Designation: D5259 – 14

Standard Test Method for Isolation and Enumeration of Enterococci from Water by the Membrane Filter Procedure[1]

This standard is issued under the fixed designation D5259; 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 a membrane filter (MF) procedure for the detection and enumeration of the enterococci bacteria in water. The enterococci, which include *Enterococcus faecalis* (*E. faecalis*), *E. faecium*, and their varieties are commonly found in the feces of humans and other warm-blooded animals. Although some strains are ubiquitous and not related to fecal pollution, enterococci in water are an indication of fecal pollution and the possible presence of enteric pathogens. These bacteria are found in water and wastewater in a wide range of densities. The detection limit is one colony forming unit (CFU)/volume filtered.

1.2 This test method has been used successfully with temperate fresh and marine ambient waters, and wastewaters. It is the user's responsibility to ensure the validity of this test method for waters of untested types.

1.3 The values stated in SI units are to be regarded as the standard.

1.4 *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 and health practices and determine the applicability of regulatory limitations prior to use.* For specific hazard statements, see Section 9.

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D1129 Terminology Relating to Water

D1193 Specification for Reagent Water

D3370 Practices for Sampling Water from Closed Conduits

D3870 Practice for Establishing Performance Characteristics for Colony Counting Methods in Microbiology (Withdrawn 2000)[3]

3. Terminology

3.1 *Definitions:*

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

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *Enterococcus*—in this test method, *Enterococcus* species are those bacteria that produce red to maroon colonies with black or reddish-brown precipitate on underside, after incubation on mE agar and subsequent transfer to EIA medium.

3.2.1.1 *Discussion*—Enterococci include *E. faecalis, E. faecium, E. avium*, and their variants.

4. Summary of Test Method

4.1 The procedure given in this test method provides a direct count of bacteria in water based on the development of colonies on the surface of the membrane filter.[4] A water sample is filtered through the membrane that retains the bacteria. Following filtration, the membrane containing the bacterial cells is placed on a selective, medium, mE agar, and incubated for 48 h at 41°C, then transferred to EIA agar and held at 41°C for 20 min. Enterococci develop as red to maroon colonies with black or reddish-brown precipitate on the underside of the filter.

5. Significance and Use

5.1 The enterococci are indicators of the bacteriological quality for potable water, shellfish growing waters, ambient, and recreational waters. A direct relationship between swimming, associated gastroenteritis, and enterococci has been established through epidemiological studies and marine and fresh water bathing beaches. These studies have led to the development of criteria that can be used to establish bathing water standards based on established health-water quality relationships.

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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] 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] Cabelli, V. J., Dufour, A. P., Levin, M. A., McCabe, L. J., and Haberman, P. W., "Relationship of Microbial Indicators to Health Effects at Marine Bathing Beaches," *American Journal of Public Health* , Vol 69, 1979, pp. 690–696.

5.2 Since small or large volumes of water or dilutions thereof, can be analyzed by the membrane filter technique, a wide range of levels of enterococci in water can be enumerated and detected.

6. Interferences

6.1 Water with high levels of colloidal or suspended materials can clog the membrane filter pores and prevent filtration. Also, suspended materials cause spreading colonies that could interfere with target colonies and thereby prevent accurate counting.

6.2 Smaller sample size or sample dilution can be used to minimize the interference of turbidity or high-background (non-target) bacterial densities. Replicates of smaller sample volumes or dilutions of sample may be filtered and the results combined. If the membrane filter technique is not applicable, the most probable number (MPN) method for fecal streptococci is recommended, with verification.

6.3 In some samples, chemicals may have toxic effects on the target organism.

7. Apparatus

7.1 *Stereoscopic Microscope,* wide-field type with magnification of 10 to 15X.

7.2 *Microscope Lamp,* producing diffuse light from a cool, white fluorescent lamp adjusted to give maximum visibility.

7.3 *Counting Device,* hand tally or electronic.

7.4 *Pipet Container,* stainless steel, aluminum, or borosilicate glass, for glass pipets.

7.5 *Pipets,* sterile tip delivery bacteriological or Mohr, glass or plastic, of appropriate volume.

7.6 *Graduated Cylinders,* 100 to 1000 mL, covered with aluminum foil or kraft paper and sterile.

7.7 *Membrane Filtration Units,* (filter base and funnel), glass plastic or stainless steel, wrapped in aluminum foil or kraft paper and sterilized.

7.8 *Ultraviolet Unit,* for disinfecting the filtration unit (optional).

7.9 *Line Vacuum, Electric Vacuum Pump, or Aspirator,* for use as a vacuum source. In an emergency or in the field, a hand pump or a syringe equipped with a check valve to prevent the return flow or air, can be used.

7.10 *Flask,* filter, vacuum, usually 1 L, with appropriate tubing. A filter manifold to hold a number of filter bases is optional.

7.11 *Forceps,* straight or curved, with smooth tips to handle filters without damage.

7.12 *Thermometer,* checked against a National Institute of Standards and Technology (NIST) certified thermometer, or one traceable to an NIST thermometer.

7.13 *Petri Dishes,* sterile, plastic, 50 by 12 mm, with tight-fitting lids.

7.14 *Bottles,* milk dilution, borosilicate glass, screw-cap with neoprene liners, marked at 99 mL for 1 to 100 dilutions. Dilution bottles marked at 90 mL or tubes marked at 9 mL may be used for 1:10 dilutions.

7.15 *Inoculation Loops,* at least 3 mm diameter, and needles, nichrome or platinum wire, 26 B and S gage, in suitable holders.

7.16 *Incubator* maintained at 41 ± 0.5°C.

7.17 *Waterbath* maintained at 44 to 46°C for tempering agar.

7.18 *Test Tubes,* 150 by 20 mm, borosilicate glass or plastic.

7.19 *Caps,* aluminum or autoclavable plastic, for 20 mm diameter test tubes.

7.20 *Test Tubes,* screw-cap, borosilicate glass, 125 by 16 mm or other appropriate size.

8. Reagents and Materials

8.1 *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 where such specifications are available.[5] Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

8.1.1 The agar used in preparation of culture media must be of microbiological grade. Whenever possible, use commercial culture media as a means of quality control.

8.1.2 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean reagent water as defined by Type III of Specification D1193.

8.1.3 *Ethanol, Methanol or Isopropanol,* in a small, wide-mouth container, for flame-sterilization of pipets.

8.2 *Membrane Filters,* sterile, white, grid marked, 47 mm diameter, with 0.45 ± 0.02 µm pore size or other pore sizes for which the manufacturer provides data demonstrating equivalency.

8.3 *Buffered Dilution Water/Buffered Rinse Water:*

8.3.1 *Composition/Litre:*

Sodium dihydrogen phosphate (NaH_2PO_4)	0.58 g
Sodium monohydrogen phosphate (Na_2HPO_4)	2.50 g
Sodium chloride	8.50 g

8.3.2 *Preparation*—Dissolve the ingredients in 1 L of water in a flask and dispense in appropriate amounts for dilutions in screw-cap bottles or culture tubes or into containers for use as rinse water, or both. Autoclave after preparation at 121°C (15 lb pressure at sea level) for 15 min. The final pH should be 7.4 ± 0.2.

8.4 *mE Agar:*

[5] *Reagent Chemicals, American Chemical Society Specifications,* American Chemical Society, Washington, DC, www.chemistry.org. For suggestions on the testing of reagents not listed by the American Chemical Society, see "Analar Standards for Laboratory Chemicals," BDH Ltd. Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary,* U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD, http://www.usp.org.

8.4.1 *Composition of Basal Medium/Litre:*

Peptone	10.0 g
Sodium chloride	15.0 g
Yeast extract	30.0 g
Esculin	1.0
Actidione	0.05 g
Sodium azide	0.15 g
Agar	15.0 g
Water	1000 mL

8.4.2 *Preparation of Basal Medium*—Add 71.2 g of the above mE basal medium to 1 L of water in a flask and heat to boiling until ingredients dissolve. Autoclave at 121°C and 15 lb pressure for 15 min and cool in a 44 to 46°C water bath.

8.4.3 *Reagents Added After Sterilization*—Mix 0.25 g nalidixic acid in 5 mL water, add 0.2 mL of NaOH solution (400 g/L) to dissolve, and add to the litre of basal medium. Add 0.15 g triphenyl tetrazolium chloride separately to the basal medium and mix.

8.4.4 *Preparation of mE Agar Plates*—Pour the mE agar into 50 mm petri plates to a 4 to 5 mm depth (approximately 4 to 6 mL), and allow to solidify. The final pH of medium should be 7.1 ± 0.2. Store in a refrigerator.

8.5 *EIA Agar:*

8.5.1 *Composition of EIA Medium/Litre:*

Esculin	1.0 g
Ferric citrate	0.5 g
Agar	15.0 g
Water	1000 mL

8.5.2 *Preparation*—Add 16.5 g of dehydrated EIA medium to 1 L of water in flask and heat to boiling until ingredients are dissolved. Autoclave the EIA medium solution at 121°C (15 lb pressure at sea level) for 15 min and cool in a 44 to 46°C water bath. After cooling, pour the medium into 50-mm petri dishes to a depth of 4 to 5 mm (approximately 4 to 6 mL and allow to solidify. The final pH should be 7.1 ± 0.2 before autoclaving. Store in a refrigerator.

8.6 *Brain Heart Infusion (BHI) Broth:*

8.6.1 *Composition:*

Calf brain infusion	200.0 g
Beef heart infusion	250.0 g
Peptone	10.0 g
Sodium chloride	5.0 g
Disodium phosphate	2.5 g
Dextrose	2.0 g
Water	1000 mL

8.6.2 *Preparation*—Dissolve 37 g of dehydrated BHI broth in 1 L of water. Dispense in 8 to 10 mL volumes in screw-cap tubes and autoclave at 121°C (15 lb pressure at sea level) for 15 min. If the medium is not used the same day as prepared and sterilized, heat in boiling water bath for several min to remove absorbed oxygen, and cool quickly without agitation, remove absorbed oxygen, and cool quickly without agitation, just prior to inoculation. The final pH should be 7.4 ± 0.2.

8.7 *BHI Broth with 6.5 % NaCl:*

8.7.1 *Composition*—BHI broth with 6.5 % NaCl is the same as BHI broth in 8.6 with additional NaCl.

8.7.2 *Preparation*—Dissolve 60.0 g NaCl per litre of prepared BHI broth. Since most commercially available dehydrated media contain sodium chloride, this amount is taken into consideration in determining the final NaCl percentage above.

8.8 *BHI Agar:*

8.8.1 *Composition*—BHI agar contains the same components as BHI (see 8.6) with the addition of 15.0 g of agar per litre of BHI Broth.

8.8.2 *Preparation*—Add 15.0 g of agar and 37.0 g of BHI dehydrated broth to 1 L of water. Heat to boiling until ingredients are dissolved. Dispense 10 to 12 mL of medium in screw-cap test tubes and sterilize for 15 min at 121°C (15 lb pressure at sea level). Slant after sterilization. The final pH should be 7.4 ± 0.2.

8.9 *Bile Esculin Agar (BEA):*

8.9.1 *Composition/Litre:*

Bacto beef extract	3.0 g
Bacto peptone	5.0 g
Bacto oxgall	40.0 g
Bacto esculin	1.0 g
Ferric citrate	0.5 g
Bacto agar	15.0
Water	1000 mL

8.9.2 *Preparation*—Add 64.5 g of dehydrated BEA to 1 L water and heat to boiling to dissolve. Dispense in 8 to 10 mL volumes in tubes for slants or into flasks for subsequent plating. Autoclave at 121°C (15 lb pressure at sea level) for 15 min. Overheating may cause darkening of the medium. Cool to 44 to 46°C and dispense into sterile petri plates. The final pH should be 6.6 ± 0.2. Store in a refrigerator.

8.10 *Gram Stain*—Prepare according to APHA document.[6]

9. Hazards

9.1 The analyst/technician must know and observe the normal good laboratory practices and safety procedures required in a microbiology laboratory while preparing, using, and disposing of cultures, reagents, and materials, and while operating sterilization and other equipment and instrumentation.

9.2 Mouth-pipetting is prohibited.

10. Sample Collection, Preservation, and Holding Times

10.1 Sampling procedures are described in detail in Section II, A of the USEPA manual[7] and Practice D3370 and adherence to sample preservation procedure and holding time limits is critical to the production of valid data. Samples should not be analyzed if these conditions are not met.

10.1.1 *Storage Temperature and Handling Conditions*—Ice or refrigerate bacteriological samples at a temperature of 1 to 4°C during transit to the laboratory. Use insulated containers to ensure proper maintenance of storage temperature. Take care that sample bottles are not totally immersed in water during transit or storage.

10.1.2 *Holding Time Limitations*—Examine samples as soon as possible after collection. Do not hold samples longer than 8 h between collection and incubation of plates.

[6] *Standard Methods for Examination of Water and Wastewater*, 18th EJ., American Public Health Association, Washington, DC, 1992, pp. 9–48.

[7] Bordner, R., Winter, J. A., and Scarpino, P. V., (eds.), "Microbiological Methods for Monitoring the Environment, Water and Wastes," EPA-600/8-78-017, U.S. Environmental Protection Agency, Office of Research and Development, Environmental Monitoring and Support Laboratory—Cincinnati, Cincinnati, Ohio, 1978.

11. Temperature Checks

11.1 Check temperatures in incubators daily to ensure operation within stated limits.

11.2 Check thermometers at least annually against an NIST certified thermometer or one traceable to NIST. Check mercury columns for breaks.

NOTE 1—It is strongly recommended to replace all mercury thermometers with an alternative source such as Non-Hazardous Liquid Filled Glass Thermometers or digital thermometers that can be calibrated against NIST thermometers.

Warning—Mercury has been designated by many regulatory agencies as a hazardous material that can cause serious medical issues. Mercury, or its vapor, has been demonstrated to be hazardous to health and corrosive to materials. Caution should be taken when handling mercury and mercury containing products. See the applicable product Safety Data Sheet (SDS) for additional information. Users should be aware that selling mercury or mercury containing products, or both, into your state or country may be prohibited by law.

11.3 See recommendations on quality control for microbiological analyses in the USEPA's *Microbiological Methods for Monitoring the Environment*, I. Water and Wastes, Part IV, C.[7]

12. Procedure

12.1 Mark the petri dishes containing the mE Agar (see 8.4) and report forms with sample identification and sample volumes.

12.2 Place a sterile membrane filter on the filter base, grid-side up and attach the funnel to the base; the membrane filter is now held between the funnel and the base.

12.3 Shake the sample bottle vigorously about 25 times to distribute the bacteria uniformly, and measure the desired volume of sample or dilution into the funnel.

12.4 For ambient surface waters and wastewaters, select sample volumes based on previous knowledge of pollution level, to produce 20 to 60 enterococci colonies on membranes. Sample volumes of 1 to 100 mL are normally tested at half log intervals, for example: 100, 30, 10, 3 mL, etc.

12.5 Use smaller sample size or sample dilution to minimize the interference of turbidity or high bacterial densities. Multiple volumes of the same sample or dilution of sample may be filtered and the results combined.

12.6 Filter the sample and rinse the sides of the funnel at least twice with 20 to 30 mL of buffered rinse water. Turn off the vacuum and remove the funnel from the filter base.

12.7 Use sterile forceps to aseptically remove the membrane filter from the filter base and roll it onto the mE agar to avoid the formation of bubbles between the membrane and the agar surface. Reseat the membrane if bubbles occur. Close the dish, invert, and incubate at 41 ± 0.5°C for 48 h.

12.8 After incubation, transfer the membranes to EIA agar plates that have been at room temperature for 20 to 30 min, and incubate at 41°C for 20 min.

12.9 After incubation, count and record colonies on those membrane filters containing, if practical, 20 to 60 red to maroon colonies with black or reddish-brown precipitate on the underside of the membrane. Use magnification for counting and a small fluorescent lamp to give maximum visibility of colonies.

13. Verification Procedure

13.1 Red to maroon colonies with black or reddish-brown precipitate on the underside of the membrane filter after incubation on EIA agar can be verified as enterococci. Verification of colonies may be required in evidence gathering, and is also recommended as a quality control procedure upon initial use of the test and with changes in sample sites, lots of commercial media, or major ingredients in media compounded in the laboratory. The verification procedure is as follows:

13.1.1 Using a sterile inoculating needle, transfer cells from the centers of at least 10 well-isolated typical colonies each into a brain heart fusion broth (BHI) tube and onto a BHI agar slant. Incubate broth tubes for 24 h and slants for 48 h at 35 ± 0.5°C.

13.1.2 After 24 h incubation, transfer a loopful of material from each BHI broth tube to:

13.1.2.1 Bile esculin agar (BEA) plate and incubate at 35 ± 0.5°C for 48 h,

13.1.2.2 BHI broth tube and incubate at 45 ± 0.5°C for 48 h, and

13.1.2.3 BHI broth tube with 6.5 % NaCl and incubate at 35 ± 0.5°C for 48 h.

13.1.3 Observe for growth.

13.1.4 After 48 h incubation, apply a gram stain to growth from each BHI agar slant.

13.1.5 Gram positive cocci that grow in BHI broth at 45°C, in BHI broth that contains 6.5 % NaCl, and in BEA and hydrolizes esculin, are verified as enterococci.

14. Calculation

14.1 Use the following general rules to calculate the enterococci count per 100 mL of sample:

14.1.1 Select and count membranes ideally with 20 to 60 red to maroon colonies developing black or reddish-brown precipitate on the underside. Calculate the final value using the following formula:

$$\text{enterococci/100 mL} = \frac{\text{number of enterococci colonies}}{\text{volume of sample filtered (mL)}} \times 100$$

15. Report

15.1 Adjust counts based on verification and report density per 100 mL of sample.

16. Performance Characteristics Based on Single Laboratory Data (Practice D3870)

16.1 *Precision*—The degree of agreement of repeated measurements of the same parameter expressed quantitatively as the standard deviation or as the 95 % confidence limits of the mean computed from the results of a series of controlled determinations. Precision of the mE method was established by

Levin et al.[8] who indicated that the test method did not exceed the expected limits for counts having the Poisson distribution.

16.2 *Bias*—The persistent positive or negative deviation of the results from the assumed or accepted true value. The bias of the enterococci MF method with mE Agar has been reported to be + 2 % of the true value.[8]

16.3 *Specificity*—The ability of a test method to select or distinguish, or both, the target bacteria from other bacteria in the same water sample. The specificity characteristic of a test method is usually reported as the percent of false positive and false negative results. The specificity for this medium as reported for various environmental water samples was 10 % false positive and 11.7 % false negative.[8]

17. Precision and Bias

17.1 *Collaborative Study Data:*

17.1.1 A collaborative study was conducted among eleven volunteer laboratories, each with two analysts who independently tested local fresh and marine recreational waters and sewage treatment plant effluent samples, in duplicate.

17.1.2 The results of the study are shown in Fig. 1 where S_o equals the pooled standard deviation among replicate counts from a single analyst for three groupings (counts less than 30, counts from 30 to 50, and counts greater than 50) and S_B equals the pooled standard deviation between means of duplicates from analysts in the same laboratory for the same groupings. The precision estimates from this study did not differ with the water types tested.

17.1.3 By linear regression, the precision of the test method can be generalized as follows:

$$S_o = 0.103\,(X) + 2.42 \text{ count/plate}$$

$$S_B = 0.152\,(X) + 5.16 \text{ count/plate}$$

where:

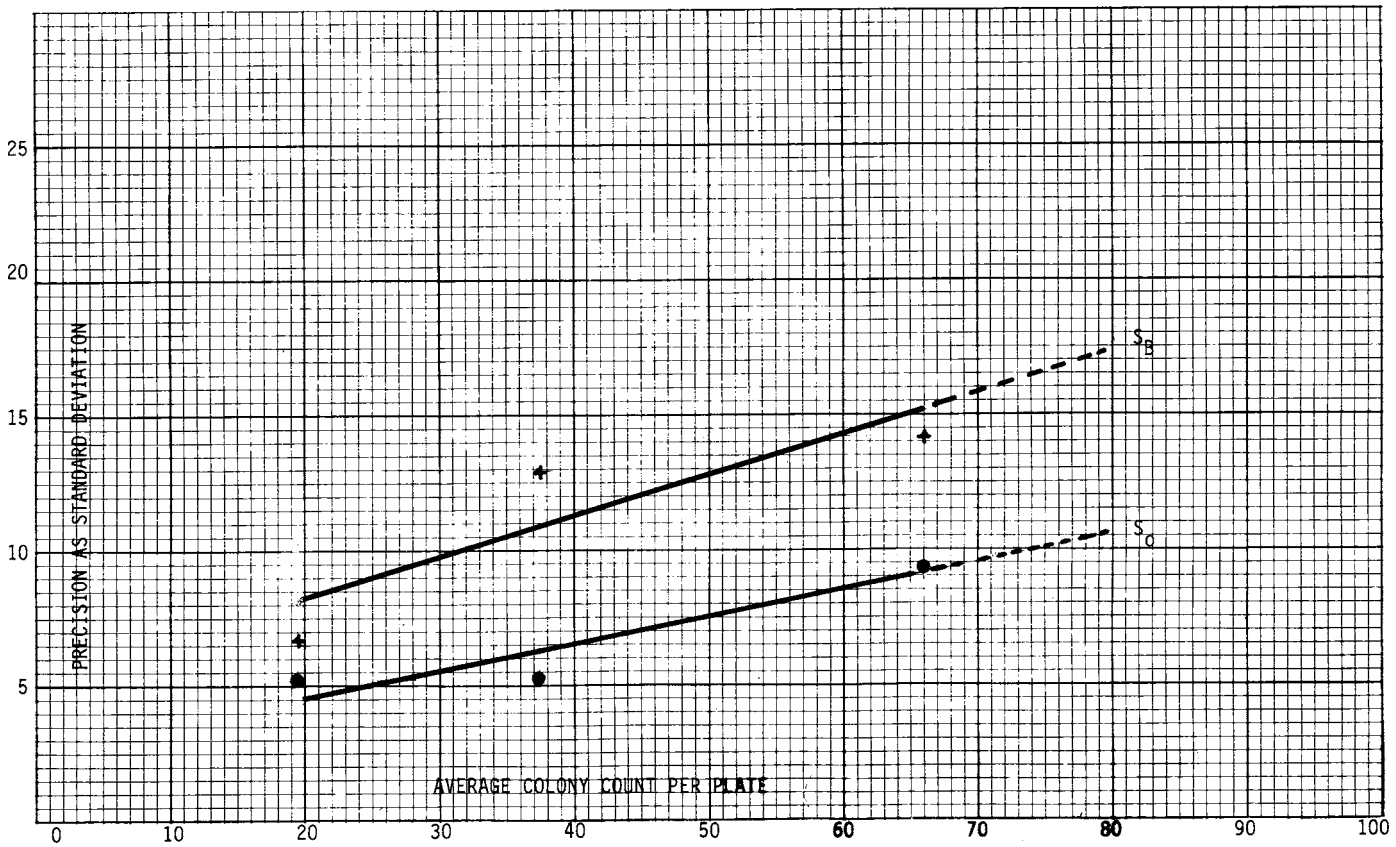
X = the count or average count/plate.

17.1.3.1 To convert the count/plate to count/100 mL, multiply the count by the dilution factor as follows:
where:

$$\text{dilution factor} = \frac{100}{\text{volume of original sample filtered}}$$

17.1.4 Because of the instability of microbial populations in water samples, each laboratory analyzed its own sample series and no full measure of recovery or bias was possible. However, all laboratories analyzed a single surrogate sample prepared from a freeze-dried culture of *Enterococcus faecalis*. The mean count (X) and the standard deviation of the counts (S_T)

[8] Levin, M. A., Fischer, J. R., and Cabelli, V. J., "Membrane Filter Technique for Enumeration of Enterococci in Marine Waters," *Applied Microbiology*, Vol 30, 1975, pp. 66–71.



NOTE 1—S_O = Standard deviation among replicate counts for a single analyst.
S_B = Standard deviation between the means of duplicate counts by analysts in the same job.

FIG. 1 Precision Estimates for Enterococci in Water by the Membrane Filter/mE Procedure

(including the variability among laboratories for this standardized enterococci sample) were 32.5 colonies/membrane and 9.42 colonies/membrane, respectively.

18. Keywords

18.1 enterococci; *Enterococcus faecalis*; *Enterococcus faecium*; *Enterococcus avium*; fecal pollution; m-E agar; two-step membrane filter procedures