Designation: D4412 – 15

Standard Test Methods for Sulfate-Reducing Bacteria in Water and Water-Formed Deposits[1]

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 and health practices and determine the applicability of regulatory limitations prior to use.*

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 Group[3]

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.

***A Summary of Changes section appears at the end of this standard**

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 by 150 mm and 20 by 150 mm.

6.4 *Test Tube Racks,* of sufficient size to contain 16 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 A*[4] *(Modified)*:

Sodium lactate ($C_3H_5NaO_3$)	3.5 g
Ammonium chloride (NH_4Cl)	1.0 g
Dipotassium, hydrogen orthophosphate (K_2HPO_4)	0.5 g
Magnesium sulfate ($MgSO_4·7H_2O$)	2.0 g
Sodium sulfate (Na_2SO_4)	0.5 g
Calcium chloride ($CaCl_2·2H_2O$)	0.1 g
Thioglycollic acid	0.1 g
Ammonium ferrous sulfate or ferrous ammonium sulfate ($(NH_4)_2SO_4·FeSO_4·6H_2O$)	0.001 g
Water (H_2O)	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* ($FeCl_3·6H_2O$)—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 ($C_8H_{12}N_2·2HCl$)	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 KH_2PO_4 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 $MgCL_2$ 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 $MgCl_2$ 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] *Reagent Chemicals, American Chemical Society Specifications*, American Chemical Society, Washington, DC. For Suggestions on the testing of reagents not listed by the American Chemical Society, see *Annual 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.

TABLE 1 Replicate Tests Summarization

Medium	Repeatability						Reproducibility		
	Analyst 1			Analyst 1					
	$\bar{X}$	s_r	r	$\bar{X}$	s_r	r	$\bar{X}$	s_r	r
Starkey's Medium A (7.3)	5.6	0.2	0.6	5.7	0.3	0.9	5.7	0.2	0.7
Starkey's Medium B (7.4)	5.5	0.1	0.2	5.4	0.3	0.8	5.4	0.2	0.6

NOTE 2—Organisms do not appear to be hypersensitive to small amounts of oxygen.

8.6 Pipet 10 mL of sample into each double-strength broth and 1 mL of sample or diluted sample into each set of five single-strength broths.

8.7 Maintain anaerobic conditions by layering 2 to 3 ml of sterile liquid paraffin in each tube.

8.8 Recap tubes and incubate at 20°C for 21 days.

8.9 Include sterile water samples with each test as negative controls.

8.10 Positive reaction is indicated by the deposit of a black precipitate (sulfide).

8.11 Confirm dubious results by the addition of 0.5 mL of ferric chloride reagent followed by 0.5 mL of p-aminodimethylaniline reagent to the MPN tube. Add reagent to the bottom of the tube using syringe or long Pasteur pipet. A positive reaction, blue color, occurs within 10 min if H_2S is present.

9. Calculation

9.1 Compute the number of positive findings resulting from multiple-portion decimal dilution planting as the combination of positives and recorded as MPN (see Standard Methods 9221).

9.2 When more than three series of tubes are employed in a decimal series of dilutions, use the results from only three of these used in computing the MPN, for example:

10 mL	1 mL	0.1 mL	0.01 mL				
5/5	5/5	2/5	0/5	=	5-2-0 × 10	=	490/100 mL
5/5	4/5	2/5	0/5	=	5-4-2	=	220/100 mL
5/5	3/5	1/5	1/5	=	5-3-2	=	140/100 mL
5/5	0/5	0/5	0/5	=	5-0-0	=	23/100 mL

10. Report

10.1 Perform a Log_{10} transformation and report the results as Log_{10} MPN SRB/100 mL of sample.

11. Precision and Bias

11.1 Microbial populations are dynamic in culture. Consequently a full interlaboratory study is infeasible. However, an evaluation of method precision was performed and reported.[6]

11.2 Unless a large number of portions of sample are examined, the precision of the MPN is rather low. For example, even when the sample contains one organism per millilitre, about 37 % of tubes inoculated with 1 mL of sample may be expected to yield negative results because of irregular distribution of the bacteria in the sample and the multiple attachment of bacteria to particles. When five tubes, each with 1 mL of sample, are employed under these conditions, a completely negative result may be expected less than 1 % of the time.

11.2.1 *Repeatability*—The difference between successive measured Log_{10} MPN SRB/100 mL values obtained by the same operator from replicate subsamples of a given sample. The repeatability coefficient, $r = 2.8\ s_r$, where s_r is the repeatability standard deviation.

11.2.2 *Reproducibility*—The difference between two single and independent Log_{10} MPN SRB/100 mL values obtained by different operators on replicate subsamples of a given sample under nominally identical test conditions. The Reproducibility coefficient, $R = 2.8\ s_R$, where s_R is the reproducibility standard deviation.

11.2.3 For both Starkey's Medium A (7.3) and Starkey's Medium B (7.4) Log_{10} MPN/100 mL, results obtained on five replicate tests by two analysts were statistically indistinguishable at the 95 % confidence level, as determined by two-way analysis of variance ($F_{obs,\ 1,1} = 0.26$; $F_{crit\ 1,1\ [\alpha=0.95]} = 4.49$). Averages ($\bar{X}$), standard deviations ($s_r$ and s_R), r and R are summarized in Table 1.

11.3 *Bias*—Since there is no accepted reference material suitable for determining the bias of the procedure, bias cannot be determined.

12. Keywords

12.1 bacteria; enumeration; most probable number; MPN; SRB; sulfate reducing bacteria

[6] Supporting data have been filed at ASTM International Headquarters and may be obtained by requesting Research Report RR:D19-1116. Contact ASTM Customer Service at service@astm.org.

SUMMARY OF CHANGES

Committee D19 has identified the location of selected changes to this standard since the last issue (D4412 – 84 (2009)) that may impact the use of this standard. (Approved July 15, 2015.)

(1) Revised Sections 2, 3, 9.1, 10.1, and 11.

(2) Added Table 1 and Section 12.