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# Method 1680: Fecal Coliforms in Biosolids by Multiple-Tube Fermentation and Membrane Filter Procedures

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## Acknowledgments

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## Disclaimer

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## Introduction

Application of treated biosolids to land is helpful as a crop nutrient and soil conditioner, but may pose the risk of release of disease-causing microorganisms into the environment if proper disinfection and use criteria are not met. The density of fecal coliforms in biosolids is used as an indicator of the average density of bacterial and viral pathogens. Under 40 CFR Part 503, a biosolid sample is classified as Class A if it contains a fecal coliform density below 1,000 most probable number (MPN)/g of total solids (dry weight basis). A biosolid sample is classified as Class B if the geometric mean fecal coliform density is less than 2 X $10^6$ MPN or colony forming units (CFU)/g of total solids (dry weight basis).

Method 1680 is adapted from *Standard Methods* 9221E and 9222D (Reference 19.1). Although these methods currently are approved for the detection of fecal coliform bacteria in biosolids, they were designed for use in water matrices. Method 1680 was developed to determine the presence of fecal coliforms reliably in biosolid matrices. This method, as well as adaptations of other existing water methods for use in biosolid matrices, will be compiled in a forthcoming EPA biosolids methods compendium document.

Depending on the physical characteristics of the sample, the multiple tube fermentation procedure or membrane filter procedure in Method 1680 is used to detect fecal coliform bacteria. Although Method 1680 is a performance-based method, fecal coliforms are considered "method-defined analytes," and requests to modify the procedures are limited, and handled on a case-by-case basis.

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## 1.0 Scope and Application

**1.1** This method describes a multiple-tube fermentation procedure (also called the most probable number (MPN) procedure) and a membrane filter (MF) procedure for the detection and enumeration of fecal coliform bacteria in biosolids. These methods use culture-specific media and elevated temperature to isolate and enumerate fecal coliform organisms. Fecal coliform bacteria, including *Escherichia coli*, are commonly found in the feces of humans and other warm-blooded animals, and indicate the potential presence of other bacterial and viral pathogens.

**1.2** This method is adapted from methods 9221E and 9222D in *Standard Methods for the Examination of Water and Wastewater*, 19th Edition, for the determination of fecal coliform bacteria in a variety of matrices (Reference 19.1).

**1.3** This method is designed to meet the survey and monitoring requirements of the U.S. Environmental Protection Agency (EPA) in regulating the use and disposal of biosolids under 40 CFR Part 503. Subpart D of the Part 503 regulation protects public health and the environment through requirements designed to reduce the potential for contact with disease-bearing microorganisms (pathogens) in biosolids applied to land or placed on a surface disposal site.

**1.4** Fecal coliform density is expected to correlate with the probability of pathogens present and document process performance (vector attraction reduction).

**1.5** A detection limit for this method has not yet been determined.

**1.6** This method may be used to determine the density of fecal coliform bacteria in biosolids. This method also may be applied specifically to determine the density of fecal coliform bacteria in Class A and Class B biosolids to satisfy the pathogen reduction requirements of Subpart D of Part 503. A biosolid sample is classified as Class A if it contains a fecal coliform density below 1,000 MPN /g of total solids (dry weight basis). A biosolid sample is classified as Class B if the geometric mean fecal coliform density is less than $2 \times 10^6$ MPN or colony forming units (CFU)/g of total solids (dry weight basis).

**1.7** To satisfy the pathogen reduction monitoring alternatives for Class B biosolids, seven samples of treated biosolids are collected at the time of use or disposal and the geometric mean fecal coliform bacterial density of these samples is confirmed not to exceed $2 \times 10^6$ MPN or CFU/g of total solids (dry weight basis). Although the Part 503 regulation does not specify the total number of samples for Class A biosolids, it is recommended that a sampling event extend over two weeks, and that at least seven samples be tested to confirm that the mean bacterial density of the samples is below 1,000 MPN/g of total solids (dry weight basis). The analysis of seven samples increases the method precision by reducing the standard error caused by inherent variations in biosolid quality.

**1.8** The presence of fecal coliforms may be determined in Class B biosolids using either the MPN or the MF procedure. Class A biosolids must be analyzed using the MPN procedures. At the low fecal coliform densities expected in Class A biosolids, the filter used in the MF procedure would be

subjected to a loading of solids too high to permit a reliable count of the number of fecal coliform colonies.

**1.9** Any modification of the method beyond those expressly permitted is subject to the application and approval of alternative test procedures under 40 CFR Part 136.4 and 136.5.

## 2.0 Summary of Method

**2.1** Fecal coliform densities of Class A and B biosolids may be determined by the MPN procedure using two media options. Fecal coliform densities in Class B biosolid samples also may be determined using an MF procedure. Each procedure is applicable within the limitations specified in Sections 11.0 and 12.0. Additional dilution schemes are available in Appendix B.

**2.2** MPN procedure

Two method options are provided in Method 1680 for the MPN procedure: (1) A presumptive step using lauryl tryptose broth (LTB) plus a confirmation step using *E. coli* (EC) media. (EC media must not be used for direct fecal coliform isolation from a biosolid sample because prior enrichment is required in LTB media for optimum recovery of fecal coliforms.) (2) A direct, single step test using A-1 media. (A-1 media may produce false positives in thermally treated biosolids.) The precision of both tests increases with increasing numbers of replicates per sample tested.

**2.2.1** Summary of the LTB/EC MPN procedure

**2.2.1.1** A minimum of four sample dilutions are required, while five or more are preferred. Each sample dilution is inoculated into five test tubes, containing sterile LTB and either bromocresol purple indicator (acid reaction) or an inverted vial (gas production). The high concentration of solids in Class A samples makes distinguishing color change difficult. As a consequence, use of bromocresol purple as an indicator of acid production is not recommended for analysis of Class A biosolids.

**2.2.1.2** LTB sample tubes are incubated in a water bath or jacketed incubator at 35°C ± 0.5°C. After 24 ± 2 hours, the tubes are examined for presumptive growth and gas or acid production. Gas production is indicated by gas bubble formation within the inverted vial. Acid production is determined by yellow color in the presence of bromocresol purple. Negative growth tubes are reincubated for an additional 24 hours before reexamination. Failure to produce gas or acid in LTB media within 48 ± 3 hours is a negative presumptive test and indicates fecal coliform bacteria are not present.

**2.2.1.3** LTB presumptive positive tubes (gas or acid production) are transferred to test tubes containing sterile EC media and inverted vials. EC tubes are incubated in a water bath at 44.5° ± 0.2°C for 24 ± 2 hours. Gas production in EC broth in 24 ± 2 hours is considered a positive fecal coliform reaction. Failure to produce gas is a negative reaction and indicates fecal coliform bacteria are not present.

**2.2.1.4** Results of the MPN procedure using LTB/EC media are reported in terms of MPN /g calculated from the number of positive EC culture tubes and percent total solids (dry weight basis, see Appendix A for determination of total solids).

**2.2.2** Summary of direct test with A-1 media MPN procedure

**2.2.2.1** A minimum of four sample dilutions are required, while five or more are preferred. Each sample dilution is inoculated into five test tubes containing sterile A-1 media and inverted vials.

**2.2.2.2** Sample tubes are incubated in a water bath or jacketed incubator at 35°C ± 0.5 °C for 3 hours, then transferred to a water bath at 44.5°C ± 0.2 °C. After 21 ± 2 hours, sample tubes are examined for growth and gas production. Gas production in 24 ± 2 hours or less is a positive reaction indicating the presence of fecal coliforms.

**2.2.2.3** Results of the MPN procedure using A-1 media are reported in terms of the most probable number (MPN)/g calculated from the number of positive A-1 culture tubes and percent total solids (dry weight basis, see Appendix A for determination of total solids).

**2.3** MF procedure (Class B only)

The MF procedure provides a direct count of fecal coliform bacteria in biosolids based on growth of colonies on the surface of a membrane filter in the presence of membrane filtration-fecal coliform (m-FC) media. This procedure is used to determine fecal coliform densities in Class B biosolids only.

**2.3.1** Culture plates are prepared with m-FC media in agar base (m-FC/agar) or m-FC broth is soaked into absorbent pads (m-FC/pads). Sample dilutions are filtered through 0.45-µm membrane filters. The filters are placed on the m-FC/agar or m-FC/pads in the prepared culture plates, placed into a waterproof bag, and submerged in a water bath at 44.5°C ± 0.2°C for 24 ± 2 hours.

**2.3.2** Colonies produced by fecal coliform bacteria in the presence of m-FC media are various shades of blue. m-FC media is specific for fecal coliform growth and normally, few non-fecal coliform bacteria will be observed. Fecal colonies are counted under low-power magnification using a dissecting microscope.

**2.3.3** Results of the membrane filter test are reported in terms of CFU/g total solids (dry weight basis) calculated from the sample quantities that produced MF counts within the desired range of 20 to 80 fecal coliform CFU/plate. For determination of total solids (dry weight basis), see Appendix A.

## 3.0 Definitions

**3.1** Fecal coliform bacteria are gram-negative, non-spore-forming rods that are found in the intestines and feces of humans and other warm-blooded animals. The predominant fecal coliform is *E. coli*. In this method, fecal coliforms are those bacteria that produce gas or acidity within 24 ± 2 hours in EC or A-1 broth media after incubation at 44.5°C ± 0.2°C. Since coliforms from other sources often cannot produce gas under these conditions, this criterion is used to define the fecal component of the coliform group. In addition, those colonies that are various shades of blue in m-FC media using the MF procedure are characterized as fecal coliforms. The MF procedure uses an enriched lactose media and incubation temperature of 44.5°C ± 0.2°C for selectivity, and is 93% accurate in

differentiating between coliforms found in the feces of warm-blooded animals and those from other environmental sources (Reference 19.1).

**3.2** Class A biosolids contain a fecal coliform density below 1,000 MPN/g of total solids (dry weight basis).

**3.3** Class B biosolids contain a geometric mean fecal coliform density of less than 2 X $10^6$ MPN or CFU/g of total solids (dry weight basis).

**3.4** Definitions for other terms are given in the glossary at the end of the method.

## 4.0 Interferences

**4.1** MPN procedure—Since the MPN tables are based on a Poisson distribution, if the sample is not adequately mixed to ensure equal bacterial cell distribution before portions are removed, the MPN value will be a misrepresentation of the bacterial density.

**4.2** MF procedure

**4.2.1** Biosolid samples containing colloidal or suspended particulate materials, such as iron, manganese, alum floc, or algae can clog the membrane filter used in the MF procedure and prevent filtration, or cause spreading of bacterial colonies that interferes with identification of target colonies.

**4.2.2** Turbidity caused by the presence of high bacterial densities, algae, and other suspended material will not permit testing of a sample volume sufficient to yield significant results.

**4.2.3** The MF procedure is not applicable to Class A biosolids. At the low fecal coliform densities expected in Class A biosolids, the filter used in the MF procedure would be subjected to a loading of solids too high to permit a reliable count of the number of fecal coliform colonies.

**4.2.4** The presence of high numbers of predatory organisms or toxic substances (metals or organics) may result in low coliform estimates. The multiple-tube fermentation method is recommended if contaminants are present.

**4.3** Percent total solids interferences: see Appendix A

## 5.0 Safety

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

**5.2** Field and laboratory staff collecting and analyzing environmental samples are under some risk of exposure to pathogenic microorganisms. Staff should apply safety procedures used for pathogens to handle all samples.

**5.3** Mouth-pipetting is prohibited.

## 6.0 Equipment and Supplies

*NOTE: Brand names, suppliers, and part numbers are for illustrative purposes only. No endorsement is implied. Equivalent performance may be achieved using apparatus and materials other than those specified here, but demonstration of equivalent performance that meets the requirements of this method is the responsibility of the laboratory.*

**6.1** Sample bottles—Sterilizable, ground-glass, or plastic wide-mouthed (stoppered or screw cap), minimum of 125 mL capacity

**6.2** Sterile waterproof plastic bags—Whirl-Pak or equivalent (may be used for collection of solid samples)

**6.3** Dilution containers

**6.3.1** Sterile, borosilicate glass or plastic, screw or snap cap, marked at 99 mL

**6.3.2** Sterile, screw cap, borosilicate glass or plastic tubes marked at 9 mL

**6.4** Pipette container—Stainless steel, aluminum or borosilicate glass, for glass pipettes

**6.5** Pipettes

**6.5.1** Sterile, to deliver (TD) bacteriological or Mohr, glass or plastic, of appropriate volume

**6.5.2** Sterile, wide-mouth

**6.6** Volumetric flasks—Borosilicate glass, screw-cap, 250- to 2000-mL volume

**6.7** Graduated cylinders—100- to 1000-mL, covered with aluminum foil or kraft paper and sterilized

**6.8** Thermometers—0°C to 50°C with 0.2°C graduations checked against a National Institute of Standards and Technology (NIST) certified thermometer, or one that meets the requirements of NIST Monograph SP 250-23

**6.9** Burner—Bunsen or Fisher type, or electric incinerator unit for sterilizing loops

**6.10** Hand tally or electronic counting device

**6.11** pH meter

**6.12** Blender and blender jar

**6.13** Micro pipettors with sterile tips may also be used for transfers

**6.14** Equipment for MPN procedure

**6.14.1** Covered water bath—With circulating system to maintain temperature of 44.5°C ± 0.2°C. Water level should be above the media.

**6.14.2** Autoclave capable of 121°C at 15 psi

**6.14.3** Covered water bath or water- or air-jacketed incubator at 35°C ± 0.5 °C

**6.14.4** Inoculation loops—Nichrome or platinum wire, disposable sterile plastic loops or sterile wooden applicator, at least 3 mm in diameter

**6.14.5** Sterile culture tubes—16 x 150 mm or 20 × 150 mm, borosilicate glass or plastic

**6.14.6** Inverted tubes or vials—10 × 50 mm

**6.14.7** Balance—Analytical balance capable of weighing 0.1 mg

**6.14.8** Caps—Loose-fitting aluminum, stainless steel, or autoclavable plastic, for 20mm-diameter test tubes

**6.14.9** Test tube racks

**6.15** Equipment for MF procedure

**6.15.1** Covered water bath with circulation system, a heat-sink incubator or an incubator that can maintain a temperature of 44.5 °C ± 0.2°C

**6.15.2** Petri dishes—Sterile, plastic, 60 × 15 mm, 50 × 12 mm, or other appropriate size, borosilicate glass or disposable plastic with tight-fitting lids

**6.15.3** Sterile waterproof plastic bags—Whirl-Pak or equivalent

**6.15.4** MF filter base and funnel— Glass, plastic or stainless steel, wrapped with aluminum foil or kraft paper and sterilized

**6.15.5** Ultraviolet unit for sanitization of the filter funnel between filtrations

**6.15.6** 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

**6.15.7** Vacuum filter flask—Usually 1 L, with appropriate tubing; filter manifold to hold a number of filter bases is optional

**6.15.8** Vacuum flask for safety trap—Place between the filter flask and the vacuum source

**6.15.9** Membrane filters—Sterile, white, grid-marked, 47-mm diameter, with 0.45-$\mu$m pore-size. The filters must meet the specifications found in Standard Methods (Reference 19.1)

**6.15.10** Presterilized absorbent pads—47-mm diameter, 0.8-mm thickness, nontoxic, capable of absorbing 2.0 to 3.0 mL broth. The pads must perform according to the ASTM Standard Test Method in D 4198 (19.3).

**6.15.11** Forceps—Sterile, straight or curved, with smooth tips to handle filters without damage

**6.15.12** Ethanol, methanol or isopropanol in a small, wide-mouth container, for sterilizing forceps

**6.15.13** Low-power (10X to 15X), binocular, wide-field dissecting microscope

**6.15.14** Microscope lamp—Producing diffuse light from a cool, white, fluorescent bulb (or equivalent) adjusted to give maximum visibility

**6.15.15** Boiling water bath for sanitization of filtration units.

**6.16** Equipment for percent total solids—see Appendix A

## 7.0 Reagents and Standards

**7.1** 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 19.2). The agar used in preparation of culture media must be of microbiological grade.

**7.2** Whenever possible, use commercial dehydrated culture media.

**7.3** Purity of water—Reagent water conforming to Specification D1193, Reagent water conforming to Type II, Annual Book of ASTM Standards (Reference 19.3).

**7.4** Dilution water

**7.4.1** Phosphate buffered dilution water

**7.4.1.1** Prepare stock phosphate buffer solution, dissolve 34.0 g potassium dihydrogen phosphate ($KH_2PO_4$), in 500 mL of reagent-grade water, adjust to pH 7.2 ± 0.5 with 1 N sodium hydroxide (NaOH), and dilute to 1 L with reagent-grade water.

**7.4.1.2** Prepare stock magnesium chloride solution by dissolving 81.1 g of magnesium chloride hexahydrate ( $MgCl_2 \cdot 6H_2O$) to 1 L of reagent-grade water.

**7.4.1.3** To prepare the buffered dilution water, add 1.25 mL stock phosphate buffer solution and 5.0 mL of the magnesium chloride solution to 1 L reagent-grade water. Dispense in appropriate amounts for dilutions in bottles or culture tubes and/or into containers for use as rinse water. After preparation, autoclave at 121 °C (15 psi) for 15 min after the buffer reaches 121 °C (15 psi). The amount of time in the autoclave must be adjusted for the volume of buffer in the containers and the size of the load.

**7.4.2** Peptone dilution water

**7.4.2.1** To prepare peptone dilution water, add 1.0 g of peptone to 1 L reagent-grade water. Adjust pH to 7.0 ± 0.1 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide, if necessary. Dispense in appropriate amounts for dilutions in bottles or culture tubes and/or into containers for use as rinse water. After preparation, autoclave at 121 °C (15 psi) for 15 min after the buffer reaches 121 °C (15 psi). The amount of time in the autoclave must be adjusted for the volume of buffer in the containers and the size of the load.

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*NOTE: When test tube racks containing 9.0 mL sterile dilution water are prepared, they are placed into an autoclavable pan with a small amount of water to contain breakage and minimize evaporation from the tubes.*

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*NOTE: Do not suspend bacteria in dilution water for more than 30 minutes at room temperature.*

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**7.5** Tryptone glucose yeast (TGY) broth/agar DIFCO# 0751-17, #0479-17 or equivalent—For preparation follow procedure as specified on bottle of media. If dehydrated media is not available, prepare media by adding 5.0 g tryptone, 2.5 g yeast extract, 1.0 g glucose, and 15.0 g agar to 1 L reagent-grade water. For broth, omit agar. Mix thoroughly and heat to dissolve. Stir well and autoclave at 121°C for 15 min. Other general growth media may be used for QA (Section 9.0) purposes.

**7.6** Media for the MPN procedure:

**7.6.1** LTB media DIFCO# 0241-17 or equivalent—For preparation follow procedure as specified on bottle of media. If dehydrated media is not available prepare the media by adding 20.0 g tryptose, 5.0 g lactose, 2.75 g dipotassium hydrogen phosphate ($K_2HPO_4$), 2.75 g potassium dihydrogen phosphate ($KH_2PO_4$), 5.0 g sodium chloride (NaCl), and 0.1 g sodium lauryl sulfate to 1 L reagent-grade water, mix thoroughly, and heat to dissolve. Adjust pH to 6.8 ± 0.2 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide, if necessary. Before sterilization, dispense 10 mL into 20 × 150 mm test tubes with inverted vials. Make sure there is enough media to cover the inverted vial at least half way after sterilization. Alternatively, for Class B samples, omit inverted vial and add 0.01 g/L bromocresol purple to presumptive media to determine acid production.

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*CAUTION: Use of bromocresol purple as an indicator of acid production is not recommended for analysis of Class A biosolids because the high concentration of solids in each tube makes it difficult to discern a color change.*

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To make double strength (2X) LTB, use 500 mL reagent-grade water. 2X LTB is needed for 10-mL inoculations, to ensure that the 10-mL sample volume does not excessively dilute the ingredient concentrations. Close tubes with metal or autoclavable plastic caps and autoclave at 121°C at 15 psi for 12 minutes. After cooling, the media should fill the inverted vials completely, leaving no air space.

**7.6.2** EC media DIFCO# 0314-17 or equivalent—Follow procedure as specified on bottle of media for preparation. If dehydrated media is not available, prepare the media by adding 20.0 g tryptose or trypticase, 5.0 g lactose, 1.5 g bile salts mixture or bile salts No.3, 4.0 g dipotassium hydrogen phosphate ($K_2HPO_4$), 1.5 g potassium dihydrogen phosphate ($KH_2PO_4$), and 5.0 g sodium chloride (NaCl) to 1 L of reagent-grade water. Mix thoroughly and heat to dissolve. Adjust pH to 6.9 ± 0.2 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide, if necessary. Before sterilization, dispense 10 mL into 20 × 150 mm test tubes, each with an inverted vial, and sufficient media to cover the inverted vial half way after sterilization. Close tubes with metal or heat-resistant plastic caps. Autoclave at 121° C at 15 psi for 15 minutes. Media should fill inverted tubes leaving no air spaces.

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*NOTE: EC-MUG media may be used in place of EC media. If inverted vials are used, they must not autofluoresce.*

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**7.6.3** A-1 media DIFCO# 1823-17 or equivalent—Follow procedure as specified on bottle of media for preparation. If dehydrated media is not available, prepare media by adding 5.0 g lactose, 20.0 g tryptone, 5.0 g sodium chloride (NaCl), and 0.5 g salicin to 1 L reagent-grade water. Heat to dissolve, then add 1.0 mL polyethylene glycol p-isooctylphenyl ether. To make double strength (2X) A-1, use 500 mL reagent-grade water. 2X A-1 is needed for 10-mL inoculations, to ensure that the 10-mL sample volume does not excessively dilute the ingredient concentrations. Adjust pH to 6.9 ± 0.1

by addition of 1.0 N hydrochloric acid or 1.0 N sodium hydroxide, if necessary. Before sterilization, dispense into 20 × 150 mm test tubes with an inverted vial. There should be sufficient media to cover the inverted vial one-half to two-thirds after sterilization. Close with metal or autoclavable plastic caps. Sterilize by autoclaving at 121 °C at 15 psi for 10 min. Ignore formation of precipitate. Media should fill inverted tubes leaving no air spaces.

**7.7** m-FC media for the membrane filter—DIFCO# 0677-17 or equivalent

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*NOTE: Do not sterilize m-FC media by autoclaving, as rosolic acid will decompose under these conditions. Also, if interference from other bacteria is not a problem, rosolic acid may be omitted from the m-FC agar.*

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**7.7.1** m-FC broth for absorbent pads—Follow procedure as specified on bottle of media for preparation. If dehydrated media is not available, prepare media by adding 10.0 g tryptose or biosate, 5.0 g proteose peptone No.3 or polypeptone, 3.0 g yeast extract, 5.0 g sodium chloride (NaCl), 12.5 g lactose, 1.5 g bile salts No. 3 or bile salts mixture, and 0.1 g aniline blue to 1 L reagent-grade water. Add 10 mL of rosolic acid solution (1% in 0.2 N NaOH) to the 1 L of broth. Heat to near boiling, promptly remove from heat, and cool to below 50°C. Do not sterilize by autoclaving, as rosolic acid will decompose under these conditions. Final pH should be 7.4 ± 0.2. Store finished media at 4 to 8 °C, preferably in sealed plastic bags or other containers to reduce moisture loss. Discard unused broth after 96 hours or unused agar after 2 weeks.

**7.7.2** m-FC agar—Agar is the same formulation as m-FC broth base with 15.0 g agar (1.5% agar) added for solidification. Rehydrate in 1 L reagent-grade water. Heat to boiling to dissolve agar, turn heat down, add the 10 mL of rosolic acid solution (1% in 0.2 N NaOH), and heat for 1 more minute. Promptly remove from heat, and cool to between 45 and 50 °C. Dispense 5 to 7 mL quantities into 60-mm, sterile glass or plastic petri dishes. If dishes of any other size are used, adjust quantity to give an equivalent depth. Final pH should be 7.2 ± 0.2.

**7.8** Bromocresol purple—DIFCO#0201 or equivalent

**7.9** Reagent water containing 1% rosolic acid (DIFCO#3228 or equivalent) in 0.2 N NaOH

**7.10** Obtain a stock culture of *E. coli* as a positive control

**7.11** Obtain a stock culture of *Pseudomonas* spp. or equivalent as a negative control

**7.12** The storage times for prepared media used in this method are provided in Table 1 below:

TABLE 1. STORAGE TIMES FOR PREPARED MEDIA

| Media | Storage Time |
|---|---|
| Membrane filter (MF) broth in screwcap flasks at 4°C | 96 hours |
| MF agar in plates with tight-fitting covers at 4°C | 2 weeks |
| Agar or broth (A-1, EC, LTB, and TGY) in loose-cap tubes at 4°C | 1 week |
| Agar or broth (A-1, EC, LTB, and TGY) in tightly closed screw-cap tubes at 4°C | 3 months |
| Poured agar plates with loose fitting covers in sealed plastic bags at 4°C | 2 weeks |
| Large volume of agar in tightly closed screw-cap flask or bottle at 4°C | 3 months |

## 8.0 Sample Collection, Preservation, and Storage

**8.1** The most appropriate location for biosolid sample collection is the point prior to leaving the wastewater treatment plant. Samples may be taken from pipes, conveyor belts, bins, compost heaps, drying beds and stockpiles.

**8.2** Collect samples in sterile non-toxic glass or plastic containers with leak-proof lids. All sampling containers and equipment must be clean and sterile.

**8.3** Equipment and container cleaning procedure

**8.3.1** Wash apparatus with laboratory-grade detergent and water

**8.3.2** Rinse with tap water

**8.3.3** Rinse with 10% HCl acid wash

**8.3.4** Rinse with distilled water

**8.3.5** Allow to air dry

**8.3.6** Cover with foil and autoclave for 15 minutes at 121°C

**8.4** Digester biosolids sampling procedure

**8.4.1** Collect digester biosolids sample from outlet pipe used to fill the truck.

**8.4.2** Purge the pipe of old biosolids and warm to the digester temperature by allowing biosolids to flow through the pipe into a bucket.

**8.4.3** Position a 1-gal. sterile bag under the flow so that only the sample touches the inside of the bag. Fill the bag, leaving 0.5 in. of head space in the bag for gas production. Leaving head room is extremely important when taking samples of biosolids that have been anaerobically digested.

**8.5** Procedure for sampling conveyor belt biosolid output

**8.5.1** Place the biosolid sample directly into the sample container without mixing or transferring to another area.

**8.5.2** Using a sterile scoop, transfer the pressed biosolids directly from the conveyer and into a sterile container.

**8.5.3** Pack sample into container. Leaving additional head space is not as important as in Section 8.4 because there is less gas formation.

**8.6** Procedure for sampling from a bin, drying bed, truck bed or similar container

**8.6.1** Remove surface material (upper six inches) and divide material to be sampled into four quadrants.

**8.6.2** Use a scoop or core the sample if material is deep.

**8.6.3** Take a sample from each of the quadrants and combine in a stainless steel or plastic bucket.

**8.6.4** After all the samples have been taken, pour the bucket out onto a plastic sheet and mix by folding the sample back onto itself several times.

**8.6.5** Reduce the sample size by "coning and quartering." Divide the bucket contents into four even piles. If sample size is still too large, divide each quarter into quarters and discard half. Put into glass or plastic sampling container.

**8.6.6** An alternate method to "coning and quartering" is to randomly take a flat shovel full of biosolids from the bucket that has been dumped onto a plastic sheet and put samples into a sampling container. (Curved scoops have been shown to favor a certain size particle and should not be used.).

**8.7** Record the following into your log book:

**8.7.1** Facility name and location

**8.7.2** Date

**8.7.3** Arrival time

**8.7.4** Name of facility and contact

**8.8** Record the following onto sample container and in log book when known:

**8.8.1** Sample number

**8.8.2** Date and time

**8.8.3** Sample name

**8.8.4** Sample location

**8.8.5** Parameters

**8.8.6** Volume

**8.8.7** Observations

**8.9** Ensure that the chain of custody form is filled out

**8.10** Sample preservation and handling—Ice or refrigerate bacteriological samples at a temperature of 1 °C to 4 °C during transit to the laboratory. Do not freeze sample. Use insulated containers to ensure proper maintenance of storage temperature. Sample bottles should be placed inside waterproof bags, excess air purged, and bags sealed to ensure that bottles remain dry during transit or storage. Refrigerate samples upon arrival in the laboratory and analyze as soon as possible after collection. Bring samples to room temperature before analysis.

**8.11** Chlorinated samples—Add a reducing agent to containers intended for the collection of biosolids containing residual chlorine or other halogen. Sodium thiosulfate ($Na_2S_2O_3$) is a satisfactory dechlorinating agent that neutralizes any residual halogen and prevents continuation of bactericidal action during sample transport. If $Na_2S_2O_3$ is used, add a sufficient volume of $Na_2S_2O_3$ to a clean sample bottle, to give a concentration of 100 mg/L in the sample. In a 120-mL sample bottle, a

volume of 0.1 mL of a 10% solution of $Na_2S_2O_3$ will neutralize a sample containing about 15 mg/L residual chlorine.

**8.12** Holding time limitations—Analyses should begin immediately, preferably, within 2 hours of collection. If it is impossible to examine samples within 2 hours, refrigerate samples at 4°C immediately after collection. Sample analysis must begin within 24 hours.

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*NOTE: Adherence to sample preservation procedures and holding time limits is critical to the production of valid data. Sample results will be considered invalid if these conditions are not met.*

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## 9.0 Quality Control

**9.1** Each laboratory that uses this method is required to operate a formal quality assurance (QA) program. The minimum requirements of this program consist of an initial demonstration of laboratory capability through analysis of positive and negative control samples and blanks (Section 9.3 and 9.4), and analysis of positive and negative control samples and blanks as tests of continued performance. Laboratory performance is compared to the performance criteria specified in Sections 9.3 and 9.4 to determine whether the results of the analyses meet the performance characteristics of the method. Specific quality control (QC) requirements for Method 1680 are provided below. General recommendations on QA and QC for facilities, personnel, and laboratory equipment, instrumentation, and supplies used in microbiological analyses are provided in the USEPA Microbiology Methods Manual, Part IV, C (Reference 19.4).

**9.2** General analytical QC procedures

**9.2.1** Check each batch (batch is defined as 20 samples or one 8-hour laboratory shift, whichever is sooner) of dilution water for sterility by adding 20 mL water to 100 mL of a nonselective broth such as TGY broth (Section 7.5). Alternatively, aseptically pass 100 mL or more dilution water through a membrane filter and place filter on TGY agar. Incubate at 35 ± 0.5 °C for 48 to 72 hours and observe growth. If any contamination is indicated, reject analytical data from samples tested with these materials.

**9.2.2** To test sterility of media, subject a representative portion of each batch to incubation at 35°C for 48 to 72 hours and observe for indications of growth. With respect to media, a batch is defined as one tube out of 50 in each lot or one tube, if the lot contains less than 50 tubes.

**9.2.3** Perform duplicate analyses on 10% of samples or one sample per test run, whichever is greater.

**9.2.4** In laboratories with more than one analyst, have each analyst conduct parallel tests on at least one positive sample monthly.

**9.2.5** Obtain reference cultures from qualified outside sources and use to establish pure cultures that are maintained for the laboratory. Use these in routine analytical runs, quarterly. Review results to correct causes of improper responses and document actions.

**9.2.6** Participate in available interlaboratory performance studies conducted by local, state, and federal agencies or commercial organizations. Review results to correct unsatisfactory performance and record corrective actions.

**9.3** Analytical QC procedures for MPN procedure—These procedures are required with each new lot of media, and in conjunction with each batch of samples.

**9.3.1** Positive and negative controls—Perform LTB/EC or A-1 MPN procedure (correlating with procedure being used by the lab) on a known positive (e.g. *E. coli*) and a known negative fecal coliform species (e.g. *Pseudomonas* sp.). Examine for appropriate responses, record results and pursue causes of irregularities.

**9.3.2** Blank—Test a 20 mL sterile water sample in the analytical scheme to verify the sterility of equipment, materials, and supplies.

**9.4** Analytical QC for MF procedure—These procedures are required with each new lot of media, and in conjunction with each batch of samples.

**9.4.1** Positive control—Prepare a stock culture of *E. coli* for testing each lot of m-FC media for satisfactory bacterial growth. Prepare dilutions of culture and filter appropriate volumes to give 20 to 80 colonies per filter. Perform MF procedure and verify 10 or more colonies obtained from several positive control filters. Presence of fecal coliform bacteria establishes that the media and procedures are working properly. Examine plates for appropriate responses, record results and pursue causes of irregularities.

**9.4.2** Negative control—Prepare dilutions of a known negative culture, such as *Pseudomonas* spp., and filter appropriate volumes to give 20 to 80 colonies per filter. Perform MF procedure and verify 10 or more colonies obtained from several negative control filters. Absence of fecal coliform bacteria establishes that the media and procedures are working properly. Examine plates for appropriate responses, record results and pursue causes of irregularities.

**9.4.3** Blank—Filter a sterile 100 mL rinse water sample and perform MF procedure. Incubate the rinse water control membrane culture under conditions identical to those of the samples. Also, perform the MF procedure with water following filtration of a series of 10 samples to check for possible cross-contamination or contaminated rinse water.

## 10.0 Equipment Calibration and Standardization

**10.1** Check temperatures in incubators twice daily, with the readings separated by at least 4 hours, to ensure operation is within stated limits of the method and record daily measurements in incubator log book.

**10.2** Calibrate thermometers and incubators at least annually against an NIST certified thermometer or one that meets requirements of NIST Monograph SP 250-23. Check mercury columns for breaks.

**10.3** Calibrate top-loading balances monthly with reference weights of ASTM Class 2.

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

## 11.0 Multiple-Tube Fermentation Procedure

**11.1** The MPN procedure may be used to determine fecal coliform densities in unknown, Class A, and Class B biosolid samples. Analysis of seven samples collected at the time of disposal using this procedure will satisfy the requirements of the monitoring alternative for demonstrating pathogen reduction in both Class A and Class B biosolids. Unknown samples should be analyzed using this

procedure with 10-fold serial dilutions, four series of five tubes. There are two method options for this procedure. The first involves the use of two media, LTB and EC. LTB is used as a presumptive media followed by EC media in a confirmed test. Do not use EC media for direct isolation from a biosolid sample because prior enrichment is required in presumptive media (LTB media) for optimum recovery of fecal coliforms. The second option is a direct test using A-1 media in a single step procedure not requiring a presumptive phase. The precision of both tests increases with increasing numbers of replicates per sample tested. For an overview of the MPN procedure refer to Figure 1.

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*NOTE: Remove medium from refrigerator 1-1.5 hours prior to inoculation, so that it reaches room temperature before use.*

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**11.2** Since sample fecal coliform densities are expected to be variable, it is recommended that at least seven biosolid samples be analyzed using this method. The geometric mean fecal coliform bacterial density of at least seven samples of biosolids should not exceed 2 X $10^6$ MPN/g of total solids to qualify as Class B biosolids. Although there is not a specific number of samples required for Class A biosolids, it is recommended that a sampling event extend over two weeks and that at least seven samples be collected and determined to be below 1,000 MPN/g of total solids to qualify as Class A biosolids.

**11.3** LTB/EC MPN procedure

**11.3.1** Presumptive phase with LTB media

**11.3.1.1** Prepare LTB media and introduce into tubes as directed in Section 7.6.1.

**11.3.1.2** Arrange test tubes in four rows of five tubes each per sample in a test tube rack. When 10 mL of sample or dilution is used, tubes should contain 10 mL of 2X LTB media (Section 11.5). Clearly label each bank of tubes to identify the sample and dilution to be inoculated.

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*NOTE: 2X LTB is needed for 10-mL inoculations, to ensure that the 10-mL inoculation volume does not excessively dilute the ingredient concentrations.*

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**11.3.1.3** Homogenize samples depending on matrix

**(A)** Liquid samples—Blend sample in a sterile blender on low speed for two minutes. Adjust pH to 7.0-7.5 by addition of 1.0 N hydrochloric acid, if necessary.

**(B)** Solid samples—Weigh out 30.0 ± 0.1 g of well-mixed sample in a sterile dish. Whenever possible, the sample tested should contain all materials that will be included in the biosolid. For example, if wood chips are part of the biosolid compost, some mixing or grinding means may be needed to achieve homogeneity before testing. Large pieces of wood that are not easily ground may be discarded before blending. Transfer the sample to a sterile blender. Alternatively, the sample may be weighed directly into the sterile blender jar. Use 270 mL of sterile dilution water

(Section 7.4) to rinse any remaining sample into the blender. Cover and blend on low speed for 2 minutes. This is the "blended" sample. A volume of 1.0-mL of the "blended" sample contains $10^{-1}$ g of the original sample. Adjust pH to 7.0-7.5 by addition of 1.0 N hydrochloric acid, if necessary.

**11.3.1.4** Based on the matrix, dilute and inoculate samples according to Section 11.5.

**11.3.1.5** Incubate inoculated tubes at 35°C ± 0.5°C. After 24 ± 2 hours, swirl each tube gently and examine it for growth, gas, and /or acidic reaction (shades of yellow). If no gas or acid has formed, reincubate for an additional 24 ± 2 hours and reexamine. Final examination should be within a total of 48 ± 3 hours. The presence of gas in the absence of growth is usually due to mishandling or improper shaking of the tubes after inoculation.

**11.3.1.6** The presence of gas in inverted vials or production of acid in the presence of bromocresol purple within 48 ± 3 hours signifies a positive presumptive reaction.

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*NOTE: Acidic reaction (color change) may be masked in some samples due to starting color of the dilution.*

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**11.3.1.7** Proceed to the confirmation phase (Section 11.3.2) for tubes with a positive presumptive reaction.

**11.3.2** Confirmation phase for fecal coliforms using EC media

**11.3.2.1** Prepare EC broth tubes as described in Section 7.6.2. For each positive LTB tube, one EC tube will be used.

**11.3.2.2** Gently shake tubes from presumptive test showing positive reaction.

**11.3.2.3** Using a sterile 3 to 3.5-mm-diameter loop or sterile wooden applicator stick, transfer growth from each positive presumptive tube to corresponding tubes containing EC broth.

**11.3.2.4** Place all EC tubes in a 44.5°C ± 0.2°C water bath within 30 min of inoculation and incubate for 24 ± 2 hours. Maintain a water depth in the water bath sufficient to immerse tubes to upper level of the media.

**11.3.2.5** After incubation, examine each tube for gas production. Gas production with growth in an EC broth culture in 24 ± 2 hours is considered a positive fecal coliform reaction. Failure to produce gas constitutes a negative reaction. The presence of gas in the absence of growth is usually due to mishandling or improper shaking of the tubes after inoculation.

**11.3.2.6** Record positive and negative reactions in the EC media tubes. Calculate MPN/g of total solids (dry weight) from the number of positive EC media tubes as described in Section 13.1.

**11.4** A-1 MPN procedure

*NOTE: A-1 media may produce false positives in thermally treated biosolids.*

**11.4.1** Prepare A-1 broth tubes as directed in Section 7.6.3.

**11.4.2** Arrange test tubes in four rows of five tubes each per sample in a test tube rack. Use 10 mL of 2X A-1 broth for 10-mL inoculations (Section 11.5). Clearly label each bank of tubes to identify the sample and dilution volume to be inoculated.

*NOTE: 2X A-1 is needed for 10-mL inoculations, to ensure that the 10-mL inoculation volume does not excessively dilute the ingredient concentrations.*

**11.4.3** Homogenize samples depending on the matrix

**11.4.3.1** Liquid samples—Blend sample in a sterile blender on low speed for two minutes. Adjust pH to 7.0-7.5 by addition of 1.0 N hydrochloric acid, if necessary.

**11.4.3.2** Solid samples—Weigh out 30.0 ± 0.1 g of well-mixed sample in a sterile dish. Whenever possible, the sample tested should contain all materials that will be included in the biosolid. For example, if wood chips are part of the biosolid compost, some mixing or grinding means may be needed to achieve homogeneity before testing. Large pieces of wood that are not easily ground may be discarded before blending. Transfer the sample to a sterile blender. Use 270 mL of sterile dilution water (Section 7.4) to rinse any remaining sample into the blender. Alternatively, the sample may be weighed directly into the sterile blender jar. Cover and blend on low speed for 2 minutes. This is the "blended" sample. A volume of 1.0-mL of the "blended" sample contains $10^{-1}$ g of the original sample. Adjust pH to 7.0-7.5 by addition of 1.0 N hydrochloric acid, if necessary.

**11.4.4** Dilute and inoculate samples depending on the matrix as described in Section 11.5.

**11.4.5** Incubate inoculated tubes at 35 ± 0.5 °C for 3 hours.

**11.4.6** Transfer tubes to a water bath at 44.5 ± 0.2 °C and incubate for an additional 21 ± 2 hours. Water level should be above the media in immersed tubes.

**11.4.7** After incubation, remove the tubes from the water bath and gently agitate. Examine tubes for growth and gas production, signifying a positive test for the presence of fecal coliforms. The presence of gas in the absence of growth is usually due to mishandling or improper shaking of the tubes after inoculation.

**11.4.8** Record positive and negative tube results. Calculate MPN/g total solids (dry weight) from the number of positive A-1 broth tubes as described in 13.1.

**11.5** MPN dilution and inoculation

The biosolid samples analyzed for fecal coliforms using this method may require dilution prior to analysis. An ideal sample volume will yield results that accurately estimate fecal coliform density. Because the number of fecal coliform bacteria in undiluted samples could easily exceed the detection limits of these procedures, the laboratory must follow the sample dilution and inoculation

procedures in Section 11.5 or the optional dilution and inoculation schemes for the MPN procedure in Section 1.0 and 2.0 of Appendix B.

*NOTE: For some transfers, it may be convenient to use a sterile, wide-mouth pipette, capable of transferring particulate matter.*

**11.5.1** Class B liquid samples—Four series of five tubes each will be used for the analysis with $10^{-3}$, $10^{-4}$, $10^{-5}$, and $10^{-6}$ mL of the original sample. The first and third series of tubes must contain 2X media. See Figure 2 for a summary of this dilution and inoculation scheme.

**11.5.1.1** Dilution

**(A)** Use a sterile pipette to transfer 11.0 mL of homogenized sample (Section 11.3.1.3(A) or 11.4.3.1) to 99 mL of sterile dilution water (Section 7.4), cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is dilution "A." A 1.0-mL volume of dilution "A" is $10^{-1}$ mL of the original sample.

**(B)** Use a sterile pipette to transfer 11.0 mL of dilution "A" to 99 mL of sterile dilution water, and mix as before. This is dilution "B." A 1.0-mL volume of dilution "B" is $10^{-2}$ mL of the original sample.

**(C)** Use a sterile pipette to transfer 1.0 mL of well-mixed dilution "B" to 99 mL of sterile dilution water, and mix as before. This is dilution "C." A 1.0-mL volume of dilution "C" is $10^{-4}$ mL of the original sample.

**(D)** Use a sterile pipette to transfer 1.0 mL of well mixed dilution "C" to 99 mL of sterile dilution water, and mix as before. This is dilution "D." A 1.0-mL volume of dilution "D" is $10^{-6}$ mL of the original sample.

**11.5.1.2** Inoculation

**(A)** Use a sterile pipette to inoculate each of the first series of five tubes with 10.0 mL of dilution "C." This series must contain 2X media. This is $10^{-3}$ mL of the original sample.

**(B)** Use a sterile pipette to inoculate each of the second series of tubes with 1.0 mL of dilution "C." This is $10^{-4}$ mL of the original sample.

**(C)** Use a sterile pipette to inoculate each of the third series of tubes with 10.0 mL of "D." This series must contain 2X media. This is $10^{-5}$ mL of the original sample.

**(D)** Use a sterile pipette to inoculate each of the fourth series of five tubes each with 1.0 mL of dilution "D." This is $10^{-6}$ mL of the original sample.

**11.5.1.3** Repeat Section 11.5.1.1 and 11.5.1.2 for each remaining Class B liquid sample. When inoculations are complete, go to Section 11.3.1.5 to continue the LTB/EC method or to Section 11.4.5 to continue the A-1 method.

**11.5.2** Class B solid samples—The four series of five tubes each will contain $10^{-3}$, $10^{-4}$, $10^{-5}$, and $10^{-6}$ g of the original sample. The first and third series of tubes must contain 2X media. See Figure 3 for a summary of this dilution and inoculation scheme.

**11.5.2.1** Dilution

**(A)** A volume of 1.0-mL of the "blended" sample (Section 11.3.1.3(B) or 11.4.3.2 ) contains $10^{-1}$ g of the original sample.

**(B)** Use a sterile pipette to transfer 11.0 mL of the blender contents to 99 mL of sterile dilution water and shake vigorously a minimum of 25 times. This is dilution "A." A volume of 1.0-mL of dilution "A" contains $10^{-2}$ g of the original sample.

**(C)** Use a sterile pipette to transfer 1.0 mL of dilution "A" to 99 mL of sterile dilution water, and mix as before. This is dilution "B." A volume of 1.0-mL of dilution "B"contains $10^{-4}$ g of the original sample.

**(D)** Use a sterile pipette to transfer 1.0 mL of dilution "B" to 99 mL of sterile dilution water, and mix as before. This is dilution "C." A volume of 1.0-mL of dilution "C" contains $10^{-6}$ g of the original sample.

**11.5.2.2** Inoculation

**(A)** Inoculate each of the first series of five tubes with 10.0 mL of dilution "B." This series of tubes must contain 2X media. This is $10^{-3}$ g of the original sample.

**(B)** Inoculate each of the second series of tubes with 1.0 mL of dilution "B." This is $10^{-4}$ g of the original sample.

**(C)** Inoculate each of the third series of tubes with 10.0 mL of "C." This series of tubes must contain 2X media. This is $10^{-5}$ g of the original sample.

**(D)** Inoculate each of the fourth series of five tubes each with 1.0 mL of dilution "C." This is $10^{-6}$ g of the original sample.

**11.5.2.3** When inoculations are complete, go to Section 11.3.1.5 to continue the LTB/EC method or to Section 11.4.5 to continue the A-1 method.

**11.5.3** Class A liquid samples—The four series of five tubes each will contain 1.0, $10^{-1}$, $10^{-2}$, and $10^{-3}$ mL of the original sample. See Figure 4 for an overview of this dilution and inoculation scheme.

**11.5.3.1** Dilution

**(A)** Use a sterile pipette to transfer 11.0 mL of homogenized sample (Section 11.3.1.3(A) or 11.4.3.1) to 99 mL of sterile dilution water (Section 7.4), cap, and mix by vigorously shaking the bottle

a minimum of 25 times. This is dilution "A." A volume of 1.0-mL of dilution "A" contains $10^{-1}$ mL of the original sample.

**(B)** Use a sterile pipette to transfer 11.0 mL of dilution "A" to 99 mL of sterile dilution water, and mix as before. This is dilution "B." A 1.0-mL volume of dilution "B" is $10^{-2}$ mL of the original sample.

**(C)** Use a sterile pipette to transfer 11.0 mL of well mixed dilution "B" to 99 mL of sterile dilution water, and mix as before. This is dilution "C." A volume of 1.0-mL of dilution "C" is $10^{-3}$ mL of the original sample.

**11.5.3.2** Inoculation

**(A)** Use a sterile pipette to inoculate each of the first series of five tubes with 1.0 mL of the original homogenized sample per tube.

**(B)** Use a sterile pipette to inoculate each of the second series of tubes with 1.0 mL of dilution "A." This is $10^{-1}$ mL of the original sample.

**(C)** Use a sterile pipette to inoculate each of the third series of tubes with 1.0 mL of dilution "B." This is $10^{-2}$ mL of the original sample.

**(D)** Use a sterile pipette to inoculate each of the fourth series of tubes with 1.0 mL of dilution "C." This is $10^{-3}$ mL of the original sample.

**11.5.3.3** Repeat steps 11.5.3.1 and 11.5.3.2 for the remaining Class A liquid samples. When inoculations are complete, go to Section 11.3.1.5 to continue the LTB/EC method or to Section 11.4.5 to continue the A-1 method.

**11.5.4** Class A solid samples—Four series of five tubes will be used for the analysis with 1.0, $10^{-1}$, $10^{-2}$ and $10^{-3}$ g of the original sample. The first series of tubes must contain 2X media. See Figure 5 for a summary of this dilution and inoculation scheme.

**11.5.4.1** Dilution

**(A)** A volume of 1.0-mL of the "blended" sample (11.3.1.3(B) or 11.4.3.2) contains $10^{-1}$ g of the original sample.

**(B)** Use a sterile pipette to transfer 11.0 mL of the blender contents to 99 mL of sterile dilution water (Section 7.4) and shake vigorously a minimum of 25 times. This is dilution "A." A volume of 1.0-mL of dilution "A" contains $10^{-2}$ g of the original sample.

**(C)** Use a sterile pipette to transfer 11.0 mL of dilution "A" to 99 mL of sterile dilution water and mix as before. This is dilution "B." A volume of 1.0-mL of dilution "B" contains $10^{-3}$ g of the original sample.

**11.5.4.2** Inoculation

**(A)** Use a sterile pipette to inoculate each of the first series of tubes with 10.0 mL of the "blended" sample. This series of tubes must contain 2X media. This is 1.0 g of the original sample. Since test tubes with inverted vials are being used, shaking is not practical. Solids that will not separate easily and/or may float should be submerged into the broth with a sterile loop.

**(B)** Use a sterile pipette to inoculate each of the second series of tubes with 1 mL of the "blended" mixture. This is $10^{-1}$ g of the original sample.

**(C)** Use a sterile pipette to inoculate each of the third series of tubes with 1.0 mL of dilution "A." This is $10^{-2}$ g of the original sample.

**(D)** Use a sterile pipette to inoculate each of the fourth series of tubes with 1.0 mL of dilution "B." This is $10^{-3}$ g of the original sample.

**11.5.4.3** Repeat Section 11.5.4.1 and 11.5.4.2 for remaining Class A solid samples. When inoculations are complete, go to Section 11.3.1.5 to continue the LTB/EC method or to Section 11.4.5 to continue the A-1 method.

## 12.0 Membrane Filter Procedure

**12.1** The MF procedure provides a direct count of fecal coliform bacteria in biosolids based on the development of colonies on the surface of the membrane filter. This procedure may be used to determine fecal coliform densities in Class B biosolid samples only. At the low fecal coliform densities expected in Class A biosolids, the filter used in the MF procedure would result in a loading of solids too high to permit a reliable count of the number of fecal coliform colonies.

*NOTE: Since chlorination stresses fecal coliforms and significantly reduces recovery, the membrane filter procedure should not be used with chlorinated biosolids. (Reference 19.8 and 19.9)*

**12.2** At least seven biosolid samples should be analyzed using this method. The geometric mean fecal coliform bacteria density of at least seven samples of biosolids should not exceed 2 X $10^6$ MPN/g of total solids to qualify as Class B biosolids.

**12.3** Mark the petri dishes with sample identification and sample dilution volume. One filtration will be performed for each sample volume.

**12.4** Place a sterile absorbent pad in each culture dish and pipette 1.8 to 2.0 mL m-FC broth (Section 7.7.1), to saturate the pad. Carefully remove any excess liquid from culture dish. Alternatively, as a substitution for the nutrient saturated absorbent pad, use m-FC agar and prepare culture dishes as described in (Section 7.7.2).

**12.5** Homogenize samples depending on the matrix

**12.5.1** Liquid samples—Blend sample in a sterile blender on low speed for two minutes. Adjust pH to 7.0-7.5 by addition of 1.0 N hydrochloric acid, if necessary.

**12.5.2** Solid samples—In a sterile dish weigh out 30.0 ± 0.1 g of well-mixed sample. Whenever possible, the sample tested should contain all materials that will be included in the

biosolid. For example, if wood chips are part of the biosolid compost, some mixing or grinding means may be needed to achieve homogeneity before testing. Large pieces of wood that are not easily ground may be discarded before blending. Transfer the sample to a sterile blender. Alternatively, the sample may be weighed directly into the sterile blender jar. Use 270 mL of sterile dilution water (Section 7.4) to rinse any remaining sample into the blender. Cover and blend on low speed for 2 minutes. This is the "blended" sample. A volume of 1.0-mL of the "blended" sample contains $10^{-1}$ g of the original sample. Adjust pH to 7.0-7.5 by addition of 1.0 N hydrochloric acid, if necessary.

**12.6** Membrane filter dilution and inoculation (filtration)—The biosolid samples analyzed for fecal coliforms using this method require dilution prior to analysis. An ideal sample volume will yield results that accurately estimate fecal coliform density. Since the number of fecal coliforms in undiluted samples could easily exceed the detection limits of these procedures, laboratories must follow the sample dilution and inoculation procedures in this section or the optional procedures in Section 3.0 of Appendix B. Additional dilutions may be added, if needed to obtain countable results.

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NOTE: *Experienced analysts may modify the dilution and inoculation schemes described in Sections 12.6.1 and 12.6.2 to obtain filters that yield ideal counts between 20 and 80 fecal coliform colonies. The membrane filter should not contain more than 200 colonies of all types. In addition, when the bacterial density of the sample is unknown, filter several 10-fold dilutions to establish fecal coliform density. Estimate volume expected to yield a countable membrane and select two additional quantities representing one-tenth and ten times this volume, respectively.*

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NOTE: *For some transfers, it may be convenient to use a sterile, wide-mouth pipette, capable of transferring particulate matter.*

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**12.6.1** Class B liquid samples—Three individual filtrations should be conducted in order, using $10^{-5}$, $10^{-4}$, and $10^{-3}$ mL of the original sample. See Figure 6 for a summary of this dilution and inoculation scheme.

**12.6.1.1** Dilution

**(A)** Use a sterile pipette to transfer 11.0 mL of homogenized sample (Section 12.5.1) to 99 mL of sterile dilution water (Section 7.4), cap, and mix by vigorously shaking the bottle a minimum of 25 times. This dilution is "A." A volume of 1.0-mL of this mixture is $10^{-1}$ mL of the original sample.

**(B)** Use a sterile pipette to transfer 11.0 mL of dilution "A" to 99 mL of sterile dilution water, and mix as before. This is dilution "B." A 1.0-mL volume of dilution "B" is $10^{-2}$ mL of the original sample.

**(C)** Use a sterile pipette to transfer 1.0 mL of dilution "B" to 99 mL of sterile dilution water, and mix as before. This is dilution "C."

A volume of 1.0-mL of dilution "C" is $10^{-4}$ mL of the original sample.

**(D)** Use a sterile pipette to transfer 1.0 mL of dilution "C" to 99 mL of sterile dilution water, and mix as before. This is dilution "D." A volume of 1.0-mL of dilution "D" is $10^{-6}$ mL of the original sample.

**12.6.1.2** Inoculation (filtration)—Samples should be filtered in order of highest dilution (least sample volume) first to avoid carry over contamination.

**(A)** Filtration 1 will use 10.0 mL of dilution "D." This is $10^{-5}$ mL of the original sample.

**(B)** Filtration 2 will use 1.0 mL of dilution "C." This is $10^{-4}$ mL of the original sample.

**(C)** Filtration 3 will use 10.0 mL of dilution "C." This is $10^{-3}$ mL of the original sample.

**12.6.1.3** Repeat Section 12.6.1.1 and 12.6.1.2 for the remaining Class B liquid samples.

**12.6.2** Class B solid samples—Three individual filtrations should be conducted using $10^{-5}$, $10^{-4}$, and $10^{-3}$ mL of the original sample. See Figure 7 for a summary of this dilution and inoculation scheme.

**12.6.2.1** Dilution

**(A)** A volume of 1.0-mL of the "blended" sample (Section 12.5.2) contains $10^{-1}$ g of the original sample.

**(B)** Use a sterile pipette to transfer 11.0 mL of the blender contents to 99 mL of sterile dilution water and shake vigorously a minimum of 25 times. This is dilution "A." A volume of 1.0-mL of dilution "A" contains $10^{-2}$ g of the original sample.

**(C)** Use a sterile pipette to transfer 1.0 mL of dilution "A" to 99 mL of sterile dilution water, and mix as before. This is dilution "B." A volume of 1.0-mL of dilution "B" is $10^{-4}$ g of the original sample.

**(D)** Use a sterile pipette to transfer 1.0 mL of dilution "B" to 99 mL of sterile dilution water, and mix as before. This is dilution "C." A volume of 1.0-mL of dilution "C" is $10^{-6}$ g of the original sample.

**12.6.2.2** Inoculation (filtration)—Samples should be filtered in order of highest dilution (least sample volume) first to avoid carry over contamination.

**(A)** Filtration 1 will use 10.0 mL of dilution "C." This is $10^{-5}$ g of the original sample.

**(B)** Filtration 2 will use 1.0 mL of dilution "B." This is $10^{-4}$ g of the original sample.

**(C)** Filtration 3 will use 10.0 mL of dilution "B." This is $10^{-3}$ g of the original sample.

**12.6.2.3** Repeat Section 12.6.2.1 and 12.6.2.2 for the remaining Class B solid samples.

**12.7** Use a sanitized filtration unit assembly at the beginning of each filtration series. Decontaminate this equipment between successive filtrations by using an UV sanitzer for 2 min, flowing steam or boiling water for 2 min. Alternatively, filtration units may be autoclaved at 121 °C and 15 psi for 15 minutes.

**12.8** Sterilize forceps with ethanol or a boiling water bath and flame before use. Using sterile forceps, place a sterile membrane filter (grid side up) over the sintered glass funnel. Carefully place top half of unit over funnel and lock it in place.

**12.9** Samples should be filtered in order of highest dilution (least sample volume) first to avoid carry over contamination.

**12.10** Measure the sample and dilution series directly into the funnel with the vacuum turned off. When less than 10 mL of sample is to be filtered, add approximately 20 mL sterile dilution water to the funnel before adding the sample and applying the vacuum or pipette the sample volume into a sterile dilution flask containing approximately 20 mL sterile dilution water , then filter the entire dilution. The increase in water volume aids in uniform dispersion of the bacterial suspension over the entire effective filtering surface.

**12.11** Filter sample under partial vacuum and rinse the funnel by filtering three, 20 to 30 mL portions of sterile dilution water.

**12.12** Upon completion of the final rinse, disengage the vacuum, and unlock and remove the funnel.

**12.13** Immediately use sterile forceps to remove the membrane filter and place it grid side up on the m-FC media-impregnated pad or m-FC agar media with a rolling motion to avoid entrapment of air. Reseat membrane if bubbles occur.

**12.14** Place prepared cultures within 30 min after filtration in waterproof plastic bags and seal. Invert, and submerge petri dishes in water bath, for 24 ± 2 hours at 44.5 ± 0.2 °C. Anchor dishes below water surface to maintain critical temperature requirements.

**12.15** After incubation, count and record colonies on the filters that contain, if practical, 20 to 80 fecal coliform colonies that are light to dark blue. Count colonies with a low-powered (10 - 15 X) binocular wide field dissecting microscope or other optical device.

**12.16** Calculate fecal coliform density from the sample quantities that produced MF counts with the desired range of 20 to 80 colonies as directed in Section 13.2.

## 13.0 Data Analysis and Calculations

Due to the extreme variability in the solid content of biosolids, fecal coliform results from biosolid samples are reported as MPN or CFU per g total solids. The density of fecal coliform bacteria following the multiple-tube fermentation procedure is estimated using the MPN procedure and results are reported as fecal coliform MPN/g total solids (dry weight basis). The MPN calculations are provided in Section 13.1. The density of fecal coliform bacterial colonies on each filter from the MF procedure is calculated by direct identification and enumeration of colonies by microscope and results are reported as fecal coliform CFU/g total solids (dry weight basis). The CFU/g calculations are provided in Section 13.2.

**13.1** Estimation of fecal coliform density using the MPN procedure

**13.1.1** The estimated density of fecal coliform bacteria, based on the confirmation test using EC media, or the direct test with A-1 media, is computed in terms of MPN. Only three of the four five-tube series will be used to estimate the MPN. The three selected dilutions are called significant dilutions and are selected according to the criteria in Section 13.1.2. MPN index values and 95% confidence limits are obtained from Table 2 using the number of positive tubes in the three significant dilutions. The MPN index from Table 2, the largest significant dilution, and % total solids (dry weight) from Appendix A are then used in Equation 1 to calculate fecal coliform MPN/g total solids (dry weight).

**13.1.2** Scenarios for selection of significant dilutions

*NOTE: A dilution refers to the mL (liquid samples) or g (solid samples) of original sample that was inoculated into each series of tubes. For example, for Class B liquid samples (Section 11.5.1), four five-tube series were used, with $10^{-3}$, $10^{-4}$, $10^{-5}$, and $10^{-6}$ mL of the original sample in each tube. (The dilutions used in these four series were $10^{-3}$, $10^{-4}$, $10^{-5}$, and $10^{-6}$ mL.) For an example of solid samples , the dilutions used for Class A solid samples (Section 11.5.4) were 1.0, $10^{-1}$, $10^{-2}$ and $10^{-3}$ g.*

**13.1.2.1** When more than three dilutions series have positive tubes, use results from only three dilution series to compute MPN. Choose the highest dilution (the most dilute, with least amount of sample, e.g., $10^{-2}$ is higher than $10^{-1}$) giving positive results in all five tubes inoculated and the two succeeding higher dilutions.

**13.1.2.2** If there are positive tubes in higher dilutions than the dilutions selected, positive results are moved up from these dilutions to increase the positive tubes in the next highest dilution selected (e.g. Section 13.1.6 sample B).

**13.1.2.3** If all tubes are positive, choose the three highest dilution series. If all tubes are negative, choose the three lowest dilution series.

**13.1.2.4** An example of significant dilution selections are given in Table 3.

**13.1.3** Use Table 2 to estimate the MPN index from the significant dilutions chosen.

**13.1.4** Use Equation 1 to calculate fecal coliform MPN/g total solids (dry weight).

*NOTE: Calculations for liquid samples (significant dilutions in mL) and solid samples (significant dilutions in g) are calculated the same way.*

**EQUATION 1**

$$\text{MPN / g total solids} = \frac{\text{MPN index from Table 2}}{(\text{largest significant dilution})(\%\ \text{total solids})}$$

**13.1.5** The MPN for combinations not appearing in the Table 2 may be estimated by Thomas' formula (Equation 2, Reference 19.6). If such unlikely tube combinations occur in more than 1% of the samples, it is an indication that the procedure is faulty or that the statistical assumptions underlying the MPN estimate are not being fulfilled.

**EQUATION 2 (THOMAS' FORMULA)**

$$MPN / 100\ mL = \frac{number\ of\ positive\ test\ tubes\ X\ 100}{\sqrt{(mL\ sample\ in\ negative\ tubes\ X\ mL\ sample\ in\ all\ tubes)}}$$

**TABLE 2. MPN INDEX AND 95% CONFIDENCE LIMITS FOR COMBINATIONS OF POSITIVE RESULTS WHEN FIVE TUBES AND THREE SIGNIFICANT DILUTIONS ARE USED.**

| Combination of Positives | MPN Index | 95% Confidence Limits | | Combination of Positives | MPN Index | 95% Confidence Limits | |
|---|---|---|---|---|---|---|---|
| | | Lower | Upper | | | Lower | Upper |
| 0-0-0 | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |
| 0-0-1 | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |
| 0-1-0 | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |
| 0-1-1 | 36 | 7 | 100 | 4-1-2 | 260 | 98 | 700 |
| 0-2-0 | 37 | 7 | 100 | 4-1-3 | 310 | 100 | 700 |
| 0-2-1 | 55 | 18 | 150 | 4-2-0 | 220 | 68 | 500 |
| 0-3-0 | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |
| 1-0-0 | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |
| 1-0-1 | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |
| 1-0-2 | 60 | 18 | 150 | 4-3-0 | 270 | 99 | 700 |
| 1-1-0 | 40 | 07 | 120 | 4-3-1 | 330 | 100 | 700 |
| 1-1-1 | 61 | 18 | 150 | 4-3-2 | 390 | 140 | 1000 |
| 1-1-2 | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |
| 1-2-0 | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |
| 1-2-1 | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |
| 1-3-0 | 83 | 34 | 220 | 4-5-0 | 410 | 140 | 1000 |
| 1-3-1 | 100 | 35 | 220 | 4-5-1 | 480 | 150 | 1200 |
| 1-4-0 | 110 | 35 | 220 | 5-0-0 | 230 | 68 | 700 |
| 2-0-0 | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |
| 2-0-1 | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |
| 2-0-2 | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |
| 2-1-0 | 68 | 18 | 170 | 5-1-0 | 330 | 100 | 1000 |
| 2-1-1 | 92 | 34 | 220 | 5-1-1 | 460 | 140 | 1200 |
| 2-1-2 | 120 | 41 | 260 | 5-1-2 | 630 | 220 | 1500 |
| 2-2-0 | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |
| 2-2-1 | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |
| 2-2-2 | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |
| 2-3-0 | 120 | 41 | 260 | 5-2-2 | 940 | 340 | 2300 |
| 2-3-1 | 140 | 59 | 360 | 5-2-3 | 1200 | 360 | 2500 |
| 2-4-0 | 150 | 59 | 360 | 5-2-4 | 1500 | 580 | 4000 |
| 3-0-0 | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |
| 3-0-1 | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |
| 3-0-2 | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |
| 3-1-0 | 110 | 35 | 260 | 5-3-3 | 1800 | 700 | 4000 |
| 3-1-1 | 140 | 56 | 360 | 5-3-4 | 2100 | 700 | 4000 |
| 3-1-2 | 170 | 60 | 360 | 5-4-0 | 1300 | 360 | 4000 |
| 3-2-0 | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |
| 3-2-1 | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |
| 3-2-2 | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |
| 3-3-0 | 170 | 68 | 400 | 5-4-4 | 3500 | 1000 | 7100 |
| 3-3-1 | 210 | 68 | 400 | 5-4-5 | 4300 | 1500 | 11000 |
| 3-3-2 | 240 | 98 | 700 | 5-5-0 | 2400 | 700 | 7100 |
| 3-4-0 | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |
| 3-4-1 | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |
| 3-4-2 | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |
| 4-0-0 | 130 | 41 | 350 | 5-5-4 | 16000 | 4000 | 46000 |
| 4-0-1 | 170 | 59 | 360 | 5-5-5 | >16000 | 7000 | — |
| 4-0-2 | 210 | 68 | 400 | | | | |

**13.1.6** In Table 3, the numerator represents the number of positive tubes per sample dilution and the denominator represents total number of tubes inoculated per sample dilution.

**TABLE 3. SIGNIFICANT DILUTIONS IN DETERMINING MPN INDEX (SIGNIFICANT DILUTIONS ARE UNDERLINED AND LARGEST SIGNIFICANT DILUTION HIGHLIGHTED)**

| *Sample (Liquid or Solid)* | *$10^{-3}$ mL or g* | *$10^{-4}$ mL or g* | *$10^{-5}$ mL or g* | *$10^{-6}$ mL or g* | *Significant Dilution Results* |
|---|---|---|---|---|---|
| *A* | 5/5 | 3/5 | 3/5 | 0/5 | 5-3-0 |
| *B* | 5/5 | 3/5 | 1/5 | 1/5 | 5-3-2 |
| *C* | 0/5 | 1/5 | 0/5 | 0/5 | 0-1-0 |

**13.1.7** MPN procedure example calculation

**13.1.7.1** In Table 3, the appropriate dilutions chosen for the MPN determination are underlined. From Table 2, the MPN indices corresponding to Samples A, B, and C are 79, 140, and 1.8 respectively. (These samples could be liquid or solid.)

**13.1.7.2** Using Equation 1, Section 13.1.4, the MPN of fecal coliforms/g total solids (dry weight) for these examples are the following. Examples assume 4% total solids content. For analysis and calculation of total solids, refer to Appendix A.

For Sample A:

MPN fecal coliforms/g total solids= $(10 \times 79) / (10^{-4} \times 4.0) = 2.0 \times 10^{6}$

For Sample B:

MPN fecal coliforms/g total solids = $(10 \times 140) / (10^{-3} \times 4.0) = 3.5 \times 10^{5}$

For Sample C:

MPN fecal coliforms/g total solids = $(10 \times 1.8) / (10^{-3} \times 4.0) = 4.5 \times 10^{3}$

**13.1.8** After the MPN of fecal coliforms/g total solids (dry weight) has been calculated, the biosolid sample can be evaluated for Class A or Class B biosolid pathogen requirements. For Class B samples, fecal coliform densities of all seven samples should be calculated and converted to $\log_{10}$ values to compute a geometric mean. The geometric mean for the seven Class B samples is determined by averaging the $\log_{10}$ values of the fecal coliform density and taking the antilog of that value, see Section 13.2.6.2 for an example.

**13.2** Calculation of fecal coliform density using data from the MF procedure —See the EPA *Microbiological Methods for Monitoring the Environment, Water and Wastes*, Part II, Section C, 3.5, for general counting rules (Reference 19.4).

**13.2.1** Three dilutions for each of the seven required samples will provide 21 individual filters plus controls. Count typical blue colonies using a low power (10X to 15X) binocular wide-field dissecting microscope or other optical device.

**13.2.2** Compute CFU from membrane filters of sample volumes that yield colony counts within the desired range of 20 to 80 per filter. However, there may be occasions when the total number of colonies on a membrane will be above or below the ideal range. If the colonies are not discrete and appear to be growing together, results should be reported as "too numerous to count" (TNTC). If no filter has a coliform count in the ideal range, total the fecal coliform counts on all countable filters and report this total as fecal coliform CFU/g total solids (dry weight basis). See Sample 2 and 3 in Table 4 for examples.

*NOTE: Experienced analysts are encouraged to modify the dilution and inoculation schemes described in Section 12.6 to obtain filters that yield between 20 and 80 CFU.*

**13.2.3** Use Equation 3 to calculate fecal coliform CFU/g total solids (dry weight basis):

**Equation 3**

$$\text{CFU / g total solids} = \frac{\text{coliform colonies counted} \times 100}{\text{mL sample filtered} \times \text{\% solids}}$$

**13.2.4** Fecal coliform densities of all seven samples should be calculated and converted to $\log_{10}$ values to compute a geometric mean. The geometric mean for the seven samples is determined by averaging the $\log_{10}$ values of the fecal coliform density and taking the antilog of that value.

**13.2.5** After the geometric mean fecal coliform density has been calculated, the biosolid sample can be evaluated against the Class B biosolid requirements.

**TABLE 4. MEMBRANE FILTER EXAMPLE DATA**

| Sample No. | % Solids | Number of Fecal Coliform Colonies on MF Plates | | |
|---|---|---|---|---|
| | | $10^{-5}$ mL Filtration | $10^{-4}$ mL Filtration | $10^{-3}$ mL Filtration |
| 1 | 3.8 | 0 | 1 | 23 |
| 2 | 4.3 | 2 | 18 | TNTC |
| 3 | 4.0 | 0 | 8 | 65 |
| 4 | 4.2 | 0 | 5 | 58 |
| 5 | 4.1 | 0 | 1 | 17 |
| 6 | 3.7 | 0 | 1 | 39 |
| 7 | 3.9 | 0 | 1 | 20 |

The antilog of 6.02 is 1.0 X $10^6$. Therefore, the geometric mean fecal coliform density is 1.0 X $10^6$ CFU/g total solids.

## 14.0 Method Performance

The method performance information presented in this section is based on the performance of the original Standard Methods procedures in water and wastewater matrices. This information will be updated when Method 1680 is tested on biosolids.

**14.1** Specificity of Media

**14.1.1** LTB media

**[Information will be added when available through testing.]**

**14.1.2** EC media

**[Information will be added when available through testing.]**

**14.1.3** A-1 media

**[Information will be added when available through testing.]**

**14.1.4** m-FC media

The m-FC media and incubation temperature of 44.5 °C ± 0.2°C is 93% accurate in differentiating between coliforms found in the feces of warm-blooded animals and those found in other environmental sources, Standard Methods 9222D.

**14.2** Bias

**14.2.1** MPN procedure

The bacterial density estimates (indices) in Table 2 have a built-in positive statistical bias of approximately 23%, Standard Methods 9222A (Reference 19.1).

**14.2.2** MF procedure

Membrane counts may underestimate the number of viable fecal coliform bacteria, Standard Methods 9222B (Reference 19.1).

**14.3** Precision

**14.3.1** MPN procedure

Unless a large number of sample portions are examined, the precision among laboratories for the test is rather low. For example, if only 1 mL is examined in a sample containing 1 coliform organism/mL, about 37% of 1 mL tubes may be expected to yield negative results because of random distribution of bacteria in the sample. When five tubes are examined, each with 1 mL of sample, a completely negative result may be expected less than 1% of the time. The precision of the MPN counts are given as 95% confidence limits in Table 2 in Section 13.1.5. Note that the precision increases with increasing numbers of replicates per sample tested, Standard Methods 9221C (Reference 19.1).

**14.3.2** MF procedure

Although the statistical reliability of the MF procedure is greater than that of the MPN procedure, membrane counts may underestimate the number of viable fecal coliform bacteria, Standard Methods 9222B (Reference 19.1). Results from the MPN test would be expected to be higher than MF results because of a built-in positive statistical bias of approximately 23%, Standard Methods 9222A (Reference 19.1). Table 6 below

illustrates some 95% confidence limits. These values are based on the assumption that bacteria are distributed randomly and follow a Poisson distribution.

For results with counts greater than 20 organisms, calculate the approximate 95% confidence limits using Equation 4.

**EQUATION 4**

$$\text{Upper limit} = c + 2\sqrt{c} \qquad \text{Lower limit} = c - 2\sqrt{c}$$

*Where, c = number of coliform colonies counted*

**TABLE 6. 95% CONFIDENCE LIMITS FOR MEMBRANE FILTER COLIFORM RESULTS USING 100 mL WATER OR WASTEWATER SAMPLE**

| Number of Coliform Colonies Counted | 95% Confidence Limits | |
|---|---|---|
| | Lower | Upper |
| 0 | 0.0 | 3.7 |
| 1 | 0.1 | 5.6 |
| 2 | 0.2 | 7.2 |
| 3 | 0.6 | 8.8 |
| 4 | 1.0 | 10.2 |
| 5 | 1.6 | 11.7 |
| 6 | 2.2 | 13.1 |
| 7 | 2.8 | 14.4 |
| 8 | 3.4 | 15.8 |
| 9 | 4.0 | 17.1 |
| 10 | 4.7 | 18.4 |
| 11 | 5.4 | 19.7 |
| 12 | 6.2 | 21.0 |
| 13 | 6.9 | 22.3 |
| 14 | 7.7 | 23.5 |
| 15 | 8.4 | 24.8 |
| 16 | 9.4 | 26.0 |
| 17 | 9.9 | 27.2 |
| 18 | 10.7 | 28.4 |
| 19 | 11.5 | 29.6 |
| 20 | 12.2 | 30.8 |

## 15.0 Reporting Results

**15.1** Report sample results as MPN fecal coliforms/g of total solids when using the multiple-tube fermentation procedures and as fecal coliform CFU/ g of solids when utilizing the membrane filter procedure. See Appendix A for determination of total solids.

## 16.0 Verification Procedures

**16.1** If the laboratory has two or more analysts, each must count typical colonies on the same membrane from one positive sample per month. Verify colonies on the media and compare the analysts counts to the verified count.

**16.2** For every 10 samples using the membrane filter procedure, verify by picking at least 10 isolated colonies from membranes on m-FC media containing typical blue colonies (positive filters) and transferring to LTB. Incubate at 35 °C for 24 and 48 hours and examine for growth and gas and/or acid production. The presence of gas in the absence of growth is usually due to mishandling or improper shaking of the tubes after inoculation. Transfer growth from positive tubes to EC broth and incubate at 44.5°C for 24 hours. Growth and gas production in EC broth verifies presence of fecal coliform organisms.

## 17.0 Pollution Prevention

**17.1** The solutions and reagents used in this method pose little threat to the environment when recycled and managed properly. Solutions and reagents should be prepared in volumes consistent with laboratory use to minimize the volume of expired materials to be disposed.

## 18.0 Waste Management

**18.1** The laboratory is responsible for complying with all Federal, State, and local regulations governing waste management, particularly hazardous waste identification rules and land disposal restrictions, and for protecting 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. An overview of requirements can be found in *Environmental Management Guide for Small Laboratories* (EPA 233-B-98-001).

**18.2** Samples, reference materials, and equipment known or suspected to have viable bacteria or viral contamination must be sterilized prior to disposal.

**18.3** 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 Government Relations and Science Policy, 1155 16th Street N.W., Washington, D.C. 20036.

## 19.0 References

**19.1** American Public Health Association, American Water Works Association, and Water Environment Federation. 1995. *Standard Methods for Water and Wastewater.* 19th Edition. Sections: 9020, 9221, 9222.

**19.2** American Society for Testing and Materials. 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 Reference 19.7.

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

**19.4** 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.

**19.5** *Environmental Regulations and Technology: Control of Pathogens and Vector Attraction in Biosolid*. 1992. EPA/625/R-92/013. Office of Research and Development. USEPA.

**19.6** Thomas, H. A. Jr. 1942. Bacterial densities from fermenation tubes. *Journal of the American Water Works Association*. 34:572.

**19.7** *Analytical Standards for Laboratory Chemicals*. BDH Ltd. Poole, Dorset, UK, and the United States Pharmacopeia.

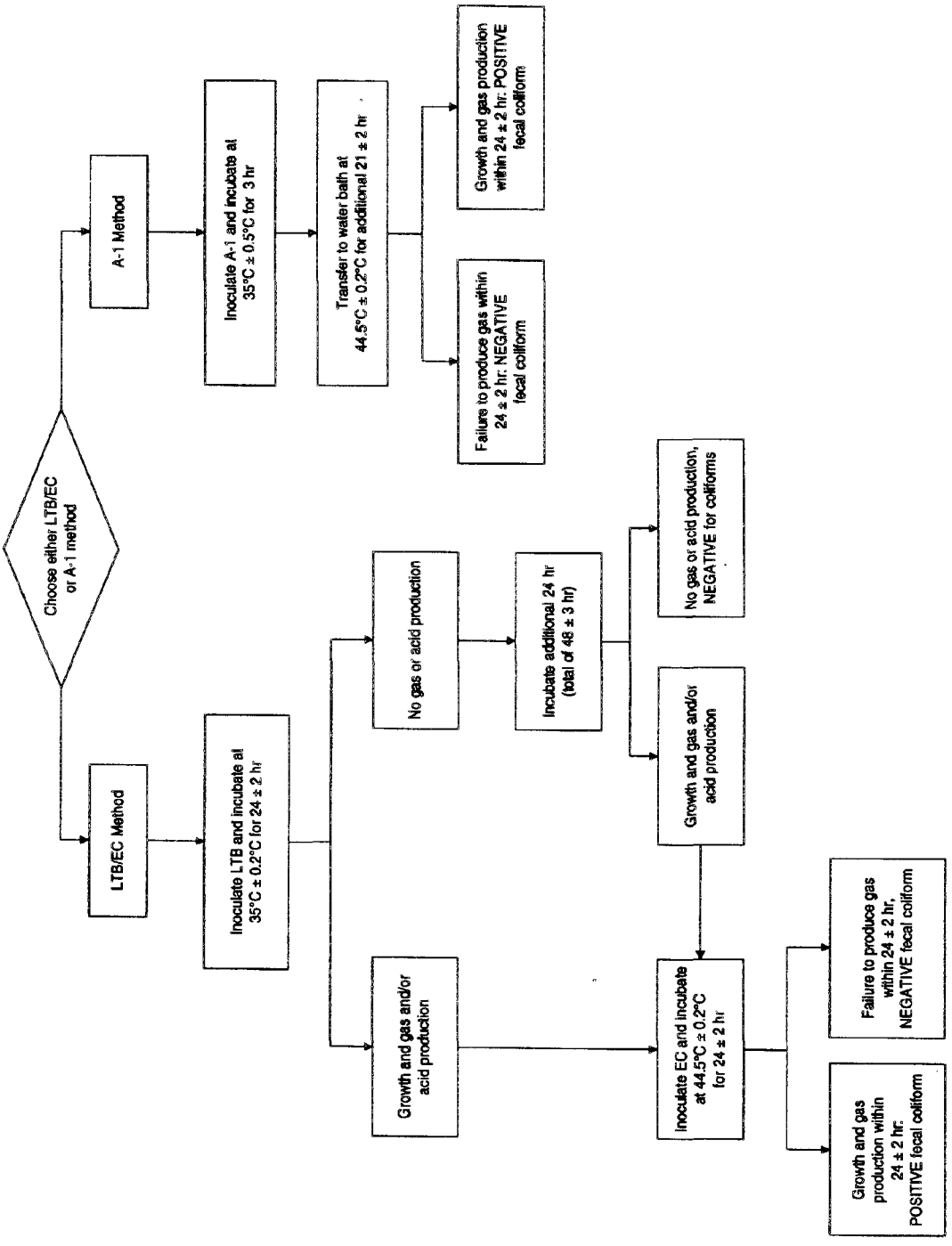
**19.8** Bordner, R. H., C. F. Frith and J. A. Winter (eds.). 1977. *Proceedings of the Symposium on the Recovery of Indicator Organisms Employing Membrane Filters*. EPA600/9-77-024. USEPA, Environmental Monitoring and Support Laboratory, Cincinnati, OH.

**19.9** Lin, S. D. 1973. Evaluation of coliform tests for chlorinated secondary effluents. *Journal of the Water and Pollution Control Federation*. 45:498.

## 20.0 Flowcharts and Validation Data

**20.1** The following pages contain flow charts of dilution and inoculation/filtration schemes for MPN, Section 11.0 and MF Section 12.0. Schemes for dilution and inoculation/filtration are dependent on Class (A or B) and matrix (solid) or (liquid).

**FIGURE 1. MULTIPLE TUBE FERMENTATION PROCEDURE**



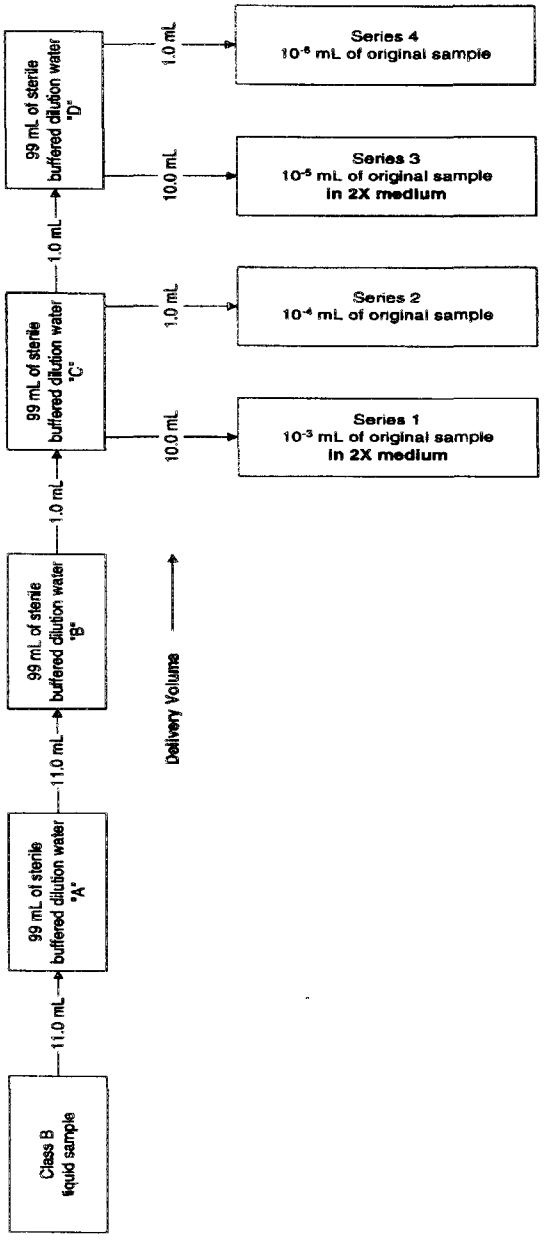



FIGURE 2. DILUTION AND INOCULATION SCHEME FOR CLASS B LIQUID SAMPLES (MULTIPLE TUBE FERMENTATION)

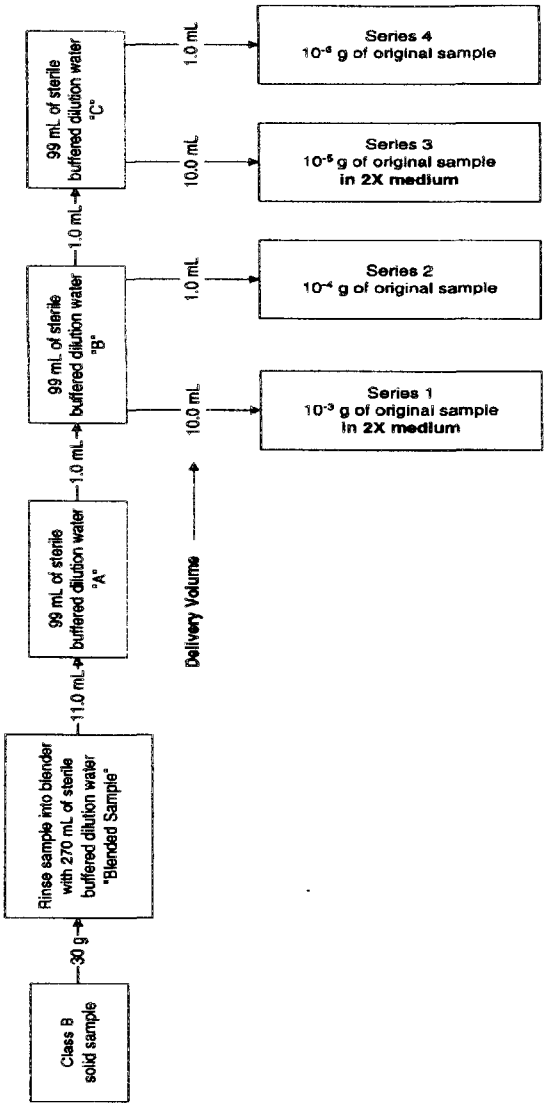



FIGURE 3. DILUTION AND INOCULATION SCHEME FOR CLASS B SOLID SAMPLES (MULTIPLE TUBE FERMENTATION)

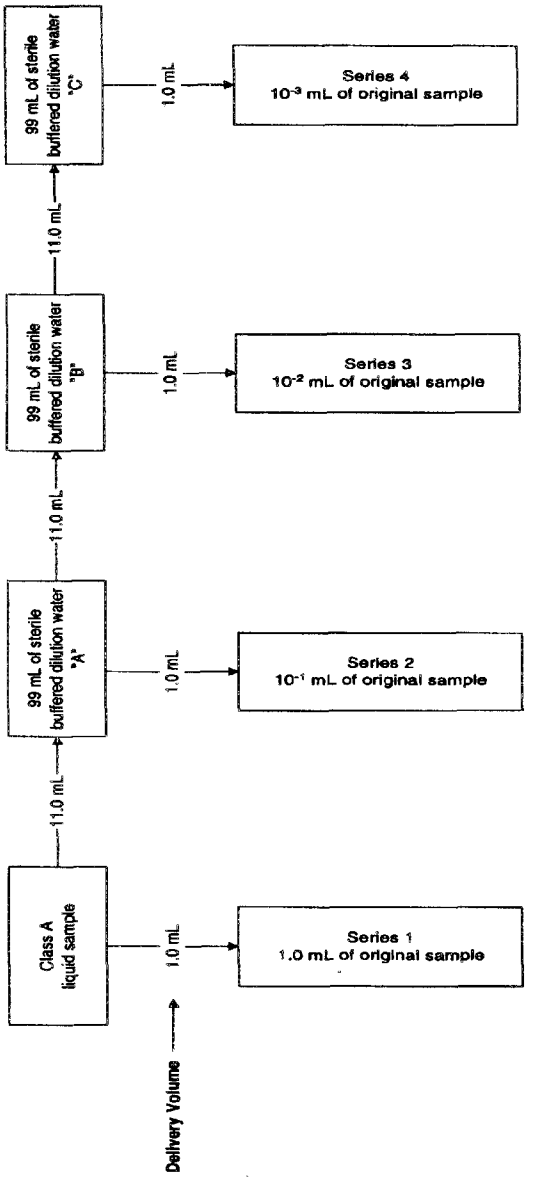



FIGURE 4. DILUTION AND INOCULATION SCHEME FOR CLASS A LIQUID SAMPLES (MULTIPLE TUBE FERMENTATION)

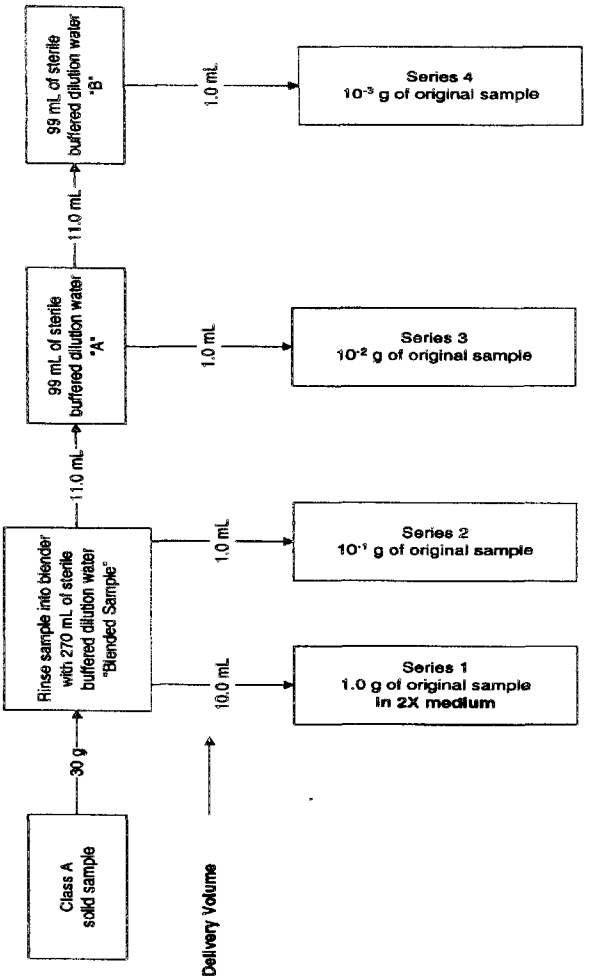



FIGURE 5. DILUTION AND INOCULATION SCHEME FOR CLASS A SOLID SAMPLES (MULTIPLE TUBE FERMENTATION)

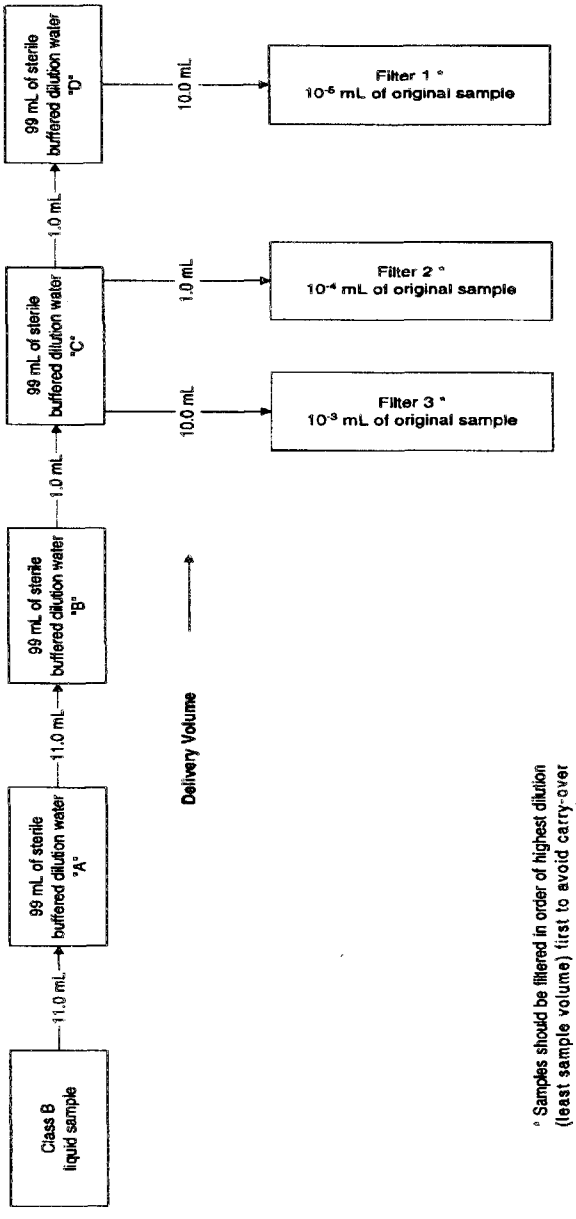
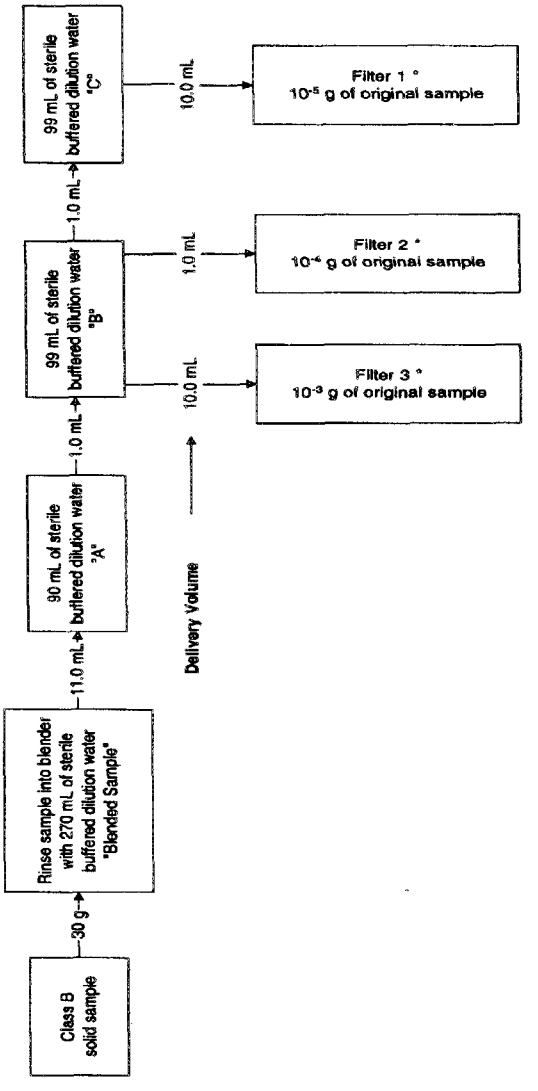



FIGURE 6. DILUTION AND FILTRATION SCHEME FOR CLASS B LIQUID SAMPLES (MEMBRANE FILTER)

° Samples should be filtered in order of highest dilution (least sample volume) first to avoid carry-over contamination. Filtration should take place in the following order: filter 1, filter 2, and filter 3.

FIGURE 7. DILUTION AND FILTRATION SCHEME FOR CLASS B: SOLID (MEMBRANE FILTER)




* Samples should be filtered in order of highest dilution (least sample volume) first to avoid carry-over contamination. Filtration should take place in the following order: filter 1, filter 2, and filter 3.

# 21.0 Glossary

The definitions and purposes are specific to this method but have been conformed to common usage as much as possible.

**21.1** Units of weight and measure and their abbreviations

**21.1.1** Symbols

| | |
|---|---|
| °C | degrees Celsius |
| μL | microliter |
| < | less than |
| > | greater than |
| % | percent |
| ± | plus or minus |

**21.1.2** Alphabetical characters

| | |
|---|---|
| CFU | colony forming units |
| EC | *Escherichia coli* |
| EPA | Environmental Protection Agency |
| g | gram |
| gal | gallon |
| L | liter |
| LTB | lauryl tryptose broth |
| M | molar |
| m-FC | membrane filtration-fecal coliform media |
| MF | membrane filter |
| mg | milligram |
| mL | milliliter |
| mm | millimeter |
| mM | milliMole |
| MPN | most probable number (in this method, multiple tube fermentation) |
| NIST | National Institute of Standards and Technology |
| nm | nanometer |
| TD | to deliver |
| TGY | tryptone glucose yeast |
| UV | ultraviolet |
| QC | quality control |

**21.2** Definitions, acronyms, and abbreviations (in alphabetical order).

Analyst—The analyst must have two years of college lecture and laboratory course work in microbiology or a closely related field. The analyst also must have at least 6 months bench experience, must have at least 3 months experience with plating procedures, and must have successfully analyzed at least 50 biosolid samples for fecal coliforms. Six months of additional experience in the above area may be substituted for two years of college. The analyst must also demonstrate acceptable performance during an on-site evaluation.

Analyte—The microorganism tested for by this method. The analytes in this method are fecal coliforms.

Differential media—A solid culture media that makes it easier to distinguish colonies of the desired organism.

Enrichment—Using a culture media for preliminary isolation that favors the growth of a particular kind of organism.

Laboratory blank—See Method blank.

Laboratory reagent blank—See Method blank.

Matrix spike (MS)—A sample prepared by adding a known quantity of organisms to a specified amount of sample matrix for which an independent estimate of target analyte concentration is available. A matrix spike is used to determine the effect of the matrix on a method's recovery efficiency.

May—This action, activity, or procedural step is neither required nor prohibited.

May not—This action, activity, or procedural step is prohibited.

Method blank—An aliquot of sterile biosolid that is treated exactly as a sample including exposure to all glassware, equipment, media, procedures that are used with samples. The method blank is used to determine if analytes or interferences are present in the laboratory environment.

Most probable number method (MPN)—A statistical determination of the number of bacteria per weight or volume of sample. It is based on the fact that the greater the number of bacteria in a sample, the more dilution is needed to reduce the density to the point at which no bacteria are left to grow in a dilution series.

Must—This action, activity, or procedural step is required.

Negative control—See Method blank

Ongoing precision and recovery standard (OPR)—a method blank spiked with known quantities of analytes. The OPR is analyzed exactly like a sample. Its purpose is to assure that the results produced by the laboratory remain within the limits specified in this method for precision and recovery.

Positive control—See Ongoing precision and recovery standard

Preferred—Optional

Preparation blank—See Method blank.

Quantitative transfer—the process of transferring a solution from one container to another using a pipette in which as much solution as possible is transferred, followed by rinsing of the walls of the source container with a small volume of rinsing solution (e.g., PBS), followed by transfer of the rinsing solution, followed by a second rinse and transfer.

Selective media—A culture media designed to suppress the growth of unwanted microorganisms and encourage the growth of desired ones.

Should—This action, activity, or procedural step is suggested but not required.

Stock suspension—A suspension containing an analyte that is prepared using a reference material traceable to EPA, the National Institute of Science and Technology (NIST), or a source that will attest to the purity and authenticity of the reference material.

Technician—See Analyst.

# Appendix A: Total Solids in Solid and Semisolid Matrices

## 1.0 Scope and Application

**1.1** This procedure is applicable to the determination of total solids in such solid and semisolid samples as soils, sediments, and biosolids separated from water and wastewater treatment processes, and biosolid cakes from vacuum filtration, centrifugation, or other biosolid dewatering processes.

**1.2** This procedure is taken from EPA Method 1684: *Total, Fixed, and Volatile Solids in Solid and Semi-Solid Matrices.*

**1.3** Method detection limits (MDLs) and minimum levels (MLs) have not been formally established for this draft procedure. These values will be determined during the validation of Method 1684.

**1.4** This procedure is performance based. The laboratory is permitted to omit any step or modify any procedure (e.g. to overcome interferences, to lower the cost of measurement), provided that all performance requirements in this procedure are met. Requirements for establishing equivalency will be provided upon validation of Method 1680.

**1.5** Each laboratory that uses this procedure must demonstrate the ability to generate acceptable results using the procedure in Section 9.2 of this appendix.

## 2.0 Summary of Method

**2.1** Sample aliquots of 25-50 g are dried at 103 °C to 105 °C to drive off water in the sample.

**2.2** The mass of total solids in the sample is determined by comparing the mass of the sample before and after each drying step.

## 3.0 Definitions

**3.1** Total Solids–The residue left in the vessel after evaporation of liquid from a sample and subsequent drying in an oven at 103 °C to 105 °C.

**3.2** Additional definitions are given in Sections 3.0 and 21.0 of Method 1680.

## 4.0 Interferences

**4.1** Sampling, subsampling, and pipetting multi-phase samples may introduce serious errors (Reference 16.1). Make and keep such samples homogeneous during transfer. Use special handling to ensure sample integrity when subsampling. Mix small samples with a magnetic stirrer. If visible suspended solids are present, pipette with wide-bore pipettes. If part of a sample adheres to the sample container, intensive homogenization is required to ensure accurate results. When dried, some samples form a crust that prevents evaporation; special handling such as extended drying times are required to deal with this. Avoid using a magnetic stirrer with samples containing magnetic particles.

**4.2** The temperature and time of residue drying has an important bearing on results (Reference 16.1). Problems such as weight losses due to volatilization of organic matter, and evolution of gases from heat-induced chemical decomposition, weight gains due to oxidation, and confounding factors like mechanical occlusion of water and water of crystallization depend on temperature and time of heating. It is therefore essential that samples be dried at a uniform temperature, and for no longer than specified. Each sample requires close attention to desiccation after drying. Minimize the time

the desiccator is open because moist air may enter and be absorbed by the samples. Some samples may be stronger desiccants than those used in the desiccator and may take on water.

**4.3** Residues dried at 103 °C to 105 °C may retain some bound water as water of crystallization or as water occluded in the interstices of crystals. They lose $CO_2$ in the conversion of bicarbonate to carbonate. The residues usually lose only slight amounts of organic matter by volatilization at this temperature. Because removal of occluded water is marginal at this temperature, attainment of constant weight may be very slow.

**4.4** Results for residues high in oil or grease may be questionable because of the difficulty of drying to constant weight in a reasonable time.

**4.5** The determination of total solids is subject to negative error due to loss of ammonium carbonate and volatile organic matter during the drying step at 103 °C to 105 °C. Carefully observe specified ignition time and temperature to control losses of volatile inorganic salts if these are a problem.

## 5.0 Safety

**5.1** Refer to Section 5.0 of Method 1680 for safety precautions

## 6.0 Equipment and Supplies

*NOTE: Brand names, suppliers, and part numbers are cited for illustrative purposes only. No endorsement is implied. Equivalent performance may be achieved using equipment and materials other than those specified here, but demonstration of equivalent performance that meets the requirements of this method is the responsibility of the laboratory.*

**6.1** Evaporating Dishes–Dishes of 100-mL capacity. The dishes may be made of porcelain (90-mm diameter), platinum, or high-silica glass.

**6.2** Watch glass–Capable of covering the evaporating dishes (Section 6.1).

**6.3** Steam bath.

**6.4** Desiccator–Moisture concentration in the desiccator should be monitored by an instrumental indicator or with a color-indicator desiccant.

**6.5** Drying oven–Thermostatically-controlled, capable of maintaining a uniform temperature of 103 °C to 105 °C throughout the drying chamber.

**6.6** Analytical balance–Capable of weighing to 0.1 mg for samples having a mass up to 200 g.

**6.7** Container handling apparatus–Gloves, tongs, or a suitable holder for moving and handling hot containers after drying.

**6.8** Bottles–Glass or plastic bottles of a suitable size for sample collection

**6.9** Rubber gloves (Optional)

**6.10** No. 7 Cork borer (Optional)

## 7.0 Reagents and Standards

7.1 Reagent water–Deionized, distilled, or otherwise purified water.

7.2 Sodium chloride-potassium hydrogen phthalate standard (NaCl-KHP).

7.2.1 Dissolve 0.10 g sodium chloride (NaCl) in 500 mL reagent water. Mix to dissolve.

7.2.2 Add 0.10 g potassium hydrogen phthalate (KHP) to the NaCl solution (Section 7.2.1) and mix. If the KHP does not dissolve readily, warm the solution while mixing. Dilute to 1 L with reagent water. Store at 4°C. Assuming 100% volatility of the acid phthalate ion, this solution contains 200 mg/L total solids, 81.0 mg/L volatile solids, and 119 mg/L fixed solids.

## 8.0 Sample Collection, Preservation, and Storage

8.1 Use resistant-glass or plastic bottles to collect sample for solids analysis, provided that the material in suspension does not adhere to container walls. Sampling should be done in accordance with Reference 13.2. Begin analysis as soon as possible after collection because of the impracticality of preserving the sample. Refrigerate the sample at 4°C up to the time of analysis to minimize microbiological decomposition of solids. Preferably do not hold samples more than 24 hours. Under no circumstances should the sample be held more than seven days. Bring samples to room temperature before analysis.

## 9.0 Quality Control

9.1 Quality control requirements and requirements for performance-based methods are given in Section 9.0 of Method 1680.

9.2 Initial demonstration of laboratory capability - The initial demonstration of laboratory capability is used to characterize laboratory performance and method detection limits.

9.2.1 Method detection limit (MDL) - The method detection limit should be established for total solids using diluted NaCl-KHP standard (Section 7.2). To determine MDL values, take seven replicate aliquots of the diluted NaCl-KHP solution and process each aliquot through each step of the analytical method. Perform all calculations and report the concentration values in the appropriate units. MDLs should be determined every year or whenever a modification to the method or analytical system is made that will affect the method detection limit.

9.2.2 Initial Precision and Recovery (IPR) - To establish the ability to generate acceptable precision and accuracy, the analyst shall perform the following operations:

9.2.2.1 Prepare four samples by diluting NaCl-KHP standard (Section 7.2) to 1-5 times the MDL. Using the procedures in Section 11, analyze these samples for total solids.

9.2.2.2 Using the results of the four analyses, compute the average percent recovery (x) and the standard deviation (s, Equation 1) of the percent recovery for total solids.

**Equation 1**

$$s = \sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x^2)}{n}}{n-1}}$$

*Where:*

*n = number of samples*
*x = % recovery in each sample*
*s = standard deviation*

**9.2.2.3** Compare s and x with the corresponding limits for initial precision and recovery in Table 2 (to be determined in validation study). If s and x meet the acceptance criteria, system performance is acceptable and analysis of samples may begin. If, however, s exceeds the precision limit or x falls outside the range for recovery, system performance is unacceptable. In this event, correct the problem, and repeat the test.

**9.3** Laboratory blanks

**9.3.1** Prepare and analyze a laboratory blank initially (i.e. with the tests in Section 9.2) and with each analytical batch. The blank must be subjected to the same procedural steps as a sample, and will consist of approximately 25 g of reagent water.

**9.3.2** If material is detected in the blank at a concentration greater than the MDL (Section 1.3), analysis of samples must be halted until the source of contamination is eliminated and a new blank shows no evidence of contamination. All samples must be associated with an uncontaminated laboratory blank before the results may be reported for regulatory compliance purposes.

**9.4** Ongoing Precision and Recovery

**9.4.1** Prepare an ongoing precision and recovery (OPR) solution identical to the IPR solution described in Section 9.2.2.1.

**9.4.2** An aliquot of the OPR solution must be analyzed with each sample batch (samples started through the sample preparation process (Section 11) on the same 8-hour shift, to a maximum of 20 samples).

**9.4.3** Compute the percent recovery of total solids in the OPR sample.

**9.4.4** Compare the results to the limits for ongoing recovery in Table 2 (to be determined in validation study). If the results meet the acceptance criteria, system performance is acceptable and analysis of blanks and samples may proceed. If, however, the recovery of total solids falls outside of the range given, the analytical processes are not being performed properly. Correct the problem, prepare another sample batch, and repeat the OPR test. All samples must be associated with an OPR analysis that passes acceptance criteria before the sample results can be reported for regulatory compliance purposes.

**9.4.5** Add results that pass the specifications in Section 9.4.4 to IPR and previous OPR data. Update QC charts to form a graphic representation of continued laboratory performance. Develop a statement of laboratory accuracy for each analyte by calculating the average percent recovery (R) and the standard deviation of percent recovery (SR). Express the accuracy as a recovery interval from R-2SR to R+2SR. For example, if R=105% and SR=5%, the accuracy is 95-115%.

**9.5** Duplicate analyses

**9.5.1** Ten percent of samples must be analyzed in duplicate. The duplicate analyses must be performed within the same sample batch (samples whose analysis is started within the same 8-hour period, to a maximum of 20 samples).

**9.5.2** The total solids of the duplicate samples must be within 10%.

## 10.0 Calibration and Standardization

**10.1** Calibrate the analytical balance at 2 mg and 1000 mg using class "S" weights.

**10.2** Calibration shall be within ± 10% (i.e. ±0.2 mg) at 2 mg and ± 0.5% (i.e. ±5 mg) at 1000 mg. If values are not within these limits, recalibrate the balance.

## 11.0 Procedure

**11.1** Preparation of evaporating dishes–Heat dishes and watch glasses at 103°C to 105°C for 1 hour in an oven. Cool and store the dried equipment in a desiccator. Weigh each dish and watch glass prior to use (record combined weight as "$W_{dish}$").

**11.2** Preparation of samples

**11.2.1** Fluid samples–If the sample contains enough moisture to flow readily, stir to homogenize, place a 25 to 50 g sample aliquot on the prepared evaporating dish. If the sample is to be analyzed in duplicate, the mass of the two aliquots may not differ by more than 10%. Spread each sample so that it is evenly distributed over the evaporating dish. Weigh and then evaporate the samples to dryness on a steam bath. Cover each sample with a watch glass, and weigh (record weight as "$W_{sample}$").

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*NOTE: Weigh wet samples quickly because wet samples tend to lose weight by evaporation. Samples should be weighed immediately after aliquots are prepared.*

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**11.2.2** Solid samples–If the sample consists of discrete pieces of solid material (dewatered biosolid, for example), take cores from each piece with a No. 7 cork borer or pulverize the entire sample coarsely on a clean surface by hand, using rubber gloves. Place a 25 to 50 g sample aliquot of the pulverized sample on the prepared evaporating dish. If the sample is to be analyzed in duplicate, the mass of the two aliquots may not differ by more than 10%. Spread each sample so that it is evenly distributed over the evaporating dish. Cover each sample with a watch glass, and weigh (record weight as "$W_{sample}$").

**11.3** Dry the samples at 103°C to 105°C for a minimum of 12 hours, cool to balance temperature in an individual desiccator containing fresh desiccant, and weigh. Heat the residue again for 1 hour, cool it to balance temperature in a desiccator, and weigh. Repeat this heating, cooling, desiccating, and

weighing procedure until the weight change is less than 5% or 50 mg, whichever is less. Record the final weight as "$W_{total}$."

*NOTE: It is imperative that dried samples be weighed quickly since residues often are very hygroscopic and rapidly absorb moisture from the air. Samples must remain in the dessicator until the analyst is ready to weigh them.*

## 12.0 Data Analysis and Calculations

**12.1** Calculate the % solids or the mg solids/kg biosolid for total solids (Equation 2).

**Equation 2**

$$\%\ total\ solids = \frac{W_{total} - W_{dish}}{W_{sample} - W_{dish}} * 100$$

*or*

$$\frac{mg\ total\ solids}{kg\ sludge} = \frac{W_{total} - W_{dish}}{W_{sample} - W_{dish}} * 1{,}000{,}000$$

*Where:*

$W_{dish}$ = *Weight of dish (mg)*
$W_{sample}$ = *Weight of wet sample and dish (mg)*
$W_{total}$ = *Weight of dried residue and dish (mg)*

**12.2** Sample results should be reported as % solids or mg/kg to three significant figures. Report results below the ML as < the ML, or as required by the permitting authority or in the permit.

## 13.0 Method Performance

**13.1** Method performance (MDL and quality control acceptance criteria) will be determined during the multi-lab validation of this method.

**13.2** Total solids duplicate determinations must agree within 10% to be reported for permitting purposes. If duplicate samples do not meet this criteria, the problem must be discovered and the sample must be run over.

## 14.0 Pollution Prevention

**14.1** Pollution prevention details are given in Section 17.0 of Method 1680.

## 15.0 Waste Management

**15.1** Waste management details are given in Section 18.0 of Method 1680.

## 16.0 References

**16.1** "*Standard Methods for the Examination of Water and Wastewater,*" 18th ed. and later revisions, American Public Health Association, 1015 15th Street NW, Washington, DC 20005. 1-35: Section 1090 (Safety), 1992.

**16.2** U.S. Environmental Protection Agency, 1992. Control of Pathogens and Vector Attraction in Sewage Biosolid. Publ 625/R-92/013. Office of Research and Development, Washington, DC.

## 17.0 Tables, Diagrams, Flowcharts, and Validation Data

**17.1** Tables containing method requirements for QA/QC will be added after the validation study has been performed.

# Appendix B: Optional Dilution and Inoculation Schemes

## 1.0 MPN Dilution and Inoculation Class B Biosolids: Option A

After initial homogenization, labs may perform 1:10 serial dilutions by transferring 11.0 mL to 99 mL sterile dilution water. This results in inoculation volumes of 1.0 mL, eliminating the need for 2X media in all but Class A solids samples. Class A solid samples will still require an initial 10.0 g inoculation volume and 2X media for the first series of tubes.

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*NOTE: For some transfers, it may be convenient to use a sterile, wide-mouth pipette, capable of transferring particulate matter.*

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**1.1** Class B liquid samples—Four series of five tubes each will be used for the analysis with $10^{-3}$, $10^{-4}$, $10^{-5}$, and $10^{-6}$ mL of the original sample. See Figure B1 for a summary of this dilution and inoculation scheme.

**1.1.1** Dilution

**1.1.1.1** Use a sterile pipette to transfer 11.0 mL of homogenized sample (Section 11.3.1.3(A) or 11.4.3.1) to 99 mL of sterile dilution water (Section 7.4), cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is dilution "A." A 1.0-mL volume of dilution "A" is $10^{-1}$ mL of the original sample.

**1.1.1.2** Use a sterile pipette to transfer 11.0 mL of dilution "A" to 99 mL of sterile dilution water, and mix as before. This is dilution "B." A 1.0-mL volume of dilution "B" is $10^{-2}$ mL of the original sample.

**1.1.1.3** Use a sterile pipette to transfer 11.0 mL of dilution "B" to 99 mL of sterile dilution water, and mix as before. This is dilution "C." A 1.0-mL volume of dilution "C" is $10^{-3}$ mL of the original sample.

**1.1.1.4** Use a sterile pipette to transfer 11.0 mL of dilution "C" to 99 mL of sterile dilution water, and mix as before. This is dilution "D." A 1.0-mL volume of dilution "D" is $10^{-4}$ mL of the original sample.

**1.1.1.5** Use a sterile pipette to transfer 11.0 mL of dilution "D" to 99 mL of sterile dilution water, and mix as before. This is dilution "E." A 1.0-mL volume of dilution "E" is $10^{-5}$ mL of the original sample.

**1.1.1.6** Use a sterile pipette to transfer 11.0 mL of dilution "E" to 99 mL of sterile dilution water, and mix as before. This is dilution "F." A 1.0-mL volume of dilution "F" is $10^{-6}$ mL of the original sample.

**1.1.2** Inoculation

**1.1.2.1** Use a sterile pipette to inoculate each of the first series of five tubes with 1.0 mL of dilution "C." This is $10^{-3}$ mL of the original sample.

**1.1.2.2** Use a sterile pipette to inoculate each of the second series of tubes with 1.0 mL of dilution "D." This is $10^{-4}$ mL of the original sample.

**1.1.2.3** Use a sterile pipette to inoculate each of the third series of tubes with 1.0 mL of "E." This is $10^{-5}$ mL of the original sample.

**1.1.2.4** Use a sterile pipette to inoculate each of the fourth series of tubes with 1.0 mL of dilution "F." This is $10^{-6}$ mL of the original sample.

**1.1.3** Repeat Section 1.1.1 and 1.1.2 of Appendix B for each remaining Class B liquid sample. When inoculations are complete, return to Section 11.3.1.5 to continue the LTB/EC method or to Section 11.4.5 to continue the A-1 method.

**1.2** Class B solid samples—The four series of five tubes each will contain $10^{-3}$, $10^{-4}$, $10^{-5}$, and $10^{-6}$ g of the original sample. See Figure B2 for a summary of this dilution and inoculation scheme.

**1.2.1** Dilution

**1.2.1.1** A volume of 1.0-mL of the "blended" sample (Section 11.3.1.3(B) or 11.4.3.2 ) contains $10^{-1}$ g of the original sample.

**1.2.1.2** Use a sterile pipette to transfer 11.0 mL of the blender contents to 99 mL of sterile dilution water, cap, and shake vigorously a minimum of 25 times. This is dilution "A." A volume of 1.0-mL of dilution "A" contains $10^{-2}$ g of the original sample.

**1.2.1.3** Use a sterile pipette to transfer 11.0 mL of dilution "A" to 99 mL of sterile dilution water and mix as before. This is dilution "B." A volume of 1.0-mL of dilution "B" contains $10^{-3}$ g of the original sample.

**1.2.1.4** Use a sterile pipette to transfer 11.0 mL of dilution"B" to 99 mL of sterile dilution water and mix as before. This is dilution "C." A volume of 1.0-mL of dilution "C" contains $10^{-4}$ g of the original sample.

**1.2.1.5** Use a sterile pipette to transfer 11.0 mL of dilution "C" to 99 mL of sterile dilution water and mix as before. This is dilution "D." A volume of 1.0-mL of dilution "D" contains $10^{-5}$ g of the original sample.

**1.2.1.6** Use a sterile pipette to transfer 11.0 mL of dilution "D" to 99 mL of sterile dilution water and mix as before. This is dilution "E." A volume of 1.0-mL of dilution "E" contains $10^{-6}$ g of the original sample.

**1.2.2** Inoculation

**1.2.2.1** Inoculate each of the first series of five tubes with 1.0 mL of dilution "B." This is $10^{-3}$ g of the original sample.

**1.2.2.2** Inoculate each of the second series of tubes with 1.0 mL of dilution "C." This is $10^{-4}$ g of the original sample.

**1.2.2.3** Inoculate each of the third series of tubes with 1.0 mL of "D." This is $10^{-5}$ g of the original sample.

**1.2.2.4** Inoculate each of the fourth series of tubes with 1.0 mL of dilution "E." This is $10^{-6}$ g of the original sample.

**1.2.3** Repeat Section 1.2.1 and 1.2.2 of Appendix B for the remaining Class B solid samples. When inoculations are complete, return to Section 11.3.1.5 to continue the LTB/EC method or to Section 11.4.5 to continue the A-1 method.

## 2.0 MPN Dilution and Inoculation Class A and B Biosolids: Option B

After initial homogenization and the first dilution (transferring 11.0 mL of the homogenized sample), labs may perform 1:10 serial dilutions by transferring 1.0 mL to 9.0 mL sterile dilution water (Section 7.4). This results in inoculation volumes of 1.0 mL, eliminating the need for 2X media in all but Class A solids samples. Class A solid samples will still require an initial 10.0 mL inoculation volume and 2X media for the first series of tubes.

*NOTE: For some transfers, it may be convenient to use a sterile, wide-mouth pipette, capable of transferring particulate matter.*

**2.1** Class B liquid samples—Four series of five tubes each will be used for the analysis with $10^{-3}$, $10^{-4}$, $10^{-5}$, and $10^{-6}$ mL of the original sample. See Figure B3 for a summary of this dilution and inoculation scheme.

**2.1.1** Dilution

**2.1.1.1** Use a sterile pipette to transfer 11.0 mL of homogenized sample (Section 11.3.1.3(A) or 11.4.3.1) to 99 mL of sterile dilution water (Section 7.4), cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is dilution "A." A 1.0-mL volume of dilution "A" is $10^{-1}$ mL of the original sample.

**2.1.1.2** Use a sterile pipette to transfer 1.0 mL of dilution "A" to 9.0 mL of sterile dilution water, and mix as before. This is dilution "B." A 1.0-mL volume of dilution "B" is $10^{-2}$ mL of the original sample.

**2.1.1.3** Use a sterile pipette to transfer 1.0 mL of dilution "B" to 9.0 mL of sterile dilution water, and mix as before. This is dilution "C." A 1.0-mL volume of dilution "C" is $10^{-3}$ mL of the original sample.

**2.1.1.4** Use a sterile pipette to transfer 1.0 mL of dilution "C" to 9.0 mL of sterile dilution water, and mix as before. This is dilution "D." A 1.0-mL volume of dilution "D" is $10^{-4}$ mL of the original sample.

**2.1.1.5** Use a sterile pipette to transfer 1.0 mL of dilution "D" to 9.0 mL of sterile dilution water, and mix as before. This is dilution "E." A 1.0-mL volume of dilution "E" is $10^{-5}$ mL of the original sample.

**2.1.1.6** Use a sterile pipette to transfer 1.0 mL of dilution "E" to 9.0 mL of sterile dilution water, and mix as before. This is dilution "F." A 1.0-mL volume of dilution "F" is $10^{-6}$ mL of the original sample.

**2.1.2** Inoculation

**2.1.2.1** Use a sterile pipette to inoculate each of the first series of five tubes with 1.0 mL of dilution "C." This is $10^{-3}$ mL of the original sample.

**2.1.2.2** Use a sterile pipette to inoculate each of the second series of tubes with 1.0 mL of dilution "D." This is $10^{-4}$ mL of the original sample.

**2.1.2.3** Use a sterile pipette to inoculate each of the third series of tubes with 1.0 mL of "E." This is $10^{-5}$ mL of the original sample.

**2.1.2.4** Use a sterile pipette to inoculate each of the fourth series of tubes with 1.0 mL of dilution "F." This is $10^{-6}$ mL of the original sample.

**2.1.3** Repeat Section 1.1.1 and 1.1.2 of Appendix B for each remaining Class B liquid sample. When inoculations are complete, return to Section 11.3.1.5 to continue the LTB/EC method or to Section 11.4.5 to continue the A-1 method.

**2.2** Class B solid samples—The four series of five tubes each will contain $10^{-3}$, $10^{-4}$, $10^{-5}$, and $10^{-6}$ g of the original sample. See Figure B4 for a summary of this dilution and inoculation scheme.

**2.2.1** Dilution

**2.2.1.1** A volume of 1.0-mL of the "blended" sample (Section 11.3.1.3(B) or 11.4.3.2 ) contains $10^{-1}$ g of the original sample.

**2.2.1.2** Use a sterile pipette to transfer 11.0 mL of the blender contents to 99 mL of sterile dilution water, cap, and shake vigorously a minimum of 25 times. This is dilution "A." A volume of 1.0-mL of dilution "A" contains $10^{-2}$ g of the original sample.

**2.2.1.3** Use a sterile pipette to transfer 1.0 mL of dilution "A" to 9.0 mL of sterile dilution water and mix as before. This is dilution "B." A volume of 1.0-mL of dilution "B" contains $10^{-3}$ g of the original sample.

**2.2.1.4** Use a sterile pipette to transfer 1.0 mL of dilution"B" to 9.0 mL of sterile dilution water and mix as before. This is dilution "C." A volume of 1.0-mL of dilution "C" contains $10^{-4}$ g of the original sample.

**2.2.1.5** Use a sterile pipette to transfer 1.0 mL of dilution "C" to 9.0 mL of sterile dilution water and mix as before. This is dilution "D." A volume of 1.0-mL of dilution "D" contains $10^{-5}$ g of the original sample.

**2.2.1.6** Use a sterile pipette to transfer 1.0 mL of dilution "D" to 9.0 mL of sterile dilution water and mix as before. This is dilution "E." A volume of 1.0-mL of dilution "E" contains $10^{-6}$ g of the original sample.

**2.2.2** Inoculation

**2.2.2.1** Inoculate each of the first series of tubes with 1.0 mL of dilution "B." This is $10^{-3}$ g of the original sample.

**2.2.2.2** Inoculate each of the second series of tubes with 1.0 mL of dilution "C." This is $10^{-4}$ g of the original sample.

**2.2.2.3** Inoculate each of the third series of tubes with 1.0 mL of "D." This is $10^{-5}$ g of the original sample.

**2.2.2.4** Inoculate each of the fourth series of tubes with 1.0 mL of dilution "E." This is $10^{-6}$ g of the original sample.

**2.2.3** Repeat Section 1.2.1 and 1.2.2 of Appendix B for the remaining Class B solid samples. When inoculations are complete, return to Section 11.3.1.5 to continue the LTB/EC method or to Section 11.4.5 to continue the A-1 method.

**2.3** Class A liquid samples—The four series of five tubes each will contain 1.0, $10^{-1}$, $10^{-2}$ and $10^{-3}$ mL of the original sample. See Figure B5 for a summary of this dilution and inoculation scheme.

**2.3.1** Dilution

**2.3.1.1** Use a sterile pipette to transfer 11.0 mL of homogenized sample (Section 11.3.1.3(A) or 11.4.3.1) to 99 mL of sterile dilution water (Section 7.4), cap, and mix by vigorously shaking the bottle a minimum of 25 times.

This is dilution "A." A volume of 1.0-mL of dilution "A" contains $10^{-1}$ mL of the original sample.

**2.3.1.2** Use a sterile pipette to transfer 1.0 mL of dilution "A" to 9.0 mL of sterile dilution water, and mix as before. This is dilution "B." A 1.0-mL volume of dilution "B" is $10^{-2}$ mL of the original sample.

**2.3.1.3** Use a sterile pipette to transfer 1.0 mL of well mixed dilution "B" to 9.0 mL of sterile dilution water, and mix as before. This is dilution "C." A volume of 1.0-mL of dilution "C" is $10^{-3}$ mL of the original sample.

**2.3.2** Inoculation

**2.3.2.1** Use a sterile pipette to inoculate each of the first series of tubes with 1.0 mL of the original homogenized sample.

**2.3.2.2** Use a sterile pipette to inoculate each of the second series of tubes with 1.0 mL of dilution "A." This is $10^{-1}$ mL of the original sample.

**2.3.2.3** Use a sterile pipette to inoculate each of the third series of tubes with 1.0 mL of dilution "B." This is $10^{-2}$ mL of the original sample.

**2.3.2.4** Use a sterile pipette to inoculate each of the fourth series of tubes with 1.0 mL of dilution "C." This is $10^{-3}$ mL of the original sample.

**2.3.3** Repeat steps 2.3.1 and 2.3.2 of Appendix B for the remaining Class A liquid samples. When inoculations are complete, return to Section 11.3.1.5 to continue the LTB/EC method or to Section 11.4.5 to continue the A-1 method.

**2.4** Class A solid samples—Four series of five tubes will be used for the analysis with 1.0, $10^{-1}$, $10^{-2}$ and $10^{-3}$ g of the original sample. The first series of tubes must contain 2X media. See Figure B6 for a summary of this dilution and inoculation scheme.

**2.4.1** Dilution

**2.4.1.1** A volume of 1.0-mL of the "blended" sample contains $10^{-1}$ g of the original sample.

**2.4.1.2** Use a sterile pipette to transfer 11.0 mL of the blender contents to 99 mL of sterile dilution water (Section 7.4), cap, and shake vigorously a minimum of 25 times. This is dilution "A." A volume of 1.0-mL of dilution "A" contains $10^{-2}$ g of the original sample.

**2.4.1.3** Use a sterile pipette to transfer 1.0 mL of dilution "A" to 9.0 mL of sterile dilution water and mix as before. This is dilution "B." A volume of 1.0-mL of dilution "B" contains $10^{-3}$ g of the original sample.

**2.4.2** Inoculation

**2.4.2.1** Use a sterile pipette to inoculate each of the first series of tubes with 10.0 mL of the "blended" sample. This series of tubes must contain 2X media. This is 1.0 g of the original sample. Since test tubes with inverted vials are being used, shaking is not practical. Solids that will not separate easily and/or may float should be submerged into the broth with a sterile loop.

**2.4.2.2** Use a sterile pipette to inoculate each of the second series of tubes with 1 mL of the blended mixture. This is $10^{-1}$ g of the original sample.

**2.4.2.3** Use a sterile pipette to inoculate each of the third series of tubes with 1.0 mL of dilution "A." This is $10^{-2}$ g of the original sample.

**2.4.2.4** Use a sterile pipette to inoculate each of the fourth series of tubes with 1.0 mL of dilution "B." This is $10^{-3}$ g of the original sample.

**2.4.3** Repeat Section 2.4.1 and 2.4.2 of Appendix B for remaining Class A solid samples. When inoculations are complete, return to Section 11.3.1.5 to continue the LTB/EC method or to Section 11.4.5 to continue the A-1 method.

## 3.0 MF Dilution and Inoculation (Filtration) Option

To avoid confusion, laboratories may eliminate 1:100 dilutions transferring 11.0 mL to 99 mL sterile dilution water (Section 7.4) for all dilution series. As a result, all dilutions will be 1:10.

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*NOTE: For some transfers, it may be convenient to use a sterile, wide-mouth pipette, capable of transferring particulate matter.*

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**3.1** Class B liquid samples—Three individual filtrations should be conducted using $10^{-5}$, $10^{-4}$, and $10^{-3}$ mL of the original sample. See Figure B7 for a summary of this dilution and inoculation scheme.

**3.1.1** Dilution

**3.1.1.1** Use a sterile pipette to transfer 11.0 mL of homogenized sample (Section 12.5.1) to 99 mL of sterile dilution water and mix by vigorously shaking the bottle a minimum of 25 times. This dilution is "A." A volume of 1.0-mL of this mixture is $10^{-1}$ mL of the original sample.

**3.1.1.2** Use a sterile pipette to transfer 11.0 mL of dilution "A" to 99 mL of sterile dilution water, and mix as before. This is dilution "B." A 1.0-mL volume of dilution "B" is $10^{-2}$ mL of the original sample.

**3.1.1.3** Use a sterile pipette to transfer 11.0 mL of dilution "B" to 99 mL of sterile dilution water, and mix as before. This is dilution "C." A 1.0-mL volume of dilution "C" is $10^{-3}$ mL of the original sample.

**3.1.1.4** Use a sterile pipette to transfer 11.0 mL of dilution "C" to 99 mL of sterile dilution water, and mix as before. This is dilution "D." A 1.0-mL volume of dilution "D" is $10^{-4}$ mL of the original sample.

**3.1.1.5** Use a sterile pipette to transfer 11.0 mL of dilution "D" to 99 mL of sterile dilution water, and mix as before. This is dilution "E." A 1.0-mL volume of dilution "E" is $10^{-5}$ mL of the original sample.

**3.1.1.6** Use a sterile pipette to transfer 11.0 mL of dilution "E" to 99 mL of sterile dilution water, and mix as before. This is dilution "F." A 1.0-mL volume of dilution "F" is $10^{-6}$ mL of the original sample.

**3.1.2** Inoculation (filtration)—Samples should be filtered in order of highest dilution (least sample volume) first to avoid carry over contamination.

**3.1.2.1** Filtration 1 will use 10.0 mL of dilution "F." This is $10^{-5}$ mL of the original sample.

**3.1.2.2** Filtration 2 will use 10.0 mL of dilution "E." This is $10^{-4}$ mL of the original sample.

**3.1.2.3** Filtration 3 will use 10.0 mL of dilution "D." This is $10^{-3}$ mL of the original sample.

**3.1.3** Complete Section 3.1.1 and 3.1.2 for the remaining Class B liquid samples.

**3.2** Class B solid samples—Three individual filtrations should be conducted using $10^{-5}$, $10^{-4}$, and $10^{-3}$ mL of the original sample. See Figure B8 for a summary of this dilution and inoculation scheme.

**3.2.1** Dilution

**3.2.1.1** A volume of 1.0-mL of the "blended" sample (Section 12.5.2) contains $10^{-1}$ g of the original sample.

**3.2.1.2** Use a sterile pipette to transfer 11.0 mL of the blender contents to 99 mL of sterile dilution water and shake vigorously a minimum of 25 times. This is dilution "A." A volume of 1.0-mL of dilution "A" contains $10^{-2}$ g of the original sample.

**3.2.1.3** Use a sterile pipette to transfer 11.0 mL of dilution "A" to 99 mL of sterile dilution water, and mix as before. This is dilution "B." A volume of 1.0-mL of dilution "B" is $10^{-3}$ g of the original sample.

**3.2.1.3** Use a sterile pipette to transfer 11.0 mL of dilution "B" to 99 mL of sterile dilution water, and mix as before. This is dilution "C." A volume of 1.0-mL of dilution "C" is $10^{-4}$ g of the original sample.

**3.2.1.3** Use a sterile pipette to transfer 11.0 mL of dilution "C" to 99 mL of sterile dilution water, and mix as before. This is dilution "D." A volume of 1.0-mL of dilution "D" is $10^{-5}$ g of the original sample.

**3.2.1.3** Use a sterile pipette to transfer 11.0 mL of dilution "D" to 99 mL of sterile dilution water, and mix as before. This is dilution "E." A volume of 1.0-mL of dilution "E" is $10^{-6}$ g of the original sample.

**3.2.2** Inoculation (filtration)—Samples should be filtered in order of highest dilution (least sample volume) first to avoid carry over contamination.

**3.2.2.1** Filtration 1 will use 10.0 mL of dilution "E." This is $10^{-5}$ g of the original sample.

**3.2.2.2** Filtration 2 will use 10.0 mL of dilution "D." This is $10^{-4}$ g of the original sample.

**3.2.2.3** Filtration 3 will use 10.0 mL of dilution "C." This is $10^{-3}$ g of the original sample.

**3.2.3** Repeat Section 3.2.1 and 3.2.2 for the remaining Class B solid samples.

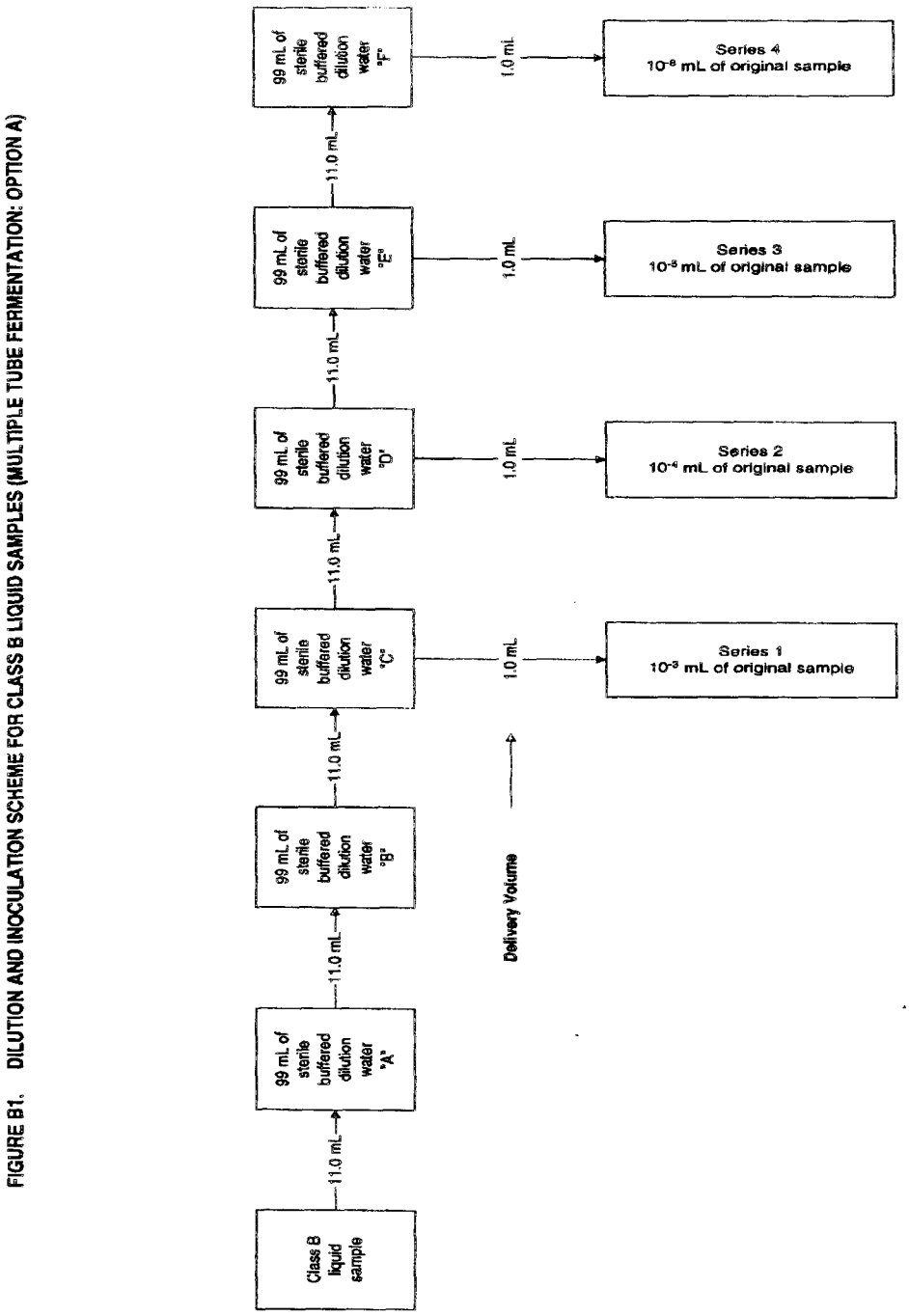



FIGURE B1. DILUTION AND INOCULATION SCHEME FOR CLASS B LIQUID SAMPLES (MULTIPLE TUBE FERMENTATION: OPTION A)

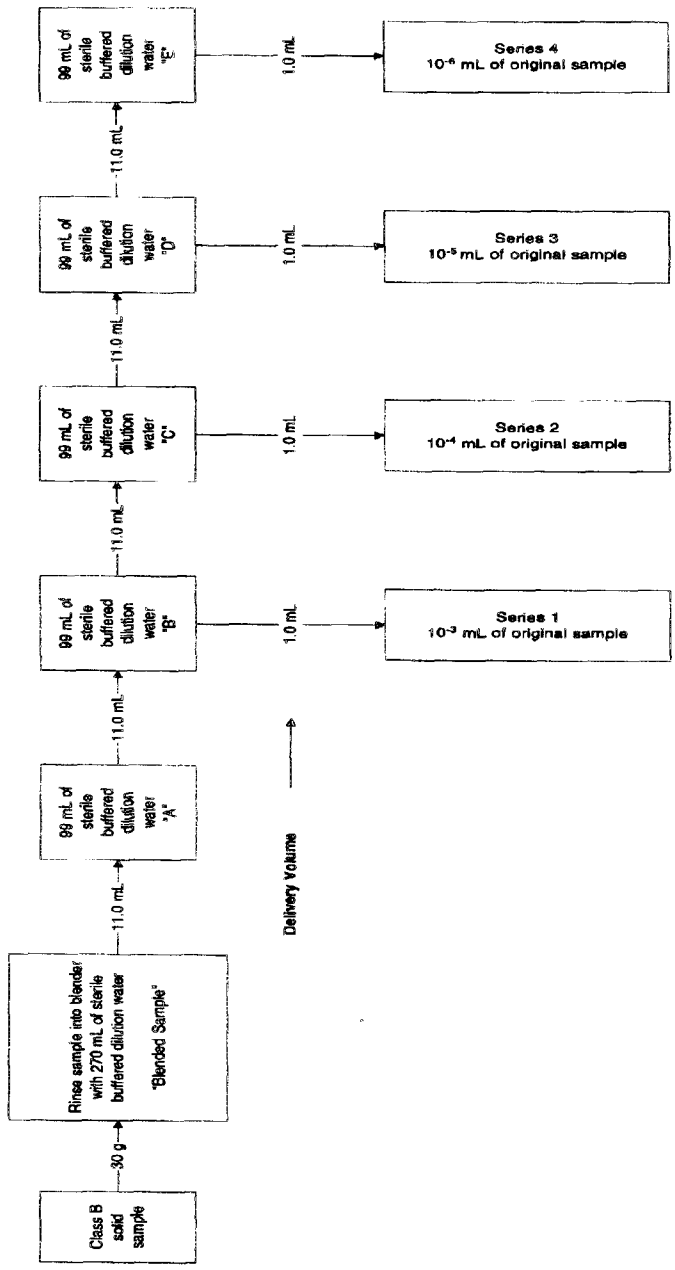



FIGURE B2. DILUTION AND INOCULATION SCHEME FOR CLASS B SOLID SAMPLES (MULTIPLE TUBE FERMENTATION: OPTION A)

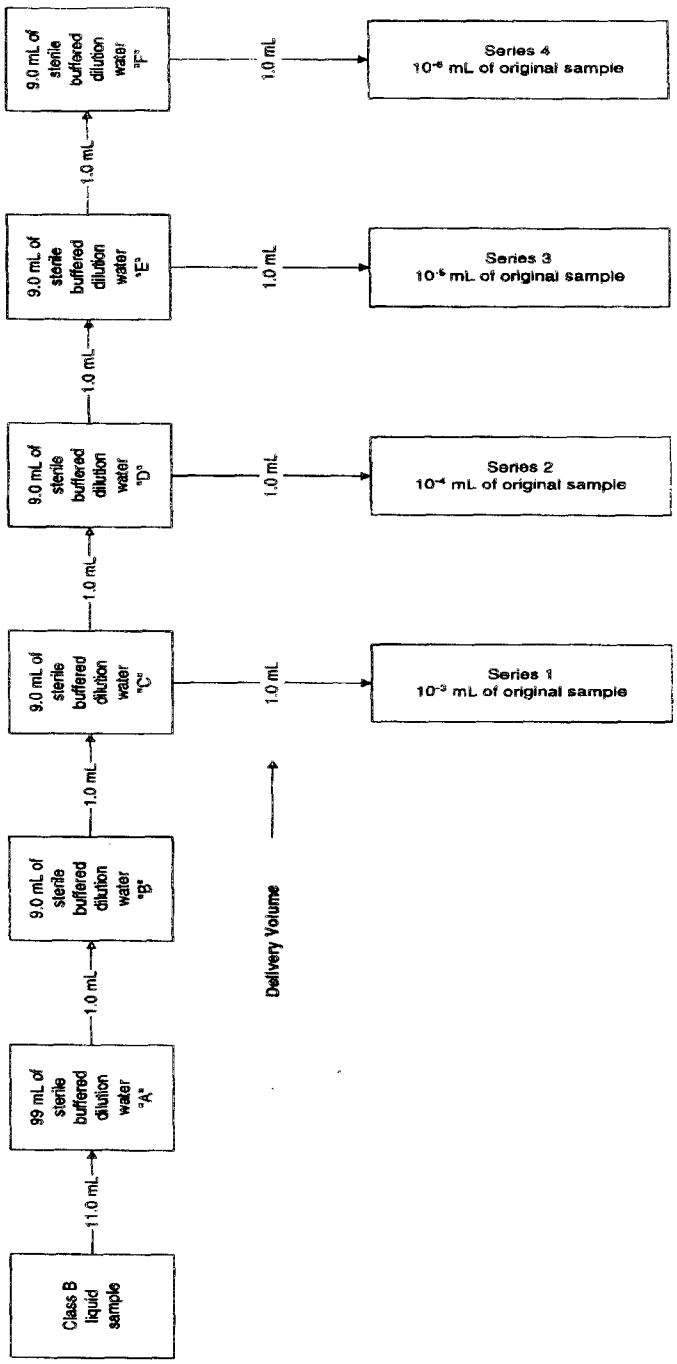



FIGURE B3. DILUTION AND INOCULATION SCHEME FOR CLASS B LIQUID SAMPLES (MULTIPLE TUBE FERMENTATION: OPTION B)

FIGURE B4. DILUTION AND INOCULATION SCHEME FOR CLASS B SOLID SAMPLES (MULTIPLE TUBE FERMENTATION: OPTION B)

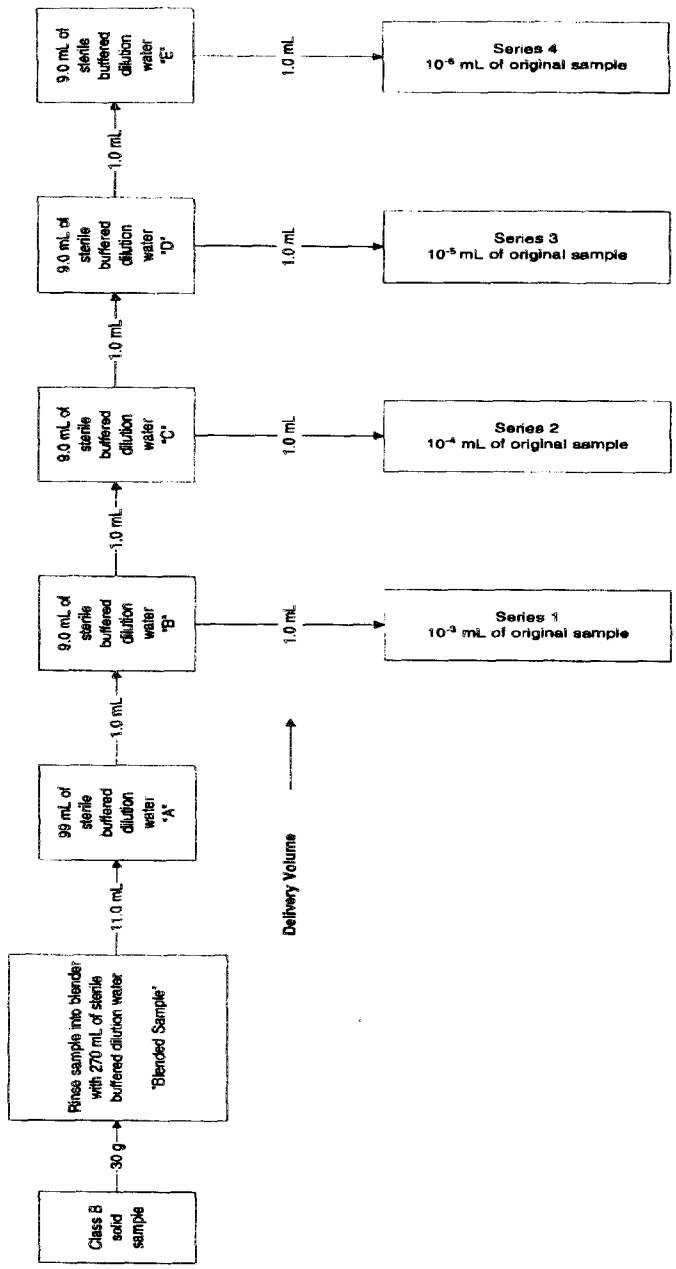
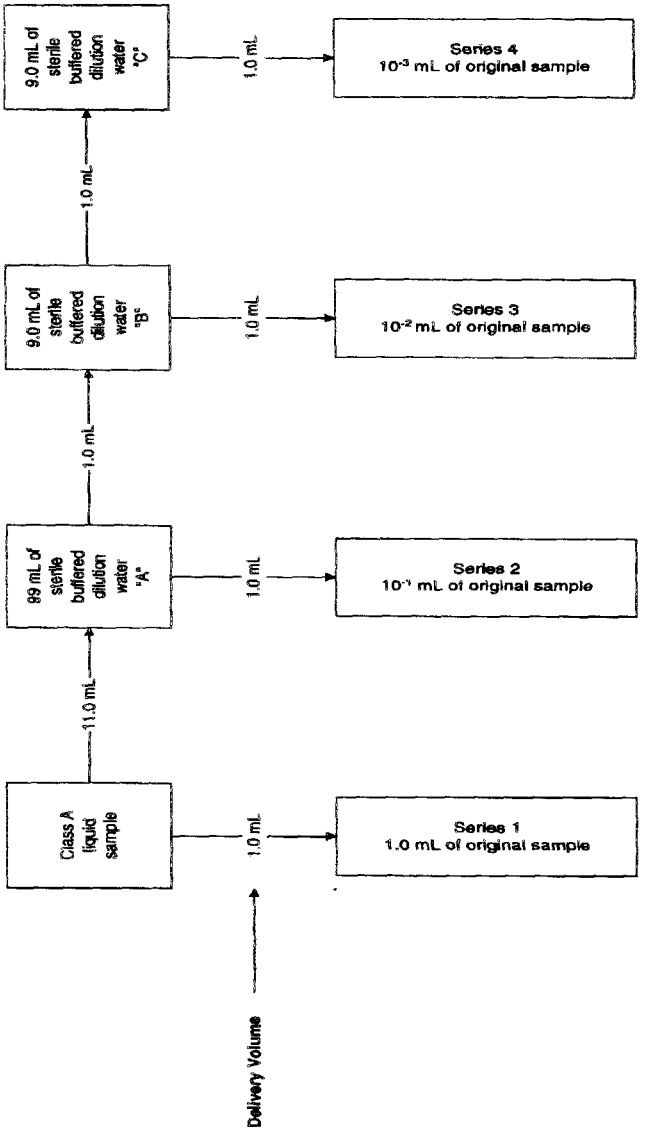


FIGURE B5. DILUTION AND INOCULATION SCHEME FOR CLASS A LIQUID SAMPLES (MULTIPLE TUBE FERMENTATION: OPTION B)



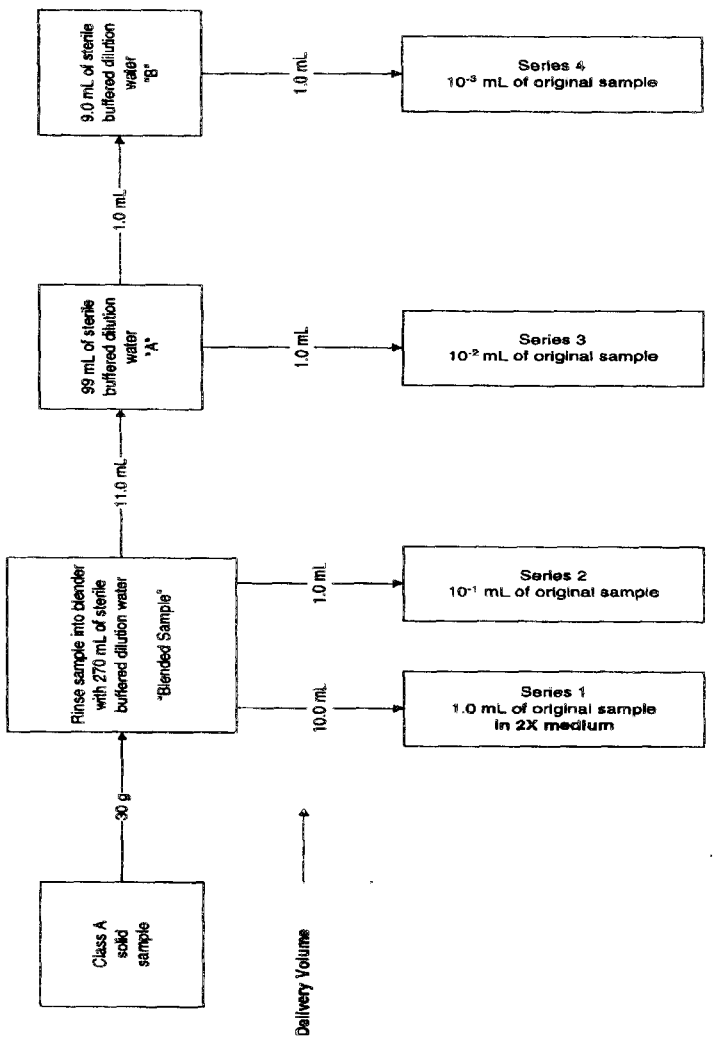



FIGURE B6. DILUTION AND INOCULATION SCHEME FOR CLASS A SOLID SAMPLES (MULTIPLE TUBE FERMENTATION; OPTION B)

**13.2.6** CFU procedure example calculation:

**13.2.6.1** In Table 4, samples 2 and 3 include filters that have colony counts out of the recommended range. In these cases, both countable plates should be used to calculate the CFU/g total solids. The fecal coliform density for Sample 2 is:

$$\text{CFU / g total solids} = \frac{(2 + 18) \times 100}{(0.000010 + 0.00010) \times 4.3} = 4.2 \times 10^6$$

The fecal coliform density for Sample 3 is:

$$\text{CFU / g total solids} = \frac{(8 + 65) \times 100}{(0.00010 + 0.0010) \times 4.0} = 1.6 \times 10^6$$

Except for Sample 5, the remaining samples have at least one membrane filter within the ideal range. For these samples, the number of colonies formed on that filter is used to calculate the coliform density. For Sample 1, the fecal coliform density is:

$$\text{CFU / g total solids} = \frac{23 \times 100}{(0.0010 \times 3.8)} = 6.0 \times 10^5$$

**13.2.6.2** Fecal coliform densities of all the samples were calculated and converted to $\log_{10}$ values to compute the geometric mean. These calculated values are presented in Table 5.

**TABLE 5. FECAL COLIFORM DENSITY OF BIOSOLID SAMPLES**

| Sample No. | Coliform Density | $\log_{10}$ |
|---|---|---|
| 1 | $6.0 \times 10^5$ | 5.78 |
| 2 | $4.2 \times 10^6$ | 6.62 |
| 3 | $1.7 \times 10^6$ | 6.23 |
| 4 | $1.4 \times 10^6$ | 6.15 |
| 5 | $4.0 \times 10^5$ | 5.60 |
| 6 | $1.1 \times 10^6$ | 6.04 |
| 7 | $5.1 \times 10^5$ | 5.71 |

The geometric mean for the seven samples is determined by averaging the $\log_{10}$ values of the coliform density and taking the antilog of that value:

$(5.78 + 6.62 + 6.23 + 6.15 + 5.60 + 6.04 + 5.71)/7 = 6.02$

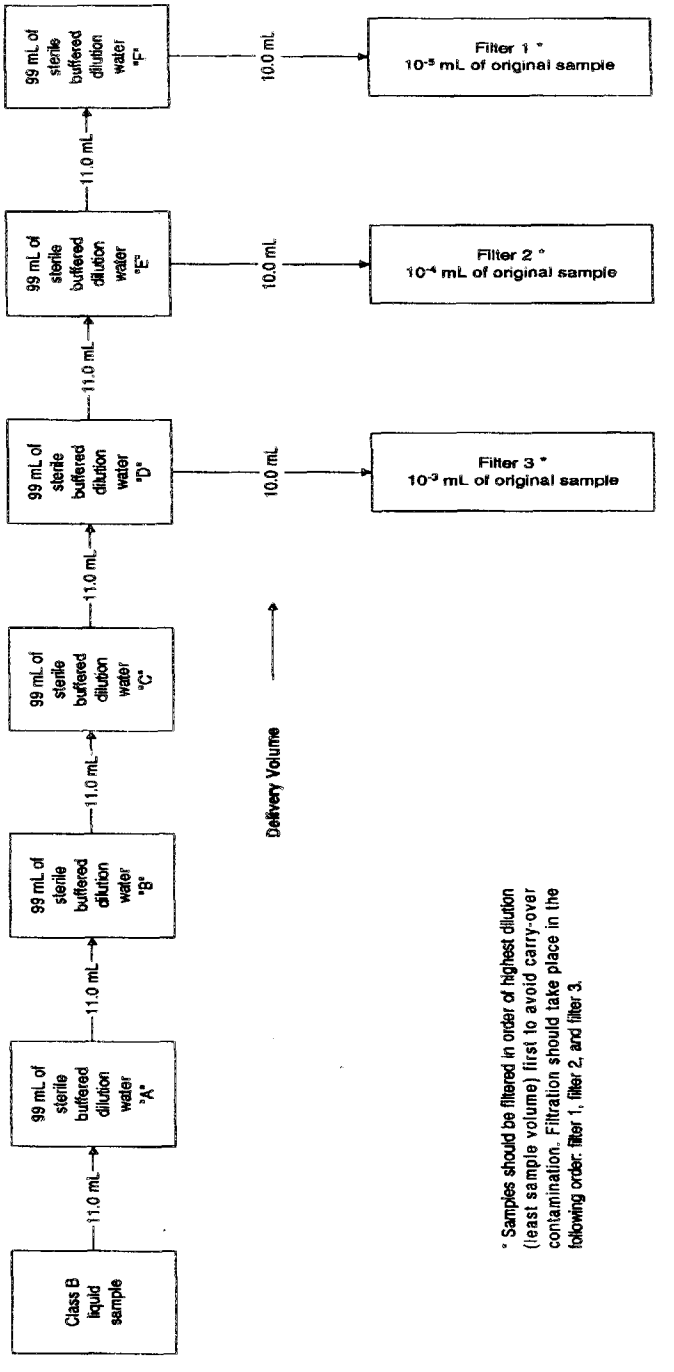
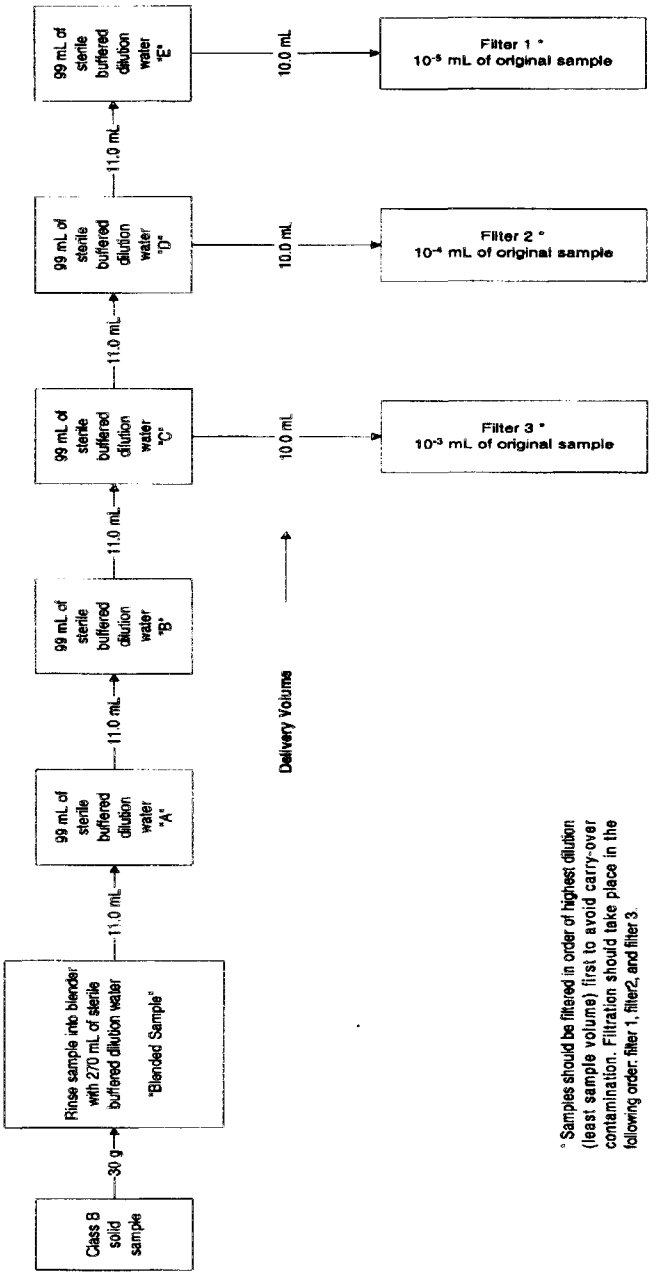



FIGURE B7. DILUTION AND FILTRATION SCHEME FOR CLASS B LIQUID SAMPLES (MEMBRANE FILTER)

**FIGURE B8. DILUTION AND FILTRATION SCHEME FOR CLASS B SOLID SAMPLES (MEMBRANE FILTER)**




* Samples should be filtered in order of highest dilution (least sample volume) first to avoid carry-over contamination. Filtration should take place in the following order: filter 1, filter2, and filter 3.