Designation: D8243 – 19

Standard Test Method for Determination of APS Reductase to Estimate Sulfate Reducing Bacterial Bioburdens in Water – Enzyme-Linked Immunosorbent Assay Method[1]

This standard is issued under the fixed designation D8243; 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 This test method provides a protocol for using enzyme-linked immunosorbent assay (ELISA) technology to test water samples for the enzyme adenosine 5'-phosphosulfate reductase (APSr) concentration.

1.1.1 APSr is present in all known sulfate reducing protists (SRP – sulfate reducing bacteria – SRB – and sulfate reducing archaea – SRA).

1.1.2 As reported in U.S. Patent 4,999,286, APS reductase concentration can be used as a surrogate parameter for estimating SRA bioburdens (Appendix X1 compares results from Test Methods D8243, D4412, and quantitative polymerase chain reaction – qPCR – testing).

1.2 This test method has been validated in tap water, oilfield produced water (salinities ranging from 100 g L^{-1} to 600 g L^{-1}), and fuel-associated water (commonly referred to as bottoms-water).

1.3 This test method detects APS reductase semi-quantitatively in the range of 0.001M to 0.1M – correlating to 10^2 SRP/mL to 10^6 SRP/mL.

1.3.1 As described in Appendix X2 test method sensitivity can be increased 10-fold to 100-fold. However, the precision statistics provided in X apply only to 10-mL specimens.

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

1.5 *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.* Some specific hazards statements are given in Section 9 on Hazards.

1.6 *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 Recommendations 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

D4412 Test Methods for Sulfate-Reducing Bacteria in Water and Water-Formed Deposits

D5847 Practice for Writing Quality Control Specifications for Standard Test Methods for Water Analysis

D6300 Practice for Determination of Precision and Bias Data for Use in Test Methods for Petroleum Products and Lubricants

D6499 Test Method for Immunological Measurement of Antigenic Protein in Hevea Natural Rubber (HNR) and its Products

D7464 Practice for Manual Sampling of Liquid Fuels, Associated Materials and Fuel System Components for Microbiological Testing

E691 Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method

E1601 Practice for Conducting an Interlaboratory Study to Evaluate the Performance of an Analytical Method

E1847 Practice for Statistical Analysis of Toxicity Tests Conducted Under ASTM Guidelines

E1914 Practice for Use of Terms Relating to the Development and Evaluation of Methods for Chemical Analysis (Withdrawn 2016)[3]

2.2 *Patents:*[4]

U.S. Patent 4,999,286 Sulfate Reducing Bacteria Determination and Control, March 12, 1991

[1] This test method is under the jurisdiction of ASTM Committee D19 on Water and is 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] The last approved version of this historical standard is referenced on www.astm.org.

[4] Available from United States Patent and Trademark Office (USPTO), Madison Building, 600 Dulany Street, Alexandria, VA 22314, https://www.uspto.gov.

3. Terminology

3.1 *Definitions:*

3.1.1 For definitions of terms used in this standard, refer to Terminology D1129.

3.1.2 *adenosine 5'-phosphosulfate reductase (APS reductase), n*—(EC 1.8.4.9 and CAS 9027-75-2) an enzyme present on all known sulfate reducing bacteria and archaea, catalyzing the two-electron reduction of the sulfate group in adenosine 5'-phosphosulfate (APS) to yield adenosine monophosphate and sulfite.

3.1.3 *categorical data, n*—variates that take on a limited number of distinct values. **E1847**

3.1.4 *chromogen, n*—a chemical or compound that reacts with an enzyme to produce a colored end-product, used to detect the presence of a substance of interest.

3.1.5 *detection limit, n*—the smallest net signal (or the derived property value, constituent mass fraction, etc.) obtained by a given measurement procedure, that can be distinguished from the background signal at a specified confidence level. **E1914**

3.1.6 *enzyme linked immunosorbent assay (ELISA), n*—an immunological test method to quantify antigen or antibody levels using an enzyme as the detection mechanism. **D6499**

3.2 *Symbols:*

APSr—APS reductase

BDL—below detection limit

GHS—Globally Harmonized System of Classification and Labeling of Chemicals

SRA—sulfate reducing archaea

SRB—sulfate reducing bacteria

SRP—sulfate reducing protists

4. Summary of Test Method

4.1 A 10-mL water specimen is filtered through a diatomaceous earth filter (8.3) that captures microbial cells. The filtrate is discarded.

4.2 The wash bottle's cap (8.5) is replaced with the filter cap from 4.1. The bottle is inverted and the 2 mL of wash solution is filtered through the filter medium to eliminate interferences.

4.3 The filter cap is removed from the wash bottle and placed onto the bottle containing the rehydrated lysing reagent (8.2). The filter cake is dispersed from the cap into the lysing reagent and allowed to stand for 2 min.

4.4 The filter cap is replaced with the final filter cap (8.7) and the lysed solution is filtered into the immunoreagent vial (8.9). The filtrate-immunoreagent mixture is allowed to stand for 2 min.

4.5 The lysate-immunoreagent mixture is dispensed into the funnel device (8.8).

4.6 After all of the fluid has passed through the funnel device's filter membrane, the contents of the chromogen reagent dropper vial (8.5) are dispensed onto the funnel device's filter membrane. A laboratory-timer (7.1), set to the appropriate time (15 min at 18 ± 2°C) is started.

4.7 At the end of the color-development period (that is, 15 min at 18 ± 2°C), compare and match the color of the funnel device's filter membrane with the colors on the QuickChek[5] color card (8.6).

4.8 Report SRB/mL based on the color match observed in 4.7.

5. Significance and Use

5.1 Sulfate reducing archaea and bacteria are known to contribute to microbiologically influenced corrosion.

5.2 Sulfate-reducing bacteria are widely distributed in marine and fresh water muds which, in consequence, frequently are laden with the hydrogen sulfide produced by these organisms during dissimilatory sulfate reduction.

5.3 Traditional, culture-dependent methods such as those described in Test Methods D4412, prescribe incubation periods of as long as 21 days before assigning a below detection limit (BDL) score to a specimen. Moreover, it is well known that not all SRP will proliferate in the nutrient media specified in Test Methods D4412.

5.4 This test method uses ELISA technology to provide semi-quantitative, culture-independent, SRP bioburden test results in less than 30 min.

5.4.1 Because all the reagents and supplies used are non-hazardous and prepackaged for single test use, this test method does not require any apparatus other than a laboratory timer. Consequently, it can be performed at or near the point of sample collection.

5.4.2 The opportunity to minimize the delay between sample collection, testing, and results availability translates into timely use of the data to drive preventive and corrective SRB control measures.

6. Interferences

6.1 SRP population densities $>10^6$ cells/mL can cause an overload effect that can cause the population density to be underestimated.

7. Apparatus

7.1 Timer, laboratory

8. Reagents and Materials

8.1 Bottle, dropper, containing 2-mL lysing reagent rehydration fluid.[6]

8.2 Bottle, lysing reagent, proprietary, freeze-dried.

[5] QuickChek is a trademark of Modern Water, Inc, New Castle, DE, https://www.modernwater.com, and is the sole source of supply known to the committee at this time. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

[6] The sole source of supply of the proprietary chromogen liquid, final filter, immunoreagent, lysing reagent, rehydrating fluid, sample bottle with filter cap, test membrane device, and wash solution is Modern Water, Inc, New Castle, DE, https://www.modernwater.com. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

8.3 Bottle, sample, with proprietary filter cap, 10 mL.

8.4 Bottle, wash, containing: wash buffer, 2 mL, proprietary.

8.5 Chromogen, proprietary, in dropper vial.

8.6 Color card, QuickChek.

8.7 Filter, final, dispensing cap, proprietary.

8.8 Funnel device, proprietary.

8.9 Immunoreagent, proprietary, in vial.

8.10 Wash reagent, final, in dropper vial.

9. Hazards

9.1 Hydrogen sulfide can accumulate in any air space in sample containers filled with SRB-contaminated water. Hydrogen sulfide is flammable (GHS02), corrosive (GHS05), and toxic (GHS06). Appropriate precautions shall be taken when handling samples likely to contain detectable SRB populations.

10. Sampling, Test Specimens, and Test Units

10.1 Samples shall be collected and handled in accordance with guidance provided in Practice D7464.

10.1.1 Because microbes continue to be metabolically active in collected samples, microbiological parameters are perishable.

10.1.2 Optimally, samples shall be tested within 4 h after collection.

10.1.3 Acceptable test results can be obtained if testing is started within 24 h on samples that have been either kept on ice or refrigerated since collection.

10.1.4 As the delay between sampling and testing increases beyond 24 h the relationship between test results and SRP bioburdens in the systems from which samples were collected becomes increasingly tenuous.

10.2 The standard specimen for this test method is 10 mL.

10.3 The sample unit size is not critical, but a minimum unit size of 100 mL is recommended for replicate testing.

11. Calibration and Standardization

11.1 No calibration or standardization is needed for this test method. However, if a user is concerned about obtaining false negative results, reagent response can be tested in accordance with the protocol provided in Appendix X2.

12. Conditioning

12.1 Enzyme reaction rates are temperature dependent. Therefore, all samples and reagents shall be permitted to equilibrate to ambient temperature before testing. As explained in 13.7.3, the time allowed for ELISA reaction color development is temperature dependent.

13. Procedure

13.1 *Rehydrate Lysing Reagent:*

13.1.1 Remove caps from rehydrating solution dropper bottle (8.1) and lysing reagent bottle (8.2).

13.1.2 Invert rehydrating solution dropper bottle (8.1) over lysing reagent bottle (8.2) and squeeze the latter to dispense rehydrating solution into lysing reagent bottle.

13.1.3 Replace lysing reagent bottle cap, and gently swirl bottle for 15 sec to facilitate reagent rehydration.

13.2 *Collect Specimen:*

13.2.1 Shake sample vigorously for 30 sec to ensure that it is mixed properly.

13.2.2 Remove dropper cap from sample bottle (8.3).

13.2.3 Dispense 10 mL of sample into sample bottle (8.3) and replace dropper cap.

13.2.4 Invert sample bottle (13.2.3) and shake for 15 sec to disperse filter cake.

13.2.5 Keeping sample bottle (13.2.4) inverted, wait 15 sec to allow filter cake to settle into dropper tip.

13.2.6 Apply steady pressure to squeeze sample bottle (13.2.5) to filter sample.

13.2.6.1 Dispense filtrate into a waste container.

13.2.6.2 Maintain pressure on sample bottle until the entire 10-mL specimen has been filtered.

NOTE 1—While performing the filtration step, do not release pressure. Doing so will create a partial vacuum in the sample bottle. This will permit air to enter the bottle, causing the filter cake to re-disperse into the specimen and potentially cause SRP to be dispensed with the filtrate.

NOTE 2—A vacuum device may be used to facilitate filtration. However, the test results used to obtain the precision statistics provided for this test method were based on manual squeezing in accordance with 13.2.6.2.

13.3 *Wash Specimen:*

13.3.1 Remove cap from wash buffer bottle (8.4).

13.3.2 Remove filter cap from sample bottle (13.2.6).

13.3.3 Place cap (13.3.2) onto wash buffer (8.4).

13.3.4 Invert wash buffer bottle (13.3.3) and shake for 15 sec to disperse filter cake.

13.3.5 Keeping wash buffer bottle (13.3.4) inverted, wait 15 sec to allow filter cake to settle into dropper tip.

13.3.6 Apply steady pressure to squeeze wash buffer bottle (13.3.5) to filter sample.

13.3.7 Dispense filtrate into a waste container.

13.3.8 Maintain pressure on sample bottle until the entire 2-mL wash buffer has been filtered.

13.4 *Lyse Cells:*

13.4.1 Remove cap from lysing reagent bottle (13.1.3).

13.4.2 Remove dropper tip from wash buffer bottle (13.3.8) and place it onto lysing reagent bottle (13.4.1).

13.4.3 Flick the side of the lysing bottle/filter cap firmly with your index finger.

13.4.3.1 If the filter cake does not become dislodged, invert the bottle and flick it firmly again.

13.4.4 After the filter cake is dislodged, strike the base on a flat surface to make the filter cake fall into the lysing fluid.

13.4.5 If any of the cake remains in the filter cap, tip the bottle upside down and allow the fluid to run into the filter cap.

NOTE 3—When the fluid will go all the way to the plug in the cap, all the filter cake has been transferred.

13.4.6 Swirl lysing bottle (13.4.5) for 15 sec to mix the filter cake and lysing reagent well.

NOTE 4—Be careful not to mix so aggressively as to cause the filter cake dispersion to foam.

13.4.7 Remove filter cap and let lysing vial stand for 2 min.

13.4.8 Snap final filter dropper top (8.7) into place on lysing bottle (13.4.7).

13.5 *React Analyte with Immunoreagent:*

13.5.1 Remove the rubber cap from the glass immunoreagent vial (8.9).

13.5.2 Invert the lysing vial (13.4.8) over the immunoreagent vial (13.5.1) and squeeze the liquid from the lysing bottle into the immunoreagent vial.

NOTE 5—Between 10 and 20 drops of fluid should flow through the final filter into the immunoreagent vial.

13.5.3 Stop squeezing the lysing bottle when all the liquid is expelled, and foam appears at the filter tip.

13.5.4 Mix the liquid in the immunoreagent vial gently and let it incubate for two minutes.

NOTE 6—During this incubation period, the antibody-coated particles bind with the APS reductase in solution.

NOTE 7—The prescribed 2 min is nominal. Incubation intervals between 2 min and 4 min do not affect test results.

13.6 *Concentrate Antigen-Antibody Complexed Particles:*

13.6.1 Decant the contents of the immunoreagent vial (13.5.4) onto the funnel device's membrane (8.8).

13.6.2 Allow all fluid to be drawn through the funnel device's membrane.

NOTE 8—In the funnel device (8.8) the filter membrane rests on an absorbent bed. The absorbent draws the fluid through the membrane. The time required for all of the fluid to drain through the funnel device's membrane can vary from 2 min to 5 min.

13.6.3 Remove yellow cap from final wash reagent vial (8.10) and squeeze vial to dispense wash reagent onto membrane.

13.6.4 Allow all fluid to be drawn through the funnel device's membrane.

13.7 *Color Development:*

13.7.1 Remove blue cap from chromogen vial (8.5).

13.7.2 Invert vial and squeeze its sides to dispense chromogen onto funnel device's membrane (13.6.4).

13.7.3 Set lab timer to appropriate time in accordance with Table 1.

13.7.4 Allow membrane color to develop for period set in 13.7.3.

13.7.4.1 Do not perform this test method if the ambient temperature is <16°C or >38°C.

13.8 *Observe Color:*

13.8.1 Place QuickChek color card (8.6) near funnel device membrane as shown in Fig. 1.

13.8.2 Align color card (8.6) with membrane to match membrane's color with appropriate color on card.

13.8.3 Record SRP/mL based on color match.

TABLE 1 Relationship Between Ambient Temperature and Chromogen Color Development Time

Temperature °C	Color Development Time (Min)
16 to 20	15
>20 to 26	10
>26 to 32	8
>32 to 38	6

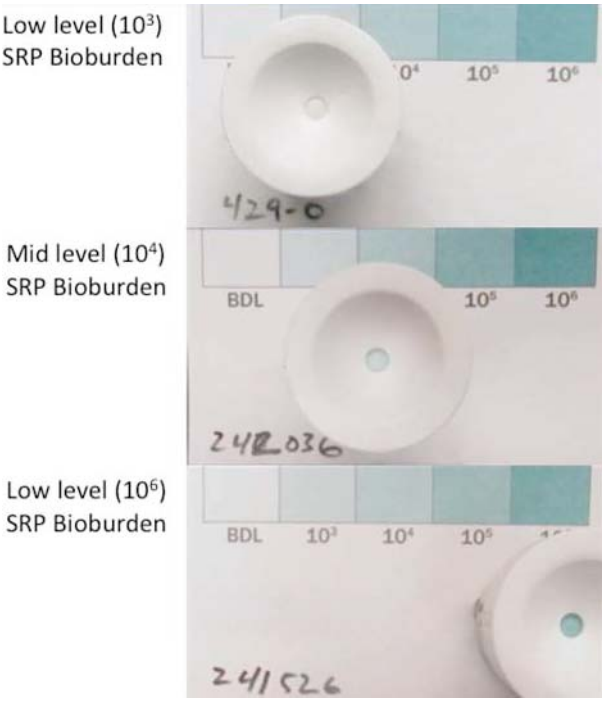

FIG. 1 QuickChek Color Card Next to Filter Device Membrane to Facilitate SRP Bioburden Scoring

14. Calculation or Interpretation of Results

14.1 For 10-mL specimens, no calculations are required. The reported SRP/mL is read directly from the QuickChek color card (8.6) in accordance with 13.8.3.

14.2 *Interpretation:*

14.2.1 SRP upper control limits can vary, depending on the system from which samples are collected.

14.2.2 In many industrial applications, any SRP bioburden that is greater than BDL triggers follow-up action.

14.2.3 Some applications set the upper control limit at $<10^3$ SRP/mL.

15. Reporting

15.1 Recognizing that SRP/mL can range from $<10^2$ SRP/mL to $>10^6$ SRP/mL, Log_{10} transformation facilitates data entry and process control plotting.

15.1.1 To transform test results, compute Log_{10} of the observed cells/mL as in Eq 1:

$$Log_{10}(10^3 \quad SRP/mL) = 3Log_{10}SRP/mL \tag{1}$$

15.1.2 After Log_{10} transformation, test results are reported as <2 (that is, BDL), 2, 3, 4, 5, 6 or >6.

16. Precision and Bias

16.1 *Precision:*

16.1.1 Test results are reported as categorical data (that is, whole numbers only). Consequently, neither Practice E691, nor Practice E1601 apply to this test method. Because results are categorical, statistical analysis assuming continuous function results overestimates variability. However, an interlaboratory study (ILS) was performed to estimate intermediate precision.