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Standard
Test Method

Field Monitoring of Bacterial Growth in Oil and Gas 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 proved to be useful in oilfield situations. This standard is intended 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 originally prepared in 1994 by NACE Task Group T-1C-21 under the direction of Unit Committee T-1C on Corrosion Monitoring in Petroleum Production. It was revised in 2004 by Task Group 214 on Bacterial Growth in Oilfield Systems—Field Monitoring: Review of NACE Standard TM0194, which is administered by Specific Technology Group (STG) 31 on Oil and Gas Production—Corrosion and Scale Inhibition and by STG 60 on Corrosion Mechanisms. It is issued by NACE under the auspices of STG 31.

(1) Institute of Corrosion (Icorr), P.O. Box 253, Leighton, Buzzard Beds, LU7 7WB, England.

In NACE standards, the terms *shall*, *must*, *should*, and *may* are used in accordance with the definitions of these terms in the NACE Publications Style Manual, 4th ed., Paragraph 7.4.1.9. *Shall* and *must* are used to state mandatory requirements. The term *should* is used to state something good and is recommended but is not mandatory. The term *may* is used to state something considered optional.

Standard
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Field Monitoring of Bacterial Growth in Oilfield Systems

Contents

Section 1: General

1.1 Scope

1.1.1 This standard describes field test methods for estimating bacterial populations commonly found in oil and gas 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 proved useful. However, caution should be exercised with any technique that is at variance from those outlined here.

1.1.2 A glossary of terms used in this standard is provided is Appendix A.

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

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

1.1.5 Media formulations for enumerating common oilfield bacteria are given.

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

1.1.7 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]

1.1.8 Emerging technologies for the rapid determination of bacterial populations and bacterial activity are addressed (See Appendix B). 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 the site/system. 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.

1.1.9 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.

1.1.10 Further information on the corrosion problems associated with bacterial growth in oilfield systems is given in NACE Publication TPC #3.[2]

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 (bulked). However, this procedure may mask fluctuations in population profiles, if determining such profiles is 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 (bulked 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.

2.1.3.5 To fully understand the ecology of a system, the entire system should be surveyed 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 sterile glass, polyethylene, or polypropylene. Sterile containers are obviously preferred, but any 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 [40°F]). For handling thermophilic bacteria, special precautions may need to be observed. Samples held for longer than 48 hours, even under refrigeration, are of dubious value.

2.2.3 The sample container should be rinsed with the system water, then 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 misunderstanding of the extent or nature of bacterial activity in the system.

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, e.g., a sampling bomb, a sample thief, or a pumped line, is required.

2.3.4 During sampling of systems containing both oil and water, phase separation should be permitted to occur before the water is used. 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 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 C 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 (H_2S) 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 H_2S), and the presence of slime and deposits.

2.4.6 Other relevant information pertaining to the sample.

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 should be used in the rare instance when such organisms are important to a particular situation. These test methods are described elsewhere.[3] Otherwise, the methods detailed here are usually sufficient.

3.1.3 Procedures for the detection or enumeration of sulfur-oxidizing bacteria[4,5] and iron bacteria[6] are not described here.

3.1.4 Only liquid culture methods are described herein. Classical methods using agar-solidified media can be found elsewhere.[7] 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 provides sufficient accuracy. For those occasions when estimates of greater precision are needed, such as for finished water quality testing, the most probable number (MPN) method[7] 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.[1]

3.2 Bacteria Testing: Media and Determinations

3.2.1 Heterotrophic Bacteria (Aerobic and Facultative Anaerobic Bacteria) Testing:

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

3.2.1.1 Phenol Red Dextrose Broth:

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 (250°F).

3.2.1.2 Standard Bacteriological Nutrient Broth:

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 (250°F).

3.2.2 Anaerobic and Facultative Anaerobic Bacteria Testing:

3.2.2.1 Thioglycolate Broth:

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 (250°F).

3.2.3 Salt composition and concentration should be formulated to approximate that of the field water being tested. The salinity should be approximated within 10%.

3.2.4 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 formulations in Paragraph 3.2.1. Some workers prefer to bottle and cap these media under reduced-oxygen conditions (See Paragraph 3.3.3).

3.2.4.1 These media can be obtained ready made (to any salinity requirement) from biological supply houses. All media should be marked with the medium preparation date and stored at 4°C (40°F) unless stated otherwise.

3.2.4.2 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.

3.2.4.2.1 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. More detail can be found in Appendix D. **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).

3.2.4.2.2 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 3.2.4.2.1, normally a 10^6 dilution factor is sufficient. Complete dilution-to-extinction determinations are not usually necessary, but may be done in special cases.

3.2.5 Incubation

3.2.5.1 The proper incubation temperature is critical to growing bacteria removed from the field system. Therefore, the incubation temperature must be within ±5°C (±9°F) 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 (176°F) or higher, special incubation procedures may be required when high-temperature fluids are encountered.

3.2.5.2 Vials that become turbid in between 1 and 14 days shall be scored as 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 may be discarded after 14 days' incubation.

3.2.5.3 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 D. 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" (≥) 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 Determination

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.[6] Also, general heterotrophic bacterial population evaluations (Paragraph 3.2) should be conducted simultaneously. Without such information, it may be difficult to estimate the contributions of SRB to the problems found.

3.3.2 Media

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

3.3.2.1 Sodium Lactate SRB Medium[(2),8,9]

Sodium lactate solution (60 to 70%)	4.0 mL
Yeast extract	1.0 g
Ascorbic acid	0.1 g
$MgSO_4 \cdot 7H_2O$	0.2 g
K_2HPO_4 (anhydrous)	0.01 g
$Fe(SO_4)_2(NH_4)_2 \cdot 6H_2O$	0.2 g
NaCl	10.0 g
Distilled water	1,000 mL

3.3.2.2 Postgate Medium B[10]

KH_2PO_4	0.5 g
NH_4Cl	1.0 g
$CaSO_4$	1.0 g
$MgSO_4 \cdot 7H_2O$	2.0 g
Sodium lactate	2.8 g
Yeast extract	1.0 g
Thioglycolic acid	0.1 g
Ascorbic acid	0.1 g
$FeSO_4 \cdot 7H_2O$	0.5 g
Distilled water	1,000 mL

3.3.2.3 Preparation of SRB Media

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

3.3.2.3.2 Because of the difficulty in growing some field strains of SRB, one of the following may be added to this medium: (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.

3.3.2.3.3 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 (40°F).

(2) Formerly referred to as API[(3)] RP 388 Medium. The RP 38 standard that previously served as a reference for this medium was not reauthorized for publication by API. Therefore, to avoid referring to a publication that is no longer in print, the medium is referred to in this standard as Sodium Lactate SRB Medium.
(3) American Petroleum Institute (API), 1220 L St. NW, Washington, DC 20005.

3.3.2.3.4 Salt composition and concentrations should be formulated to approximate that of the field water being tested. The salinity should be approximated within 10%.

3.3.2.3.5 The medium 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 medium preparation (because the medium is black on initial preparation). In such cases, the H_2S may be removed by boiling the water prior to medium preparation. Likewise, other source waters may contain high levels of CO_2 that may lead to pH instability. Buffering may be needed.

3.3.2.3.6 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."

3.3.2.3.7 Some workers report that the addition of 20 to 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 the medium described in Paragraph 3.3.2.1 with 15 g/L agar added.

3.3.2.4 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 in which high SRB populations are expected (as indicated by sulfide production, microbiologically influenced corrosion, etc.), other media options should be screened to determine the most appropriate one for a particular system. Appendix E lists an alternative SRB growth medium that has given improved SRB recovery in some situations. In addition, several rapid methods are available (one commercially) for estimating SRB populations (see Appendix B).

3.3.3 Medium Bottling Procedure

3.3.3.1 To limit oxygen contamination, fill serum vials (nominal 10-mL capacity) with 9 mL of hot bacterial growth medium 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.

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 for 0.5 hours, water rinsing to remove all acid, and then transferring into a container of acetone for storage. 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 3.2.4.2.

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 ±5°C (9°F) 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 (176°F) or higher, special incubation procedures may be required when high-temperature fluids are encountered.

3.3.5.2 Vials that turn black shall be scored as positive. Vials shall not be scored as negative until 28 days. Vials that turn black within two hours are discounted (i.e., not scored) because the blackening is caused by the presence of sulfide in the water sample. If these vials are the only ones blackening after 28 days, subcultures shall be made into fresh medium to serve as a check. (**NOTE:** It is acceptable to make these subcultures after only 7 days to reduce the turnaround time for obtaining results. However, backup subcultures must be made after 28 days to confirm these results in case they are negative.) The time that it takes each vial to blacken shall be noted because this can be used as an indication of the "strength" (i.e., activity) of the growing culture. Other bacteria (e.g., *Shewanella putrefaciens*[11]) 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 3.2.5.3).

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 may 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 recommended) 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 bottles, 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 medium 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 may 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 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. Techniques for sessile bacterial study produce variable results. 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 components are referred to as "coupons" in this standard. Standard corrosion coupons are a good example. Another alternative is the use of removed pipe sections (spools).[12] Alternatively, coupons specially 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 representative of sessile bacterial growth. For example, coupons are often located at the "6 o'clock" position in oil and gas 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 effects.

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, the bacteria shall be removed 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) solution 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 PBS 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.

5.3.1.1.1 PBS Solution

NaCl	8.7 g
KH_2PO_4	0.4 g
K_2HPO_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 Solution

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.[13,14]

5.4.2 In recognition of the importance of biofilm growth, many different test methods to evaluate biocide effectiveness are under development. 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
GLOSSARY

4-6-diamidino-pheynylindole hydrochloride (DAPI): A DNA-binding molecule that will fluoresce when illuminated with appropriate excitation wavelength of light, allowing visualization and enumeration of bacterial cells in a sample. Compare *acridine orange*.

Acridine orange: A fluorochome that binds to DNA and RNA and fluoresces when excited with UV light. This reagent can be used to stain bacterial cells for enumeration by fluorescence microscopy.

Adenosine triphosphate (ATP): A molecule that provides energy to living cells via hydrolysis of the high energy bond to the terminal phosphate.

Aerobic bacteria: Bacteria that grow and reproduce in the presence of oxygen.

Agar: A dried polysaccharide extract of red algae used as a solidifying agent in microbiological media.

Algae: Unicellular to multi-cellular plants that occur in fresh water, marine water, and damp terrestrial environments. All algae possess chlorophyll for photosynthesis.

Anaerobic bacteria: Bacteria that grow and reproduce in the absence of oxygen.

APS-reductase: An enzyme specific to sulfate-reducing bacteria that is involved in the reduction of sulfate to sulfide.

Archaebacteria: The kingdom of monerans that consists of methanogenic bacteria, halophilic bacteria, and thermoacidophilic bacteria. These bacteria evolved over 3.5 billion years ago and exist in extreme environments, are anaerobic, and derive energy from inorganic molecules or light. Compare *Eubacteria*.

ATP photometry: A method of bacterial enumeration that quantifies the amount of ATP present in a sample and thereby provides an estimate of bacteria present based on the assumption that the concentration of ATP is proportional to number of bacterial cells.

Autoclave: A chamber that utilizes pressure and heat to sterilize solutions, media, instruments, and glassware by killing all microorganisms present. See *sterilize.*

Bacteria: Prokaryotic microorganisms enclosed by a cell membrane without a fully differentiated nucleus.

Bacterial culturing: Techniques used to grow bacteria present in a sample inoculum in select growth media in the laboratory. See *culture medium*.

Biocide: A chemical product that is intended to kill or render harmless biological organisms. Also termed *antimicrobial pesticide*.

Biocide efficacy: The degree of performance a biocide exhibits in killing bacteria. This is usually based on concentration and contact time relative to other biocides being screened.

Biofilm: A matrix of bacteria, exopolymer, debris, and particulate matter that adheres to a surface.

Biomass: The mass per sample volume of microorganisms present. When referring to sessile biofilms, this term may also include the solids formed by bacterial growth such as exopolymer.

Broth: An alternative term for liquid medium used to culture bacteria in the laboratory. See *culture medium*.

Copepods: Aquatic crustaceans comprising the most numerous group of metazoans in the water community. Adults average 1 to 2 mm in size. They represent an important link between phytoplankton and fish in the food chain.

Cost-effective biocide: A biocide that provides superior kill of microorganisms based on cost per gallon or pound.

Coupon: A removable system component used to sample sessile bacteria growth. Standard corrosion coupons are an example.

Culture medium: Formulated solution of organic and inorganic nutrients that facilitate bacterial growth in the laboratory.

Dilution-to-extinction method: During microbial enumeration using the serial dilution method, dilution-to-extinction refers to continuing the serial dilutions to a point at which no growth will be encountered, i.e., to a point at which no microorganisms are transferred in the final dilution. This ensures that a full estimate of the original population in the sample can be determined. See *serial dilution method*.

Dose-response test: Biocide screening test to establish concentration and contact time for effective bacterial kill. See *time-kill test*.

Duplicate culturing: Performing replicate cultures, for instance with the serial dilution method, in order to obtain more reliable interpretation of the results.

Emulsion: A mixture in which one liquid, termed the *dispersed phase*, is uniformly distributed (usually as minute globules) in another liquid, called the continuous phase or dispersion medium. In the oil field, typically water is dispersed as droplets in oil (water-in-oil emulsion). A reverse emulsion refers to oil dispersed in water (oil-in-water emulsion).

Facultative anaerobic bacteria: Bacteria that are able to carry out both aerobic and anaerobic metabolism and therefore are able to grow and reproduce in both the presence and absence of oxygen.

Filter backwash: A process of forcing a water stream back through a filter in order to dislodge particles from the filter media. Oftentimes biocide may be introduced during this cycle to treat sessile bacteria buildup on the filter media.

Fluorescence microscopy: A microscopic method that utilizes a specific illuminating wavelength of light to excite a fluorescent stain or probe added to a sample for specific detection of cells, structures, or molecules present in the sample. Specific filters are used to select for proper emission spectra of the illuminated probe.

Fluorescent antibody: An antibody or immunoglobulin that has been raised against a specific antigen being investigated, such as a protein or cell component, and is coupled to a fluorescein molecule to allow its detection.

Fluorescein isothiocyanate: A very common fluorochome that is excited at 420 to 480 nm and fluorescesces at 530 to 540 nm. This protein dye is often used in fluorescence microscopy and can be conjugated with an antibody for use in immunofluorescence methods.

Fungi: A group of plants that lacks chlorophyll and includes molds, rusts, mildews, smuts, and mushrooms.

Heterotrophic bacteria: Bacteria that are unable to use carbon dioxide as their sole source of carbon and require one or more organic compounds.

Hydrocarbon oxidizing organisms: Heterotrophic microorganisms capable of using hydrocarbons as their energy source as well as a carbon source for growth. This metabolic process is generally aerobic, requiring the presence of oxygen.

Hydrogenase: An enzyme that catalyzes the oxidation of hydrogen and is possibly involved in cathodic depolarization by sulfate-reducing bacteria.

Immunoassay: A detection method that takes advantage of antibody specificity to a protein or cell component being analyzed. The antibody is usually conjugated to a fluorescein dye or chomogenic substrate, which allows quantification of the molecule being investigated.

Inoculum: A medium or sample containing microorganisms that is introduced into a culture.

Iron bacteria: The so-called iron-oxidizing bacteria. These bacteria oxidize ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}), which generally precipitates as iron hydroxide.

Membrane filter technique: An enumeration technique for waters with low bacterial concentrations in which a volume of sample is passed through a 0.45-µm filter using a filter funnel and vacuum system. Any organisms in the sample are concentrated on the surface of the membrane. The filter is then placed in nutrient medium. The passage of nutrients through the filter facilitates the growth of organisms on the upper surface of the membrane. The discrete colonies that form on the surface of the membrane can be easily transferred to confirmation media.

Microbial ecology: Encompasses a wide range of disciplines and focuses on the interactions of microorganisms with one another and with their environment.

Microorganisms: Common term used for unicellular organisms of the plant or animal kingdoms that are structurally related. These cannot be seen without magnification and generally range from 0.2 to 200 µm in size.

Most probable number (MPN) method: The essence of this method is the dilution of a sample to such a degree that inocula will sometimes but not always contain viable organisms. The "outcome," i.e., the numbers of inocula producing growth at each dilution, will imply an estimate of the original, undiluted concentration of bacteria in the sample. In order to obtain estimates over a broad range of possible concentrations, microbiologists use serial dilutions, incubating several tubes or plates (replicates) at each dilution.

Osmotic balance: In the context used here, the proper salt concentration of a medium required for the bacteria being cultured to be able to maintain proper osmoregulation.

pH: The negative logarithm of the hydrogen ion activitiy written as:

$$pH = -\log_{10} (a_{H^+})$$

Where a_{H^+} = hydrogen ion activity = the molar concentration of hydrogen ions multiplied by the mean ion-activity coefficient.

Phase separation: Specifically used here to refer to the macroscopic separation of oil and water in a sample into the two respective fluids by a allowing time for the fluids to "settle."

Phenol red dextrose broth: Culture medium used to grow heterotrophic bacteria.

Phytoplankton: A collective term for free-floating aquatic plants and plant-like organisms. Compare *zooplankton*.

Pig run: The process of launching a pig device in a pipeline segment for the purpose of cleaning or to monitor pipeline integrity.

Pigging: A procedure used for cleaning pipeline scale, deposits, and solids or to monitor pipeline integrity. The pig consists of a cylindrical device that forms a seal with the inner pipe surface and is launched through a segment of the pipeline using differential pressure. Pigs can be equipped with brushes or various adaptations to facilitate cleaning or permit inspection of the pipe wall.

Planktonic bacteria: Bacteria that are freely floating in brine. Planktonic bacteria can become sessile bacteria by adhering to a surface.

Postgate medium B: Specific medium designed for culturing sulfate-reducing bacteria characterized by lactate that is used as a carbon source and sulfate to provide the terminal electron acceptor required for SRB metabolism.

Protozoa: Single-celled eukaryotic microorganisms that feed heterotrophically and exhibit diverse forms of motility.

Radiorespirometry: Sensitive method for bacterial enumeration whereby radioactive nutrients are metabolized by bacteria in a sample and the amount of radiolabeled gases that are generated are measured to give an estimate of the number of viable bacteria in the sample.

Redox potential: A measure of the relative oxidation-reduction potential of an environment. Aerobic bacteria grow best in systems with highly positive redox potentials (oxidizing environments) while anaerobic bacteria, including SRB, will grow much better in reducing environments where the redox potential is less than -100 mV.

Salinity: The measure of dissolved salts in the system water, usually reported as total dissolved salts (TDS) or chlorides. The salinity should be approximated when media for culturing microorganisms from the system water are prepared.

Sampling bomb: A sample device that can be used to take liquid samples at discrete depths in drums, tanks, and surface water bodies.

Sampling thief: Another term used for a liquid sampling device used to take samples at discrete depths. See *sampling bomb*.

Serial dilution method: Method of enumerating bacteria in a sample via transfer to a series of growth media vials using successive 1:10 dilutions in each successive vial. Following an incubation period, the number of positive cultures provides an estimate of the number of bacteria in the original sample. For statistical validity this test is done with replicates and the population estimate is derived from a statistical table. See *Most Probable Number method*.

Serum vial: A glass vial used for culturing bacteria. It contains a septum that can be sealed with a metal ring. The septum can be accessed with a syringe needle for inoculating bacteria. The vial assembly can be filled with culture media and autoclaved for sterilization.

Sessile bacteria: Bacteria that are attached to surfaces. Bacteria that live in biofilms are sessile bacteria.

Shewanella putrefaciens: Although not true SRB, these sulfidogenic bacteria exist in biofilms and can act synergistically with SRB to facilitate MIC and hydrogen sulfide formation.

Shut-in: In general, refers to closing the valves to a well to shut off production. The term also refers to closing down a segment of a system, vessels, piping, or injection wells. During the shut-in period, the fluids in that part of the system are stagnant and amenable to increased bacterial growth.

Sodium lactate SRB medium: Medium for culturing SRB. It is derived from the original Postgate B medium and provides similar nutrients and salts but in a slightly different formulation. See *Postgate B medium*.

Spool: A monitoring device for obtaining sessile bacterial samples that often consists of a removable pipe section inserted in a side-stream flow loop, whereby a representative sample of sessile biofilm growth can be acquired.

Standard bacteriological nutrient broth: Basic culture medium for heterotrophic bacteria containing beef extract and peptone.

Sterile: Free of living organisms. To sterilize a medium or material is to kill all microorganisms that are present. See *autoclave*.

Strict anaerobe: A microorganism that grows only in the absence of oxygen.

Subculture: For the purposes of this standard, used to evaluate false positives for the presence of SRB in the first two vials of a dilution series that have turned black within two hours due to the presence of hydrogen sulfide. After the 28-day incubation period, a 1-mL aliquot can be taken from these vials and re-tested by serial dilution into SRB media (subculturing) to determine whether SRB are present.

Sulfur oxidizing organisms: A broad group of aerobic bacteria that derives energy from the oxidation of sulfide or elemental sulfur to sulfate.

Sulfate-reducing bacteria: A diverse variety of heterotrophic microorganisms characterized by its metabolism of sulfate to sulfide.

Thermophilic bacteria: Bacteria that grow and reproduce in high temperature environments, above 113°F (45°C).

Thioglycolate broth: A culture medium used to grow anaerobic bacteria in the laboratory.

Time-kill test: A biocide screening test that determines the efficacy of a biocide against microorganisms cultured from the system and identifies optimum contact times and concentrations for effective bacterial kill.

Zooplankton: Collective term for nonphotosynthetic organisms present in plankton.

APPENDIX B
RAPID METHODS FOR ASSESSING BACTERIAL POPULATIONS

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 enzymatic 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.[15] In view of these disadvantages, ATP photometry is not used for routine monitoring. It has been used successfully, however, to monitor trends, particularly following biocide treatments.[16]

Fluorescence Microscopy. The total number of bacteria in the sample can be determined, and live and dead cells can be distinguished, by fluorescence microscopy. Specific stains that fluoresce when irradiated with ultraviolet light are used. Stains such as acridine orange, fluorescein isothiocyanate (FITC), and 4, 6- diamidino-2-phenylindole hydrochloride (DAPI) are used for total bacteria counts because they stain both the live and the dead cells. Recent developments in fluorescence stain technology have resulted in methods using a combination of dual fluorescent dyes, with different emission spectra, that can distinguish between live and dead bacteria. A fluorescence microscope is used to allow cells to be counted.[17] 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.[18]

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,[19] 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 ^{35}S-labeled sulfate. After incubation, the reaction should be terminated with acid to kill the cells and to release any ^{35}S-sulfide produced by SRB. Such sulfides should be fixed in zinc acetate prior to quantification, using a liquid scintillation counter. Once the ^{35}S-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.[20] However, it is a highly specialized technique involving expensive laboratory equipment. Also, the handling of radioactive substances is strictly 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.[21] The 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 as well as viable SRB. **NOTE:** While this method, as cited, 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 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. 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.[22]

APPENDIX C
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 filter[23] 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 µm. When the membrane is handled, 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[24] gives details of membrane filtration, including the most commonly used membrane types.

APPENDIX D
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. This is accomplished by a stepwise 1:10 dilution scheme until, by theory, no bacteria are transferred. The growth medium in the serial-dilution vials provides 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 1 and 10 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 ±5°C [±9°F]). This is important.

NOTE: See alternative inoculation procedure described in Paragraph 3.2.4.2.2.

RESULTS INTERPRETATION AND REPORTING

Following incubation, visually examine all vials and report the results. Turn the vials over several times to resuspend 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 D1 through D3. 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 underestimate 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 growth medium. 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

<u>All Vials Show Growth</u>

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 ≥1,000,000 or $\geq 10^6$ bacteria/mL.

<u>There Is a Gap in the Positive Vials</u>

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 to 99, and the other 100 to 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 populations 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 the 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 3.2.5.3), 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 D1 through D3 (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.[7] It should also be noted that these tables are based on statistical estimates and are not empirical.

TABLE D1
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 D2
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 D3
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 E
ALTERNATIVE SRB GROWTH MEDIUM FORMULATION

Postgate Medium G[11]	g/L
KH_2PO_4	0.2
NH_4Cl	0.3
Na_2SO_4	3.0
$CaCl_2{\cdot}2H_2O$	0.15
$MgCl_2{\cdot}6H_2O$	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 Na_2O_3Se + 0.5 g/L NaOH).

b. Trace elements, 1 mL (from autoclaved stock of $FeCl_2{\cdot}4H_2O$, 1.5 g; H_3BO_3, 60 mg; $MnCl_2{\cdot}4H_2O$, 100 mg; $CoCl_2{\cdot}6H_2O$, 120 mg; $ZnCl_2$, 70 mg; $NiCl_2{\cdot}6H_2O$, 25 mg; $CuCl_2{\cdot}2H_2O$, 15 mg; $NaMoO_4{\cdot}2H_2O$, 25 mg/L).

c. $NaHCO_3$, 2.55 mg (30 mL of 8.5% w/v solution, filter-sterilized after saturation with CO_2).

d. $Na_2S{\cdot}9H_2O$, 0.36 g (3 mL of 12% w/v solution autoclaved under N_2).

e. Vitamins, 0.1 mL (from filter-sterilized stock of biotin, 1 mg; p-aminobenzoic acid, 5 mg; vitamin B_{I2}, 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).

Carbon sources, 1 mL/100 mL final medium of autoclaved stocks (e.g., 20% sodium acetate· tri-hydrate, and/or 7% propionic acid).