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AMERICAN SOCIETY FOR TESTING AND MATERIALS
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Standard Test Method for Simultaneous Enumeration of Total and Respiring Bacteria in Aquatic Systems by Microscopy[1]

This standard is issued under the fixed designation D 4454; 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 covers the detection and enumeration of aquatic bacteria by the use of an acridine-orange epifluorescence direct-microscopic counting procedure. This test method is applicable to environmental waters and potable waters.

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

1.3 The procedure described 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 Minimal cell size which allows the detection of formazan deposits is represented by bacteria of 0.4 µm.[2]

1.7 *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]

4.1 A water sample is treated with an aqueous solution of INT-dye (2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride) for 20 min. The reaction then is stopped by adding a 37 % solution of formaldehyde. Sample is filtered through a 0.1-µm pore size polycarbonate membrane filter (presoaked in sudan black solution or equivalent), and stained with acridine orange for 3 min.

4.2 The filter is then air-dried and examined under oil immersion for total bacteria under epifluorescence illumination and for respiring bacteria under transmitted bright light illumination.

5. Significance and Use

5.1 Measurement of bacterial densities is generally the first step in establishing a relationship between bacteria and other biochemical processes.[5] It is known that the classical plate count procedure underestimates bacterial densities while the epifluorescence direct microscopic procedure more accurately depicts the total numbers of nonviable or dormant and viable cells in a water sample. The acridine-orange INT-formazan reduction technique provides information on the total concentrations of bacteria as well as that proportion which are actively respiring and thus involved in degradative processes.

5.2 The acridine-orange INT-formazan reduction technique is both quantitative and precise.

5.3 This procedure is ideal for enumerating both pelagic and epibenthic bacteria in all fresh water and marine environments.

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

6. Apparatus

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

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

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

6.4 *High-Pressure Mercury Lamp*, 200-W, on a UV light

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[1] This test method is under the jurisdiction of ASTM Committee D-19 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] Zimmerman, *et al*, "Simultaneous Determination of Total Number of Aquatic Bacteria and the Number Thereof Involved in Respiration," *Applied and Environmental Microbiology*, Vol 36, 1978, pp. 926–935

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

source giving vertical illumination, and a filter unit H2 (Leitz) [6] with BG12 and BG38 transmission filters or equivalents.

6.5 *Stage Micrometer*, 2 by 200 parts.

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

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

6.8 *Membrane Filter*, sterile plain regular polycarbonate, 25-mm (0.1-µm pore size).

6.9 *Filter Apparatus*, that should contain 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 IA reagent water (Type I reagent water which has been 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 the 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 *Aqueous INT-Dye* (0.2 %)—Dissolve 200 mg of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride in 100 mL of water.

7.6 *Sudan Dye Solution*—Dissolve 100 mg of Sudan Black B or equivalent in 75 mL of absolute ethanol then add 75 mL of water and mix.

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

7.8 *Formaldehyde*, 37 % solution.

8. Procedure

8.1 *Sample Processing*:

8.1.1 Place 10 mL of the sample into a clean, sterile test tube. Add 1 mL of 0.2 % aqueous INT-dye 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride.

8.1.2 Mix carefully and hold the sample in the dark at *in situ* temperature for approximately 20 min.

8.1.3 Stop the reaction by adding 0.1 mL of 37 % formaldehyde that also acts as preservative (at this stage the sample can be stored at 4°C up to one month).

8.2 *Membrane Filtration and Microscopic Examination*:

8.2.1 Filter 1 mL of the (INT) treated/preserved sample through 0.1-µm polycarbonate membrane which has been presoaked for 24 h in a solution of sudan black B (BDH) in 50 % ethanol.

8.2.2 Stain the filter with 3 mL of acridine orange for 3 min.

8.2.3 Filter the acridine orange.

8.2.4 Remove the filter, and air-dry for 15 s.

8.2.5 Place the membrane on a clean slide on which has been added ≃1 to 2 drops of very low fluorescing immersion oil.

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

8.2.7 Count cells using incident fluorescent illumination in a violet light wavelength range (410 nm) for total bacteria.

8.2.8 Switch to bright field illumination and count cells showing only bright red spots (indication of respiring bacteria).

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

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

8.2.11 Calculate the average number of both total and respiring bacteria per micrometer area.

8.2.12 Use the procedure outlined below to determine bacterial densities per millilitre of water sample.

8.2.13 Use Type IA water as a negative control and as a control against autofluorescing particle interference.

9. Enumeration and Density Calculation

9.1 Bacterial densities are calculated as follows:

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

where:

n = average number of bacteria per net micrometer field, that is [(total number of bacteria counted)/(number of micrometre 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×) × (Leitz Ploempak unit 1.25×).

9.2 The conversion factor of 2.37×10^4 for the 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 Report results as total number of bacteria per millilitre of sample and as total number of active bacteria per millilitre.

10.2 The results can also be expressed as the percentage of microbial populations that are actively respiring.

11. Precision and Bias [8]

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

[6] Filter unit H2 with BG12 and BG38 transmission filters is available from Leitz Inc., 24 Link Dr., Rockleigh, NJ 07647.

[7] "Reagent Chemicals, American Chemical Society Specifications," Am. Chemical Soc., 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., NY, and the "United States Pharmacopeia."

[8] Supporting data for this test method have been filed at ASTM Headquarters. Request RR: D19-1117.

TABLE 1 Summary of Precision and Bias—Acridine-Orange INT-Formazan Reduction Technique to Estimate Total and Respiring Aquatic Bacteria

NOTE 1—Two separate predetermined samples (A and B) were prepared and dispatched to three independent laboratories for conducting an interlaboratory study to obtain a precision statement. The information from these laboratories is summarized in the table. The bias statement cannot be included here because the persistent 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 ($\times 10^6$)	Respiring ($\times 10^4$)		Total ($\times 10^6$)	Respiring ($\times 10^4$)
Repeatability:[B]			*Repeatability:*[B]		
n	5	5	*n*	5	5
mean	1.4	4.2	mean	9.6	2.9
S_T, Overall Precision	0.25	4.2	S_T, Overall Precision	2.9	2.3×10^6
S_O, Single Operator Precision	0.14	3.1	S_O, Single Operator Precision	1.8	2.3
Reproducibility:[C]			*Reproducibility:*[C]		
n	5	5	*n*	5	5
mean	1.2	4.0	mean	6.96	0.4
S_T, Overall Precision	0.54	3.8	S_T, Overall Precision	4.6	2.8×10^6
S_O, Single Operator Precision	0.11	1.4	S_O, Single Operator Precision	0.4	0.1

[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.

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