



Designation: D 4455 – 85 (Reapproved 1997)

## Standard Test Method for Enumeration of Aquatic Bacteria by Epifluorescence Microscopy Counting Procedure<sup>1</sup>

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 ( $\epsilon$ ) 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.<sup>2</sup>

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.<sup>2</sup>

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<sup>3</sup>

D 1193 Specification for Reagent Water<sup>3</sup>

D 3370 Practices for Sampling Water from Closed Conduits<sup>3</sup>

### 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- $\mu$ m 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.<sup>4</sup>

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.<sup>5</sup>

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 $\times$ ).

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

<sup>1</sup> 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. Current edition approved Jan. 25, 1985. Published March 1985.

<sup>2</sup> DIFCO Technical Information—Bacto Acridine Orange Stain, is available from Difco Laboratories, P.O. Box 1058, Detroit, MI 48201.

<sup>3</sup> *Annual Book of ASTM Standards*, Vol 11.01.

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

<sup>5</sup> 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.