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Method 1106.1: Enterococci in Water by Membrane Filtration Using membrane-Enterococcus-Esculin Iron Agar (mE-EIA)

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Method 1106.1: *Enterococci* in Water by Membrane Filtration Using membrane-Enterococcus-Esculin Iron Agar (mE-EIA)

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1.0 Scope and Application

1.1 This method describes a membrane filter (MF) procedure for the detection and enumeration of the enterococci bacteria in water. The enterococci are commonly found in the feces of humans and other warm-blooded animals. Although some strains are ubiquitous and not related to fecal pollution, the presence of enterococci in water is an indication of fecal pollution and the possible presence of enteric pathogens.

1.2 The enterococci test measures the bacteriological quality of recreational waters. Epidemiological studies have led to the development of criteria which can be used to promulgate recreational water standards based on the established relationship between health effects and water quality. The significance of finding enterococci in recreational water samples is the direct relationship between the density of enterococci in the water and swimming-associated gastroenteritis studies of marine and fresh water bathing beaches (Reference 18.3).

1.3 The test for enterococci can be applied to potable, fresh, estuarine, marine, and shellfish growing waters.

1.4 Since a wide range of sample volumes or dilutions can be analyzed by the MF technique, a wide range of enterococci levels in water can be detected and enumerated.

2.0 Summary of Method

2.1 The MF method provides a direct count of bacteria in water based on the development of colonies on the surface of the membrane filter (Reference 18.4). A water sample is filtered through the membrane which 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$\pm$ 0.5°C. Following incubation, the filter is transferred to a differential medium, EIA agar, and incubated at 41$\pm$ 0.5°C for and additional 20 min. Pink to red enterococci colonies will develop a black or reddish-brown precipitate on the underside of the filter. These colonies are counted with a fluorescent lamp and a magnifying lens.

3.0 Definitions

3.1 In this method, enterococci are those bacteria which produce pink to red colonies after incubation on mE agar and that form a with black or reddish-brown precipitate after subsequent transfer to EIA medium. Enterococci include *Streptococcus faecalis*, *Streptococcus faecium*, *Streptococcus avium*, and their variants.

4.0 Interferences and Contamination

4.1 Water samples containing collodial or suspended particulate materials can clog the membrane filter and prevent filtration, or cause spreading of bacterial colonies which could interfere with identification of target colonies.

5.0 Safety

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

5.2 Mouth-pipetting is prohibited.

6.0 Equipment and Supplies

6.1 Glass lens with magnification of 2-5x or stereoscopic microscope.

6.2 Lamp, with a cool, white fluorescent tube.

6.3 Hand tally or electronic counting device.

6.4 Pipet container, stainless steel, aluminum or borosilicate glass, for glass pipets.

6.5 Pipets, sterile, T.D. bacteriological or Mohr, glass or plastic, of appropriate volume.

6.6 Graduated cylinders, 100-1000 mL, covered with aluminum foil or kraft paper and sterile.

6.7 Membrane filtration units (filter base and funnel), glass, plastic or stainless steel, wrapped with aluminum foil or kraft paper and sterilized.

6.8 Ultraviolet unit for sanitization of the filter funnel between filtrations (optional).

6.9 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 of air, can be used.

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

6.11 Flask for safety trap placed between the filter flask and the vacuum source.

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

6.13 Ethanol, methanol or isopropanol in a small, wide-mouth container, for flame-sterilizing forceps.

6.14 Burner, Bunsen or Fisher type, or electric incinerator unit for sterilizing loops and needles.

6.15 Thermometer, checked against a National Institute of Standards and Technology (NIST) certified thermometer, or one that meets the requirements of NIST Monograph SP 250-23.

6.16 Petri dishes, sterile, plastic, 9 x 50 mm, with tight-fitting lids.

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

6.18 Flasks, borosilicate glass, screw-cap, 250-2000 mL volume.

6.19 Membrane filters, sterile, white, grid marked, 47 mm diameter, with 0.45 ± 0.02 µm pore size.

6.20 Inoculation loops, at least 3 mm diameter, and needles, nichrome or platinum wire, 26 B & S gauge, in suitable holders. Sterile disposable applicator sticks or plastic loops are alternatives to inoculation loops.

6.21 Incubator maintained at 41 ± 0.5°C.

6.22 Waterbath maintained at 50°C for tempering agar.

6.23 Test tubes, 20 x 150 mm, borosilicate glass or plastic.

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

6.25 Test tubes, screw-cap, borosilicate glass, 16 x 125 mm or other appropriate size.

6.26 Whirl-Pak® bags.

7.0 Reagents and Standards

7.1 Purity of Reagents: Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society (Reference 18.6). The agar used in preparation of culture media must be of microbiological grade.

7.2 Whenever possible, use commercial culture media as a means of quality control.

7.3 Purity of Water: Reagent water conforming to Specification D1193, reagent water conforming Type II, Annual Book of ASTM Standards (Reference 18.1).

7.4 Phosphate Buffered Saline

7.4.1 Composition:

Sodium Dihydrogen Phosphate	0.58 g
Sodium Monohydrogen Phosphate	2.5 g
Sodium Chloride	8.5 g
Reagent-Grade Distilled Water	1.0 L

7.4.2 Preparation: Dissolve the ingredients above in 1 L of reagent-grade distilled water in a flask, and dispense in appropriate amounts for dilutions in screw-cap bottles or culture tubes, and/or into containers for use as rinse water. Autoclave at 121°C (15 lb pressure) for 15 min. Final pH should be 7.4 ± 0.2.

7.5 Phosphate Buffered Dilution Water (Reference 18.2)

7.5.1 Composition of Stock Phosphate Buffer Solution:

Phosphate dihydrogen phosphate	34.0 g
Reagent-Grade distilled water	500.0 mL

7.5.2 Preparation: Adjust the pH of the solution to 7.2 with 1 N NaOH, and bring the volume to 1 L with reagent-grade distilled water. Sterilize by filtration or autoclave at 121°C (15 lb pressure) for 15 min.

7.5.3 Preparation of Stock Magnesium Chloride Solution: Add 38 g anhydrous $MgCl_2$ or 81.1 g $MgCl_2 \cdot 6H_2O$ to 1 L reagent-grade distilled water. Sterilize by filtration or autoclave at 121°C (15 lb pressure) for 15 min.

7.5.4 Storage of Stock Solutions: After sterilization, store the stock solutions in the refrigerator until used. Handle aseptically. If evidence of mold or other contamination appears, the affected stock solution should be discarded and a fresh solution should be prepared.

7.5.5 Working Phosphate Buffered Dilution Water: Mix 1.25 mL of the stock phosphate buffer and 5 mL of the $MgCl_2$ stock per liter of reagent-grade distilled water. Dispense in appropriate amounts for dilutions in screw-cap bottles or culture tubes, and/or into containers for use as rinse water. Autoclave at 121°C (15 lb pressure) for 15 min. Final pH should be 7.0 ± 0.2.

7.6 mE Agar

7.6.1 Composition of Basal Medium Ingredients:

Peptone	10.0 g
Sodium Chloride	15.0 g
Yeast Extract	30.0 g
Esculin	1.0 g
Actidione (Cycloheximide)	0.05 g
Sodium Azide	0.15 g
Agar	15.0 g
Reagent-Grade Distilled Water	1.0 L

7.6.2 Preparation of Basal Medium: Add dry ingredients to 1 L of reagent-grade distilled water in a flask, and heat to boiling until the ingredients dissolve using a magnetic stirrer. Autoclave at 121°C (15 lb pressure) for 15 min, and cool in a 50°C waterbath.

7.6.3 Reagents Added After Sterilization: Mix 0.24 g nalidixic acid in 5 mL of reagent-grade distilled water, add 0.2 mL of 10 N NaOH. Allow the mixture to dissolve, and add the mixture to the basal medium. Add 0.15 g triphenyltetrazolium chloride to the basal medium and mix.

7.6.4 Alternately, the following solutions may be used:

7.6.4.1 Nalidixic acid: Add 0.48 g of nalidixic acid and 0.4 mL 10 N NaOH to 10 mL of reagent-grade distilled water and mix. Filter-sterilize the solution, and add 5.2 mL per liter of medium.

7.6.4.2 Triphenyltetrazolium chloride (TTC): Add 0.25 g of TTC to 25 mL of reagent-grade distilled water, and warm to dissolve. Filter-sterilize the solution, and add 15 mL per liter of medium.

7.6.5 Preparation of mE Agar Plates: Pour the mE Agar into 9x50 mm petri dishes to a 4–5 mm depth (approximately 4–6 mL), and allow to solidify. Final pH of medium should be 7.1 ± 0.2. Store in a refrigerator.

7.7 Esculin Iron Agar (EIA)

7.7.1 Composition:

Esculin	1.0 g
Ferric Citrate	0.5 g
Agar	15.0 g
Reagent-Grade Distilled Water	1.0 L

7.7.2 Preparation: Add dry ingredients to 1 L of reagent-grade distilled water in a flask, and heat to boiling until the ingredients are dissolved. Autoclave the medium at 121°C (15 lb pressure) for 15 min, and cool in a 50°C waterbath. After cooling, pour the medium into 9x50 mm petri dishes to a depth of 4–5 mm (approximately 4–6 mL), and allow to solidify. Final pH should be 7.1 ± 0.2. Store in a refrigerator.

7.8 Brain Heart Infusion Broth (BHIB)

7.8.1 Composition:

Calf Brain Infusion	200.0 g
Beef Heart Infusion	250.0 g
Proteose Peptone	10.0 g
Sodium Chloride	5.0 g
Disodium Phosphate	2.5 g
Dextrose	2.0 g
Reagent-Grade Distilled Water	1.0 L

7.8.2 Preparation: Combine ingredients except reagent-grade distilled water. Dissolve 37 g of combined ingredients in 1 L of reagent grade water. Dispense in 10-mL volumes in screwcap tubes, and autoclave at 121°C (15 lb pressure) 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, just prior to inoculation. Final pH should be 7.4 ± 0.2.

7.9 Brain Heart Infusion Broth (BHIB) with 6.5% NaCl

7.9.1 Composition:

BHIB with 6.5% NaCl is the same as BHIB broth above, but with additional NaCl.

7.9.2 Preparation: Add 60.0 g NaCl per liter of medium. Since most commercially available dehydrated media already contain sodium chloride, that amount is subtracted from the 65 g per liter required to make a final concentration of 6.5% NaCl.

7.10 Brain Heart Infusion Agar (BHIA)

7.10.1 Composition:

BHIA contains the same components as BHIB (see above) with the addition of 15.0 g agar per liter of BHIB.

7.10.2 Preparation: Suspend 52 g dehydrated BHIA in 1 L of reagent-grade distilled water. Heat to boiling until the ingredients are dissolved. Dispense 10 mL of medium in screwcap test tubes, and sterilize for 15 min at 121°C (15 lb pressure). After sterilization, slant until solid. Final pH should be 7.4 ± 0.2.

7.11 Bile Esculin Agar (BEA)

7.11.1 Composition:

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 g
Reagent-Grade Distilled Water	1.0 L

7.11.2 Preparation: Combine dry ingredients. Add 64.0 g of dry ingredients to 1 L reagent-grade distilled water, and heat to boiling to dissolve completely. Dispense 10-mL volumes in tubes for slants or larger volumes into flasks for subsequent plating. Autoclave at 121°C (15 lb pressure) for 15 min. Overheating may cause darkening of the medium. Cool in a 50°C waterbath, and dispense into sterile petri dishes. Final pH should be 6.6 ± 0.2. Store in a refrigerator.

8.0 Sample Collection, Preservation, and Storage

8.1 Sampling procedures are described in detail in the USEPA microbiology methods manual, Section II, A (Reference 18.2). Adherence to sample preservation procedures and holding time limits is critical to the production of valid data. Samples should not be analyzed if these conditions are not met.

8.1.1 Storage Temperature and Handling Conditions

Ice or refrigerate bacteriological samples at a temperature of 1-4°C during transit to the laboratory. Use insulated containers to assure proper maintenance of storage temperature. Take care that sample bottles are not totally immersed in water during transit or storage.

8.1.2 Holding Time Limitations

Examine samples as soon as possible after collection. Do not hold samples longer than 6 h between collection and initiation of analyses.

9.0 Quality Control

9.1 See recommendations on quality control for microbiological analyses in the USEPA microbiology methods manual, Part IV, C (Reference 18.2).

10.0 Calibration and Standardization

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

10.2 Check thermometers at least annually against a NIST certified thermometer or one that meets the requirements of NIST monograph SP 250-23. Check mercury columns for breaks.

11.0 Procedure

11.1 Prepare the mE Agar as directed in section 7.6.

11.2 Mark the petri dishes and report form with the sample identification and volume.

11.3 Place a sterile membrane filter on the filter base, grid side up, and attach the funnel to the base so that the membrane filter is held between the funnel and the base.

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

11.5 Select sample volumes based on previous knowledge of the pollution level, to produce 20–60 enterococci colonies on the membranes. Sample volumes of 1–100 mL are normally tested at half-log intervals (*e.g.*, 100, 30, 10, 3 mL).

11.6 Smaller sample sizes or sample dilutions can be used to minimize the interference of turbidity or for high bacterial densities. Multiple volumes of the same sample or sample dilutions may be filtered, and the results may be combined.

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

11.8 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. Run the forceps around the edge of the filter to be sure that the filter is properly seated on the agar. Close the dish, invert, and incubate at 41 ± 0.5°C for 48 h (See Photo 1.).

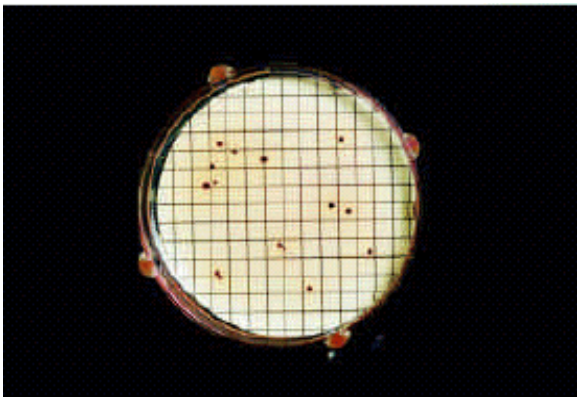

Photo 1. Enterococci on ME Agar. Colonies that are pink to dark red are considered to be presumptive enterococci.

11.9 After incubation, transfer the membranes to EIA plates that have been warmed up to room temperature for 20–30 min, and incubate at 41 ± 0.5/C for an additional 20–30 min. (See Photo 2.)

11.10 After the second incubation, count and record colonies on those membrane filters containing, if practical, 20–60 pink-to-red 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.

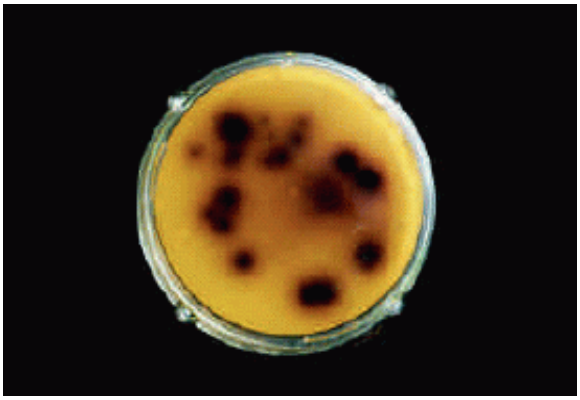

Photo 2. Enterococci on Esculin Iron Agar (EIA). Colonies that are pink to dark red on mE Agar and have a reddish brown to black precipitate on the underside of the filter when placed on EIA are confirmed as enterococci.

12.0 Data Analysis and Calculations

12.1 Use the following general rules to calculate the enterococci count per 100 ml of sample:

12.1.1 Select the membrane filter with an acceptable number of pink-to-red colonies (20-60) that form a black or reddish-brown precipitate on the underside of the filter when placed on EIA. Calculate the number of enterococci per 100 mL according to the following general formula:

$$\text{Enterococci/100 mL} = \frac{\text{Number of enterococci colonies}}{\text{Volume of sample filtered (mL)}} \text{ X } 100$$

12.1.2 See the USEPA microbiology methods manual, Part II, Section C, 3.5, for general counting rules (Reference 18.2).

13.0 Method Performance

13.1 Performance characteristics

13.1.1 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.* (Reference 18.4) who indicated that the method did not exceed the expected limits for counts having the Poisson distribution.

13.1.2 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 the mE Agar has been reported to be +2% of the true value (Reference 18.4).

13.1.3 Specificity - The ability of a method to select and/or distinguish the target bacteria from other bacteria in the same water sample. The specificity characteristic of a 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 (Reference 18.4).

13.2 Collaborative study data

13.2.1 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. That data were reported to the Environmental Monitoring and Support Laboratory - Cincinnati, U.S. Environmental Protection Agency, for statistical analyses.

13.2.2 The results of the study are shown in Figure 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.

13.2.3 By linear regression, the precision of the method can be generalized as:

S_O = 0.013 count/100 mL + 2.42 (dilution factor) and

S_B = 0.152 count/100 mL + 5.16 (dilution factor)

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

13.2.4 Because of the instability of microbial populations in water samples, each laboratory analyzed is 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 *Streptococcus faecalis*. The mean count ($\bar{O}$) and the standard deviation of the counts (S_y) (including the variability among laboratories for this standardized enterococci sample) were 32.5 colonies/membrane and 9.42 colonies/membrane, respectively.

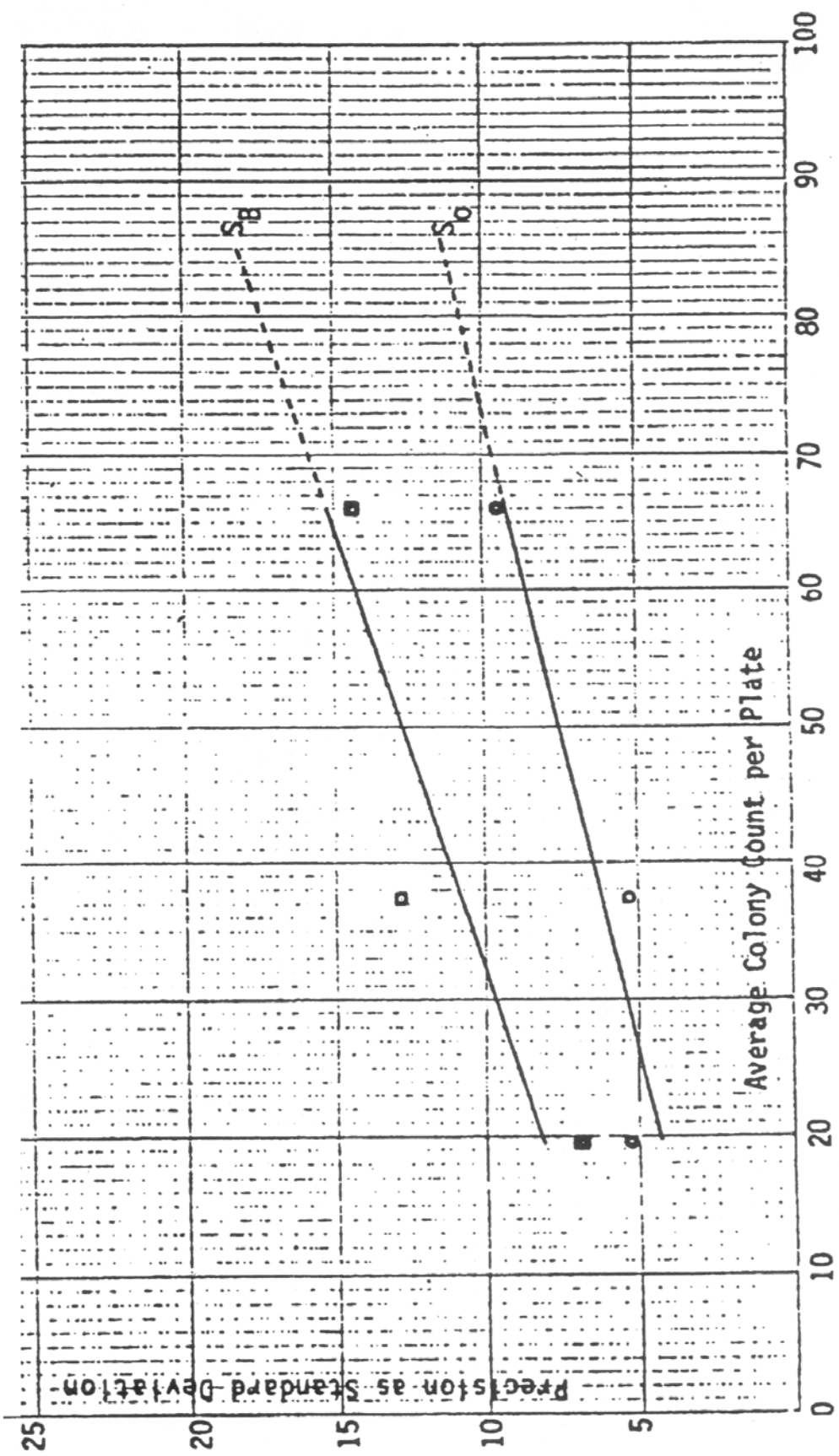

FIGURE 1. Precision Estimates for Enterococci in Water by the Membrane Filter/mE Procedure

14.0 Reporting Results

14.1 There should be at least three volumes tested per sample. Report the results as enterococci per 100 mL of sample.

15.0 Verification Procedure

15.1 Pink-to-red colonies on mE Agar that produce a black or reddish-brown precipitate after incubation on EIA agar can be verified as enterococci. Verification of colonies may be required in evidence gathering and it is also recommended as a means of quality control for the initial use of the test and for changes in sample sites, lots of commercial media, or major ingredients in media compounded in the laboratory. The verification procedure follows.

15.2 Using a sterile inoculating needle, transfer cells from the centers of at least 10 well-isolated typical colonies into a BHIB tube and onto a BHIA slant. Incubate broth tubes for 24 h and agar slants for 48 h at 35 ± 0.5°C.

15.3 After a 24 h incubation, transfer a loopful of material from each BHIB tube to BEA, BHIB and BHIB with 6.5% NaCl.

15.3.1 Incubate the BEA and BHIB with 6.5% NaCl at 35 ± 0.5°C for 48 h.

15.3.2 Incubate the BHIB at 45 ± 0.5°C for 48 h.

15.4 Observe for growth on all media.

15.5 After 48 h incubation, apply a Gram stain to growth from each BHIA slant.

15.6 Gram-positive cocci that grow and hydrolyze esculin on BEA (*i.e.*, produce a black or brown precipitate), and grow in BHIB at 45 ± 0.5°C and BHIB with 6.5% NaCl at 35 ± 0.5°C are verified as enterococci.

16.0 Pollution Prevention

16.1 The solutions and reagents used in this method pose little threat to the environment when recycled and managed properly.

16.2 Solutions and reagents should be prepared in volumes consistent with laboratory use to minimize the volume of expired materials to be disposed.

17.0 Waste Management

17.1 It is the laboratory's responsibility to comply with all federal, state, and local regulations governing waste management, particularly the biohazard and hazardous waste identification rules and land disposal restrictions, and to protect the air, water, and land by minimizing and controlling all releases from fume hoods and bench operations. Compliance with all sewage discharge permits and regulations is also required.

17.2 Samples, reference materials, and equipment known or suspected to have viable enterococci attached or contained must be sterilized prior to disposal.

17.3 Samples preserved with HCl to pH <2 are hazardous and must be neutralized before being disposed, or must be handled as hazardous waste.

17.4 For further information on waste management, consult "The Waste Management Manual for Laboratory Personnel" and "Less Is Better: Laboratory Chemical Management for Waste Reduction," both available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street NW, Washington, DC 20036.

18.0 References

18.1 Annual Book of ASTM Standards, Vol. 11.01, American Society for Testing and Materials, Philadelphia, PA 19103.

18.2 Bordner, R., J.A. Winter and P.V. Scarpino (eds.). 1978. Microbiological Methods for Monitoring the Environment, Water and Wastes, EPA-600/8-78-017. Office of Research and Development, USEPA.

18.3 Cabelli, V. J., A. P. Dufour, M. A. Levin, L. J. McCabe, and P. W. Haberman, 1979. Relationship of Microbial Indicators to Health Effects at Marine Bathing Beaches. *Amer. Jour. Public Health*. 69:690-696.

18.4 Levin, M. A., J. R. Fischer and V. J. Cabelli. 1975. Membrane Filter Technique for Enumeration of Enterococci in Marine Waters. *Appl. Microbiol*. 30:66-71.

18.5 Improved Enumeration Methods for the Recreational Water Quality Indicators: Enterococci and *Escherichia coli*. 2000. EPA/821/R-97/004. Office of Science and Technology, Washington D.C.

18.6 Reagent Chemicals, American Chemical Society Specifications, American Chemical Society, Washington, DC. For suggestions of the testing of reagents not listed by the American Chemical Society, see Analar Standards for Laboratory Chemicals, BDH Ltd., Poole, Dorset, UK and the United States Pharmacopeia.

18.7 Test Methods for *Escherichia coli* and Enterococci in Water by the Membrane Filter Procedure. 1985. EPA-600/4-85/076. Environmental Monitoring and Support Laboratory, Cincinnati, USEPA.