Designation: D 4454 – 85 (Reapproved 2002)

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×).

[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] 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.