



Standard Test Method

Field Monitoring of Bacterial Growth in Oilfield Systems

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Foreword

This standard describes field test methods that are useful for estimating bacterial populations, including sessile bacterial populations, commonly found in oilfield systems. The described test methods are those that can be done on site and that require a minimum of laboratory equipment or supplies. The described test methods are not the only methods that can be used, but they are methods that have been proven to be useful in oilfield situations. This standard is meant to be used by technical field and service personnel, including those who do not necessarily have extensive or specific training in microbiology. However, because microbiology is a specialized field, some pertinent and specific technical information and explanation are provided to the user. Finally, the implications of the results obtained by these test methods are beyond the scope of this standard. The interpretation of the results is site- and system-specific and may require more expertise than can be provided by this standard.

This standard is loosely based on a document produced by the former Corrosion Engineering Association (CEA). CEA operated in the United Kingdom under the auspices of NACE and the Institute of Corrosion (ICorr).⁽¹⁾ This NACE International standard was developed by NACE Task Group T-1C-21 under the direction of Unit Committee T-1C on Corrosion Monitoring in Petroleum Production and is issued under the auspices of Group Committee T-1 on Corrosion Control in Petroleum Production.

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Section 1: General

1.1 Scope

1.1.1 This standard describes field test methods for estimating bacterial populations commonly found in oilfield systems. Although these techniques have been successful in the oil field, they are not the only methods that are used. It is not the intent of this standard to exclude additional techniques that can be proven useful. However, caution should be exercised with any technique that is at variance from those outlined here.

1.1.2 This standard deals only with bacteria and does not consider other organisms that may be found in oilfield fluids, such as phytoplankton (algae), protozoa, or fungi. In addition, these methods are not applicable to marine organisms such as zooplankton (copepods).

1.1.3 Because effective sampling is essential to any successful analysis, emphasis is given to sampling methods that are suitable for use in oilfield conditions.

1.1.4 Media formulae for enumerating common oilfield bacteria are given.

1.1.5 This standard describes dose-response (time-kill) testing for evaluating biocides used in oilfield applications.

1.1.6 Methods for evaluating surface attached (sessile) bacteria are addressed. The importance of these bacteria in oilfield problems is usually not adequately considered.

Attached bacterial populations are often the most important component of a system's microbial ecology.

1.1.7 Emerging technologies for the rapid determination of bacterial populations and bacterial activity are addressed (see Appendix A). While these technologies are not specifically recommended, it is not the intent of this standard to prevent the use of any technology that can be useful. However, the user must determine the applicability of these new methods to his needs. Similarly, there are a number of commercially available "test kits" for detecting various types of microorganisms; these are not discussed in this standard. However, the user could use this standard to evaluate the suitability of these test kits for any particular situation.

1.1.8 The simple presence of bacteria in a system does not necessarily indicate that they are causing a problem. In addition, bacterial populations causing problems in one situation, or system, may be harmless in another. Therefore, "action" concentrations for bacterial contamination cannot be given. Rather, bacterial population determinations are one more diagnostic tool useful in assessing oilfield problems.

1.1.9 Further information on the corrosion problems associated with bacterial growth in oilfield systems is given in NACE International publication TPC #3.¹

Section 2: Sampling Procedures for Planktonic Bacteria

2.1 Baseline Sampling

2.1.1 Natural bacterial population fluctuation and uneven bacterial distribution within water systems may hamper accurate assessment of bacteria numbers. If baseline studies described here show a large variation in reported bacterial populations, several samples should be taken on each occasion and combined (bulk). However, this procedure may mask fluctuations in population profiles, should determining such profiles be a goal of the work.

2.1.2 Field operators should be solicited for valuable information. These operators can often provide, or obtain, critical past biological monitoring (background) data taken from the system. Communication with operators can also ensure that baseline sampling occurs during normal operations and not during excursions (pigging, shut-ins, biocide treatments, etc). In addition, selection of proper sample sites can best be made in cooperation with operators.

2.1.3 Sampling Frequency

2.1.3.1 Sampling frequency depends on how the field system operates and should encompass the various stages of its operation.

2.1.3.2 Some systems may exhibit large population variations over a short time. To establish the natural variation in bacteria numbers, samples (bulk or otherwise) should normally be taken randomly over several days to establish a baseline. This work should also establish the sample points that are representative of the system. As an example of what sample frequency might be required, twice-daily sampling over three to five days is often used. In other cases, greater sample frequencies over longer time periods may be required.

2.1.3.3 If the evaluation spans several months, it is important to account for any system variables that are related to seasonal changes. Usually, these variables can only be established with extensive background monitoring.

2.1.3.4 During biocide treatments, additional samples should normally be taken immediately prior to treatment and at random intervals over several days after each treatment. A good procedure would be to match the sampling schedule used with the baseline sampling for the system.

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2.1.3.5 To fully understand the ecology of a system, it is important to survey the entire system rather than only areas where elevated bacterial populations are expected or where obvious bacterial problems are occurring.

2.2 Sampling Bottles

2.2.1 It should be assumed that bacterial populations undergo both qualitative and quantitative changes with time while being held in any sample container. Sample containers should be made of glass, polyethylene, or polypropylene. Sterile containers are obviously preferred, but new containers usually suffice. In the latter case, the samples that were collected in nonsterile containers should be so noted.

2.2.2 To minimize changes, the sample should be analyzed without delay, preferably on site. If a delay of more than one hour is unavoidable, a glass container should be used; however, it should be noted that errors in bacterial population estimates still could result. The time delay occurring between sampling and analysis should be held constant for all testing. For example, if some samples are normally analyzed four hours after collection, all samples should be held for four hours before testing. This practice helps minimize population variability caused by the sample handling procedure. Samples to be held more than four hours should be refrigerated (4°C). Samples held for longer than 48 hours, even under refrigeration, are of dubious value.

2.2.3 The sample container should be completely filled to flush out air and then closed with a screw cap (preferably with an airtight liner). The cap should only be removed just prior to sampling and replaced immediately afterwards. Touching the internal surfaces of the container neck and cap should be avoided.

2.3 Possible Sampling Problems

2.3.1 *These sampling procedures pertain only to planktonic bacteria.* Special procedures are required for sampling sessile bacteria (see Section 5). Relying on only planktonic bacterial testing for problem solving may lead to serious errors.

2.3.2 The available sampling points may not be suitable for identifying a microbiological problem (i.e., at or close to the suspected location of the problem). Prior consultation with operators may identify alternatives to avoid some sampling problems. Ideally, consultation on sample point location should take place during the design and construction phase of the facility.

2.3.3 Samples may be taken from either flowing (e.g., pipeline) or static (e.g., storage tank) systems. Usually, samples should be obtained by cracking a valve and allowing the fluids to flow for several minutes (to thoroughly

flush out dead-space fluids) before collecting the sample. In some instances (such as with tank bottoms or when sampling from open waters), a specially designed sampling apparatus is required, e.g., a sampling bomb or a pumped line.

2.3.4 During sampling of systems containing both oil and water, phase separation should be permitted to occur before using the water. Samples with low water cuts (i.e., low percentage of water) or those with tight emulsions may not contain enough water for testing. If an additional sample is necessary to obtain enough water for a particular test, caution should be exercised to prevent contamination during sample bulking. It is usually satisfactory to directly use an emulsion for bacterial isolation. The recorded water cut may be used to estimate the water volume used in the culturing procedure (for those workers who feel more accurate bacterial population estimates will result).

2.3.5 Occasionally, there are insufficient sterile sample containers available. In an emergency, the same containers may be used several times as long as microbiological analyses are carried out immediately. The container should be rinsed thoroughly with several changes of the water being sampled. Samples that were collected in reused containers should be so noted (in case of contamination). Obviously contaminated (e.g., oily, sludgy, etc.) containers should not be reused. Alternatively, it is possible to carefully fill a sterile syringe from a running sample stream. **Caution is required to prevent contamination of the sample.** NOTE: This should be an emergency measure only, because the smaller the sample taken, the less it represents the system.

2.3.6 If the detection of very low bacterial populations is required (i.e., less than one viable cell per mL), special means to increase the bacteria numbers must be used. One common method for doing this is the membrane filtration technique. See Appendix B for more detail. Sterile sample containers must be used with the membrane filtration technique.

2.4 The following information should be recorded when taking samples:

2.4.1 Date, time, and location of the sample.

2.4.2 Sample temperature and pH.

2.4.3 Dissolved oxygen and hydrogen sulfide (H₂S) content.

2.4.4 Any production chemicals present, with concentration noted.

2.4.5 Observations on color (particularly suspended metallic sulfide or black water), turbidity, odor (particularly H₂S), and the presence of slime and deposits.

Section 3: Culture Techniques

3.1 General

3.1.1 Bacterial culturing in artificial growth media is accepted as the standard technique for the estimation of bacteria numbers. However, users should be aware of the limitations of the culture technique:

3.1.1.1 Any culture medium grows only those bacteria able to use the nutrients provided.

3.1.1.2 Culture medium conditions (pH, osmotic balance, redox potential, etc.) prevent the growth of some bacteria and enhance the growth of others.

3.1.1.3 Conditions induced by sampling and culturing procedures, such as exposure to oxygen, may hamper the growth of strict anaerobes.

3.1.1.4 Only a small percentage of the viable bacteria in a sample can be recovered by any single medium; i.e., culture media methods may underestimate the number of bacteria in a sample.

3.1.1.5 Some bacteria cannot be grown on culture media at all.

3.1.2 A test for hydrocarbon-oxidizing organisms can be used in the rare instance when such organisms are important to a particular situation. These test methods are described elsewhere.² Otherwise, the methods detailed here are usually sufficient.

3.1.3 Procedures for the detection or enumeration of sulfur-oxidizing bacteria^{3,4} and iron bacteria⁵ are not described here. These organisms are seldom of interest in the typical anaerobic oilfield system. Additionally, suitable on-site culture techniques are not available for these bacteria.

3.1.4 Only liquid culture methods are described herein. Classical methods using agar-solidified media can be found elsewhere.⁶ Such methods are impractical for routine field use. In addition, because only population estimates to the nearest order of magnitude are required, duplicate culturing in liquid media represents sufficient accuracy for the task. For those rare occasions when estimates of greater precision are needed, such as for finished water quality testing, the most probable number technique (MPN)⁶ can be used. However, the large amount of bench space, glassware, incubator space, and operator time required for this method also makes it impractical for routine field work.⁷

3.2 General Heterotrophic Bacteria Testing: Media and Determinations

3.2.1 Several different liquid bacterial culture media are widely used for enumerating heterotrophic bacteria in oilfield waters. Examples are:

3.2.1.1 Standard Bacteriological Nutrient Broth (for aerobic and facultative anaerobic heterotrophic bacteria):

Beef extract	3.0 g
Peptone	5.0 g
Distilled water	1,000 mL

The pH should be adjusted to 7.0 with NaOH.

The broth should be distributed to tubes or vials and autoclaved for 15 min at 121°C.

3.2.1.2 Phenol Red Dextrose Broth (for aerobic and facultative anaerobic acid-producing heterotrophic bacteria):

Beef extract	1.0 g
Peptone	10.0 g
Phenol Red	0.018 g
Dextrose	5.0 g
NaCl	5.0 g
Distilled water	1,000 mL

The pH should be adjusted to 7.0 with NaOH.

The broth should be distributed to tubes or vials and autoclaved for 15 min at 121°C.

3.2.1.3 Thioglycolate Medium (for anaerobic and facultative anaerobic heterotrophic bacteria):

Yeast extract	5.0 g
Casitone	15.0 g
Sodium chloride	2.5 g
L-cystine	0.25 g
Thioglycolic acid	0.3 mL
Agar	0.75 g
Dextrose	5.0 g
Distilled water	1,000 mL

The pH should be adjusted to 7.0 with NaOH.

The medium should be heated to boiling, distributed to tubes or vials, and autoclaved for 15 min at 121°C.

3.2.2 For saline waters with greater than 2,500 ppm total dissolved solids (the usual case), enough sodium chloride must be added to match the salinity of the water being tested.

3.2.3 Fill serum vials, 10 mL nominal capacity, with 9 mL of media. Stopper the vials with butyl or natural latex rubber stoppers. Protect and seal the rubber stopper with a disposable metallic cap. Steam sterilize the filled and sealed vials in accordance with the media formulae in Paragraph 3.2.1. Some workers prefer to bottle and cap these media under reduced-oxygen conditions (see 3.3.3).

3.2.3.1 These media can be obtained premixed (to any salinity requirement) from biological supply houses. All media should be marked with the medium preparation date and stored at 4°C unless stated otherwise.

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3.2.3.2 Arrange media vials into a "dilution series." The media temperature should approximate the temperature of the sample to avoid "shock" effects on the microbes in the sample. Inoculate the first dilution vial with a sterile disposable syringe containing 1 mL of sample collected as described in Section 2; discard the syringe. Then complete the serial dilution with one of the following procedures. All work must be done in duplicate. **NOTE:** Disposable 3-mL plastic syringes with 25-mm (1.0-in.) 22-gauge needles are convenient.

3.2.3.2.1 Classical Procedure: Vigorously agitate the inoculated vial and, using another sterile syringe, withdraw 1 mL of the inoculated broth. Inject this 1 mL of inoculated broth into the second dilution vial. Vigorously agitate this vial; then use another sterile syringe to transfer 1 mL to the third dilution vial. Repeat this procedure in the same manner until an appropriate dilution factor is reached. The appropriate dilution factor depends on the expected bacterial population. Normally a 10^6 dilution factor, with six dilution vials, is sufficient. Because bacterial populations of this magnitude are indicative of serious problems, it is not usually productive to carry out longer dilution series. More detail can be found in Appendix C. **NOTE:** In cases of severe bacterial contamination, the user may wish to periodically determine the bacterial population by a complete dilution-to-extinction procedure. This may require a 10^9 dilution factor (or greater).

3.2.3.2.2 Alternative Procedure (widely practiced): Vigorously agitate the initially inoculated vial as before. Using another sterile syringe, withdraw 1 mL from this vial and inject it into the second vial of the dilution series. Keeping that syringe needle in this vial, invert the vial, and rinse the syringe thoroughly by drawing up and expelling several milliliters of broth three times. Then withdraw 1 mL of this well-mixed broth and inject it into the third vial of the dilution series. Cleanse the syringe again by rinsing three times (in the inverted third dilution vial) before using it to transfer 1 mL to the next dilution vial. Repeat this procedure for each dilution desired. **NOTE:** The occasional spurious result is more likely when

using this method. However, because of inherent inaccuracies of culturing, an occasional spurious result is usually acceptable. If this is not felt to be the case or spurious results are common, then the previous (i.e., classical) dilution method should be used. As with the classical procedure outlined in Paragraph 3.2.3.2.1, normally a 10^6 dilution factor is sufficient. Complete dilution-to-extinction determinations are not usually necessary, but can be done in special cases.

3.2.4 Incubation

3.2.4.1 The proper incubation temperature is critical to growing bacteria removed from the field system. Therefore, incubation must be within $\pm 5^\circ\text{C}$ of the recorded temperature of the water when sampled. This incubation temperature must be recorded. Because oilfield bacteria can grow in produced fluids at temperatures of 80°C or higher, special incubation procedures may be required when high-temperature fluids are encountered.

3.2.4.2 Vials that become turbid in between one and seven days should be considered positive. With phenol red dextrose media, a color change from red to yellow accompanying the turbidity is a positive for acid-producing bacteria. These vials can be discarded after seven days' incubation.

3.2.4.3 Estimate bacteria numbers using Table 1. However, it must be noted that using this table is simplistic. Estimating bacterial populations by the serial dilution method is a subject for statistical analysis. The more replicate samples done, the tighter the statistical distribution, and the more precise the estimate. With the duplicate testing prescribed in this standard, the ranges of bacterial populations shown in Table 1 are actually too narrow. Adding to the confusion is the fact that bacterial media inherently underestimate bacterial populations. However, by convention, the values reported in Table 1 are considered acceptable for oilfield situations. For more details, see Appendix C. The bacterial estimate reported is the one shown in the fourth column. If all the serial dilution vials used are positive, then report the results as "equal to or greater than" ($\geq$) the highest dilution used in the testing.

TABLE 1

RESULTS INTERPRETATION TABLE

Number of Positive Vials	Actual Dilution of Sample	Growth (+) Indicates (Bacteria per mL)	Reported Bacteria per mL
1	1:10	1 to 9	10
2	1:100	10 to 99	100
3	1:1,000	100 to 999	1,000
4	1:10,000	1,000 to 9,999	10,000
5	1:100,000	10,000 to 99,999	100,000
6	1:1,000,000	100,000 to 999,999	1,000,000

3.3 Sulfate-Reducing Bacteria (SRB) Testing: Media and De-termination

3.3.1 SRB testing should be conducted in association with other analyses, such as pH, redox potential, oxygen content, total dissolved solids, and whenever possible, sulfide and sulfate content.⁵ It is also helpful to simultaneously conduct general heterotrophic bacterial population evaluations (Paragraph 3.2). Without such information, it may be difficult to estimate the contributions of SRB to the problems found.

3.3.2 Media

3.3.2.1 As with heterotrophic culturing, serial dilution in a liquid medium should be used to estimate SRB to the nearest order of magnitude. Two widely used media formulae for SRB estimation are given below:

3.3.2.1.1 American Petroleum Institute (API)⁽²⁾ Medium⁸

Sodium lactate solution (60 to 70%)	4.0 mL
Yeast extract	1.0 g
Ascorbic acid	0.1 g
MgSO ₄ ·7H ₂ O	0.2 g
K ₂ HPO ₄ (anhydrous)	0.01 g
Fe(SO ₄) ₂ (NH ₄) ₂ ·6H ₂ O	0.2 g
NaCl	10.0 g
Distilled water	1,000 mL

3.3.2.1.2 Modified Postgate Medium B⁹

KH ₂ PO ₄	0.5 g
NH ₄ Cl	1.0 g
Na ₂ SO ₄	1.0 g
CaCl ₂ ·6H ₂ O	0.1 g
MgSO ₄ ·7H ₂ O	2.0 g
Sodium lactate solution (60 to 70%)	5.0 mL
Yeast extract	1.0 g
Sodium thioglycolate	0.1 g
Sodium ascorbate	0.1 g
FeSO ₄ ·7H ₂ O	0.5 g
Distilled water	1,000 mL

3.3.2.1.3 Preparation of API and Modified Postgate B Media

3.3.2.1.3.1 Dissolve the ingredients with gentle heating and adjust the pH to 7.3 ± 0.3 with NaOH solution.

3.3.2.1.3.2 Because of the difficulty in growing some field strains of SRB, 1) 0.05 mL thioglycolic acid for additional redox reduction, 2) an acid-etched iron nail to provide adequate iron concentrations, and/or 3) 2.5 g sodium acetate may be added to these media.

3.3.2.1.3.3 All vials should be marked with the date that the medium was prepared and then examined periodically for deterioration. They should be stored at 4°C.

3.3.2.1.3.4 Additional NaCl should be added to match the salinity of the test water.

3.3.2.1.3.5 Media may be made up in source water, i.e., substituting filtered source water for distilled water in the medium formula. Some source waters may contain sulfide concentrations that make them inappropriate for use in media preparation (because the media is black on initial preparation). In such cases, the H₂S can be removed by boiling the water prior to media preparation. Likewise, other source waters may contain high levels of CO₂ that may lead to pH instability. Buffering may be needed.

3.3.2.1.3.6 Adding sulfur-containing compounds other than sulfate (i.e., sulfite, bisulfite, thiosulfate, etc.) should be avoided. These compounds can allow non-SRB to grow in these media and be reported as SRB rather than more appropriately as "sulfide-producing bacteria."

3.3.2.1.3.7 Some workers report that the addition of 20-30% melted SRB agar to a sample of field water improves SRB recovery from the water. This method should be considered qualitative. The SRB agar is a commercial product similar to API medium (Paragraph 3.3.2.1) with 15 g/L agar added.

3.3.2.2 There are SRB that use carbon sources other than lactate, specifically acetate, propionate, and butyrate. These nonlactate-utilizing SRB may be present in some oilfield systems and may not grow in media containing only lactate. In these cases, SRB culturing in traditional media can seriously underestimate the total SRB population present. If lactate-based media invariably and unexpectedly yield low SRB populations in situations where high SRB populations are expected (as indicated by sulfide production, microbiologically influenced corrosion, etc.), it is advantageous to screen other media options to determine the most appropriate one for a particular system. Appendix D lists two alternative SRB growth media that have given improved SRB recovery in some situations. NOTE: These media are not generally commercially available. In addition, several rapid methods are available (one commercially) for estimating SRB populations (see Appendix A).

3.3.3 Media Bottling Procedure

3.3.3.1 To limit oxygen contamination, fill serum vials (nominal 10 mL capacity) with 9 mL of hot bacterial growth media while maintaining an inert gas atmosphere (e.g., nitrogen or argon). Seal the vials with stoppers made of butyl or natural latex rubber and cap them with disposable metallic covers. After sealing, sterilize the filled vials at 100 kPa (15 psig) steam pressure for 15 minutes.

⁽²⁾ American Petroleum Institute, 1220 L Street, N.W., Washington, DC 20005.

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3.3.3.2 If iron nails are used, they should be added prior to filling and stoppering. The nails should be prepared by degreasing in acetone, soaking in 2 N HCl (0.5 hr), water rinsing to remove all acid, and then transferring into a container of acetone for storage. Alternatively, the nails may be sandblasted. Iron wire or reduced iron powder, reagent grade, may be substituted for the iron nail.

3.3.4 Inoculation

3.3.4.1 Collect water samples according to the technique described in Section 2. Make serial dilutions according to Paragraph 3.2.3.2.

3.3.5 Incubation

3.3.5.1 Proper incubation temperature is critical for growing the bacteria present in the field system. Incubation must be within $\pm 5^{\circ}\text{C}$ of the recorded temperature of the water when sampled. The incubation temperature must be recorded. Because oilfield bacteria can grow in produced fluids at temperatures of 80°C or

higher, special incubation procedures may be required when high-temperature fluids are encountered.

3.3.5.2 Vials that turn black within 14 days should be considered positive. However, occasionally vials should be retained for a full 28 days to check for late positives. Such lengthy incubation is not necessary for situations (or systems) in which it is known that late positives do not develop. Vials that are positive within two hours are discounted because the blackening is caused by the presence of sulfide in the water sample. If these vials are the only ones blackening after 14 days, subcultures made into fresh medium can serve as a check. It is frequently useful to note the time that it takes the vials to blacken. This can be used as an indication of the "strength" (i.e., activity) of the growing culture. Other bacteria (e.g., *Shewanella putrefaciens*¹⁰) can produce sulfide (and cause media blackening) in some cases, especially when sulfur sources other than sulfate are present.

3.3.5.3 Estimate bacteria numbers using Table 1 (also see Paragraph 3.2.4.3).

Section 4: Evaluation of Chemicals for Control of Planktonic Bacteria

4.1 When a chemical inhibitor (biocide) is desired to control microbial activity in a system, it is necessary to select an effective chemical agent that is compatible with the fluids and components in the system. On-site dose-response (time-kill) testing is often used as a guide for selecting biocides.

4.2 Biocide Time-Kill Testing for Planktonic Bacteria

4.2.1 To assess a potential biocide application, adapt the following basic test procedure. A goal is to match test conditions to those prevailing in the system under scrutiny. It is unrealistic to describe a single, standard procedure for biocide testing; therefore only the basic test design is outlined. These biocide tests must be done in duplicate, as a minimum.

4.2.2 Basic Test Procedure

4.2.2.1 Obtain field water samples as previously described (see Section 2). Begin testing immediately after sample collection. Make testing conditions as similar as possible to those prevailing in the system. For example, for anaerobic systems (typical), the tests should be performed in nitrogen- or argon-purged bottles.

4.2.2.2 The organisms used to challenge the test biocides should be the population normally found in the test fluid. Alternatively, up to a 1% inoculum of a fully grown culture originating from the field system can be used. Use no more than 1% inoculum to prevent the undue addition of organic material to the test systems.

4.2.2.3 Add distilled water-based biocide stock solutions (10,000 mg/L suggested) to small sterile bottles (30 to 200 mL). The stock solution volume added to

each bottle (test system) should be the amount calculated to provide one of the dose rates expected to be useful in the system (once the bottle is filled with field water). The total biocide stock solution added should not exceed 1% of the final volume. Add distilled water instead of biocide stock solution to several bottles to serve as controls for the field water.

4.2.2.4 Fill the above test systems, both those containing the biocide dilutions and the control bottles, with the test fluid (containing bacteria). Mix thoroughly and immediately withdraw 1-mL samples from the control bottles to determine the number of viable bacteria initially present in the test bottles. Use the methods described previously for general heterotrophic bacteria (see Paragraph 3.2), for SRB (see Paragraph 3.3), or both (depending on the objectives of the biocide application) to make this determination. Septum seals should be used to limit oxygen ingress into the test systems.

4.2.2.5 Choose biocide exposure times (test system holding times) to match the likely contact times for the biocide within the field system. At the end of these times, withdraw 1-mL samples from each dilution of each biocide being tested (and the controls) and determine viable bacterial populations, as described in Paragraph 4.2.2.4.

4.2.2.6 Following growth media incubation, tabulate the surviving bacterial populations for each biocide dose rate and each exposure time. Use this tabulation to determine the minimum effective biocide dose rate. Use this dose rate and the biocide unit cost to calculate the most cost-effective biocide.

4.2.2.7 Examine the test systems for evidence of biocide/water incompatibilities. However, the lack of apparent incompatibilities in these systems does not preclude compatibility problems in the field system.

4.2.2.8 Field experience shows that time-kill testing can only serve as a guide for the field application of the biocide. Therefore, biocide effectiveness must be confirmed once the chemical is added to the actual field system. Some fine adjustment of biocide dose rates is almost always required. In addition, biocide/system compatibility problems may not become apparent until field trials are performed.

4.2.2.9 Notes

4.2.2.9.1 This testing is most reliable when the test procedure most closely matches the normal operating condition of the field system, including

the presence of normal amounts of production chemicals. Therefore, the user must modify the procedure to suit a particular system.

4.2.2.9.2 False results might be encountered in the first or second serial dilutions with the higher biocide concentrations used because of the transfer of significant biocide concentrations from the test fluid to the growth medium.

4.2.2.9.3 *The tests described here are only for planktonic organisms.* The ability of biocides to control sessile bacteria in the system cannot be determined by this technique. See "Section 5: Assessment of Sessile Bacteria" for more detail. In general, *biocides are much less effective against sessile bacteria than against planktonic bacteria.*

Section 5: Assessment of Sessile Bacteria

5.1 Attached microbes (sessile bacteria) are normally the most important biological component of the bacterial ecology of an oilfield system. The previously discussed planktonic techniques are of limited value for assaying these bacteria. Unfortunately, techniques for sessile bacterial study are still in the developmental stage. Consequently, few routine procedures can be described. However, the following guidelines should provide a basis for analytical work that yields valuable information about sessile bacteria within an oilfield system.

5.2 Sampling Biofilms

5.2.1 Any removable field system component can potentially be used to sample for sessile bacteria. These removable materials are referred to as "coupons" in this discussion. Standard corrosion coupons are a good example. Another alternative is the use of removed pipe sections (spools).¹¹ Alternatively, coupons especially designed for microbiological use are available from suppliers of corrosion-monitoring systems, as well as service companies.

5.2.2 The coupons may be located in suitably designed side streams or they may be placed within actual system flow paths by employing properly designed coupons and access fittings. The coupons must be located such that they are in flow patterns most representative of the system. For this reason, coupons are often located at the "6 o'clock" position in piping.

5.2.3 When metal coupons are used, they must be similar in composition to the pipework of the system and electrically isolated to prevent galvanic corrosion.

5.2.4 During any baseline or investigation survey, sessile samples should always be collected. Good sources are filter backwashes, pig runs, pipe walls at unions, etc. Corrosion failures should always be tested for sessile bacterial populations.

5.2.5 While clean coupons inserted in the system may be rapidly colonized by bacteria, the time taken for the development of a dense biofilm is variable and depends on the system. A major obstacle in working with sessile bacteria samples is the uneven nature of sessile growth within the system (patchiness). For this reason, multiple sessile samples (or large surface areas) should be removed during each sampling episode.

5.3 Monitoring of Sessile Bacteria

5.3.1 The above sampling devices can be used to monitor biofilm development by periodically removing them and then applying the techniques described earlier to count the bacteria (Section 3). However, with sessile bacteria, it is first necessary to remove the bacteria from the coupon by scraping with a sterile scalpel, swabbing, shaking with glass beads, or using ultrasonic devices. If scalpels or swabs are used, the biofilm and associated products must be completely dispersed using a vortex mixer with glass beads or by a sonic bath. In each case, sterile phosphate-buffered saline (or ultra-filtered field water) shall be used to collect the removed bacteria. It is necessary to establish that the collecting method used is effective and that the assay methods allow efficient recovery of the bacteria being analyzed.

5.3.1.1 Phosphate-buffered saline (PBS) is commonly used to process sessile samples. This solution can also be used to suspend deposits from corrosion failures, coupons, pig run specimens, or biofilm probes. The saline solution provides an environment for maintaining viable bacteria without providing nutrients for growth. Furthermore, some investigators have reported benefits from using anaerobic PBS solutions in processing sessile specimens from gas or oil pipelines.

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5.3.1.1.1 PBS

NaCl	8.7 g
KH ₂ PO ₄	0.4 g
K ₂ HPO ₄	1.23 g
Distilled water	1,000 mL

Bottle and autoclave (100 kPa [15 psig]/20 minutes) (for brines, greater amounts of NaCl should be added to avoid osmotic shock effects).

5.3.1.1.2 Anaerobic PBS

Prepare as above and add:

20 mL of 2.5% cysteine-HCl
and/or

20 mL of 5% ascorbic acid

1 mL of 0.1% Resazurin indicator (optional)

5.4 Assessment of Biocide Efficiency

5.4.1 Coupons bearing biofilms can be used to assess the efficiency of biocide treatments against sessile bacteria. Coupon-based biofilm samples should be removed before, during, and after biocide treatment. Surviving bacteria should be assayed as above. For time-kill testing, sessile bacteria on coupons should be exposed to biocides either under static conditions or by being placed in dynamic flow loops.^{12,13}

5.4.2 In recognition of the importance of biofilm growth, many different test methods are under development to test for biocide effectiveness. Such tests will undoubtedly become more widely used in the future, but no single recommended procedure can be given at this time.

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APPENDIX A

RAPID METHODS FOR ASSESSING BACTERIAL NUMBERS

The procedures for bacterial analysis outlined in the main body of this standard rely on growth of bacteria in nutrient media. Such techniques generally do not allow rapid evaluation of bacterial contamination. Many techniques have been used to obtain rapid information about microbial populations in oilfield systems. These include measurement of adenosine triphosphate (ATP), general fluorescent microscopy, and measurement of hydrogenase. Methods specific for SRB are as follows: radiorespirometry, fluorescent antibody microscopy, and measurement of APS-reductase. These techniques are outlined below, together with literature references to specific applications. Users are responsible for determining the appropriateness of any of these methods for their needs.

GENERAL BACTERIA

ATP Photometry. ATP is present in all living cells and is involved in energy metabolism. Because it rapidly disappears on cell death, ATP can give an indication of the viable biomass present in a sample. ATP can be measured using an enzymic reaction that generates flashes of light when ATP is present. These flashes are detected in a photomultiplier, the output being proportional to the amount of ATP. Many analytical kits are currently available.

Disadvantages of ATP photometry include the following: 1) sulfide, chloride, and chemical additives interfere with the reaction; 2) the results exhibit unacceptable scatter with low bacteria numbers; 3) the method does not differentiate between the various types of organisms; and 4) the method requires a sensitive instrument.¹⁴ In view of these disadvantages, ATP photometry is not used for random monitoring. It has been used successfully, however, to monitor trends, particularly following biocide treatments.¹⁵

Fluorescent Microscopy. The total number of bacteria in a sample can be determined by the use of specific stains that fluoresce when irradiated with ultraviolet light. Stains such as acridine orange, fluorescein isothiocyanate (FITC), and 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) are examples. A fluorescent microscope is used to allow cells to be counted.¹⁶ As with ATP photometry, this method requires a delicate instrument and is best suited for the laboratory. Only total bacteria counts can be determined. Some interferences can result from organic and inorganic material suspended in the sample.

Hydrogenase Measurement. The hydrogenase test analyzes for the hydrogenase enzyme that is produced by bacteria able to use hydrogen as an energy source. Because it is believed that the use of cathodic hydrogen is an important factor in microbiologically influenced corrosion, the presence of hydrogenase may indicate a potential for this corrosion. A strong hydrogenase activity can also indicate the presence of a microbial biofilm community.¹⁷

Hydrogenase testing is best performed on sessile samples. Hydrogenase should be measured by first collecting the bacteria in a sample (e.g., by filtration), exposing to an enzyme-extracting solution, then noting the degree of hydrogen oxidation in an oxygen-free atmosphere (as evidenced by a color reaction with a dye). A response can be expected in 0.5 to 4 hours; a 12-hour exposure is generally used to allow the system to equilibrate for comparison purposes.

SRB TESTS

Radiorespirometry. This method, as currently proposed,¹⁸ is specific to SRB. Like the culture methods described elsewhere in this standard, it requires bacterial growth for detection. Unlike other culture methods for SRB, however, it produces results in one to two days of total testing time. The sample should first be incubated with a known trace amount of ³⁵S-labeled sulfate. After incubation, the reaction should be terminated with acid to kill the cells and to release any ³⁵S-sulfide produced by SRB. Such sulfides should be fixed in zinc acetate prior to quantification, using a liquid scintillation counter. Once the ³⁵S-sulfides are fixed, they can be quantified in laboratories away from the site. When the natural concentration of sulfate is known, the overall activity of the SRB population can be calculated.

Radiorespirometry has been applied to quantify SRB in the field and for testing biocide efficiency in the laboratory.¹⁹ However, it is a highly specialized technique involving expensive laboratory equipment. Also, the handling of radioactive substances is highly regulated.

Fluorescent Antibody Microscopy. This method is similar to the general fluorescent microscopy described above, except that FITC (the fluorescent dye used) is bound to antibodies specific to SRB cells; consequently, only those bacteria recognized by the antibodies fluoresce under the microscope.²⁰ The

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major advantage is speed, because results are obtained within two hours. The major limitation of this method is that, because the antibodies are developed against whole SRB cells, they are specific only to the type of SRB used in their manufacture. While a large number of SRB antibodies can be combined to make the test fairly general, there is always the possibility that new strains that are not detected will be encountered. Other than that, the disadvantages are similar to those for other microscope techniques: a high degree of training required, difficulty in dealing with samples containing a lot of debris, the need for a laboratory facility, and the detection of nonviable SRB as well as viable. **NOTE:** While this method, as referenced, is used to detect SRB, it can be used for other microbes as well. However, separate antibody "pools" must be developed for each microbe to be tested.

APS-Reductase Measurement. This novel immunoassay takes advantage of the functional definition of SRB, which is "any bacteria capable of anaerobically reducing sulfate to sulfide." A unique requirement for this process is the presence of an enzyme, APS-reductase. Measurement of the amount of APS reductase in a sample, therefore, gives an estimation of the total number of SRB present. Because this internal enzyme is common to all SRB, there is little concern that "unusual" strains present in a given sample will go undetected. The test does not require bacterial growth to occur (no medium is used) and is independent of sample temperature, salinity, and redox condition. The test should be carried out using disposable "kits" that are fully contained and usable either in the field or the laboratory. The entire test takes 15 to 20 minutes.²¹

APPENDIX B MEMBRANE FILTRATION-AIDED BACTERIAL ANALYSES

Occasionally, it is desirable to test for microbial contamination in waters that contain very low bacterial populations (<1 to 10 cells/mL). In these situations, either large volumes of sample must be inoculated into culture media, or the cells in these samples must be concentrated. The membrane filter²² technique can be used to test relatively large volumes of sample and generally yields numerical results rapidly. However, this technique is limited to low-turbidity waters.

A known volume of water (usually one liter) should be filtered through a presterilized cellulose acetate membrane with a pore size no larger than 0.45 μ . When handling the membrane, sterilized forceps should always be used and the filter should never be touched to nonsterile objects. Forceps should normally be cleaned with 70% alcohol, such as isopropanol or methanol, followed by flaming. **NOTE:** Permission for using an open flame must be obtained from the local operations management. If

flaming is not permitted, then enough presterilized forceps should be provided. If many samples are to be taken, the parts of the filter holder in direct contact with the membrane should also be sterilized between samples.

The filter should then be placed on a suitable agar surface (for heterotrophic bacteria) or inserted into a bottle containing the appropriate SRB medium. **NOTE:** This method is most applicable to aerobic or facultative anaerobic heterotrophic bacteria. Recovery efficiencies for strict anaerobes and SRB are likely to be low.

NACE Standard TM0173, "Methods for Determining Water Quality for Subsurface Injection Using Membrane Filters," gives details of membrane filtration, including the most commonly used membrane types.²³

APPENDIX C BACTERIAL CULTURING BY SERIAL DILUTION

In the serial-dilution approach to bacterial culturing, an attempt is made to transfer smaller and smaller portions of the original fluid to each successive vial of media. This is accomplished by a stepwise 1:10 dilution scheme until, by theory, no bacteria are transferred. Growth media in the serial-dilution vials provide nutrients for prolific growth of the transferred bacteria. A growing bacterial population causes turbidity (cloudiness) in general-count heterotrophic vials and a black precipitate in sulfate-reducer vials. The final vial of a dilution series to show these conditions should be the vial that received between one and ten bacteria and represents the dilution factor necessary to reduce the original inoculum to these concentrations. Multiplying by the dilution factor gives the approximate number of bacteria per mL present in the original sample (see note below).

SAMPLING

Collect samples according to Section 2.

GENERAL CULTURING PROCEDURE (Performed in duplicate)

Preliminary Steps	Label General-Count Vials (heterotrophic medium) 1 through 6.
	Label Sulfate-Reducer Vials 1 through 6.
Syringe 1	Fill syringe with 2 mL of sample. Inject 1 mL into General-Count Vial 1, and 1 mL into Sulfate-Reducer Vial 1. Shake vials or aspirate fluid vigorously with the syringe. Discard syringe. Do not touch any object (other than the vials' rubber septums) with the needle during this operation.

Syringe 2	Remove 1 mL from General-Count Vial 1 and inject into General-Count Vial 2. Remove 1 mL from Sulfate-Reducer Vial 1, and inject into Sulfate-Reducer Vial 2. Shake vials. Discard syringe.
Syringe 3	Repeat as above from Vial 2 to Vial 3.
Syringe 4	Repeat as above from Vial 3 to Vial 4.
Syringe 5	Repeat as above from Vial 4 to Vial 5.
Syringe 6	Repeat as above from Vial 5 to Vial 6.
Incubation	Incubate the dilution series at a temperature approximating the field temperatures (within $\pm 5^{\circ}\text{C}$). This is important.

NOTE: See alternative inoculation procedure described in Paragraph 3.2.3.2.2.

RESULTS INTERPRETATION AND REPORTING

Following incubation, visually examine the media vials and report the results. Turn the media vials over several times to re-suspend growth that has settled to the bottom. Count the number of positive vials (the ones that show growth), and use Table 1 to approximate the number of bacteria in the original sample. **NOTE:** Table 1 gives a simplistic approximation of the bacterial population. Estimating bacterial populations by the serial-dilution method is a subject for statistical analysis. The more replicate samples done, the tighter the statistical distribution, and the more precise the estimate. This is illustrated in Tables C1 through C3. Therefore, with the duplicate testing prescribed in this standard, the ranges of bacterial populations shown in Table 1 are actually too narrow. Adding to the confusion is that bacterial media inherently underestimates bacterial populations. However, by convention, the values reported in Table 1 are considered acceptable for oilfield situations.

SERIAL DILUTION-TO-EXTINCTION THEORY

The basis for estimating the bacterial population is as follows: As supplied, each vial contains 9 mL of media. When 1 mL of a water sample is added to Vial 1 of a set, the sample is thereby diluted tenfold. On transferring 1 mL of fluid to Vial 2, a tenfold dilution of Vial 1 is effected; i.e., each vial in the series is a tenfold dilution of the preceding one. For example, when growth occurs in Vials 1, 2, and 3, but not in Vials 4, 5, and 6 of a series, it follows that no bacteria were transferred into Vial 4 from Vial 3. Theoretically, Vial 3 must have received at least 1 bacterium from Vial 2, but presumably no more than 10. Because three tenfold dilutions were involved, the results indicate a range of 100 to 999 bacteria per mL in the original sample. By convention, the upper limit number of this range is reported as the estimation result. In this case, 1,000 bacteria/mL are reported.

COMMON GROWTH INTERPRETATION PROBLEMS

All Vials Show Growth

Sometimes, severely infected waters produce growth in all vials of a test series. This indicates that a true end point was not reached. For example, if all the vials in a series of six show growth, then the population is 1,000,000 or more per mL. Record the population as $\geq 1,000,000$ or $\geq 10^6$ bacteria/mL.

There Is a Gap in the Positive Vials

Occasionally, after incubation of a set of inoculated vials, a vial that is clear (no growth) may be followed by one that is turbid, indicating growth. One might, for example, find turbidity in Vials 1, 2, 3, and 5 of a set, but not in Vial 4. There are several likely explanations:

1. Accidental contamination of Vial 5 occurred. Perhaps the syringe needle touched some contaminated object in the process of transferring fluid from Vial 4 to Vial 5.
2. Only a few living bacterial cells may have been transferred into Vial 4 from Vial 3, and these same cells could have been picked up in the 1 mL of fluid transferred from Vial 4 to Vial 5. The result would be growth in Vial 5, but not in Vial 4.
3. The bacteria left in Vial 4 did not survive for unknown reasons, whereas the bacteria transferred to Vial 5 did.

The interpretation when a gap occurs is still based on the number of positive vials; i.e., the population in this example would be 10,000 per mL, not 100,000 per mL. If more than one negative vial occurs between positive vials, the chances that contamination has occurred are more likely. In this case, it is usual to ignore the odd positive.

Duplicates Show Different Results

Quite often duplicate serial dilutions provide different estimates for the bacterial population in a water sample. For example, one serial dilution reports 10-99, and the other 100-999. Both results may be tabulated for the sample, or more often, only the higher population range is reported.

BACTERIAL GROWTH MEDIA

The selection of the proper growth media to analyze bacterial population in oilfield systems is often taken for granted. It should not be. Media selection is often critical. A variety of bacterial media formulations are offered by various media suppliers. Some effort at assessing several of these media in each individual system to find the best medium to use generally pays dividends. What is equally critical, and often overlooked, is that the total dissolved solids (TDS) in the media must approximate the TDS found in the system water. Some vendors prepare

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bacterial media in the actual field water to approximate more closely the conditions that the field bacteria are accustomed to seeing. Those experiencing problems in assessing the bacterial populations in a system may wish to try this latter media preparation technique with alternative media formulations.

RESULTS INTERPRETATION

As noted earlier (Paragraph 3.2.4.3), a generalized table is routinely used to report the results of serial dilution testing. However, sampling large-volume oilfield systems and then estimating the bacterial population in those systems by using small-volume serial-dilution testing is an inherently inaccurate process. For this reason, this standard specifies duplicate

testing as a minimum requirement for all growth testing. Precision can be increased further by using even more replicate samples. However, costs associated with increased replicate testing (including testing time) must be weighed against the practical value derived from the increased precision.

To illustrate, Tables C1-C3 (below) show the impact of increased sample replication in serial-dilution testing on the precision of the results. These tables were derived from *Laboratory Methods in Food and Dairy Microbiology*, by W.F. Harrigan and M.E. McCance.⁶ It should also be noted that these tables are based on statistical estimates and are not empirical.

TABLE C1**SINGLE SERIAL DILUTION**

Number of Positive Vials	Actual Dilution of Sample	Estimated Range of Bacteria per mL
1	1:10	1 to 145
2	1:100	7 to 1,450
3	1:1,000	69 to 14,500
4	1:10,000	690 to 145,000
5	1:100,000	6,900 to 1,450,000
6	1:1,000,000	69,000 to 14,500,000

TABLE C2**DUPLICATE SERIAL DILUTION**

Number of Positive Vials	Actual Dilution of Sample	Estimated Range of Bacteria per mL
1	1:10	1 to 66
2	1:100	15 to 660
3	1:1,000	150 to 6,600
4	1:10,000	1,500 to 66,000
5	1:100,000	15,000 to 660,000
6	1:1,000,000	150,000 to 6,600,000

TABLE C3**FIVE REPLICATE SERIAL DILUTION**

Number of Positive Vials	Actual Dilution of Sample	Estimated Range of Bacteria per mL
1	1:10	1 to 33
2	1:100	33 to 330
3	1:1,000	330 to 3,300
4	1:10,000	3,300 to 33,000
5	1:100,000	33,000 to 330,000
6	1:1,000,000	330,000 to 3,300,000

APPENDIX D ALTERNATIVE SRB GROWTH MEDIA FORMULATIONS

Postgate Medium B⁹

	<u>g/L</u>
KH ₂ PO ₄	0.5
NH ₄ Cl	1.0
CaSO ₄	1.0
MgSO ₄ ·7H ₂ O	2.0
Sodium lactate	2.8
Yeast extract	1.0
Ascorbic acid	0.1
Thioglycolic acid	0.1
FeSO ₄ ·7H ₂ O	0.5
Distilled water	1,000 mL

Adjust pH to 7 to 7.5. Autoclave.

Postgate Medium G⁹

	<u>g/L</u>
KH ₂ PO ₄	0.2
NH ₄ Cl	0.3
Na ₂ SO ₄	3.0
CaCl ₂ ·2H ₂ O	0.15
MgCl ₂ ·6H ₂ O	0.4
KCl	0.3
NaCl	1.2
Distilled water	970 mL

Sterilize by autoclaving; components marked below are added aseptically later. Adjust pH to 7.2 with 2 N HCl.

Additions to Postgate Medium G:

- a. Selenite, 3 g (from autoclaved stock of 3 mg Na₂O₃Se + 0.5 g/L NaOH).
- b. Trace elements, 1 mL (from autoclaved stock of FeCl₂·4H₂O, 1.5 g; H₃BO₃, 60 mg; MnCl₂·4H₂O, 100 mg; CoCl₂·6H₂O, 120 mg; ZnCl₂, 70 mg; NiCl₂·6H₂O, 25 mg; CuCl₂·2H₂O, 15 mg; NaMoO₄·2H₂O, 25 mg/L).
- c. NaHCO₃, 2.55 mg (30 mL of 8.5% w/v solution, filter-sterilized after saturation with CO₂).
- d. Na₂S·9H₂O, 0.36 g (3 mL of 12% w/v solution autoclaved under N₂).
- e. Vitamins, 0.1 mL (from filter-sterilized stock of biotin, 1 mg; p-aminobenzoic acid, 5 mg; vitamin B₁₂, 5 mg; thiamine, 10 mg/100 mL).
- f. Growth stimulants, 0.1 mL (from autoclaved stock of isobutyric acid, valeric acid, 2-methylbutyric acid, 3-methylbutyric acid, 0.5 g of each; caproic acid, 0.2 g; succinic acid, 0.6 g/100 mL NaOH to pH 9).
- g. Carbon sources, 1 mL/100 mL final medium of autoclaved stocks (e.g., 20% sodium acetate trihydrate, and/or 7 % propionic acid).