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An American National Standard

Standard Test Method for Enumeration of Aquatic Bacteria by Epifluorescence Microscopy Counting Procedure[1]

This standard is issued under the fixed designation D 4455; 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 describes a procedure for detection and enumeration of aquatic bacteria by the use of an acridine-orange epifluorescence direct-microscopic counting procedure. It is applicable to environmental waters.

1.2 Certain types of debris and other microorganisms may fluoresce in acridine orange-stained smears.

1.3 The test method requires a trained microbiologist or technician who is capable of distinguishing bacteria from other fluorescing bodies on the basis of morphology when viewed at higher magnifications.[2]

1.4 Use of bright light permits differentiation of single bacteria where reduced formazan is deposited at the polar ends.

1.5 Approximately 10^4 cells/mL are required for detection by this test method.[2]

1.6 *This standard does not purport to address 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:*

D 1129 Terminology Relating to Water[3]

D 1193 Specification for Reagent Water[3]

D 3370 Practices for Sampling Water from Closed Conduits[3]

3. Terminology

3.1 *Definitions*—For definitions of terms used in this test method, refer to Terminology D 1129.

4. Summary of Test Method

4.1 Enumeration of aquatic bacteria is obtained by passing a water sample through a 0.2-μm polycarbonate membrane filter.

4.2 The membrane filter is stained with acridine orange solution.

4.3 The stained filter is examined for fluorescing bacteria cells using a fluorescent microscope.

4.4 The fluorescent bacteria are counted. Dilutions are taken into consideration and bacterial concentrations established.

5. Significance and Use

5.1 Bacterial populations, as part of the microbial community in aquatic systems are actively involved in nutrient cycling. The significance of these populations is often difficult to ascertain because of the presence of many physiological types. However, measurement of bacterial densities is usually the first step in trying to establish any relationship that might exist between bacteria and other biochemical processes.[4]

5.2 Acridine-orange epifluorescence direct-counting procedure cannot differentiate between viable and nonviable cells.

5.3 This procedure cannot be used to convert directly the numbers to total carbon biomass because of the natural variations in bacterial cell size.

5.4 The acridine-orange epifluorescence direct-microscopic count is both quantitative and precise.

5.5 This procedure is ideal for enumerating both pelagic and epibenthic bacteria in all fresh water and marine environments.[5]

5.6 The process can be employed in survey activities to characterize the bacteriological densities of environmental waters.

5.7 The procedure can also be used to estimate bacterial densities in cooling tower waters, process waters, and waters associated with oil drilling wells.

6. Apparatus

6.1 *Fluorescence Microscope*, with oil-immersion objective lens (100×).

6.2 *Eye pieces*, 12.5×, equipped with a net micrometer (10 by 10 mm) (25 by 2-mm squares).

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[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] DIFCO Technical Information—Bacto Acridine Orange Stain, is available from Difco Laboratories, P.O. Box 1058, Detroit, MI 48201.

[3] *Annual Book of ASTM Standards*, Vol 11.01.

[4] Cherry, et al, "Temperature Influence on Bacterial Populations in Aquatic Systems," *Water Research*, Vol 8, 1974, pp. 149–155.

[5] Daley, R. J., "Direct Epifluorescence Enumeration of Native Aquatic Bacteria," *Native Aquatic Bacteria: Enumeration, Activity, and Ecology*, ASTM STP 695, ASTM, 1979, pp. 29–45.

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6.3 *Condenser*, 1.25×, suitable for the microscope.

6.4 *High-Pressure Mercury Lamp*, 200 W, on a UV light source giving vertical illumination and a filter unit H2 (Leitz)[6] with BG12 and BG38 transmission filters or equivalent.

6.5 *Stage Micrometer*, 2 by 200 parts.

6.6 *Membrane Filter Support* (25 mm), sterile, particle-free, fritted-glass.

6.7 *Funnel*, 15-mL capacity or equivalent.

6.8 *Membrane Filter*, sterile plain regular polycarbonate-25 mm, (0.2-µm pore size).

6.9 *Filter Apparatus*, containing vacuum source, filtering flask, and a filtering flask as a water trap.

6.10 *Forceps* (flat tip), *Alcohol*, *Bunsen Burner, Clean Glass Slides*, and *Cover Slips*.

7. Reagents and Materials

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, when such specifications are available.[7]

7.2 *Purity of Water*—Unless otherwise indicated, references to water shall conform to Specification D 1193 Type 1A reagent water (Type I reagent water filtered twice through a 0.2-µm filter to produce bacteria-free water).

7.3 *Phosphate Buffer Solution*—Dissolve 34.0 g of potassium dihydrogen phosphate (KH_2PO_4) in 500 mL of water. Adjust to pH 7.2 ± 0.05 with NaOH solution (40 g/L) and dilute to 1 L with water.

7.4 *Acridine Orange Solution*—Dissolve 10 mg of acridine orange in 100 mL of phosphate buffer. Filter small portions of the acridine orange solution through a 0.2-µm filter before use.

7.5 *Isopropanol*.

7.6 *Xylene*.

7.7 *Immersion Oil*, very low fluorescing (equivalent to Cargille Type A).

8. Procedure

8.1 Place a 0.2-µm membrane filter on the filter base and attach the funnel. Add 10 mL of buffered water to the funnel then add 1 mL of the water sample or dilution (use 9-mL dilution blanks). Turn on the vacuum.

8.2 Rinse the membrane with 5 mL of sterile reagent water.

8.3 Turn off the vacuum and flood the membrane with the acridine orange solution. Allow to stand for 3 to 4 min, then turn on the vacuum and filter through.

8.4 Rinse the membrane with 0.5 mL of isopropanol. Do not exceed 10-s contact time.

8.5 Rinse the membrane with 0.4 mL of xylene.

8.6 Remove the membrane and air dry for 15 s.

8.7 Place membrane on a clean microscope slide on which has been added 2 drops of fluorescence-free immersion oil.

8.8 Place another drop of immersion oil on top of membrane and apply cover slip.

8.9 Count cells using incident fluorescent illumination in violet light wavelength range (410 nm).

8.10 Count 20 fields at random within the stained portion of the membrane.

8.11 Count only that portion of the field which lies within the micrometer area.

8.12 Calculate the average number of bacteria per micrometer area.

8.13 Use the procedure outlined in Section 9 to determine bacterial density per millilitre of water sample.

8.14 Type IA water is used as a negative control and as a control against autofluorescing particle interferences.

8.15 Water sample may be preserved with 0.2 mL of 10 % formaldehyde per 10 mL of the sample.

9. Enumeration and Density Calculation

9.1 Bacterial densities are calculated for 25-mm filters as follows:

$$\text{Bacterial Density/mL} = (2.37 \times 10^4 n/d)$$

where:

n = average number of bacteria per net micrometer field; that is [(total number of bacteria counted)/(number of micrometer fields counted)], and

d = dilution factor.

2.37×10^4 is the membrane conversion factor based on a magnification of 1562.5 (eyepiece 12.5×) × (objective 100×) × (condexer unit 1.25×).

9.2 The membrane conversion factor of 2.37×10^4 for the above magnification is obtained as follows:

$$\text{(Wet area of 25 mm membrane/Area of micrometer)}$$

$$= (204.3 \text{ mm}^2/0.0086 \text{ mm}^2)$$
$$= 2.37 \times 10^4$$

Wet area is determined by measuring internal diameter of the funnel.

10. Report

10.1 The results are reported as number of bacteria per 1 mL of the sample.

11. Precision and Bias [8]

11.1 See Table 1 for the expression of single operator precision as S_O and overall precision as S_T.

11.2 See Table 1 for a statement on the bias of the test method.

[6] Filter unit H2 with BG12 and BG 38 transmission filters is available from Leitz Inc., 24 Link Dr., Rockleigh, NJ 07647.

[7] "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 "Reagent Chemicals and Standards," by Joseph Rosin, D. Van Nostrand Co., Inc., New York, NY, and the "United States Pharmacopeia."

[8] Supporting data for this test method have been filed at ASTM Headquarters. Request RR: D19-1118.

TABLE 1 Precision and Bias of Acridine Orange Epifluorescence Technique

NOTE 1—Two separate predetermined samples (A and B) were prepared and dispatched to six laboratories for conducting an interlaboratory study to obtain a precision statement. A bias statement cannot be included here because the positive or negative deviation of the method value from the accepted true value cannot be estimated.

Sample A [A]	Bacteria/mL	Sample B [A]	Bacteria/mL
	Total (×10 [4])		Total (×10 [6])
Repeatability: [B]		*Repeatability:* [B]	
n	5	*n*	5
mean	0.62	mean	8.6
S_T, Overall Precision [B]	0.28	S_T, Overall Precision	1.5
S_O, Single Operator [B] Precision	0.14	S_O, Single Operator Precision	0.52
Reproducibility: [C]		Reproducibility: [C]	
n	3.25	*n*	3.8
mean	0.73	mean	9.7
S_T, Overall Precision	0.2	S_T, Overall Precision	0.75
S_O, Single Operator Precision	0.37	S_O, Single Operator Precision	0.89

[A] where:

S_T=the average standard deviation calculated by pooling the sum of the squares, and

S_O=the square root of the quotient extracted from the sum of the individual analyst variances divided by the number of analysts.

[B] Reading of five (5) slides from a sample.

[C] Reading of one (1) slide five times from a sample.

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