



Designation: D4412 – 19 (Reapproved 2024)

Standard Test Methods for Sulfate-Reducing Bacteria in Water and Water-Formed Deposits¹

This standard is issued under the fixed designation D4412; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ϵ) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 These test methods cover the procedure for the detection and enumeration by the most probable number (MPN) technique of sulfate-reducing bacteria in water or water-formed deposits.

1.2 Two media preparations are provided. Medium A which is prepared with reagent grade water, and Medium B which is prepared using the water to be sampled as the water source. Medium B is offered for those special conditions where sulfate-reducing bacterial strains have adapted to atypical non-fresh water environment.

1.3 For the isolation and enumeration of thermophilic sulfate-reducing bacteria encountered in waters associated with oil and gas production, all broths, dilution blanks, and incubations must be maintained at temperatures of at least 45 °C and preferably within 5 °C at the sample temperature.

1.4 The sensitivity of these test methods can be increased by purging the dilution blanks and tubes of media with nitrogen immediately prior to use.

1.5 The analyst should be aware that adequate collaborative data for precision and bias statements as required by Practice D2777 are not provided. See Section 11 for details.

1.6 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.7 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.8 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recom-*

mendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.

2. Referenced Documents

2.1 *ASTM Standards:*²

D1129 Terminology Relating to Water

D1193 Specification for Reagent Water

D2777 Practice for Determination of Precision and Bias of Applicable Test Methods of Committee D19 on Water

D6503 Test Method for Enterococci in Water Using Enterolert

2.2 *Other Standards:*

Standard Methods 9221 Multiple-Tube Fermentation Technique for Members of the Coliform Group³

3. Terminology

3.1 *Definitions*—For definitions of terms used in this standard, refer to Terminology D1129.

3.1.1 *most probable number, n*—statistical method for determining bacterial density based on the Poisson distribution. **D6503**

3.2 *Acronyms:*

3.2.1 *SRB, n*—sulfate-reducing bacteria

4. Summary of Test Methods

4.1 Water and water deposit samples and dilutions of these samples are dispensed into tubes of Starkey's medium (A or B) following five tube MPN procedures. The tubes are sealed with liquid paraffin, and incubated at 20 °C for 21 days.⁴ Positive reactions are indicated by the deposit of a black precipitate.

5. Significance and Use

5.1 Sulfate-reducing bacteria are widely distributed in marine and fresh water muds which, in consequence, frequently

¹ These test methods are under the jurisdiction of ASTM Committee D19 on Water and are the direct responsibility of Subcommittee D19.24 on Water Microbiology.

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² For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

³ Available from Standard Methods, <http://standardmethods.org>.

⁴ Starkey, R. L., "Characteristics and Cultivation of Sulfate-Reducing Bacteria," *Journal of the American Water Works Association*, Vol 40, 1948, pp. 1291–1298.

are laden with the hydrogen sulfide produced by these organisms during dissimilatory sulfate reduction.

5.2 It has been reported that *Desulfovibrio* spp. can form as much as 10 g of sulfide per litre during active multiplication. Sulfate-reducing bacteria can cause the external or internal corrosion of water or wastewater pipelines and pipelines for petroleum and natural gas. The formation of galvanic cells by massive growth of sulfate-reducing bacteria under suitable conditions makes the corrosion much worse than just the effect of the hydrogen sulfide on the metal or concrete.

6. Apparatus and Materials

6.1 *Anaerobic Incubator*, 20 °C, if available, or conventional 20 °C incubator.

NOTE 1—For thermophilic organisms use a 45 °C incubator.

6.2 *Pipets*, sterile, 1 mL and 10 mL, “calibrated” to deliver.

6.3 *Test Tubes*, with close fitting or airtight caps; 16 mm by 150 mm and 20 mm by 150 mm.

6.4 *Test Tube Racks*, of sufficient size to contain 16 mm and 20 mm tubes.

7. Reagents

7.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society,⁵ when such specifications are available.

7.2 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean Reagent Water Type II conforming to Specification D1193. In addition, reagent water used for these test methods must be sterile.

7.3 Starkey’s Medium A⁴ (Modified):

Sodium lactate (C ₃ H ₅ NaO ₃)	3.5 g
Ammonium chloride (NH ₄ Cl)	1.0 g
Dipotassium, hydrogen orthophosphate (K ₂ HPO ₄)	0.5 g
Magnesium sulfate (MgSO ₄ ·7H ₂ O)	2.0 g
Sodium sulfate (Na ₂ SO ₄)	0.5 g
Calcium chloride (CaCl ₂ ·2H ₂ O)	0.1 g
Thioglycolic acid	0.1 g
Ammonium ferrous sulfate or ferrous ammonium sulfate ((NH ₄) ₂ SO ₄ ·FeSO ₄ ·6H ₂ O)	0.001 g
Water (H ₂ O)	1 L

7.3.1 Double strength medium (2×) is prepared as above except 500 mL of water are used instead of 1 L.

7.3.2 Heat to dissolve and dispense 9 mL of medium per single strength tube, and 10 mL per double strength tube.

7.3.3 Tubes should be of sufficient capacity to contain 1 mL of inoculum plus 9 mL of single strength medium or 10 mL of inoculum plus 10 mL of 2× medium.

7.3.4 pH of medium should be 7.2 after autoclave sterilization, at 121 °C for 15 min.

7.4 *Starkey’s Medium B*—The medium is similar to that described in 7.3, 7.3.1, and 7.3.2 with the following modification:

7.4.1 Water collected from the sample collection site is used to prepare the medium outlined in 7.3. The water sample is filtered to remove particulates (1.2 µm membrane filter) and the pH is recorded.

7.4.1.1 After preparing the Medium B following 7.3.1, 7.3.2, and 7.3.3, and prior to dispensing, check and adjust pH, if necessary to that of the original water used, then filter sterilize the medium by passage through 0.2 µm filter and aseptically dispense into presterilized tubes.

7.5 Hydrogen Sulfide Test Reagent:

7.5.1 *Ferric Chloride Stock Solution* (FeCl₃·6H₂O)—Dissolve 13.5 g of ferric chloride in a mixture of 250 mL of water and 250 mL of HCl (sp gr 1.19). Store in an airtight amber container. Prepare fresh monthly.

7.5.2 *p-Aminodimethylaniline Dihydrochloride Stock Solution*:

p-Aminodimethylaniline dihydrochloride (C ₈ H ₁₂ N ₂ ·2HCl)	1.0 g
HCl (6 N)	500 mL

Dissolve 1 g of p-aminodimethylaniline dihydrochloride in 500 mL of 6 N HCl. Store for up to 1 month in an amber airtight container.

7.6 *Liquid Paraffin*—Heavy, sterile, or sterile mineral oil.

7.7 Buffered Dilution Water, Stock Solution:

7.7.1 Dissolve 34.0 g of KH₂PO₄ in 500 mL of water, adjust pH to 7.2 with 1 N NaOH and dilute to 1 L with distilled water. This is called the stock phosphate solution.

7.7.2 Dissolve 38 g of MgCl₂ in 1 L of distilled water.

7.8 *Buffered Dilution Water, Working Solution*—Add 1.25 mL of stock buffered dilution water and 5 mL of MgCl₂ solution to 500 mL of water. Bring to 1 L with water. Mix well and dispense as 90 mL dilution blanks in screw-capped bottles. Sterilize by autoclaving at 121 °C for 15 min.

8. Procedure

8.1 Clean and disinfect the area with a cleaning solution that leaves no residue.

8.2 Set out and label five replicate tubes of 10 mL double-strength Starkey’s medium, A or B, in the test tube rack.

8.3 Set out and label five replicate tubes of 10 mL single-strength Starkey’s medium, A or B, for each mL of sample or mL of sample dilution to be tested. Use two sets of five replicate 10 mL tubes, each to contain 1 mL of sample or 1 mL of 1/10 dilution of sample.

8.4 Prior to sample inoculation, heat tubes of media and dilution blanks in a water bath to 60 °C then cool rapidly to 20 °C to ensure minimal oxygen levels.

8.5 Shake sample thoroughly, at least 25 times; make dilutions starting with 10 mL of sample into one 90 mL dilution blank.

⁵ ACS Reagent Chemicals, Specifications and Procedures for Reagents and Standard-Grade Reference Materials, American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.