United States Environmental Protection Agency
Office of Water
EPA-821-R-23-006
www.epa.gov
September 2023

Method 1600.1: Enterococci in Water by Membrane Filtration Using membrane-*Enterococcus* Indoxyl-β-D-Glucoside Agar (mEI)

U.S. Environmental Protection Agency
Office of Water (4303T)
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Washington, DC 20460

EPA-821-R-23-006

Acknowledgments

This method was developed under the direction of James W. Messer and Alfred P. Dufour of the U.S. Environmental Protection Agency's (EPA) Human Exposure Research Division, National Exposure Research Laboratory, Cincinnati, Ohio.

The following laboratories are gratefully acknowledged for their participation in the validation of this method in wastewater effluents:

Volunteer Research Laboratories

- EPA Office of Research and Development, National Risk Management Research Lab: Mark C. Meckes
- U.S. Army Corps of Engineers, Washington Aqueduct: Elizabeth A. Turner, Michael L. Chicoine, and Lisa Neal

Volunteer Verification Laboratories

- City of Los Angeles Bureau of Sanitation: Farhana Mohamed, Ann Dalkey, Ioannice Lee, Genevieve Espineda, and Zora Bahariance
- Orange County Sanitation District, Environmental Sciences Laboratory: Charles McGee, Michael von Winckelmann, Kim Patton, Linda Kirchner, James Campbell, Arturo Diaz, and Lisa McMath

Volunteer Participant Laboratories

- City of Los Angeles Bureau of Sanitation: Farhana Mohamed, Ann Dalkey, Ioannice Lee, Genevieve Espineda, and Zora Bahariance
- County Sanitation Districts of Los Angeles County (JWPCP): Kathy Walker, Michele Padilla, and Albert Soof
- County Sanitation Districts of Los Angeles County (SJC): Shawn Thompson and Julie Millenbach
- Environmental Associates (EA): Susan Boutros and John Chandler
- Hampton Roads Sanitation District (HRSD): Anna Rule, Paula Hogg, and Bob Maunz
- Hoosier Microbiological Laboratories (HML): Don Hendrickson, Katy Bilger, and Lindsey Shelton
- Massachusetts Water Resources Authority (MWRA): Steve Rhode and Mariya Gofhsteyn
- North Shore Sanitation District (NSSD): Robert Flood
- Texas A&M University: Suresh Pillai and Reema Singh
- University of Iowa Hygienic Laboratory: Nancy Hall and Cathy Lord
- Wisconsin State Laboratory of Hygiene (WSLH): Jon Standridge, Sharon Kluender, Linda Peterson, and Jeremy Olstadt
- Utah Department of Health: Sanwat Chaudhuri and Devon Cole

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Method 1600.1 revisions include incorporation of editorial changes, standardizing language between the EPA membrane filtration methods, current lab practices and clarifying edits.

Questions concerning this method, or its application should be addressed to:

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Table of Contents

List of Appendices

Appendices A and B are taken from *Microbiological Methods for Monitoring the Environment. Water and Wastes* (Reference 18.6).

Appendix A: Part II (General Operations), Section A (Sample Collection, Preservation, and Storage).

Appendix B: Part II (General Operations), Sections C.3.5 (Counting Colonies) and C.3.6 (Calculation of Results).

Method 1600.1: Enterococci in Water by Membrane Filtration Using membrane-*Enterococcus* Indoxyl-β-D-Glucoside Agar (mEI)

September 2023

1.0 Scope and Application

1.1 Method 1600.1 describes a membrane filter (MF) procedure for the detection and enumeration of enterococci bacteria in ambient (fresh and marine) water and wastewater. This is a single-step method that is a modification of EPA Method 1106.1 (mE-EIA). Unlike the mE-EIA method, it does not require the transfer of the membrane filter to another medium. The modified medium has a reduced amount of triphenyltetrazolium chloride (TTC) and includes indoxyl β-D-glucoside, a chromogenic cellobiose analog used in place of esculin. In this procedure, β-glucosidase-positive enterococci produce an insoluble indigo blue complex which diffuses into the surrounding media, forming a blue halo around the colony.

1.2 Enterococci bacteria 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.

1.3 Epidemiological studies have led to the development of criteria which can be used for recreational water quality standards based on established relationships between health effects and water quality. The significance of finding enterococci in ambient fresh or marine water samples is the direct relationship between the density of enterococci and the risk of gastrointestinal illness associated with swimming in the water (References 18.1 and 18.2).

1.4 For method application please refer to Title 40 Code of Federal Regulations Part 136 (40 CFR Part 136).

2.0 Summary of Method

Method 1600.1 provides a direct count of bacteria in ambient waters (fresh and marine) and wastewater based on the development of colonies on the surface of the membrane filter (Reference 18.3). A water sample is filtered through the membrane filter which retains the bacteria. Following filtration, the membrane filter containing the bacterial cells is placed on a selective medium, mEI agar, and incubated for 24 ± 2 hours at 41°C ± 0.5°C. All colonies greater than or equal to (≥) 0.5 mm in diameter (regardless of color) with a blue halo are recorded as enterococci colonies. A fluorescent lamp with a magnifying lens is used for counting to give maximum visibility of colonies.

3.0 Definitions

In Method 1600.1, enterococci are those bacteria which produce colonies greater than or equal to 0.5 mm in diameter with a blue halo, regardless of color, after incubation on mEI agar. The blue halo should not be included in the colony diameter measurement. Enterococci include *Enterococcus faecalis (E. faecalis)*, *E. faecium*, *E. avium*, *E. gallinarium*, and their variants. The genus *Enterococcus* includes the enterococci formerly assigned to the Group D fecal streptococci.

4.0 Interferences and Contamination

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

4.2 The presence of high numbers of competing or inhibitory organisms, background debris, or toxic substances (e.g., metals or organic compounds) could interfere with enumeration of target colonies.

5.0 Safety

5.1 The analyst 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 The selective medium (mEI) and azide-dextrose broth used in this method contain sodium azide as well as other potentially toxic components. Caution must be exercised during the preparation, use, and disposal of these media to prevent inhalation or contact with the medium or reagents.

5.3 Mouth-pipetting is prohibited.

5.4 This method does not address all of the safety issues associated with its use. It is the responsibility of the laboratory to establish appropriate safety and health practices prior to use of this method. A reference file of safety data sheets (SDSs) should be available to all personnel involved in Method 1600.1 analyses.

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 or aluminum for reusable pipets

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

6.6 Sterile graduated cylinders, 100-1000 mL, covered with aluminum foil or kraft paper

***Note**: Graduated cylinders should be accurate to within 2.5% (Class B or better) .*

6.7 Sterile membrane filtration units (filter base and funnel), glass, plastic, disposable, or stainless steel, wrapped with aluminum foil or kraft paper, or in sterilization pouches

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

6.11 A filter manifold to hold a number of filter bases or vacuum filter flask (1 L) with appropriate tubing

6.12 Flask for safety trap placed between the filtration apparatus and the vacuum source

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

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

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

6.16 Thermometer (digital or non-mercury), verified against a National Institute of Standards and Technology (NIST) certified thermometer, or one that meets the requirements of NIST Handbook 105-6

6.17 Balance, analytical balance capable of weighing 0.1 mg

6.18 pH meter

6.19 Petri dishes, sterile, plastic, 15 × 60 mm with loose fitting lids or 15 × 100 mm with loose fitting lids

6.20 Dilution bottles (e.g., milk dilution), borosilicate glass, screw cap with neoprene liners, 125 mL volume with markings at 99 mL or 90 mL to prepare 1:100 and 1:10 dilutions, respectively

6.21 Flasks, borosilicate glass, screw cap, 250-2000 mL volume

6.22 Cellulose ester membrane filters, sterile, white, grid marked, 47 mm diameter, with 0.45 µm pore size

6.23 Platinum wire inoculation loops, at least 3 mm diameter in suitable holders; or sterile plastic loops

6.24 Incubator maintained at 41°C ± 0.5°C

6.25 Water bath for tempering media

6.26 Cornwall syringe, sterile, to deliver at least 5 mL or media dispensing pump

6.27 Test tubes, borosilicate glass, 16 × 125 mm or other appropriate size(s) and autoclavable caps

6.28 Autoclave or steam sterilizer capable of achieving 121°C (15 lb pressure per square inch [PSI]) for 15 minutes

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 18.4). 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 reagent water: Reagent-grade water conforming to specifications in: *Standard Methods for the Examination of Water and Wastewater* (latest edition approved by EPA in 40 CFR Part 136), Section 9020 (Reference 18.5).

7.4 Phosphate buffered saline (PBS)

7.4.1 Composition:

Sodium dihydrogen phosphate (NaH_2PO_4)	0.58 g
Disodium hydrogen phosphate (Na_2HPO_4)	2.5 g
Sodium chloride (NaCl)	8.5 g
Reagent-grade water	1.0 L

7.4.2 Dissolve the reagents in 1 L of reagent-grade water and dispense in appropriate amounts for dilutions in screw cap bottles or culture tubes, and/or into containers for use as rinse water. Autoclave after preparation at 121°C (15 PSI) for 15 min. Final pH should be 7.4 ± 0.2. Store at <10°C, but above freezing, for up to three months in screw cap containers.

7.5 mEI Agar

7.5.1 Composition:

Peptone	10.0 g
Sodium chloride (NaCl)	15.0 g
Yeast extract	30.0 g
Esculin	1.0 g
Actidione (Cycloheximide)	0.05 g
Sodium azide	0.15 g
Indoxyl ß-D-glucoside	0.75 g
Agar	15.0 g
Reagent-grade water	1.0 L

7.5.2 Add reagents to 1 L of reagent-grade water, mix thoroughly, and heat to dissolve completely. Autoclave at 121°C (15 PSI) for 15 minutes and cool to 45°C -50°C in a water bath or according to manufacturer's instructions.

7.5.3 Prepare a solution of 0.24 g of nalidixic acid (sodium salt) in 5 mL of purified water. Add a few drops of 0.1 N NaOH to dissolve.

7.5.4 After cooling the mEI medium add the nalidixic acid solution (Section 7.5.3) and 0.02 g triphenyltetrazolium chloride (TTC) and mix thoroughly.

***Note**: The amount of TTC used in this medium (mEI) is less than the amount used for mE agar in Method 1106.2.*

7.5.5 Dispense ~ 4-6 mL of mEI agar into 15 × 60 mm sterile Petri dishes to a 4-5 mm depth and allow to solidify. Final pH of medium should be 7.1 ± 0.2. Invert and store prepared plates at <10°C, but above freezing, for up to two weeks in the dark, as exposure to light can cause degradation of the medium.

7.6 Tryptic soy agar (TSA)

7.6.1 Composition:

Pancreatic digest of casein	15.0 g
Enzymatic digest of soybean meal	5.0 g
Sodium chloride (NaCl)	5.0 g
Agar	15.0 g
Reagent-grade water	1.0 L

7.6.2 Add reagents to 1 L of reagent-grade water, mix thoroughly, and heat to dissolve completely. Autoclave at 121°C (15 PSI) for 15 minutes and cool to 45°C -50°C in a water bath or according to manufacturer's instructions. After cooling, dispense ~ 12-15 mL of TSA into each sterile Petri dish to a 4-5 mm depth, and allow to solidify. Final pH should be 7.3 ± 0.2. Invert and store prepared plates at <10°C, but above freezing, for up to two weeks.

7.7 Brain heart infusion broth (BHIB)

7.7.1 Composition:

Calf brains, infusion from 200.0 g	7.7 g
Beef heart, infusion from 250.0 g	9.8 g
Proteose peptone	10.0 g
Sodium chloride (NaCl)	5.0 g
Disodium hydrogen phosphate (Na_2HPO_4)	2.5 g
Dextrose	2.0 g
Reagent-grade water	1.0 L

7.7.2 Add reagents to 1 L of reagent-grade water, mix thoroughly, and heat to dissolve completely. Dispense 10-mL volumes in screw cap tubes, and autoclave at 121°C (15 PSI) for 15 minutes. Final pH should be 7.4 ± 0.2. Store at <10°C, but above freezing, for up to three months in screw cap tubes or up to two weeks in loose cap tubes.

7.8 Brain heart infusion broth (BHIB) with 6.5% NaC1

7.8.1 Composition:

BHIB with 6.5% NaC1 is the same as BHIB above (Section 7.7), but with additional NaC1.

7.8.2 Add NaCl to formula provided in Section 7.7 above, such that the final concentration is 6.5% (65 g NaCl/L). Typically, for commercial BHIB media, an additional 60.0 g NaCl per liter of medium will need to be added to the medium. Prepare and store as in Section 7.7.2.

7.9 Brain heart infusion agar (BHIA)

7.9.1 Composition:

BHIA contains the same components as BHIB (Section 7.7), with the addition of 15.0 g agar per liter of BHIB.

7.9.2 Add agar to formula for BHIB provided in Section 7.7 above. Prepare as in Section 7.7.2. After sterilization, slant until solid. Final pH should be 7.4 ± 0.2. Store slants at <10°C, but above freezing, for up to three months in screw cap tubes.

7.10 Bile esculin agar (BEA)

7.10.1 Composition:

Beef extract	3.0 g
Pancreatic digest of gelatin	5.0 g
Oxgall	20.0 g
Esculin	1.0 g
Ferric citrate	0.5 g
Bacto agar	14.0 g
Reagent-grade water	1.0 L

7.10.2 Add reagents to 1 L reagent-grade water, heat with frequent mixing, and boil 1 minute to dissolve completely. Dispense 10-mL volumes in tubes for slants or larger volumes into flasks for subsequent plating. Autoclave at 121°C (15 PSI) for 15 minutes. Overheating may cause darkening of the medium. To prepare plates, cool medium in a 45 - 50°C water bath or according to manufacturer's instructions. After cooling, dispense ~ 12-15 mL of BEA into sterile Petri dishes to a 4-5 mm depth and allow to solidify. Final pH should be 6.8 ± 0.2. Store at <10°C, but above freezing, for up to two weeks (plates) or up to three months in screw cap tubes (slants).

7.11 Azide dextrose broth (ADB)

7.11.1 Composition:

Beef extract	4.5 g
Pancreatic digest of casein	7.5 g
Proteose peptone No. 3	7.5 g
Dextrose	7.5 g
Sodium chloride (NaCl)	7.5 g
Sodium azide	0.2 g
Reagent-grade water	1.0 L

7.11.2 Add reagents to 1 L of reagent-grade water and dispense in screw cap bottles. Autoclave at 121°C (15 PSI) for 15 minutes. Final pH should be 7.2 ± 0.2. Store at <10°C, but above freezing, for up to three months in screw cap bottles.

7.12 Control cultures

7.12.1 Positive control and/or spiking organism (either of the following are acceptable)

- Stock cultures of *Enterococcus faecalis* (*E. faecalis*) ATCC® # 19433™
- *E. faecalis* ATCC® # 19433™ BioBalls (bioMérieux Inc., Durham NC)

7.12.2 Negative control organism (either of the following are acceptable)

- Stock cultures of *Escherichia coli* (*E. coli*) ATCC® # 11775™
- *E. coli* ATCC® # 11775™ BioBalls (bioMérieux Inc., Durham NC)

8.0 Sample Collection, Handling, and Storage

Sampling procedures are briefly described below. Detailed sampling methods can be found in *Microbiological Methods for Monitoring the Environment. Water and Wastes*, Part II, Section A Reference 18.6, and Appendix A. Adherence to sample preservation procedures and holding time limits is critical to the production of valid data. Samples should only be analyzed if the conditions below are met.

8.1 Sampling techniques

Samples are collected by hand or with a sampling device if the sampling site has difficult access such as a dock, bridge, or bank adjacent to a surface water. Composite samples should not be collected, since such samples do not display the range of values found in individual samples.

The sampling depth for surface water samples should be 6-12 inches below the water surface. Sample containers should be positioned such that the mouth of the container is pointed away from the sampler or sample point. After removal of the container from the water, a small portion of the sample should be discarded to allow for proper mixing before analyses.

8.2 Storage temperature and handling conditions

Ice or refrigerate water samples at a temperature of <10°C during transit to the laboratory. Do not freeze the samples. 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.

8.3 Holding time limitations

Sample analysis should begin immediately, preferably within 2 hours of collection. Sample incubation must be started no later than 8 hours from time of collection.

9.0 Quality Control

9.1 Each laboratory that uses Method 1600.1 is required to operate a formal quality assurance (QA) program that addresses and documents instrument and equipment maintenance and performance, reagent quality and performance, analyst training and certification, and records storage and

retrieval. Additional recommendations for QA and quality control (QC) procedures for microbiological laboratories are provided in Reference 18.6.

9.2 The minimum analytical QC requirements for the analysis of samples using Method 1600.1 include an initial demonstration of laboratory capability through performance of the initial precision and recovery (IPR) analyses (Section 9.3), ongoing demonstration of laboratory capability through performance of the ongoing precision and recovery (OPR) analysis (Section 9.4) and matrix spike (MS) analysis (Section 9.5, disinfected wastewater only), and the routine analysis of positive and negative controls (Section 9.6), filter sterility checks (Section 9.8), method blanks (Section 9.9), and media sterility checks (Section 9.11). For the IPR, OPR and MS analyses, it is necessary to spike samples with either laboratory-prepared spiking suspensions or BioBalls as described in Section 14.

***Note**: Performance criteria for Method 1600.1 are based on the results of the interlaboratory validation of Method 1600 in PBS and disinfected wastewater matrices. The IPR (Section 9.3) and OPR (Section 9.4) recovery criteria (**Table 1**) are valid method performance criteria that should be met, regardless of the matrix being evaluated, the matrix spike recovery criteria (Section 9.5, **Table 2**) pertain only to disinfected wastewaters.*

9.3 Initial precision and recovery (IPR) – The IPR analyses are used to demonstrate acceptable method performance (recovery and precision) and should be performed by each laboratory before the method is used for analyzing field samples. EPA recommends but does not require that an IPR be performed by each analyst. IPR samples should be accompanied by an acceptable method blank (Section 9.9) and appropriate media sterility checks (Section 9.11). IPR analyses are performed as follows:

9.3.1 Prepare four, 100-mL samples of sterile PBS and spike each sample with *E. faecalis* ATCC® # 19433™ according to the spiking procedure in Section 14. Spiking with laboratory-prepared suspensions is described in Section 14.2 and spiking with BioBalls is described in Section 14.3. Filter and process each IPR sample according to the procedures in Section 11 and calculate the number of enterococci per 100 mL according to Section 13.

9.3.2 Calculate the percent recovery (R) for each IPR sample using the appropriate equation in Section 14.2.4.3 or 14.3.2 for samples spiked with laboratory-prepared spiking suspensions or BioBalls, respectively.

9.3.3 Using the percent recoveries of the four analyses, calculate the mean percent recovery and the relative standard deviation (RSD) of the recoveries. The RSD is the standard deviation divided by the mean, multiplied by 100.

9.3.4 Compare the mean recovery and RSD with the corresponding IPR criteria in **Table 1**, below. If the mean and RSD for recovery of enterococci meet acceptance criteria, performance is acceptable, and analysis of field samples may begin. If the mean recovery or the RSD fall outside of the required range for recovery, performance is unacceptable. In this event, identify the problem by evaluating each step of the analytical process, media, reagents, and controls, correct the problem and repeat the IPR analyses.

Table 1. Initial and Ongoing Precision and Recovery (IPR and OPR) Acceptance Criteria

Performance test	Lab-prepared spike acceptance criteria	BioBall® acceptance criteria
Initial precision and recovery (IPR)		
• Mean percent recovery	31% - 127%	85% - 106%
• Precision (as maximum relative standard deviation)	28%	14%
Ongoing precision and recovery (OPR) as percent recovery	27% - 131%	78% - 113%

9.4 **Ongoing precision and recovery (OPR)** – To demonstrate ongoing control of the analytical system, the laboratory should routinely process and analyze spiked PBS samples. The laboratory should analyze one OPR sample after every 20 field and matrix spike samples or one per week that samples are analyzed, whichever occurs more frequently. OPR samples must be accompanied by an acceptable method blank (Section 9.9) and appropriate media sterility checks (Section 9.11). The OPR analysis is performed as follows:

9.4.1 Spike a 100-mL sterile PBS sample with *E. faecalis* ATCC® # 19433™ according to the spiking procedure in Section 14. Spiking with laboratory-prepared suspensions is described in Section 14.2 and spiking with BioBalls is described in Section 14.3. Filter and process each OPR sample according to the procedures in Section 11 and calculate the number of enterococci per 100 mL according to Section 13.

9.4.2 Calculate the percent recovery (R) for the OPR sample using the appropriate equation in Section 14.2.4.3 or 14.3.2 for samples spiked with laboratory-prepared spiking suspensions or BioBalls, respectively.

9.4.3 Compare the OPR result (percent recovery) with the corresponding OPR recovery criteria in Table 1, above. If the OPR result meets the acceptance criteria for recovery, method performance is acceptable, and analysis of field samples may continue. If the OPR result falls outside of the acceptance criteria, system performance is unacceptable. In this event, identify the problem by evaluating each step of the analytical process, media, reagents, and controls, correct the problem and repeat the OPR analysis.

9.4.4 As part of the laboratory QA program, results for OPR and IPR samples should be charted, and updated records maintained in order to monitor ongoing method performance. The laboratory should also develop a statement of accuracy for Method 1600.1 by calculating the average percent recovery (R) and the standard deviation of the percent recovery (s_r). Express accuracy as a recovery interval from $R - 2s_r$ to $R + 2s_r$.

9.5 **Matrix spikes (MS)** – MS analysis are performed to determine the effect of a particular matrix on enterococci recoveries. The laboratory should analyze one MS sample when disinfected wastewater samples are first received from a source from which the laboratory has not previously analyzed samples. Subsequently, 5% of field samples (1 per 20) from a given disinfected wastewater source should include a MS sample. MS samples must be accompanied by the analysis of an unspiked field sample sequentially collected from the same sampling site, an acceptable method blank (Section 9.9), and appropriate media sterility checks (Section 9.11). When possible, MS analyses should also be accompanied by an OPR sample (Section 9.4), using

the same spiking procedure (laboratory-prepared spiking suspension or BioBalls). The MS analysis is performed as follows:

9.5.1 Prepare two, 100-mL field samples that were sequentially collected from the same site. One sample will remain unspiked and will be analyzed to determine the background or ambient concentration of enterococci for calculating MS recoveries (Section 9.5.3). The other sample will serve as the MS sample and will be spiked with *E. faecalis* ATCC® # 19433™ according to the spiking procedure in Section 14.

9.5.2 Select sample volumes based on previous analytical results or anticipated levels of enterococci in the field sample to achieve the recommended target range of enterococci (20-60 colony forming unit [CFU], including spike) per filter. If the laboratory is not familiar with the matrix being analyzed, it is recommended that a minimum of three dilutions be analyzed to ensure that a countable plate is obtained for the MS and associated unspiked sample. If possible, 100-mL of sample should be analyzed.

9.5.3 Spike the MS sample volume(s) with a laboratory-prepared suspension as described in Section 14.2 or with BioBalls as described in Section 14.3. Immediately filter and process the unspiked and spiked field samples according to the procedures in Section 11.

***Note**: When analyzing smaller sample volumes (e.g., <20 mL), 20-30 mL of PBS should be added to the funnel, or an aliquot of sample should be dispensed into a 20-30 mL dilution blank prior to filtration. This will allow even distribution of the sample on the membrane.*

9.5.4 For the MS sample, calculate the number of enterococci (CFU/100 mL) according to Section 13 and adjust the colony counts based on any background enterococci observed in the unspiked matrix sample.

9.5.5 Calculate the percent recovery (R) for the MS sample (adjusted based on ambient enterococci in the unspiked sample) using the appropriate equation in Section 14.2.4.3 or 14.3.2 for samples spiked with laboratory-prepared spiking suspensions or BioBalls, respectively.

9.5.6 Compare the MS result (percent recovery) with the appropriate method performance criteria in Table 2, below. If the MS recovery meets the acceptance criteria, system performance is acceptable and analysis of field samples from this disinfected wastewater source may continue. If the MS recovery is unacceptable and the OPR sample result associated with this batch of samples is acceptable, a matrix interference may be causing the poor results. If the MS recovery is unacceptable, all associated field data should be flagged.

9.5.7 Acceptance criteria for MS recovery (**Table 2**) are based on data from spiked disinfected wastewater matrices and are not appropriate for use with other matrices (e.g., ambient waters).

Table 2. Matrix Spike Precision and Recovery Acceptance Criteria

Performance test	Lab-prepared acceptance criteria	BioBall® acceptance criteria
Percent recovery for MS	29% - 122%	63% - 110%

9.5.8 Laboratories should record and maintain a control chart comparing MS recoveries for all matrices to batch-specific and cumulative OPR sample results analyzed using Method 1600.1. These comparisons should help laboratories recognize matrix effects on method recovery and may also help to recognize inconsistent or sporadic matrix effects from a particular source.

9.6 Culture controls

9.6.1 **Negative controls** – The laboratory should analyze negative controls to ensure that the mEI agar is performing properly. Negative controls should be analyzed whenever a new batch of media or reagents is used. On an ongoing basis, the laboratory should perform a negative control every day that samples are analyzed.

9.6.1.1 Negative controls are conducted by filtering a dilute suspension of viable *E. coli* (e.g., ATCC® # 11775™) and analyzing as described in Section 11. Viability of the negative controls should be demonstrated using a non-selective media (e.g., nutrient agar or TSA).

9.6.1.2 If the negative control fails to exhibit the appropriate response, check and/or replace the associated media or reagents, and/or the negative control, and reanalyze the negative control.

9.6.2 **Positive controls** – The laboratory should analyze positive controls to ensure that the mEI agar is performing properly. Positive controls should be analyzed whenever a new batch of media or reagents is used. On an ongoing basis, the laboratory should perform a positive control every day that samples are analyzed. An OPR sample (Section 9.4) may take the place of a positive control.

9.6.2.1 Positive controls are conducted by filtering a dilute suspension of viable *E. faecalis* (e.g., ATCC® # 19433™) and analyzing as described in Section 11.

9.6.2.2 If the positive control fails to exhibit the appropriate response, check and/or replace the associated media or reagents, and/or the positive control, and reanalyze the positive control.

9.6.3 **Controls for verification media** – All verification media should be tested with appropriate positive and negative controls whenever a new batch of media and/or reagents are used. On an ongoing basis, the laboratory should perform positive and negative controls on each of the verification media with each batch of samples submitted to verification. Examples of appropriate controls for verification media are provided in**Table 3**.

Table 3. Verification Controls

Medium	Positive Control	Negative Control
Bile esculin agar (BEA)	*E. faecalis*	*E. coli*
Brain heart infusion broth (BHIB) with 6.5% NaCl	*E. faecalis*	*E. coli*
Brain heart infusion broth (BHIB) incubated at 45°C	*E. faecalis*	*E. coli*

9.7 **Colony verification** – The laboratory should verify 10 typical colonies (positive) and 10 atypical colonies (negative) per month or 1 typical colony and 1 atypical colony from 10% of all positive samples, whichever is greater, to identify potential errors in lab procedure or change in matrix chemistry or biology. Verification procedures are provided in Section 12.0.

9.8 **Filter sterility check** – Place at least one membrane filter per lot of filters on a TSA plate and incubate for 24 ± 2 hours at 35°C ± 0.5°C. Absence of growth indicates sterility of the filter. On an ongoing basis, the laboratory should perform a filter sterility check every day that samples are analyzed.

9.9 **Method blank** – Filter a 50-mL volume of sterile PBS and place the filter on a mEI agar plate and incubate according to Section 11.0. Absence of growth indicates freedom of contamination from the target organism. On an ongoing basis, the laboratory should perform a method blank every day that samples are analyzed.

9.10 **Filtration blank** – Filter a 50-mL volume of sterile PBS before beginning sample filtrations.Place the filter on a TSA plate and incubate for 24 ± 2 hours at 35°C ± 0.5°C. Absence of growth indicates sterility of the PBS buffer and filtration assembly.

***Note**: If using UV to sanitize funnels in between samples, consider adding additional filtration blanks.*

9.11 **Media sterility check** – The laboratory should test media sterility by incubating one unit (tube or plate) from each batch of medium (TSA, mEI agar, and verification media) as appropriate and observing for growth. Absence of growth indicates media sterility. On an ongoing basis, the laboratory should perform a media sterility check, for each medium used, every day that samples are analyzed.

9.12 **Analyst colony counting variability** – Laboratories with two or more analysts should compare each analyst's colony counts from one positive field sample per month. Colony counts should be within 10% between analysts. Laboratories with a single analyst should have that analyst perform duplicate colony counts of a single membrane filter each month. Duplicate colony counts should be within 5% for a single analyst. If no positive field samples are available, an OPR sample may be substituted for these determinations.

10.0 Calibration and Standardization

10.1 Check temperatures in incubators twice daily with a minimum of 4 hours between each reading 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 Handbook 105-6.

10.3 Refrigerators used to store media and reagents should be monitored daily to ensure proper temperature control.

10.4 Calibrate the pH meter prior to each use period with the two standards (e.g., pH 4.0, 7.0, 10.0) closest to the range being tested.

10.5 Calibrate top-loading balances monthly with reference weights of ASTM Class 2 (Standard E617-18).

11.0 Procedure

11.1 Prepare the mEI agar as directed in Section 7.6. If media was prepared in advance and refrigerated, bring to room temperature prior to use.

11.2 Label Petri dishes and report forms with sample identification (e.g., field and QC samples) and sample 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 now held between the funnel and the base.

11.4 Shake the sample bottle vigorously about 25 times in 7 seconds in a one foot arc 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 enterococci level, to produce 20-60 enterococci colonies on membranes. It is recommended that a minimum of three dilutions be analyzed to ensure that a countable plate (20-60 enterococci colonies) is obtained.

11.6 Smaller sample size 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.

***Note**: When analyzing smaller sample volumes (e.g., <20 mL), add 20-30 mL of sterile PBS to the funnel prior to adding the sample, or add sample volume to a PBS dilution blank (e.g., 20-30 mL), prior to filtration. This will allow even distribution of the sample on the membrane.*

11.7 Filter the sample and rinse the sides of the funnel at least twice with 20-30 mL of sterile PBS . 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 mEI 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 outside the area of filtration, close to the edge of the dish, to be sure that the filter is properly seated on the agar. Close the dish, invert, and incubate at 41°C ± 0.5°C for 24 ± 2 hours.

***Note**: If the medium is prepared in 15 × 60 mm loose lid Petri dishes, they should be incubated in a tightfitting container (e.g., plastic vegetable crisper) containing a moistened paper towel to prevent dehydration of the membrane filter and medium.*

11.9 After incubation, count and record colonies on those membrane filters containing, if practical, 20-60 colonies ≥0.5 mm in diameter with a blue halo regardless of colony color, as an enterococci (see **Photo 1**). ***Note**: When measuring colony size do not include the halo. Use magnification for counting and a small fluorescent lamp to give maximum visibility of colonies.*

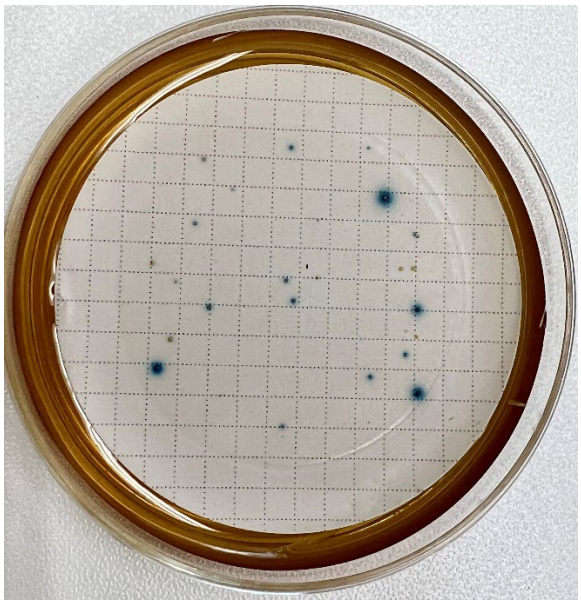

Photo 1. Enterococci colonies on mEI produce blue halos.

12.0 Verification Procedure

12.1 Colonies ≥0.5 mm in diameter of any color having a blue halo after incubation on mEI agar are considered "typical" enterococci colonies. Verification of colonies may be required in evidence gathering and it is also recommended as a means to identify potential errors in lab procedure or change in matrix chemistry or biology. The verification procedure is described below.

***Note**: When evaluating wastewater using Method 1600.1, it is recommended that the false negative rate for each matrix be evaluated through biochemical confirmation (see 15.2.6) and negative results adjusted accordingly, especially if large numbers of atypical colonies are observed in a particular matrix.*

12.2 Using a sterile inoculating loop or needle, transfer growth from the centers of at least 10 well-isolated typical and at least 10 well-isolated atypical colonies into BHIB tubes and onto BHIA slants. Incubate broth for 24 ± 2 hours and agar slants for 48 ± 3 hours at 35°C ± 0.5°C.

12.3 After a 24-hour incubation, transfer a loopful of broth from each BHIB tube to BEA, BHIB, and BHIB with 6.5% NaCl. Streak for isolation on BEA plates.

12.3.1 Incubate BEA and BHIB with 6.5% NaCl at 35°C ± 0.5°C for 48 ± 3 hours.

12.3.2 Incubate BHIB at 45°C ± 0.5°C for 48 ± 3 hours.

12.4 Observe all verification media for growth.

12.5 After a 48-hour incubation, perform a Gram stain using growth from each BHIA slant.

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

12.7 Alternately, commercially available multi-test identification systems (e.g., Vitek®) that include esculin hydrolysis and growth in 6.5% NaCl test reactions may be used to verify colonies

13.0 Data Analysis and Calculations

Use the following general rules to calculate the enterococci count (CFU) per 100 mL of sample:

13.1 If possible, select a membrane filter with 20-60 colonies ≥0.5 mm in diameter (regardless of colony color) with a blue halo. Calculate the number of enterococci CFU per 100 mL according to thefollowing general formula:

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

13.2 Refer to general counting rules in Reference 18.6 and Appendix B.

13.3 Report results as enterococci CFU per 100 mL of sample.

14.0 Sample Spiking Procedure

14.1 QC requirements (Section 9.0) include the preparation and analysis of spiked reference (PBS) and matrix samples in order to monitor initial and ongoing method performance. For the IPR (Section 9.3), OPR (Section 9.4), and MS (Section 9.5) analyses it is necessary to spike samples with either laboratory-prepared spiking suspensions (Section 14.2) or BioBalls (Section 14.3) as described below.

14.2 Laboratory-Prepared Spiking Suspensions

14.2.1 Preparation

14.2.1.1 Stock Culture. Prepare a stock culture by inoculating a TSA slant (or other non-selective media) with *E. faecalis* ATCC® # 19433™ and incubating at 35°C ± 3°C for 20 ± 4 hours. This stock culture may be stored in the dark at room temperature for up to 30 days.

14.2.1.2 Undiluted Spiking Suspension. Prepare a 1% solution of ADB by combining 99 mL of sterile phosphate buffered saline and 1 mL of sterile single strength azide dextrose broth in a sterile screw cap bottle or re-sealable dilution water container. From the stock culture of *E. faecalis* ATCC® # 19433™ in Section 14.2.1.1, transfer a small loopful of growth to the 1% ADB solution and vigorously shake a minimum of 25 times. Disperse the inoculum by vigorously shaking the broth culture and incubate at 35°C ± 3°C for 20 ± 4 hours. This culture is referred to as the undiluted spiking suspension and should contain approximately 1.0×10^6 - 1.0×10^7 *E. faecalis* CFU per mL of culture.

14.2.1.3 Mix the undiluted spiking suspension (Section 14.2.1.2) thoroughly by shaking the bottle a minimum of 25 times and prepare a series of dilutions (4 total) in the following manner:

14.2.1.3.1 Dilution "A" – Aseptically transfer 1.0 mL of the undiluted spiking suspension to 99 mL of sterile PBS and mix thoroughly by shaking the bottle a minimum of 25 times. This is spiking suspension dilution "A" and 1 mL contains 10^{-2} mL of the original undiluted spiking suspension.

14.2.1.3.2 Dilution "B" – Aseptically transfer 1.0 mL of dilution "A" to 99 mL of sterile PBS and mix thoroughly by shaking the bottle a minimum of 25 times. This is spiking suspension dilution "B" and 1 mL contains 10^{-4} mL of the original undiluted spiking suspension.

14.2.1.3.3 Dilution "C" – Aseptically transfer 11.0 mL of dilution "B" to 99 mL of sterile PBS and mix thoroughly by shaking the bottle a minimum of 25 times. This is spiking suspension dilution "C" and 1 mL contains 10^{-5} mL of the original undiluted spiking suspension.

14.2.1.3.4 Dilution "D" – Aseptically transfer 11.0 mL of dilution "C" to 99 mL of sterile PBS and mix thoroughly by shaking the bottle a

minimum of 25 times. This is spiking suspension dilution "D" and 1 mL contains 10^{-6} mL of the original undiluted spiking suspension.

14.2.2 Sample spiking

Add 3.0 mL of the spiking suspension dilution "D" (Section 14.2.1.3.4) to 100 mL of PBS or appropriate volume of sample and mix thoroughly by shaking the bottle a minimum of 25 times. The volume of undiluted spiking suspension added to each 100 mL sample is 3.0×10^{-6} mL [(0.3 mL × 10^{-5} mL) per 100 mL of sample] which is referred to as $V_{\text{spiked per 100 mL sample}}$ in Section 14.2.4.1 below. Filter the spiked sample and analyze the filter according to the procedures in Section 11.

14.2.3 Enumeration of spiking suspension

14.2.3.1 Prepare TSA spread plates, in triplicate, for spiking suspension dilutions "B", "C", and "D".

***Note**: Agar plates must be dry prior to use. To ensure that the agar surface is dry, plates should be made several days in advance and stored inverted at room temperature or dried using a laminar-flow hood.*

14.2.3.2 Mix dilution "B" by shaking the bottle a minimum of 25 times. Pipet 0.1 mL of dilution "B" onto the surface of each TSA plate in triplicate.

14.2.3.3 Mix dilution "C" by shaking the bottle a minimum of 25 times. Pipet 0.1 mL of dilution "C" onto the surface of each TSA plate in triplicate.

14.2.3.4 Mix dilution "D" by shaking the bottle a minimum of 25 times. Pipet 0.1 mL of dilution "D" onto the surface of each TSA plate in triplicate.

14.2.3.5 Use a sterile bent glass rod or spreader to distribute the inoculum over the surface of plates by rotating the dish by hand or on a turntable.

***Note**: Ensure that the inoculum is evenly distributed over the entire surface of the plate.*

14.2.3.6 Allow the inoculum to completely absorb into the medium of each plate. Invert plates and incubate at 35°C ± 0.5°C for 20 ± 4 hours.

14.2.3.7 Count and record number of colonies per plate. The number of enterococci (CFU/mL) in the undiluted spiking suspension will be calculated using all TSA plates yielding counts within the countable range of 30 to 300 CFU per plate.

14.2.4 Recovery calculations for spiked samples

14.2.4.1 Calculate the concentration of enterococci (CFU/mL) in the undiluted spiking suspension (Section 14.2.1.2) according to the following equation. Example calculations are provided in **Table 4**, below.

$$Enterococci_{\,undiluted\ spike} = \frac{(CFU_1 + CFU_2 + \ldots + CFU_n)}{(V_1 + V_2 + \ldots + V_n)}$$

Where,

CFU = Number of colony forming units from TSA plates yielding counts within the countable range of 30 to 300 CFU per plate

V = Volume of undiluted sample on each TSA plate yielding counts within the countable range of 30 to 300 CFU per plate

n = Number of plates with counts within the countable range of 30 to 300 CFU per plate

***Note**: The example calculated numbers provided in the tables below have been rounded at the end of each step for simplification purposes. Generally, rounding should only occur after the final calculation.*

Table 4. Example Calculations of Laboratory-prepared Enterococci Spiking Concentration

Examples	CFU/plate (triplicate analyses) from TSA plates			Enterococci CFU/mL in undiluted spiking suspension (Enterococci $_{\text{undiluted spike}}$) [a]
	10^{-5} mL plates	10^{-6} mL plates	10^{-7} mL plates	
Example 1	94, 106, 89	9, 11, 28	1, 0, 4	(94+106+89) / (10^{-5}+10^{-5}+10^{-5}) = 289 / (3.0 × 10^{-5}) = 9,633,333 = **9.6 × 10^6 CFU/mL**
Example 2	32, 55, 72	8, 5, 3	0, 0, 0	(32+55+72) / (10^{-5}+10^{-5}+10^{-5}) = 159 / (3.0 × 10^{-5}) = 5,300,000 = **5.3 × 10^6 CFU/mL**

[a] Enterococci undiluted spike is calculated using all plates yielding counts within the countable range of 30 to 300 CFU per plate

14.2.4.2 Calculate true concentration of spiked enterococci (CFU/100 mL) according to the following equation. Example calculations are provided in **Table 5,** below.

$$T_{spiked\ Enterococci} = Enterococci_{\,undiluted\ spike}\ x\ V_{spiked\ per\ 100\ mL\ sample}$$

Where,

$\mathbf{T}_{\text{spiked Enterococci}}$ = Number of spiked Enterococci (CFU/100 mL)

$\mathbf{Enterococci}_{\text{undiluted spike}}$ = Enterococci (CFU/mL) in undiluted spiking suspension

$\mathbf{V}_{\text{spiked per 100 mL sample}}$ = mL of undiluted spiking suspension per 100 mL sample

Table 5. Example Calculations for Determination "True" Spiked Enterococci Concentration

$Enterococci_{undiluted\ spike}$	$V_{spiked\ per\ 100\ mL\ sample}$	$T_{spiked\ Enterococci}$
9.6×10^6 CFU/mL	3.0×10^{-6} mL per 100 mL of sample	(9.6×10^6 CFU/mL) × (3.0×10^{-6} mL / 100 mL) = **28.8 CFU/100 mL**
5.3×10^6 CFU/mL	3.0×10^{-6} mL per 100 mL of sample	(2.8×10^6 CFU/mL) × (3.0×10^{-6} mL / 100 mL) = **8.4 CFU/100 mL**

14.2.4.3 Calculate percent recovery (R) of spiked enterococci (CFU/100 mL) according to the following equation. Example calculations are provided in **Table 6**, below.

$$R = 100\ x\ \frac{N_s - N_u}{T}$$

Where,

R = Percent recovery

$\mathbf{N_s}$ = Enterococci (CFU/100 mL) in the spiked sample (Section 13)

$\mathbf{N_u}$ = Enterococci (CFU/100 mL) in the unspiked sample (Section 13)

T = True spiked Enterococci (CFU/100 mL) in spiked sample (Section 14.2.4.2)

Table 6. Example Percent Recovery Calculations for Lab-prepared Spiked Samples

N_s (CFU/100 mL)	N_u (CFU/100 mL)	$T_{spiked\ Enterococci}$ (CFU/100 mL)	Percent recovery (R)
42	<1	28.8	100 × (42 - 1) / 28.8 = **142%**
34	10	28.8	100 × (34 - 10) / 28.8 = **83%**
10	<1	8.4	100 × (10 - 1) / 8.4 = **107%**

14.3 BioBall® spiking procedure

14.3.1 Aseptically add 1 BioBall® to 100 mL of PBS or appropriate volume of sample and mix by vigorously shaking the sample bottle a minimum of 25 times. Analyze the spiked sample according to the procedures in Section 11.

14.3.2 Recovery calculations for samples spiked with BioBalls – Calculate percent recovery (R)of spiked enterococci (CFU/100 mL) according to the following equation. Example calculations are provided in **Table 7**, below.

$$R = 100\ x\ \frac{N_s - N_u}{T}$$

Where,

R = Percent recovery

$\mathbf{N_s}$ = Enterococci (CFU/100 mL) in the spiked sample (Section 13)

$\mathbf{N_u}$ = Enterococci (CFU/100 mL) in the unspiked sample (Section 13)

T = True spiked enterococci (CFU/100 mL) in spiked sample based on the lot mean value provided by manufacturer

Table 7. Example BioBall® Percent Recovery Calculations

N_s (CFU/100 mL)	N_u (CFU/100 mL)	T (CFU/100 mL)	Percent recovery (R)
24	<1	32	100 × (24 - 1) / 32 = **72%**
36	0	32	100 × (36 - 10) / 32 = **81%**

15.0 Method Performance

15.1 Performance characteristics (Reference 18.3)

15.1.1 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 themean computed from the results of a series of controlled determinations. The precision among laboratories for marine water and surface water was 2.2% and 18.9%, respectively.

15.1.2 Bias – The persistent positive or negative deviation of the results from the assumed or accepted true value. The persistent positive or negative deviation of the results from the assumed or accepted true value was not significant.

15.1.3 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 6.0% false positive and 6.5% false negative.

15.1.4 Multi-laboratory variability – A collaborative study was conducted among fourteen collaborators at twelve laboratories to examine the interlaboratory reproducibility of the method. Reproducibility among laboratories (RSD_R) for freshwater, marine water, chlorinated secondary effluent, and non-chlorinated primary effluent ranged from 2.2% for marine water to 18.9% for freshwater with a low enterococcal density.

15.2 Interlaboratory validation of Method 1600 in disinfected wastewater (Reference 18.7)

15.2.1 Twelve volunteer participant laboratories, two enterococci verification laboratories, and two research laboratories participated in the U.S. Environmental Protection Agency's (EPA's) interlaboratory validation study of EPA Method 1600. The purposes of the study were to characterize method performance across multiple laboratories and

disinfected wastewater matrices and to develop quantitative quality control (QC) acceptance criteria. A detailed description of the of the study and results are provided in the validation study report (Reference 18.7).

15.2.2 Recovery – Method 1600 was characterized by mean laboratory-specific recoveries of enterococci from disinfected wastewater samples spiked with BioBalls ranging from 77.1% to 114.9%, with an overall mean recovery of 90.8%. Mean laboratory-specific recoveries of enterococci from PBS samples spiked with BioBalls ranged from 88.0% to105.1%, with an overall mean recovery of 95.4%.

15.2.3 Precision – Method 1600 was characterized by laboratory-specific relative standard deviations (RSDs) from disinfected wastewater samples spiked with BioBalls ranging from 0% to 69.5%, with an overall pooled, within-laboratory RSD of 22.6%. For PBS samples spiked with BioBalls, laboratory-specific RSDs ranged from 3.1% to 13.7%, with an overall pooled, within-laboratory RSD of 8.1%.

15.2.4 False positive confirmation rates – Method 1600 laboratory-specific false positive confirmation rates for unspiked disinfected/secondary results combined, ranging from 0.0% to 10.0%. For secondary wastewater (excluding disinfected results), only 2 of 123 typical colonies submitted to verification were non-enterococci, resulting in a false positive confirmation rate of 1.6%. For disinfected wastewater (excluding secondary results), none of the 66 typical colonies submitted to verification were non-enterococci, resulting in a false positive confirmation rate of 0.0%. Since all 2184 typical colonies observed during the study could not be submitted to confirmation, the percent of total colonies that would have resulted in a false positive result was estimated (see Table 6, Reference 18.7). It is estimated that 0.0% and 1.2% of the total colonies would have resulted in a false positive for disinfected wastewater and secondary wastewater, respectively.

15.2.5 False negative rates – Method 1600 laboratory-specific false negative confirmation rates for unspiked disinfected/secondary results combined, ranged from 28.6% to 100.0%. For secondary wastewater (excluding disinfected results), 62 of 79 atypical colonies submitted to verification were identified as enterococci, resulting in a false negative confirmation rate of 78.5% for secondary wastewater. For disinfected wastewater (excluding secondary results), eight of eight atypical colonies submitted to verification were identified as enterococci, resulting in a false negative confirmation rate of 100.0% for disinfected wastewater. Since all 839 atypical colonies observed during the study could not be submitted to confirmation, the percent of total colonies that would have resulted in a false negative result was estimated. It is estimated that 21.2% and 22.8% of the total colonies would have resulted in a false negative for disinfected wastewater and secondary wastewater, respectively. The false positive and false negative assessments are provided in **Table 8**.

Table 8. False Positive and False Negative Assessment for Unspiked Disinfected and Unspiked Secondary Wastewater Effluents

Matrix	Total colonies		False positive (FP) assessment				False negative (FN) assessment			
	Typical	Atypical	Typical colonies submitted	No. FP colonies	FP Confirmation rate (%) [a]	Estimated % of total colonies that would have been a FP [b]	Atypical colonies submitted	No. FN colonies	FN Confirmation rate (%) [c]	Estimated % of total colonies that would have been a FN [d]
Disinfected	391	105	66	0	0.0	0.0	8	8	100.0	21.2
Secondary	1793	734	123	2	1.6	1.2	79	62	78.5	22.8
Disinfected + Secondary	2184	839	189	2	1.1	0.8	87	70	80.5	22.3

[a] False positive confirmation rate = number of false positive colonies / number of typical colonies submitted

[b] Percent of total colonies estimated to be false positives = [(total typical colonies FP confirmation rate) / (total number of typical and atypical colonies observed); e.g., [(1793 × (2/123)) / (1793+734)] × 100 = 1.2%

[c] False negative confirmation rate = number of false negative colonies / number of atypical colonies submitted

[d] Percent of total colonies estimated to be false negatives = [(total atypical colonies* FN confirmation rate) / (total number of typical and atypical colonies observed)] × 100; e.g., [(734 × (62/79)) / (1793+734)] × 100 = 22.8%

15.2.6 During evaluation of the study results, it was noted that many of the false negatives (atypical colonies submitted to verification which were identified as enterococci) were pink to red in color but simply lacked a blue halo. The predecessor to EPA Method 1600 for enterococci is EPA Method 1106.1 which uses mE and EIA media. For EPA Method1106.1, pink to red colonies on mE, which produce a brown precipitate after transfer to EIA are considered positive for enterococci. Tetrazolium chloride (TTC), the reagent responsible for producing pink to red enterococci colonies on mE, is also included as a reagent in mEI. A follow-on study was conducted, for which pink to red colonies without halos from unspiked secondary wastewaters were submitted to verification. For pink to red colonies without halos that were ≥0.5 mm colony size, 54 of 90 colonies submitted were identified as enterococci, resulting in a 60.0% verification rate.

Results of the verification analyses from the initial study were assessed with pink to red colonies without halos being counted as enterococci. When pink to red colonies without halos are counted as enterococci, the estimated percent of total colonies that would have resulted in false positives increases slightly from 0.8% to 2.7%, for combined disinfectedand secondary results. More importantly, the estimated percent of total colonies that would have resulted in false negatives decreased from 22.3% to 7.0% for combined disinfected and secondary results and from 21.2% to 2.9% for disinfected wastewater.

The re-assessment of false positive and false negative initial study results with pink to red colonies without halos counted as enterococci are provided in **Table 9**.

Table 9. Re-Assessment of False Positive and False Negative Initial Study Results with Pinkto Red Colonies without Halos Counted as Enterococci

Matrix (sample no.)	Total colonies		False positive (FP) assessment				False negative (FN) assessment			
	Typical	Atypical	Typical colonies submitted	No. FP colonies	FP confirmation rate (%) [a]	Estimated % of total colonies that would have been a FP [b]	Atypical colonies submitted	No. FN colonies	FN confirmation rate (%) [c]	Estimated % of total colonies that would have been a FN [d]
Disinfected (Samples 1-4)	477	19	69	0	0.0	0.0	4	3	75.0	2.9
Secondary (Samples 5, 6)	2291	236	166	7	4.2	3.8	32	27	84.4	7.9
Disinfected & Secondary (Samples 1-6)	2768	255	235	7	3.0	2.7	36	30	83.3	7.0

a False positive confirmation rate = number of false positive colonies / number of typical colonies submitted

b Percent of total colonies estimated to be false positives = [(total typical colonies × FP confirmation rate) / (total number of typical and atypical colonies observed)] × 100; e.g., [(2291 × (7/166)) / (2291+236)] × 100 = 3.8%

c False negative confirmation rate = number of false negative colonies / number of atypical colonies submitted

d Percent of total colonies estimated to be false negatives = [(total atypical colonies × FN confirmation rate) / (total number of typical and atypical colonies observed)] × 100; e.g., [(236 × (27/32)) / (2291+236)] × 100 = 7.9%

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 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 GovernmentRelations and Science Policy, 1155 16th Street NW, Washington, DC 20036.

18.0 References

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Appendix A:
Part II (General Operations), Section A (Sample Collection, Preservation, and Storage)

Sample Collection[1]

1.0 Sample Containers

1.1 Sample Bottles: bottles must be resistant to sterilizing conditions and the solvent action of water. Wide-mouth borosilicate glass bottles with screw cap or ground-glass stopper or heat-resistant plastic bottles may be used if they can be sterilized without producing toxic materials (see examples A and C in Figure 1). Screw caps must not produce bacteriostatic or nutritive compounds upon sterilization.

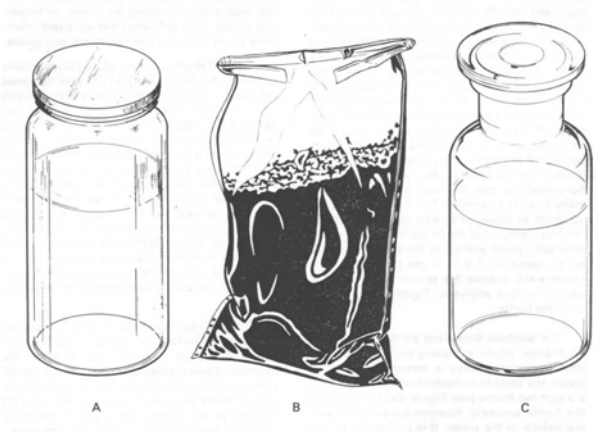

Figure 1. Suggested sample containers.

1.2 Selection and Cleaning of Bottles: Samples bottles should be at least 125 mL volume for adequate sampling and for good mixing. Bottles of 250 mL, 500 mL, and 1000 mL volume are often used for multiple analyses. Discard bottles which have chips, cracks, and etched surfaces. Bottle closures must be water-tight. Before use, thoroughly cleanse bottles and closures with detergent and hot water, followed by a hot water rinse to remove all trace of detergent. Then rinse them three times with laboratory-pure water.

1.3 Dechlorinating Agent: The agent must be placed in the bottle when water and wastewater samples containing residual chlorine are anticipated. Add sodium thiosulfate to the bottle before sterilization at a concentration of 0.1 mL of a 10% solution for each 125 mL sample volume. This concentration will neutralize approximately 15 mg/L of residue chlorine.

1.4 Chelating Agent: A chelating agent should be added to sample bottles used to collect samples suspected of containing >0.01 mg/L concentrations of heavy metals such as copper, nickel or zinc, etc. Add 0.3 mL of a 15% solution of ethylenediaminetetraacetic acid (EDTA) tetrasodium salt, for each 125 mL sample volume prior to sterilization.

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[1]The text is largely taken from Part II, Section A, of the EPA publication *Microbiological Methods for Monitoring the Environment. Water and Wastes.* EPA-600/8-78-017, December 1978.

1.5 Wrapping Bottles: Protect the tops and necks of glass stoppered bottles from contamination by covering them before sterilization with aluminum foil or kraft paper.

1.6 Sterilization of Bottles: Autoclave glass or heat-resistant plastic bottles at 121°C (15 PSI) for 15 minutes. Alternatively, dry glassware may be sterilized in a hot oven at 170°C for not less than two hours. Ethylene oxide gas sterilization is acceptable for plastic containers that are not heat-resistant. Sample bottles sterilized by gas should be stored overnight before being used to allow the last traces of gas to dissipate.

1.7 Plastic Bags: The commercially available bags (Whirl-pak®) (see example B in Figure 1) are a practical substitute for plastic or glass samples bottles in sampling soil, sediment, or biosolids. The bags are sealed in manufacture and opened only at time of sampling. The manufacturer states that such bags are sterilized.

2.0 Sampling Techniques

Samples are collected by hand or with a sampling device if the sampling site has difficult access such as a bridge or bank adjacent to a surface water.

2.1 Chlorinated Samples: When samples such as treated waters, chlorinated wastewaters or recreational waters are collected, the sample bottle must contain a dechlorinating agent (see Section 1.3 above).

2.2 Composite Sampling: In no case should a composite sample be collected for bacteriologic examination. Data from individual samples show a range of values. A composite sample will not display this range. Individual results will give information about industrial process variations in flow and composition. Also, one or more portions that make up a composite sample may contain toxic or nutritive materials and cause erroneous results.

2.3 Surface Sampling by Hand: A grab sample is obtained using a sample bottle prepared as described in (1) above. Identify the sampling site on the bottle label and on a field log sheet. Remove the bottle covering and closure and protect from contamination. Grasp the bottle at the base with one hand and plunge the bottle mouth down into the water to avoid introducing surface scum (Figure 2). Position the mouth of the bottle into the current away from the hand of the collector and, if applicable, away from the side of the sampling platform. The sampling depth should be 15-30 cm (6-12 inches) below the water surface. If the water body is static, an artificial current can be created, by moving the bottle horizontally in the direction it is pointed and away from the sampler. Tip the bottle slightly upwards to allow air to exit and the bottle to fill. After removal of the bottle from the stream, pour out a small portion of the sample to allow an air space of 2.5-5 cm (1-2 inches) above each sample for proper mixing of the sample before analyses. Tightly stopper the bottle and place on ice (do not freeze) for transport to the laboratory.

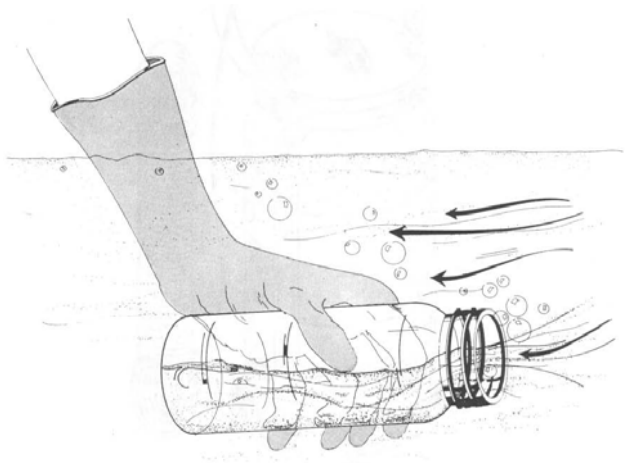

Figure 2. Grab sampling technique for surface waters.

3.0 Selection of Sampling Sites and Frequency

These sites will be described for streams, rivers, estuarine, marine, and recreational waters as well as domestic and industrial wastewaters.

3.1 Stream Sampling: The objectives of the initial survey dictate the location, frequency and number of samples to be collected.

3.1.1 Selection of Sampling Sites: A typical stream sampling program includes sampling locations upstream of the area of concern, upstream and downstream of waste discharges, upstream and downstream from tributary entrances to the river and upstream of the mouth of the tributary. For more complex situations, where several waste discharges are involved, sampling includes sites upstream and downstream from the combined discharge area and samples taken directly from each industrial or municipal waste discharge. Using available bacteriological, chemical and discharge rate data, the contribution of each pollution source can be determined.

3.1.2 Small Streams: Small streams should be sampled at background stations upstream of the pollution sources and at stations downstream from pollution sources. Additional sampling sites should be located downstream to delineate the zones of pollution. Avoid sampling areas where stagnation may occur (e.g., backwater of a tributary) and areas located near the inside bank of a curve in the stream which may not be representative of the main channel.

3.1.3 Large Streams and Rivers: Large streams are usually not well mixed laterally for long distances downstream from the pollution sources. Sampling sites below point source pollution should be established to provide desired downstream travel time and dispersal as determined by flow rate measurements. Particular care must be taken to establish the proper sampling points. Occasionally, depth samples are necessary to determine vertical mixing patterns.

3.2 Estuarine and Marine Sampling: Sampling estuarine and marine waters requires the consideration of other factors in addition to those usually recognized in fresh water sampling. They include tidal cycles, current patterns, bottom currents and counter-currents, stratification, seasonal fluctuations, dispersion of discharges and multi-depth samplings.

The frequency of sampling varies with the objectives. When a sampling program is started, it may be necessary to sample every hour around the clock to establish pollution loads and dispersion patterns. The sewage discharges may occur continuously or intermittently.

When the sampling strategy for a survey is planned, data may be available from previous hydrological studies done by the Coast Guard, Corps of Engineers, National Oceanic and Atmospheric Administration (NOAA), U.S. Geological Survey, or university and private research investigations. In a survey, float studies and dye studies are often carried out to determine surface and undercurrents. Initially depth samples are taken on the bottom and at five feet increments between surface and bottom. A random grid pattern for selecting sampling sites is established statistically.

3.2.1 Estuarine Sampling: When a survey is made on an estuary, samples are often taken from a boat, usually making an end to end traverse of the estuary. Another method involves taking samples throughout a tidal cycle, every hour or two hours from a bridge or from an anchored boat at a number of fixed points.

In a large bay or estuary where many square miles of area are involved, a grid or series of stations may be necessary. Two sets of samples are usually taken from an area on a given day, one at ebb or flood slack water, and the other three hours earlier, or later, at the half tidal interval. Sampling is scheduled so that the mid-sampling time of each run coincides with the calculated occurrence of the tidal condition.

In location sampling sites, one must consider points at which tributary waters enter the main stream or estuary, location of shellfish beds and bathing beaches. The sampling stations can be adjusted as data accumulate. For example, if a series of stations half mile apart consistently show similar values, some of these stations may be dropped and other stations added in areas where data shows more variability.

Considerable stratification can occur between the salt water from the sea and the fresh water supplied by a river. It is essential when starting a survey of an unknown estuary to find out whether there is any marked stratification. This can be done by chloride determinations at different locations and depths. It is possible for stratification to occur in one part of an estuary and not in another.

On a flood tide, the more dense salt water pushing up into the less dense fresh river water will cause an overlapping with the fresh water flowing on top. A phenomenon called a salt water wedge can form. As a result, stratification occurs. If the discharge of pollution is in the salt water layer, the contamination will be concentrated near the bottom at the flood tide. The flow or velocity of the fresh water will influence the degree of stratification which occurs. If one is sampling only at the surface, it is possible that the data will not show the polluted underflowing water which was contaminated at the point below the fresh water

river. Therefore, where stratification is suspected, samples at different depths will be needed to measure vertical distribution.

3.2.2 Marine Sampling: In ocean studies, the environmental conditions are most diverse along the coast where shore, atmosphere and the surf are strong influences. The shallow coastal waters are particularly susceptible to daily fluctuations in temperature and seasonal changes.

Sampling during the entire tidal cycle or during a half cycle may be required. Many ocean studies such as sampling over the continental shelf involve huge areas and no two areas of water are the same.

Selection of sampling sites and depths are most critical in marine waters. In winter, cooling of coastal waters can result in water layers which approach 0°C. In summer, the shallow waters warm much faster than the deeper waters. Despite the higher temperature, oxygen concentrations are higher in shallow than in deeper waters due to greater water movement, surf action and photosynthetic activity from macrophytes and the plankton.

Moving from the shallow waters to the intermediate depths, one observes a moderation of these shallow water characteristics. In the deeper waters, there is a marked stabilization of conditions. Water temperatures are lower and more stable. There is limited turbulence, little penetration of light, sparse vegetation and the ocean floor is covered with a layer of silts and sediments.

3.3 Recreational Waters (Bathing Beaches): Sampling sites at bathing beaches or other recreational areas should include upstream or peripheral areas and locations adjacent to natural drains that would discharge stormwater, or run-off areas draining septic wastes from restaurants, boat marinas, or garbage collection areas. Samples of bathing beach water should be collected at locations and times of heaviest use. Daily sampling is the optimum frequency during the season. Weekends and holidays which are periods of highest use must be included in the sampling program. Samples of estuarine bathing waters should be obtained at high tide, ebb tide and low tide in order to determine the cyclic water quality and deterioration that must be monitored during the swimming season.

3.4 Domestic and Industrial Waste Discharges: It is often necessary to sample secondary and tertiary wastes from municipal waste treatment plants and various industrial waste treatment operations. In situations where the plant treatment efficiency varies considerably, grab samples are collected around the clock at selected intervals for a three to five day period. If it is known that the process displays little variation, fewer samples are needed. In no case should a composite sample be collected for bacteriological examination. The National Pollution Discharge Elimination System (NPDES) has established wastewater treatment plant effluent limits for all dischargers. These are often based on maximum and mean values. A sufficient number of samples must be collected to satisfy the permit and/or to provide statistically sound data and give a fair representation of the bacteriological quality of the discharge.

Appendix B:
Part II (General Operations), Sections C.3.5 (Counting Colonies) and C.3.6 (Calculation of Results)

Counting Colonies[1]

1.0 Counting Colonies

Colonies should be counted using a fluorescent lamp with a magnifying lens. The fluorescent lamp should be nearly perpendicular to the membrane filter. Count colonies individually, even if they are in contact with each other. The analyst must learn to recognize the difference between two or more colonies which have grown into contact with each other and single, irregularly shaped colonies which sometimes develop on membrane filters. The latter colonies are usually associated with a fiber or particulate material and the colonies conform to the shape and size of the fiber or particulates. Colonies which have grown together almost invariably show a very fine line of contact.

2.0 Calculation of Results

2.1 Select the membrane filter with the number of colonies in the acceptable range and calculate count per 100 mL according to the general formula:

Count per 100 mL = (No. of colonies counted/Volume of sample filtered, in mL) × 100

2.2 Counts Within the Acceptable Limits

The acceptable range of colonies that are countable on a membrane is a function of the method. Different methods may have varying acceptable count ranges. All examples in this appendix assume that the acceptable range of counts is between 20-60 colonies per membrane.

For example, assume that filtration of volumes of 50, 15, 5, 1.5, and 0.5 mL produced colony counts of 200, 110, 40, 10, and 5, respectively.

An analyst would not actually count the colonies on all filters. By inspection the analyst would select the membrane filter with the acceptable range of target colonies, as defined by the method, and then limit the actual counting to such membranes.

After selecting the best membrane filter for counting, the analyst counts colonies and applies the general formula as in Section 2.1 above to calculate the count/100 mL.

2.3 More Than One Acceptable Count

2.3.1 If there are acceptable counts on replicate plates, carry counts independently to final reporting units, then calculate the arithmetic mean of these counts to obtain the final reporting value.

[1]The text is largely taken from Part II, Section C, of the EPA publication *Microbiological Methods for Monitoring the Environment. Water and Wastes.* EPA-600/8-78-017, December 1978.

Some examples were kindly provided by Kristen Brenner, US EPA.

Example, if the counts are 24 and 36 for replicate plates of 100 mL each, then the arithmetic mean is calculated as follows:

$$\frac{24\ CFU/100\ mL + \ 36\ CFU/100\ \ mL}{2} = \ 30\ \ CFU/100\ \ mL$$

2.3.2 If there is more than one dilution having an acceptable range of counts, independently carry counts to final reporting units, and then average for final reported value.

For example, if volumes of 100, 10, 1 and 0.1 mL produced colony counts of too numerous to count (TNTC), 55, 30, and 1, respectively, then two volumes, 10 mL and 1 mL, produced colonies in the acceptable counting range.

Independently carry each MF count to a count per 100 mL:

$$\frac{55}{10}\ x\ 100 = 550\ \ CFU/100\ mL$$

and

$$\frac{30}{1}\ x\ 100 = \ 3000\ \ CFU/100\ mL$$

Calculate the arithmetic mean as in Section 2.3.1 above:

$$\frac{550\ CFU/100\ \ mL\ +\ 3000\ CFU/100\ \ mL}{2} = \ 1775\ CFU/100\ mL$$

Report this as 1775 CFU/100 mL.

2.4 If all MF counts are below the lower acceptable count limit, select the most nearly acceptable count.

2.4.1 For example, sample volumes of 100, 10, and 1 mL produced colony counts of 17, 1, and 0, respectively.

Here, no colony count falls within recommended limits. Calculate on the basis of the most nearly acceptable plate count, 17, and report as 17 CFU/100 mL.

***Note**: In this case, because no calculations were done (i.e., this is the count for 100 mL), the count is reported as 17 CFU/100 mL rather than an "estimated count of 17 CFU/100 mL"*

2.4.2 As a second example, assume a count in which sample volumes of 10 and 1 mL produced colony counts of 18 and 0, respectively.

Here, no colony count falls within recommended limits. Calculate on the basis of the most nearly acceptable plate count, 18, and calculate as in Section 2.3.2 above.

$$\frac{18}{10}\ x\ 100 = 180\ CFU/100\ mL$$

Report this as an estimated count of 180 CFU/100 mL.

2.5 If counts from all membranes are zero, calculate using count from largest filtration volume.

For example, sample volumes of 25, 10, and 2 mL produced colony counts of 0, 0, and 0, respectively, and no actual calculation is possible, even as an estimated report. Calculate the number of colonies per 100 mL that would have been reported if there had been one colony on the filter representing the largest filtration volume. In this example, the largest volume filtered was 25 mL and thus the calculation would be:

$$\frac{1}{25}\ x\ 100 = 4\ CFU/100\ mL$$

Report this as < (less than) 4 CFU/100 mL.

2.6 If all membrane counts are above the upper acceptable limit, calculate count using the smallest volume filtered.

For example, assume that the volumes 1, 0.3, and 0.01 mL produced colony counts of TNTC, 150, and 110 colonies, respectively. Since all colony counts are above the acceptable limit, use the colony count from the smallest sample volume filtered and estimate the count as:

$$\frac{110}{0.01}\ x\ 100 =\ 1{,}100{,}000\ CFU/100\ mL$$

Report this as estimated count 1.1×10^6 CFU/100 mL

2.7 If typical colonies are too numerous to count (TNTC), use upper limit count with smallest filtration volume.

For example, assume that the volumes 1, 0.3, and 0.01 mL all produced too many typical colonies, and that the laboratory bench record indicated TNTC.

Use the upper acceptable count for the method (60 colonies in this example) as the basis of calculation with the smallest filtration volume and estimate the count as:

$$\frac{60}{0.01}\ x\ 100 = 600{,}000\ CFU/100\ mL$$

Report this as > (greater than) 6×10^5 CFU/100 mL

2.8 If colonies are both above and below the upper and lower acceptable limits (i.e., no counts are within the acceptable limits), select the most nearly acceptable count.

2.8.1 For example, sample volumes of 100, 10, and 1 mL produced colony counts of 64, 6, and 0, respectively.

Here, no colony count falls within recommended limits. Calculate on the basis of

the most nearly acceptable plate count, 64, and report as 64 CFU/100 mL.

***Note**: In this case, because no calculations were done (i.e., this is the count for 100 mL), the count is reported as 64 CFU/100 mL rather than an "estimated count of 64 CFU/100 mL"*

2.8.2 As a second example, assume a count in which sample volumes of 100, 10 and 1 mL produced colony counts of 98, 18, and 0, respectively.

Here, no colony count falls within recommended limits. Calculate on the basis of the most nearly acceptable plate count, 18, and calculate as in section 2.3.2 above.

$$\frac{18}{10}\, x\, 100 = 180\ \ CFU/\,100\, mL$$

Report this as estimated count 180 CFU/100 mL.

2.9 If there is no result because of a confluent growth, > 200 atypical colonies (TNTC), lab accident, etc., report as No Data and specify the reason.