as medical equipment, textiles, fuel additives, cosmetics, and plastics. The unique properties of nanomaterials compelled the EPA to research more on them to fill in the "knowledge gaps" that were preventing the proper regulation of nanomaterials. The EPA has a comprehensive research program for nanomaterials where scientists research the most prevalent nanomaterials that may have human and environmental health implications. The research develops a scientific foundation to better understand, predict, and manage the challenges of nanomaterials. Currently, the EPA is focusing on few major nanomaterials, namely, nanosilver, carbon nanotubes, cerium oxide, titanium oxide, iron, and microsized copper. The EPA will use this research to develop research protocols for characterizing ENMs and for evaluating exposure and toxicity in complex biological or environmental systems. The research focuses on mapping the environmental fate and the effect of nanomaterials on ecosystems and wildlife health and on developing successful nanomaterials. This research allows the EPA scientists to evaluate the relationships between the physical and chemical properties of ENMs and their fate, transport, and effects. It would help in the creation of safer and more sustainable nanomaterials.

The EPA tends to be media specific in regulating nanomaterials with separate regulations for different classes of materials such as the Clean Air Act (CAA) to address air pollution, the Clean Water Act for water pollution, the Resource Conservation and Recovery Act for hazardous and solid wastes, and the Federal Insecticide, Fungicide and Rodenticide Act for pesticides. It has relied on these media-specific regulations for regulation of nanotechnology or the use of nanomaterials in a very limited way. Instead, it relies more on the Toxic Substance Control Act (TSCA) to screen and regulate nanotechnology-related products (US EPA, 2016b). The TSCA was adopted in 1986 as the "gap-filling" statue intended to provide EPA with a complete authority to regulate all chemical risks that were not covered under the media-specific acts (Regulations.gov, 2017; US EPA, 2016dTSCA#).

TSCA provides four distinct advantages to EPA:

1. The TSCA is unlike most US environmental regulations due to its ability to be a multimedia and therefore has the authority to regulate all potential uses and exposures of nanotechnology.
2. The TSCA provides premarket screening of new toxic substances. This helps in the screening of toxins even before they are emitted into the environment. Most media-specific regulations kick in after the pollutant has been emitted into the environment.

3. The TSCA represents an unmatched variety of regulatory tools that EPA can select from regulating nanotechnology, including testing requirements, reporting requirements, labeling, restrictions, and prohibitions.
4. Focusing on multiple regulations to regulate a single class of products is resource intensive. The TSCA provides a narrower focus that is more effective than the media-specific regulations.

The EPA ensures that the products manufactured and used do not cause unreasonable risk to human health and the environment. To achieve this aim, it takes two major steps under TSCA:

1. Premanufacture notifications for new nanomaterials
2. An information-gathering rule on new and existing nanomaterials

16.3.2.2.1 Premanufacture Notifications for New Nanomaterials

Under Section 5 of the TSCA, manufacturers and importers of new chemical substance are required to provide specific information to EPA for review prior to manufacturing chemicals and introducing them into the market. This notice is known as a premanufacture notice. The EPA can take necessary action to ensure that the chemicals that may or will pose a threat to the environment or human health is effectively controlled. They are also encouraged to contact the EPA if they need any assistance in determining whether their nanoscale materials are subject to the "New Chemical Review Program."

The New Chemicals Review Program of the EPA is mandated by Section 5 of the TSCA and helps to manage the potential risk to human health and the environment from chemicals new to the marketplace. The program acts as a "gatekeeper" that identifies the conditions to be placed on a new chemical before it can enter the market.

Section 8 (b) of the TSCA requires the EPA to compile, keep current, and publish a list of each chemical substance that is manufactured or processed, including imports, in the United States for uses under the TSCA. Also called the "TSCA Inventory" or simply "the Inventory," it plays a central role in the regulation of most industrial chemicals in the United States (US EPA, 2016a). It was established in 1978 and now lists about 85,000 chemicals. A chemical substance that is listed in the TSCA inventory is considered as an "existing" chemical substance in the US market, and a substance not listed in the TSCA inventory is considered as a "new" substance. It contains substances including organics, inorganics, polymers, and chemicals of unknown or variable composition, complex reaction products, and biological materials. The chemicals that are not regulated under the TSCA are not listed on the inventory. They include substances such as pesticides, food and food additives, drugs, cosmetics, tobacco and tobacco products, nuclear materials, and munitions.

The EPA decides whether a nanoscale substance is new or existing on a case-by-case approach. Given the several circumstances that can contribute to the unique properties of nanomaterials, the mere mention of the name of a substance in the inventory is not enough to determine whether the nanomaterial is new or existing.

Thus, this case-by-case approach is better suited for nanomaterials. Since 2005, the EPA has received 160 new chemical notices under the TSCA for nanoscale materials. It has taken the following actions to control and limit exposures to these chemicals, including the following:

- Limiting the uses of the nanoscale materials
- Requiring the use of personal protective equipment (PPE) and engineering controls
- Limiting environmental releases
- Requiring testing to generate health and environmental effects data

The EPA has significantly limited the manufacture of substances at the nanoscale through the use of consent orders or Significant New Use Rules under TSCA. It has also allowed the manufacture of new chemical nanoscale materials but only in circumstances where exposures were tightly controlled to protect against unreasonable risks.

16.3.2.2.2 Information Gathering Rule on New and Existing Nanomaterials

On April 6, 2015, the EPA proposed a new law based on one-time reporting and recordkeeping requirements under TSCA section 8(a). This proposed rule would require that companies that manufacture certain chemical substances already in commerce as nanoscale materials notify the EPA of information including production volume, methods of manufacture and processing, exposure and release information, and available health and safety data. The information gathered under the new rule, after enforcement, would facilitate EPA's evaluation of the materials and help in the determination of whether further action, including additional information collection, is needed.

Along with this on June 22, 2016, President Obama signed a law, the Frank R. Lautenberg Chemical Safety for the 21st Century Act (US EPA, 2016e), which amends the TSCA. It requires the EPA to mandatorily evaluate existing chemicals with clear and enforceable deadlines. It also enforces a new risk-based safety standard and warrants an increased public transparency for chemical information.

The EPA also coordinated its activity internationally with international organizations such as the International Organization for Standardization and the Organisation for Economic Co-operation and Development to further understand the properties and potential risks of nanomaterials and develop international standards for nanotechnology. On February 4, 2011, the United States and Canada jointly created the Canada–US Regulatory Cooperation Council to better align their regulatory approaches in various areas, including nanotechnology. As part of this initiative, a Nanotechnology Work Plan was developed to increase regulatory transparency and coordination between both countries with respect to nanomaterials.

16.3.2.3 National Institute of Occupational Safety and Health

The debate on the safety of nanotechnology and usage on nanomaterials is an ongoing issue. There are products that have approved nanomaterials in them. While there are not enough data to completely map the toxicity of these nanoparticles, there are

human workers that work in the industry to produce them. The NIOSH focuses on the health and safety of these workers as they are susceptible to high exposures that have health and safety effects (CDC-NIOSH, 2007; NIOSH, 2016a, 2016d).

NIOSH was created in 1970 under the Occupational Health and Safety Act with the mandate to assure that "every man and woman in the Nation safe and healthful working conditions and to preserve our human resources." It is the "federal agency responsible for conducting research and making recommendations to prevent work-related injury illness or health." NIOSH has been instrumental in discussing the possible human health and environmental consequences of nanotechnologies and practices. NIOSH is working in partnership with many other government agencies through participation in the NNI. It estimates that there are 400,000 working globally in the field of nanotechnology and 150,000 in the United States alone (NIOSH, 2016a, 2016c).

NIOSH is largely a nonregulatory agency, but the guidance and recommendations issued by NIOSH are often used by other agencies responsible for developing and enforcing workplace safety and health regulations. It, however, is also has a degree of authority to regulate using procedures and requirements for specific health and safety-related matters involving a particular industry. These include the Coal Workers Surveillance Program, NIOSH grants and educational training programs, approval of respiratory protective equipment and Health Hazard Evaluation at places of employment, and implementation of the World Trade Center Health Care Program.

In 2004, the NIOSH founded the Nanotechnology Research Center (NTRC) to "identify critical issues, create a strategic plan for investigating these issues, coordinate the NIOSH research effort, develop research partnerships, and disseminate information gained." In September 2005, NTRC released its strategic plan for NIOSH nanotechnology research that affirmed that there was a knowledge gap between existing possible risks and permissive uses of nanomaterials in the workplace as nanomaterials may have "biological properties distinct from fine particles of similar dimensions." NIOSH has identified 10 critical topic areas to help in addressing knowledge gaps, develop strategies. and provide recommendations (NIOSH, 2016b, 2016e). They are the following:

- Toxicity and internal dose
- Risk assessment
- Epidemiology and surveillance
- Engineering controls and PPE
- Measuring methods
- Exposure assessment
- Fire and explosion safety
- Recommendations and guidance
- Global collaborations
- Uses of nanotechnology for application in occupational safety and health

NIOSH conducts research on each of these topics and has issued reports and guidance for the industry to ensure the safe production or usage of nanomaterials in the industry.

16.3.2.4 Drawbacks of the US Regulatory Approach on Nanotechnology and Nanomaterials

16.3.2.4.1 "Fit" Problem

The EPA primarily relies on the TSCA to regulate a nanomaterial before it enters the environment, but if a nanomaterial is already in the environment, the media-specific acts kick in. There is a "fit" problem in applying the regulations of existing regulations for the regulation of nanotechnology or nanomaterials. For example, while applying the CAA, to stationary sources to regulate nanomaterial-based emissions, the regulation recommends using the "best available technology" (BAT) to set up standards to regulate the nanomaterials. But there are no existing technologies on which BAT can be based. Also, CAA employs the measurement of the mass of a particle in a particular size range to determine the ambient levels of the criterion of air pollutants such as particulates. Such mass-based techniques are not applicable for nanomaterials as the threat-determining factor is the surface area rather than weight.

The FDA regulation mostly distinguishes between different categories based on their dominant mode of action. For example, if a product acts by chemical mode of action, it is a drug, but if a product acts by a mechanical mode of action, it is a medical device. This distinction may become obsolete for a nanomaterial that operates at a molecular level and may be categorized as chemical, mechanical, and even biological.

16.3.2.4.2 Defining Nanotechnology

The coordinating central agency of the NNI has been instrumental in helping various agencies in adopting a similar definition of nanotechnology. Most of the definitions specify nanotechnology as the usage of materials that have at least one dimension in the range of 1–100 nm. This puts an ambiguity for the materials with sizes above 100 nm. Would they not be considered as a nanomaterial? Also, a question that arises is what percentage of the size distribution should be in the nano range for the material to qualify as a nanoproduct. The FDA has not adopted any central definition on nanomaterials or nanotechnology and evaluates products on a case-by-case approach using its existing regulations. The EPA and NIOSH have a definition of a nanomaterial by the size range of 1–100 nm. This definition contributes to a big ambiguity in the usage of nanomaterials.

16.3.2.4.3 Uncertainty Problem

There is no doubt that there is a significant knowledge gap in the regulation of nanotechnology due to the lack of proper data. The US government has launched several initiatives to address this issue. However, the accurate compilation of data takes time. The industry often argues that nanotechnology should not be considered intrinsically dangerous and that a possible threat should be supplemented by adequate data. The Federal Agencies, on the other hand, tend to have a more precautionary principle and have often advised companies to give up commercial production of nanomaterials until there are adequate data to support the safe nature of the nanomaterial in question. However, the agencies also limited by existing regulations such the EPA can regulate a nanotechnology product under Section 6 of the TSCA if it can demonstrate that the product imposes "unreasonable" risk, and if it wants to test a product in order to demonstrate the risk, the EPA must show that the product has the potential for a

"substantial" risk. EPA, in such cases, gets trapped within its own regulatory framework. Therefore, the EPA has now opted for a voluntary disclosure of the nanomaterial and its risk by the manufacturer or the marketer.

16.3.2.4.4 Other Factors

There are numerous other factors that contribute to the problems in regulating a nanomaterial effectively. Politically, even if a regulation is obsolete and it is agreed upon by all legislators, it may not be replaced due to a political gridlock in trying to replace the regulation with a newer, better regulation. The effectiveness of the new regulation in regulating the nanotechnology product must be demonstrated and must be accepted by the majority. If this is not the case, it may take a lot of time in regulating a product.

The views of the legislators are often views of the public. If the public wants the successful regulation of a product, it can pressurize the government to do so. There have been examples in history where the public initially took a stand on curbing a harmful product nanomaterial, but when they found out that the product did not contain a nanomaterial, the controversy died out. This double standard of the public is also responsible for a slower action in regulation of a nanomaterial. For example, the tests that are done to demonstrate that a product does not contain a nanomaterial are often not communicated to the public. This lack of transparency, vastly influenced by the media they are in contact with, adds to the problem.

16.4 Conclusion

Nanotechnology has, with no doubt, demonstrated that there is a range of applications that are novel and highly. The main reason for the novel properties of nanotechnology is their increased surface area. Also, the properties that they demonstrate is size dependent; i.e., the properties can change if the size of the nanoparticles changes. The properties also depend on how many dimensions the confinement there is in the nanoscale range. Along with the increased applications has come the plethora of uncertainties in their uses.

The EU and the United States both have developed stringent procedures to combat the increasing risks associated with nanotechnology. They have also taken a lead in developing regulatory approaches for dealing with potential nanomaterials risks. REACH is the leading legislation that deals with chemicals used in the EU. It has strived to regulate nanomaterials with its existing regulations but is plagued by its approach toward nanomaterials that fails to recognize nanomaterials as separate entities that have properties significantly different from their parent bulk material (Greenhalgh, 2012). This is in contrast to the approach of the EU toward the nanomaterials used in the food and pesticide sectors where EFSA aids in the development and implementation of regulations. The EU regulates the nanomaterials used in this sector on a case-by-case basis, and much attention is given if the active ingredient of the product used is a nanomaterial. The regulation of medicines using nanomaterials in the EU is aided by the EMA. The EMA is the decentralized agency of the EU that oversees the approval of medicines and advises the EU on approval of development of drugs for human and veterinary use based on scientific data. The fast pace of development of nanomedicines and the increasing complexity of the subsequent generation of

drugs are major challenges that the EMA faces. It has to steadily develop its knowledge database for the existing nanomaterials and be ready for the next generation of nanomedicines. The greatest challenge faced by the EU is in terms of regulating the level of nanomaterials in its natural waters. The scale that the WFD is expected to work is large. It aims to regulate the chemical levels in waterbodies to maintain a "good status." However, the state of one water body may differ greatly from another due to a different geographical location and hence the methods used to evaluate the good status may also vary greatly. This makes the regulation of nanomaterials in the natural waters quite challenging.

The United States has three major agencies that regulate the nanomaterials in their market: the FDA for food and drugs, the US EPA to oversee and regulate the effect of nanomaterials in the environment (US EPA, 2016c), and the NIOSH that looks into the safety of the workers employed in the industry manufacturing nanomaterials. The United States on the whole has adopted a much more flexible strategy in dealing with nanomaterials that has helped it to encompass the existing nanomaterials and has aided in its preparation for the next generation of nanomaterials. However, there are a few problems with the regulations like there may be ambiguity in the definition of nanomaterials used by different agencies (Boverhof et al., 2015). But overall, the United States has developed a strong framework of nanomaterial regulation in its market.

The EU and the United States have also been cooperating their work at different levels to develop uniform standards and international trade. Still, there is a gap of extending this international cooperation to other nations that are still not part of the union. It is imperative to do so as there are many third-world nations that are using nanomaterials but are vastly unaware of their possible implications. Even if they are aware, they lack the resources to fully regulate these nanomaterials. Also, many Asian and South American nations that have developed joint programs for gathering scientific information and developing regulations related to nanotechnology. Without a global cooperation, the free flow of important information will not be there and the aim of a holistic approach to using nanotechnology cannot be fulfilled easily. A first, and critical, step in this process is the creation of sound and internationally agreed on scientific building blocks for risk assessment. There has to be increased cooperation between various agencies across nations to develop uniform standards of regulation of nanomaterials. This international cooperation has to be extended to risk management and promotion of international trade. Also, there is an urgent need to close the gaps related to nanotechnology and establish a comprehensive market to register products related to nanotechnology. This will not only help in creating robust regulations for the current nanomaterials but also help in preparing future challenges in the regulation. This can be achieved only through increased research not only in the application of nanomaterials but also on the toxicity associated with them and their overall effect on the environment. Special emphasis should be paid to their long-term effect on the environment and the human health. Any minor change in the nanomaterial should be investigated in detail for the possible change in the toxicity and its effect on the environment. Above all, a flexible approach is the need of the hour. The nanomaterials should always be studied on a case-by-case basis rather than regulating them through the set of laws that govern bulk materials. The market for nanomaterials is expected to grow, not just linearly but exponentially. It is more on our side and on the side of the government to balance the risks with the benefits.

REFERENCES

Amenta, V., K. Aschberger, M. Arena, H. Bouwmeester, F. Botelho Moniz, P. Brandhoff, S. Gottardo et al. 2015. Regulatory aspects of nanotechnology in the agri/feed/food sector in EU and Non-EU countries. *Regulatory Toxicology and Pharmacology* 73, no. 1 (October): 463–476.

Azoulay, D. 2012. Just Out of Reach. Center for International Environmental Law (CIEL): 1–37. http://www.ciel.org/Publications/Nano_Reach_Study_Feb2012.pdf.

Baun, A., S.N. Sørensen, R.F. Rasmussen, N.B. Hartmann, and C.B. Koch. 2008. Toxicity and bioaccumulation of xenobiotic organic compounds in the presence of aqueous suspensions of aggregates of nano-C60. *Aquatic Toxicology* 86, no. 3 (February): 379–387. http://www.sciencedirect.com/science/article/pii/S0166445X07004250.

Beaudrie, C.E.H., M. Kandlikar, and T. Satterfield. 2013. From cradle-to-grave at the nanoscale: Gaps in U.S. regulatory oversight along the nanomaterial life cycle. *Environmental Science and Technology* 47, no. 11: 5524–5534.

Berger, M. 2013. Nanotechnology Policy Making—Voluntary Tools. Nanotechnology and Emerging Technologies News from Nanowerk. http://www.nanowerk.com/spotlight/spotid=29846.php (accessed December 29, 2016).

Boverhof, D.R., C.M. Bramante, J.H. Butala, S.F. Clancy, W.M. Lafranconi, J. West, and S.C. Gordon. 2015. Comparative Assessment of Nanomaterial Definitions and Safety Evaluation Considerations. Regulatory Toxicology and Pharmacology 73, no. 1: 137–150.

Boverhof, D.R., C.M. Bramante, J.H. Butala, S.F. Clancy, W.M. Lafranconi, J. West, and S.C. Gordon. 2015. Comparative assessment of nanomaterial definitions and safety evaluation considerations. *Regulatory Toxicology and Pharmacology* 73, no. 1 (October): 137–150.

CDC-NIOSH (Centers for Disease Control and Prevention–National Institute for Occupational Safety and Health). 2007. Progress toward Safe Nanotechnology in the Workplace. DHHS (NIOSH) Publication No. 123: 1–199. https://www.cdc.gov/niosh/docs/2010-104/pdfs/2010-104.pdf.

Chellaram, C., G. Murugaboopathi, A.A. John, R. Sivakumar, S. Ganesan, S. Krithika, and G. Priya. 2014. Significance of Nanotechnology in Food Industry. APCBEE Procedia 8, no. 1: 109–113. http://www.sciencedirect.com/science/article/pii/S2212670814000906.

Chen, H., M.C. Roco, X. Li, and Y. Lin. 2008. Trends in nanotechnology patents. *Nature Nanotechnology*, no. 3 (March): 123–125. https://yllin.mis.nsysu.edu.tw/paper/yllin/Trends%20in%20nanotechnology%20patents.pdf.

CIEL (Center for International Environmental Law). 2012. Revision of REACH Annexes for Nanomaterials. Center for International Environmental Law (CIEL)—Position Paper. http://www.ciel.org/wp-content/uploads/2015/10/Position-Paper-REACH-Annexes-Final.pdf (accessed December 29, 2016).

CONTAM (EFSA Panel on Contaminants in the Food Chain). 2016. Presence of microplastics and nanoplastics in food, with particular focus on seafood. *EFSA Journal* 14, no.1 (May): 4501–4531.

Dang, Y., Y. Zhang, L. Fan, H. Chen, and M.C. Roco. 2010. Trends in worldwide nanotechnology patent applications: 1991 to 2008. *Journal of Nanoparticle Research* 12, no. 3: 687–706. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2988206/.

de la Ossa, D.H.P. 2014. Quality Aspects of Nano-Based Medicines. Workshop, S.M.E.: Focus on Quality for Medicines Containing Chemical Entities. http://www.ema.europa.eu/docs/en_GB/document_library/Presentation/2014/04/WC500165444.pdf.

de la Ossa, D.H.P. 2015. Nanomedicines: EMA Experience and Perspective. Horizon 2020 European Union Funding for Research & Innovation. http://euronanoforum2015.eu/wp-content/uploads/2015/06/2_NanomedicinesEMA-experienceperspective_DoloresHernan_10042015.pdf.

Dowling, A., R. Clift, N. Grobert, D. Hutton et al., 2004. Nanoscience and nanotechnologies: Opportunities and uncertainties. *The Royal Society and The Royal Academy of Engineering Report* 46, no. July: 618–618. https://royalsociety.org/~/media/Royal_Society_Content/policy/publications/2004/9693.pdf.

ECHA (European Chemicals Agency). 2012. Updated Guidance on Information Requirements and Chemical Safety Assessment for Nanomaterials. ECHA news. https://echa.europa.eu/web/guest/view-article/-/journal_content/3df5b7b9-a36d-4e74-811b-3aeee23366f8 (accessed November 12, 2016).

ECHA. 2016. Understanding REACH—ECHA. ECHA web page. https://echa.europa.eu/regulations/reach/understanding-reach (accessed November 5, 2016).

EFSA (European Food and Safety Authority) Scientific Committee. 2011. Scientific opinion on guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain. *EFSA Journal* 9, no. 5 (April): 2140. https://www.efsa.europa.eu/en/efsajournal/pub/2140.

EFSA. 2012. Application of the Definition of Nanomaterial in Food. *Letter from EFSA to Director General of EU* (October): 1–5. https://www.efsa.europa.eu/sites/default/files/assets/corporatenanotechnology121003.pdf.

EFSA. 2016. Novel Food. EFSA web page, Novel Food Topic. http://www.efsa.europa.eu/en/topics/topic/novel-food.

Ehmann, F., K. Sakai-Kato, R. Duncan et al. 2013. Next-generation nanomedicines and nanosimilars: EU regulators' initiatives relating to the development and evaluation of nanomedicines. *Nanomedicine (London)* 8, no. 5: 849–56. http://www.ncbi.nlm.nih.gov/pubmed/23656268.

Etheridge, M.L., S.A. Campbell, A.G. Erdman, C.L. Haynes, S.M. Wolf, and J. McCullough. 2013. The big picture on small medicine: The state of nanomedicine products approved for use or in clinical trials. *Nanomedicine* 9, no. 1 (January): 1–14. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4467093/.

European Commission. 2006. Regulation (EC) 1907/2006 of the European Parliament and of The Council of 18 December 2006—REACH. *Official Journal of the European Union*. http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32006R1907&from=en.

European Commission. 2009. Commission Regulation (EC) No. 450/2009 of 29 May 2009 on Active and Intelligent Materials and Articles Intended to Come into Contact with Food. *Official Journal of European Union*, no. 450: L 135, 3–11.

European Commission. 2012. Second Regulatory Review on Nanomaterials. Communication from the Commission to the European Parliament, the Council and the European Economic and Social Committee, Brussels. http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:52012DC0572&from=EN (accessed November 13, 2016).

European Commission. 2016a. Food Contact Material. European Commission web page. https://ec.europa.eu/food/safety/chemical_safety/food_contact_materials_en (accessed November 13, 2016).

European Commission. 2016b. Food Information to Consumers—Legislation. European Commission. http://ec.europa.eu/food/safety/labelling_nutrition/labelling_legislation_en (Accessed December 11, 2016).

European Commission. 2016c. REACH Implementation Projects. European Commission, Environment web page. http://ec.europa.eu/environment/chemicals/reach/preparing/index_en.htm (accessed November 12, 2016).

European Commission. 2016d. REACH. European Commission, Environment web page. http://ec.europa.eu/environment/chemicals/reach/reach_en.htm (accessed November 12, 2016).

European Commission. 2016e. RIPoN. European Commission, Environment web page. http://ec.europa.eu/environment/chemicals/nanotech/reach-clp/ripon_en.htm (accessed November 12, 2016).

European Commission. 2017. Definition—Nanomaterials—Environment—European Commission. http://ec.europa.eu/environment/chemicals/nanotech/faq/definition_en.htm. European Commission (accessed December 29, 2016).

European Medicines Agency. 2017. European Medicines Agency Pre-authorisation Procedural Advice for Users of the Centralised Procedure. European Medicines Agency web page, Pre Authorization Guidance Topic. http://www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/q_and_a/q_and_a_detail_000167.jsp&mid=WC0b01ac0580b18196 (accessed June 15, 2017).

European Parliament and the Council of the European Union. 2002a. Commission Regulation (EC) No 172/2002 of 30 January 2002 on the issue of system B export licenses in the fruit and vegetables sector. *Official Journal of the European Communities*. http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32002R0172&from=EN (accessed November 13, 2016).

European Parliament and the Council of the European Union. 2002b. Regulation (EC) No 178/2002 of The European Parliament and of The Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. *Official Journal of the European Communities*. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2002:031:0001:0024:en:PDF (accessed November 13, 2016).

European Parliament and the Council of the European Union. 2009. Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. *Official Journal of the European Union*. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:309:0001:0050:en:PDF (accessed November 13, 2016).

European Parliament and the Council of the European Union. 2011. Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the Provision of Food Information to Consumers, Amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council. *Official Journal of the European Union*. http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32011R1169&from=EN (accessed December 16, 2016).

European Parliament and the Council of the European Union. 2015. Regulation (EU) 2015/2283 of the European Parliament and of the Council. *Official Journal of the European Union*. https://www.food.gov.uk/sites/default/files/annexd_regulation_eu2015_2283.pdf.

Falkner, R., L. Breggin, N. Jaspers, J. Pendergrass, and J. Porter. 2010. International coordination and cooperation: The next agenda in nanomaterials regulation. In *International Handbook on Regulating Nanotechnologies*, ed. G.A. Hodge, D.M. Bowman, and A.D. Maynard, Edward Elgar, Cheltenham, UK, pp. 508–524.

Fiorino, D.J. 2010. Voluntary Initiatives, Regulation and Nanotechnology Oversight: Charting a path. Project on Emerging Nanotechnologies Pen-19. http://www.nanotechproject.org/events/archive/voluntary/.

Gallocchio, F., S. Belluco, and A. Ricci. 2015. Nanotechnology and food: Brief overview of the current scenario. *Procedia Food Science* 5: 85–88. http://www.sciencedirect.com/science/article/pii/S2211601X15001121.

Ganzleben, C. and S.F. Hansen. 2012a. Final Report Environmental Exposure to Nanomaterials—Data Scoping Study. European Commission, Environment. http://ec.europa.eu/environment/chemicals/nanotech/pdf/exposure_nanomaterials.pdf.

Ganzleben, C. and S. Hansen. 2012b. Nanomaterials as priority substances under the Water Framework Directive. *Elni Review* 2: 28–45. http://www.elni.org/fileadmin/elni/dokumente/Archiv/2012/Heft_2/elni2012-2_AC-Ganzleben-Hansen.pdf.

Gatof, J. and D.M. Bowman. 2015. Reviewing the regulatory barriers for nanomedicine: Global questions and challenges. *Nanomedicine (London)* 10: 3275–3286.

Gorjiara, T. and C. Baldock. 2014. Nanoscience and nanotechnology research publications: A comparison between Australia and the rest of the world. *Scientometrics* 100, no. 1 (April): 121–48. https://link.springer.com/article/10.1007/s11192-014-1287-6.

Gottschalk, F., E. Kost, and B. Nowack. 2013. Engineered nanomaterials in water and soils: A risk quantification based on probabilistic exposure and effect modeling. *Environmental Toxicology and Chemistry* 32, no. 6 (June): 1278–1287. https://www.ncbi.nlm.nih.gov/pubmed/23418073, https://www.researchgate.net/publication/235650213_Engineered_nanomaterials_in_water_and_soils_A_risk_quantification_based_on_probabilistic_exposure_and_effect_modeling.

Greenhalgh, L. 2012. EU JRC Finds Significant Gaps on Nano in REACH Dossiers. Chemical Watch, news item. https://chemicalwatch.com/11373/eu-jrc-finds-significant-gaps-on-nano-in-reach-dossiers (accessed December 29, 2016).

Hafner, A., J. Lovrić, G.P. Lakoš, and I. Pepić. 2014. Nanotherapeutics in the EU: An overview on current state and future directions. *International Journal of Nanomedicine* 9: 1005–1023. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3933707&tool=pmcentrez&rendertype=abstract.

Hamburg, M.A. 2012. FDA's Approach to regulation of products of nanotechnology. *Science* 336, no. 6079: 299–300. http://science.sciencemag.org/content/336/6079/299.long.

Hansen, S.F., A. Maynard, A. Baun, and J.A. Tickner. 2008. Late lessons from early warnings for nanotechnology. *Nature Nanotechnology* 3 (July). Nature Publishing Group: 444. http://dx.doi.org/10.1038/nnano.2008.198.

Hansen, S.F. 2009. Environmental Science & Policy Regulation and Risk Assessment of Nanomaterials: Too Little, Too Late? PhD Thesis. http://www2.er.dtu.dk/publications/fulltext/2009/ENV2009-069.pdf (accessed December 29, 2016).

Hansen, S.F., C. Ganzleben, and A. Baun. 2011. Nanomaterials and the European Water Framework Directive. *European Journal of Law and Technology* 2, no. 3. http://ejlt.org/article/view/85/154.

IBM (International Business Machines). 2016. IBM Spelled with 35 Xenon Atoms. IBM News Room. https://www-03.ibm.com/press/us/en/photo/28500.wss (accessed December 29, 2016).

Kah, M. and T. Hofmann. 2014. Nanopesticide research: Current trends and future priorities. *Environment International* 63 (February): 224–235. http://www.sciencedirect.com/science/article/pii/S0160412013002754.

Lux Research. 2014. Nanotechnology update: Corporations up their spending as revenues for nano-enabled products increase. https://members.luxresearchinc.com/research/report/13748.

Marchant, G., D. Sylvester, and K.W. Abbott. 2007. Nanotechnology regulation: The United States approach. In *New Global Frontiers in Regulation: The Age of Nanotechnology*, ed. Hodge, G.A., D.M. Bowmann, and K. Ludlow, 189–211. Edward Elgar, Cheltenham, UK.

Maria, A. and P. Del. 2010. The EU Approach to Regulating Nanotechnology. European Trade Union Institute, Working Paper 2010.05, 1–46. http://www.etui.org/content/download/4963/49794/file/Nano-working-paper.pdf.

Maynard, A. 2009. Nanotechnologies—Five Years on. SafeNano. 2020. http://2020science.org/2009/07/29/nanotechnologies-five-years-on/Science (accessed December 29, 2016).

McDermott Will & Emery. 2013. 2013 Nanotechnology Patent Literature Review: Graphitic Carbon-Based Nanotechnology and Energy Applications Are on the Rise. McDermott Will & Emery, Special Report (February): 111–126. http://www.lexology.com/library/detail.aspx?g=cba6a112-bf7c-48d4-824c-c1df3cc6831b.

Milmo, S. Seeking Harmonization in Nanomedicines Regulatory Framework. BioPhar9m International, (August) 1, 2013. http://www.biopharminternational.com/seeking-harmonization-nanomedicines-regulatory-framework-0.

Mühlebach, S., G. Borchard, and S. Yildiz. 2015. Regulatory challenges and approaches to characterize nanomedicines and their follow-on similars. *Nanomedicine* 10, no. 4: 659–674.

Nature Nanotechnology. 2009. "Plenty of room" revisited. *Nature Nanotechnology* 4:781. http://www.nature.com/nnano/journal/v4/n12/full/nnano.2009.356.html (accessed December 29, 2016).

NIOSH. 2016a. About NIOSH. NIOSH Workplace Safety and Health Topic, CDC web page. https://www.cdc.gov/niosh/about/default.html (accessed December 10, 2016).

NIOSH. 2016b. Nanotechnology—10 Critical Topic Areas. NIOSH Workplace Safety and Health Topic, CDC web page. https://www.cdc.gov/niosh/topics/nanotech/critical.html (accessed December 10, 2016).

NIOSH. 2016c. Nanotechnology—Frequently Asked Questions. NIOSH Workplace Safety and Health Topic, CDC web page. https://www.cdc.gov/niosh/topics/nanotech/faq.html (accessed December 10, 2016).

NIOSH. 2016d. Nanotechnology—Introduction. NIOSH Workplace Safety and Health Topic, CDC web page. https://www.cdc.gov/niosh/topics/nanotech/ (accessed December 11, 2016).

NIOSH. 2016e. Nanotechnology—Nanotechnology at NIOSH. NIOSH Workplace Safety and Health Topic, CDC web page. https://www.cdc.gov/niosh/topics/nanotech/nanotechnology-research-center.html (accessed December 10, 2016).

NIOSH/CDC. 2016. Building a Safety Program to Protect the Nanotechnology Workforce: A Guide for Small to Medium-Sized Enterprises. CDC web page. https://www.cdc.gov/niosh/docs/2016-102/pdfs/2016-102.pdf.

Noorlander, C.W., M.W. Kooi, A.G. Oomen, M.V. Park, R.J. Vandebriel, R.E. Geertsma, and G. Agnes. 2015. Horizon scan of nanomedicinal products. *Nanomedicine (London)* 10: 1–10.

Oberdörster, E. 2004. Manufactured nanomaterials (fullerenes, C (60)) induce oxidative stress in the brain of juvenile largemouth bass. *Environmental Health Perspectives* 112, no. 10 (July 7): 1058–1062. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1247377/.

Oberdörster, G., E. Oberdörster, and J. Oberdörster. 2005. Nanotoxicology: An emerging discipline evolving from studies of ultrafine particles. *Environmental Health Perspectives* 113, no. 7 (July): 823–839. https://www.ncbi.nlm.nih.gov/pubmed/16002369.

Oldenburg, S.J. 2011. Silver Nanoparticles: Properties and Applications. Sigma-Aldrich. http://www.sigmaaldrich.com/technical-documents/articles/materials-science/nanomaterials/silver-nanoparticles.html (accessed December 29, 2016).

Praetorius, A., N. Tufenkji, K.-U. Goss et al. 2014. The road to nowhere: Equilibrium partition coefficients for nanoparticles. *Environmental Science: Nano* 1, no. 4: 317–323. http://pubs.rsc.org/en/content/articlehtml/2014/en/c4en00043a.

Prakash, A., S. Sen, and R. Dixit. 2013. The emerging usage and applications of nanotechnology in food processing industries: The new age of nanofood. *International Journal of Pharmaceutical Sciences Review and Research* 22, no. 21: 107–111. https://www.researchgate.net/publication/259291103_The_Emerging_Usage_and_Applications_of_Nanotechnology_in_Food_Processing_Industries_The_new_age_of_Nanofood.

Prinz, M.J. 2014. Current Legislative Framework for Nanomaterials Introduction to the Impact Assessment on Transparency Measures. Validation Workshop Brussels (June): 34. http://ec.europa.eu/DocsRoom/documents/5716/attachments/1/translations/en/renditions/native.

Radomski, A., P. Jurasz, D. Alonso-Escolano et al. 2005. Nanoparticle-induced platelet aggregation and vascular thrombosis. *British Journal of Pharmacology* 146, no. 6 (November 12): 882–893. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1751219/.

Regulations.gov. 2015. Chemical Substances When Manufactured or Processed as Nanoscale Materials: TSCA Reporting and Recordkeeping Requirements. Regulations.gov, web page. https://www.regulations.gov/document?D=EPA-HQ-OPPT-2010-0572-0001 (accessed December 10, 2016).

SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks). 2007. Opinion on the Appropriateness of the Risk Assessment Methodology in Accordance with the Technical Guidance Documents for New and Existing Substances for Assessing the Risks of Nanomaterials. European Commission. http://ec.europa.eu/health/archive/ph_risk/committees/04_scenihr/docs/scenihr_o_010.pdf (accessed December 29, 2016).

StatNano. 2016a. Nanotechnology Products Database. http://product.statnano.com/ (accessed December 29, 2016).

StatNano. 2016b. StatNano Annual Report 2015. http://statnano.com/publications/3864 (accessed December 29, 2016).

Royal Society and The Royal Academy of Engineering. 2004. Nanoscience and nanotechnologies: Opportunities and uncertainties. *Nanoscience and Nanotechnologies* (July): 69–78. https://royalsociety.org/~/media/Royal_Society_Content/policy/publications/2004/9693.pdf.

US EPA (US Environmental Protection Agency). 2008. TSCA Inventory Status of Nanoscale Substances—General Approach. TSCA Chemical Substance Inventory (January): 1–7. https://www.epa.gov/sites/production/files/2015-10/documents/nmsp-inventorypaper2008.pdf.

US EPA. 2016a. About the TSCA Chemical Substance Inventory. TSCA Chemical Substance Inventory, USEPA web page. https://www.epa.gov/tsca-inventory/about-tsca-chemical-substance-inventory (accessed December 7, 2016).

US EPA. 2016b. Control of Nanoscale Materials Under the Toxic Substances Control Act. Reviewing New Chemicals Under the Toxic Substances Control Act Topic, UDEPA web page. https://www.epa.gov/reviewing-new-chemicals-under-toxic-substances-control-act-tsca/control-nanoscale-materials-under (accessed December 7, 2016).

US EPA. 2016c. Research on Nanomaterials. US EPA web page. https://www.epa.gov/chemical-research/research-nanomaterials (accessed December 7, 2016).

US EPA. 2016d. Reviewing New Chemicals Under the Toxic Substances Control Act (TSCA). USEPA web page. https://www.epa.gov/reviewing-new-chemicals-under-toxic-substances-control-act-tsca/basic-information-review-new (accessed December 10, 2016).

US EPA. 2016e. The Frank R. Lautenberg Chemical Safety for the 21st Century Act. Assessing and Managing Chemicals Under TSCA, US EPA web page. https://www.epa.gov/assessing-and-managing-chemicals-under-tsca/frank-r-lautenberg-chemical-safety-21st-century-act (accessed December 10, 2016).

US FDA (US Food and Drug Authority). 2014. FDA Issues Guidance to Support the Responsible Development of Nanotechnology Products. US FDA News Archives. https://wayback.archive-it.org/7993/20161022070001/http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm402499.htm (accessed June 12, 2017).

US FDA. 2016a. Nanotechnology Fact Sheet. US FDA web page, Science and Research Topic. http://www.fda.gov/ScienceResearch/SpecialTopics/Nanotechnology/ucm402230.htm (accessed December 7, 2016).

US FDA. 2016b. Nanotechnology Programs at FDA. FDA web page, Science and Research Special Topics. http://www.fda.gov/ScienceResearch/SpecialTopics/Nanotechnology/default.htm (accessed December 10, 2016).

US FDA. 2016c. FDA's Approach to Regulation of Nanotechnology Products. US FDA web page. http://www.fda.gov/ScienceResearch/SpecialTopics/Nanotechnology/ucm301114.htm (accessed December 7, 2016).

ADDITIONAL READINGS

Dorbeck-Jung, B.R. and N. Chowdhury. 2011. Is the European medical products authorisation regulation equipped to cope with the challenges of nanomedicines? *Law and Policy* 33, no. 2: 276–303.

ECHA. 2016. Information from the Existing Substances Regulation (ESR). European Chemicals Agency web page. https://echa.europa.eu/information-on-chemicals/information-from-existing-substances-regulation (accessed November 12, 2016).

European Commission. 2016. Questions and Answers: New Regulation on Novel Food. European Union Website. http://europa.eu/rapid/press-release_MEMO-15-5875_en.htm (accessed November 12, 2016).

Fernandes, J.A., P. Kauppila, L. Uusitalo, V. Fleming-Lehtinen, S. Kuikka, and H. Pitkänen. 2012. Evaluation of reaching the targets of the Water Framework Directive in the Gulf of Finland. *Environmental Science and Technology* 46, no. 15: 8220–8228.

Fiorino, D.J. 2010. Voluntary Initiatives, Regulation and Nanotechnology Oversight: Charting a path. Project on Emerging Nanotechnologies Pen-19. http://www.nanotechproject.org/events/archive/voluntary/.

Hristozov, D.R., S. Gottardo, A. Critto, and A. Marcomini. 2012. Risk assessment of engineered nanomaterials: A review of available data and approaches from a regulatory perspective. *Nanotoxicology* 6, no. 1 (January): 880–898.

Jones, P. 2011. Nanomaterials: Now We Have a Definition, What Next? Chemical Watch, news item. https://chemicalwatch.com/9030/nanomaterials-now-we-have-a-definition-what-next (accessed November 13, 2016).

Jones, P. 2013. EU Nano Group Debates REACH Annex Options. Chemical Watch, news item. https://chemicalwatch.com/14579/eu-nano-group-debates-reach-annex-options (accessed November 12, 2016).

Lam, C.W., J.T. James, R. Mc Cluskey and R.L. Hunter. 2004. Pulmonary toxicity of single-wall carbon nanotubes in mice 7 and 90 days after intratracheal instillation.

Toxicological Sciences 77, no. 1 (January): 126–134. https://www.ncbi.nlm.nih.gov/pubmed/14514958

Maynard, R. 2002. Late lessons from early warnings: The precautionary principle 1896–2000. *Occupational and Environmental Medicine* 59: 789–790.

Maynard, A. 2009. Nanotechnologies—Five Years on. SafeNano. 2020. http://2020science.org/2009/07/29/nanotechnologies-five-years-on/Science (accessed December 29, 2016).

Nanotechnology 101. 2012. Size of the nanoscale. National Nanotechnology Initiative. https://www.nano.gov/nanotech-101/what/nano-size (accessed December 29, 2016).

NIOSH. 2016a. Nanotechnology—Guidance and Publications. NIOSH Workplace Safety and Health Topic, CDC web page. https://www.cdc.gov/niosh/topics/nanotech/pubs.html (accessed December 11, 2016).

NIOSH. 2016b. NIOSH Regulations. NIOSH Workplace Safety and Health Topic, CDC web page. https://www.cdc.gov/niosh/regulations.html (accessed December 10, 2016).

OECD (Organisation for Economic Co-operation and Development). 2007. Opportunities and Risks of Nanotechnologies. Report in co-operation with the OECD International Futures Programme. http://www.oecd.org/science/nanosafety/44108334.pdf.

Roco, M.C. 2011. The long view of nanotechnology development: The National Nanotechnology Initiative at 10 years. In *Nanotechnology Research Directions for Societal Needs in 2020*, ed. M.C. Roco, M.C. Hersam, and C.A. Mirkin, 1–28. Dordrecht, Springer.

SCEHINR. 2010. Scientific Basis for the Definition of the Term "Nanomaterial." European Commission. European Commission. http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_030.pdf (accessed December 12, 2016).

Wijnhoven, P., S. Dekkers, and W.I. Hagens. 2009. Exposure to Nanomaterials in Consumer Products Exposure to Nanomaterials in Consumer Products. RIVM Letter Report 340370001: 6–45. http://www.rivm.nl/bibliotheek/rapporten/340370001.pdf.

Wijnhoven, S.W.P., A.G. Oomen, F.C. Bourgeois et al. 2011. Development of an Inventory for Consumer Products Containing Nanomaterials. Final Report prepared by Centre for Substances and Integrated Risk Assessment, European Commission web page. http://ec.europa.eu/environment/chemicals/nanotech/pdf/study_inventory.pdf.

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