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Chapter A4

METHODS FOR COLLECTION AND ANALYSIS OF AQUATIC BIOLOGICAL AND MICROBIOLOGICAL SAMPLES

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LABORATORY ANALYSIS

Total coliform bacteria (membrane-filter method)

Immediate incubation test (B-0025-85)

Parameter and Code:
Coliform, membrane filter, immediate M-Endo medium (colonies/100 mL): 31501

The standard test for presence of members of the coliform group may be made by using the following membrane-filter method or by using the multiple-tube test described in the "Presumptive Test," "Presumptive Onsite Test," and "Confirmation Test" subsections in the "Total Coliform Bacteria (Most-Probable-Number, MPN, Method)" section, or in Bordner and others (1978) and American Public Health Association and others (1985).

The coliform group is defined as the aerobic and facultative anaerobic, gram-negative, nonspore-forming, rod-shaped bacteria that ferment lactose with gas formation at 35 °C within 48 hours. For the purposes of the methods described in the following paragraphs, the coliform group is defined as all the organisms that produce colonies with a golden-green metallic sheen when incubated at 35 °C on M-Endo medium within 24 hours.

1. Applications

The membrane-filter method is applicable to fresh and saline water. The test is performed using the agar-plate method.

2. Summary of method

The sample is filtered onsite immediately after collection, and the filter is placed on a nutrient medium designed to stimulate the growth of members of the coliform group and to suppress the growth of most noncoliform organisms. After incubation at 35±0.5 °C for 22 to 24 hours, the colonies are counted.

3. Interferences

3.1 Suspended materials may inhibit the filtration of sample volumes sufficient to produce significant results. Coliform colony formation on the filter may be inhibited by large numbers of noncoliform colonies, by the presence of algal filaments and detritus, or by toxic substances.

3.2 Water samples having a large suspended-solids concentration may be divided between two or more membrane filters. The multiple-tube test, which is described in this chapter, will give the most reliable results when suspended-solids concentrations are large and coliform counts are small.

4. Apparatus

All materials used in microbiological testing need to be free of agents that inhibit bacterial growth. Most of the materials and apparatus listed in this section are available from scientific supply companies.

The following apparatus list assumes the use of an onsite kit for microbiological water tests, such as the portable water laboratory (Millipore, or equivalent). If other means of sample filtration are used, refer to the manufacturer's instructions for proper operation of the equipment. Items marked with an asterisk (*) in the list are included in the portable water laboratory (fig. 1).

4.1 *Alcohol burner*, glass or metal, containing ethyl alcohol for flame sterilizing of forceps.

4.2 *Aluminum seals*, one piece, 20 mm.

4.3 *Bottles*, milk dilution, screwcap.

4.4 *Bottles*, serum.

4.5 *Crimper*, for attaching aluminum seals.

4.6 *Decapper*, for removing aluminum seals from spent tubes.

4.7 *Filter-holder assembly** and *syringe* that has a *two-way valve** or *vacuum hand pump*.

4.8 *Forceps**, stainless steel, smooth tips.

4.9 *Graduated cylinders*, 100-mL capacity.

4.10 *Hypodermic syringes*, sterile, 1-mL capacity, equipped with 26-gauge, ⅜-in. needles.

4.11 *Hypodermic syringes*, sterile, 10-mL capacity, equipped with 22-gauge, 1- to 1½-in. needles.

4.12 *Incubator**, for operation at a temperature of 35±0.5 °C. A portable incubator as provided in the portable water laboratory, or *heaterblock* (fig. 2), which operates on either 115 V ac or 12 V dc, is convenient for onsite use. A larger incubator, having more precise temperature regulation, is satisfactory for laboratory use.

4.13 *Membrane filters*, white, grid, sterile, 0.45- or 0.7-μm mean pore size, 47-mm diameter, and *absorbent pads*.

4.14 *Microscope*, binocular wide-field dissecting-type, and *fluorescent lamp*.

4.15 *Pipets*, 1-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.16 *Pipets*, 10-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.17 *Pipettor*, or *pi-pump*, for use with 1- and 10-mL pipets.

4.18 *Plastic petri dishes with covers*, disposable, sterile, 50×12 mm.

4.19 *Rubber stoppers*, 13×20 mm.

4.20 *Sample-collection apparatus.* Use an appropriate device for collecting a representative sample from the environment to be tested, following guidelines in the "Collection" subsection of the "Bacteria" section.

4.21 *Sterilizer,* horizontal steam autoclave, or vertical steam autoclave.

CAUTION.—If vertical autoclaves or pressure cookers are used, they need to be equipped with an accurate pressure gauge, a thermometer with the bulb 2.5 cm above the water level, automatic thermostatic control, metal air-release tubing for quick exhaust of air in the sterilizer, metal-to-metal-seal eliminating gaskets, automatic pressure-release valve, and clamping locks preventing removal of lid while pressure exists. These features are necessary in maintaining sterilization conditions and decreasing safety hazards.

To obtain adequate sterilization, do not overload sterilizer. Use a sterilization indicator to ensure that the correct combination of time, temperature, and saturated steam has been obtained.

4.22 *Thermometer,* having a temperature range of at least 40 to 100 °C.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 *Buffered dilution water.* Dissolve 34 g potassium dihydrogen phosphate (KH_2PO_4) in 500 mL distilled water. Adjust to pH 7.2 using 1 *N* sodium hydroxide (NaOH). Dilute to 1 L using distilled water. Sterilize in dilution bottles at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Add 1.25 mL KH_2PO_4 solution to 1 L distilled water containing 0.1 percent peptone. (Do not store KH_2PO_4 solutions for more than 3 months.) Dispense in milk dilution or serum bottles (capped with rubber stoppers and crimped with aluminum seals) in quantities that will provide 99±2 mL after autoclaving at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Allow enough space between bottles for steam to circulate during autoclaving. Loosen caps prior to sterilizing and tighten when bottles have cooled.

5.2 *Distilled or deonized water.*

5.3 *Ethyl alcohol,* 95-percent denatured or absolute ethyl alcohol for sterilizing equipment. Absolute methyl alcohol also may be used for sterilization.

5.4 *M-Endo agar.* Add 4.8 g of M-Endo broth MF to 100 mL 2 percent nondenatured ethyl alcohol, then add 1.5 g agar. Stir well and place the beaker containing the medium in a boiling water bath and heat the medium to 96 °C, stirring constantly. Do not autoclave the medium. When the medium begins to boil, promptly remove from heat and cool to 45 to 50 °C. Pour to a depth of 4 mm (6–7 mL) in 50-mm petri dish bottoms. When the medium solidifies, store the prepared petri dishes at 2 to 10 °C for a maximum period of 4 to 5 days.

5.5 *Methyl alcohol,* absolute, for sterilizing filter-holder assembly.

6. Analysis

The volumes of the sample to be filtered depend on the expected bacterial density of the water being tested, but the volumes should be enough that, after incubation, at least one of the membrane filters will contain from 20 to 80 total coliform colonies and not more than 200 of all types (total coliform plus noncoliform colonies). It is extremely important that the limitation on total coliform colonies be observed, otherwise the medium used in the method may not support development of the characteristic metallic sheen. If the upper limit of 80 total coliform colonies per membrane filter is exceeded, interferences from crowding, deposits of extraneous material, and other factors will give questionable results.

The lower limit of 20 total coliform colonies per membrane filter is arbitrarily set as a number below which statistical validity becomes questionable. However, even with a bacterial population of 200 or fewer colonies (coliform plus noncoliform) per 100 mL of sample, fewer than 20 total coliform colonies will be present on the membrane filter of some samples.

The following sample volumes are suggested for filtration:

1. Unpolluted raw surface water: 0.1-, 0.4-, 1.5-, 6-, 25-, and 100-mL samples will include a range of 20 to 80,000 total coliform colonies per 100 mL using the criterion of 20 to 80 total coliform colonies on a filter as an ideal determination.
2. Polluted raw surface water: 0.002-, 0.006-, 0.025-, 0.1-, 0.4-, and 1.6-mL samples will include a range of 1,200 to 4,000,000 total coliform colonies per 100 mL.

6.1 Sterilize filter-holder assembly (Note 1). In the laboratory, wrap the funnel and filter base parts of the assembly separately in kraft paper or polypropylene bags and sterilize in the autoclave at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes. Steam must contact all surfaces to ensure complete sterilization. Cool to room temperature before use.

Note 1: Onsite sterilization of filter-holder assembly needs to be in accordance with the manufacturer's instructions but usually involves application and ignition of methyl alcohol to produce formaldehyde. Autoclave sterilization in the laboratory prior to the trip to the sampling site is preferred. Sterilization must be performed at all sites.

6.2 Assemble the filter holder and, using flame-sterilized forceps (Note 2), place a sterile membrane filter over the porous plate of the assembly, grid side up. Carefully place funnel on filter to avoid tearing or creasing the membrane.

Note 2: Flame-sterilized forceps—Dip forceps in ethyl or methyl alcohol, pass through flame to ignite alcohol, and allow to burn out. Do not hold forceps in flame.

6.3 Shake the sample vigorously about 25 times to obtain an equal distribution of bacteria throughout the sample before transferring a measured portion of the sample to the filter-holder assembly.

6.3.1 If the volume of sample to be filtered is 10 mL or more, transfer the measured sample directly onto the dry membrane.

6.3.2 If the volume of the sample is between 1 and 10 mL, pour about 20 mL sterilized buffered dilution water into the funnel before transferring the measured sample onto the membrane. This facilitates distribution of bacteria.

6.3.3 If the volume of original water sample is less than 1 mL, proceed as in 6.3.1 after preparing appropriate dilutions by adding the sample to buffered dilution water in a sterile milk dilution bottle (Note 3) in the following volumes:

Dilution	Volume of sample added to 99-milliliter milk dilution bottle	Filter this volume
1:10	11 milliliters of original sample	1 milliliter of 1:10 dilution
1:100	1 milliliter of original sample	1 milliliter of 1:100 dilution
1:1,000	1 milliliter of 1:10 dilution	1 milliliter of 1:1,000 dilution
1:10,000	1 milliliter of 1:100 dilution	1 milliliter of 1:10,000 dilution

Note 3: Use a sterile pipet or hypodermic syringe for each bottle. After each transfer, close and shake the bottle vigorously at least 25 times to maintain distribution of the organisms in the sample. Diluted samples need to be filtered within 20 minutes after preparation.

6.4 Apply vacuum and filter the sample. When vacuum is applied using a syringe fitted with a two-way valve, proceed as follows: Attach the filter-holder assembly to the inlet of the two-way valve with plastic tubing. Draw the syringe plunger very slowly on the initial stroke to avoid the danger of air lock before the assembly fills with water. Push the plunger forward to expel air from the syringe. Continue until the entire sample has been filtered. If the filter balloons or develops bubbles during sample filtration, disassemble the two-way valve and lubricate the rubber valve plugs lightly with stopcock grease. If a vacuum hand pump is used, do not exceed a pressure of 25 cm to avoid damage to bacteria.

6.5 Rinse sides of funnel twice with 20 to 30 mL of sterile buffered dilution water while applying vacuum.

6.6 Maintaining the vacuum, remove the funnel from the base of the filter-holder assembly and, using flame-sterilized forceps, remove the membrane filter from the base and place it on the agar in the plastic petri dish, grid side up, using a rolling action at one edge. Use care to avoid trapping air bubbles under the membrane (Note 4).

Note 4: Hold the funnel while removing the membrane filter and place it back on the base of the assembly when the membrane filter has been removed. Placement of the funnel on anything but the base of the assembly may result in contamination of the funnel.

6.7 Place top on petri dish and proceed with filtration of the next volume of water. Filter in order of increasing sample volume, rinsing with sterile buffered dilution water between filtrations.

6.8 Clearly mark the lid of each plastic petri dish indicating location, time of collection, time of incubation, sample number, and sample volume. Use a waterproof felt-tip marker or grease pencil.

6.9 Inspect the membrane in each petri dish for uniform contact with the agar. If air bubbles (indicated by bulges) are present under the filter, remove the filter using sterile forceps and roll onto the agar again.

6.10 Close the plastic petri dish by firmly pressing down on the top.

6.11 Incubate the filters in the tightly closed petri dishes in an inverted position (agar and filter at the top) at 35 ± 0.5 °C for 22 to 24 hours. Filters need to be incubated within 20 minutes after placement on medium.

6.12 Using forceps, remove the filters and allow to dry for at least 1 minute on an absorbent surface. Membranes that have colonies with poor sheen production can be allowed to dry completely. This will enhance sheen production.

6.13 Count the number of coliform sheen colonies, that is, dark colonies having a golden-green metallic sheen. The sheen may cover the entire colony or appear only in a central area or on the periphery. The color plate in Millipore Corp. (1973, p. 42) may be helpful in identifying total coliform colonies. The counts are best made using 10× to 15× magnification. Place the illuminator (fluorescent) as directly above the filter as possible.

6.14 Autoclave all cultures at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 to 30 minutes before discarding.

7. Calculations

7.1 If only one filter has a colony count between the ideal of 20 and 80, use the equation:

$$\text{Total coliform colonies/100 mL} = \frac{\text{Number of colonies counted} \times 100}{\text{Volume of original sample filtered (milliliters)}}.$$

7.2 If all filters have counts less than the ideal of 20 colonies or greater than 80 colonies, calculate using the equations in 7.5 for only those filters having at least one colony and not having colonies too numerous to count. Report results as number per 100 mL, followed by the statement, "Estimated count based on nonideal colony count."

7.3 If no filters develop characteristic total coliform colonies, report a maximum estimated value. Assume a count of one colony for the largest sample volume filtered, then calculate using the equation in 7.1. Report the results as less than (<) the calculated value per 100 mL.

7.4 If all filters have colonies too numerous to count, report a minimum estimated value. Assume a count of 80 total coliform colonies for the smallest sample volume filtered, then calculate using the equation in 7.1. Report the results as greater than (>) the calculated value per 100 mL.

7.5 Sometimes two or more filters of a series will produce colony counts within the ideal counting range. Make colony counts for all such filters. The method for calculating and averaging is as follows (Note 5):

$$\frac{\text{Volume filter 1} + \text{Volume filter 2}}{\text{Volume sum}} \qquad \frac{\text{Colony count filter 1} + \text{Colony count filter 2}}{\text{Colony count sum}}$$

Total coliform colonies/100 mL =

$$\frac{\text{Colony count sum} \times 100}{\text{Volume sum (milliliters)}}.$$

Note 5: Do not calculate the total coliform colonies per 100 mL for each volume filtered and then average the results.

8. Reporting of results

Report total coliform concentration as total coliform colonies per 100 mL, M-Endo immediate incubation at 35 °C as follows: less than 10 colonies, whole numbers; 10 or more colonies, two significant figures.

9. Precision

No numerical precision data are available.

10. Sources of information

American Public Health Association, American Water Works Association, and Water Pollution Control Federation, 1985, Standard methods for the examination of water and wastewater (16th ed.): Washington, D.C., American Public Health Association, 1,268 p.

Bordner, R.H., Winter, J.A., and Scarpino, Pasquale, eds., 1978, Microbiological methods for monitoring the environment, water and wastes: Cincinnati, Ohio, U.S. Environmental Protection Agency, EPA-600/8-78-017, 338 p.

Millipore Corp., 1973, Biological analysis of water and wastewater: Bedford, Mass., Application Manual AM302, 84 p.

Total coliform bacteria (membrane-filter method)

Delayed incubation test (B-0030-85)

Parameter and Code:
Coliform, membrane filter, delayed M-Endo medium (colonies/100 mL): 31503

The delayed incubation test is not a substitute for the immediate incubation test. Results obtained from these two tests are not comparable.

1. Applications

The method is applicable to fresh and saline water. It is used when it is not possible to begin incubation of samples at the specified temperature within 6 hours of collection. Within 72 hours, the membranes must be transferred to a nutrient medium and normal incubation started. The applicability of the delayed incubation test for a specific water source can be determined by comparative test procedures with conventional methods.

2. Summary of method

The sample is filtered onsite immediately after collection, and the filter is placed on a holding medium and shipped to the laboratory. The holding medium maintains the viability of the coliform organisms and generally does not permit visible growth during the time of transit. The coliform determination is completed in the laboratory by transferring the membrane to a growth medium, incubating at 35±0.5 °C for 20 to 22 hours, and counting the typical coliform colonies.

3. Interferences

3.1 Suspended materials may inhibit the filtration of sample volumes sufficient to produce significant results. Coliform colony formation on the filter may be inhibited by large numbers of noncoliform colonies, by the presence of algal filaments and detritus, or by toxic substances.

3.2 Water samples having a large suspended-solids concentration may be divided between two or more membrane filters.

4. Apparatus

All materials used in microbiological testing need to be free of agents that inhibit bacterial growth. Most of the materials and apparatus listed in this section are available from scientific supply companies.

The following apparatus list assumes the use of an onsite kit for microbiological water tests, such as the portable water laboratory (Millipore, or equivalent). If other means of sample filtration are used, refer to the manufacturer's instructions for proper operation of the equipment. Items marked with an asterisk (*) in the list are included in the portable water laboratory (fig. 1).

4.1 *Alcohol burner*, glass or metal, containing ethyl alcohol for flame sterilizing of forceps.

4.2 *Aluminum seals*, one piece, 20 mm.

4.3 *Bottles*, milk dilution, screwcap.

4.4 *Bottles*, serum.

4.5 *Crimper*, for attaching aluminum seals.

4.6 *Decapper*, for removing aluminum seals from spent tubes.

4.7 *Filter-holder assembly** and *syringe* that has a *two-way valve** or *vacuum hand pump*.

4.8 *Forceps**, stainless steel, smooth tips.

4.9 *Graduated cylinders*, 100-mL capacity.

4.10 *Hypodermic syringes*, sterile, 1-mL capacity, equipped with 26-gauge, ⅜-in. needles.

4.11 *Hypodermic syringes*, sterile, 10-mL capacity, equipped with 22-gauge, 1- to 1½-in. needles.

4.12 *Incubator**, for operation at a temperature of 35±0.5 °C. A portable incubator as provided in the portable water laboratory, or *heaterblock* (fig. 2), which operates on either 115 V ac or 12 V dc, is convenient for onsite use. A larger incubator, having more precise temperature regulation, is satisfactory for laboratory use.

4.13 *Membrane filters*, white, grid, sterile, 0.45- or 0.7-μm mean pore size, 47-mm diameter, and *absorbent pads*.

4.14 *Microscope*, binocular wide-field dissecting-type, and *fluorescent lamp*.

4.15 *Pipets*, 1-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.16 *Pipets*, 10-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.17 *Pipettor*, or *pi-pump*, for use with 1- and 10-mL pipets.

4.18 *Plastic petri dishes with covers*, disposable, sterile, 50×12 mm.

4.19 *Rubber stoppers*, 13×20 mm.

4.20 *Sample-collection apparatus*. Use an appropriate device for collecting a representative sample from the

environment to be tested, following guidelines in the "Collection" subsection of the "Bacteria" section.

4.21 *Sterilizer*, horizontal steam autoclave, or vertical steam autoclave.

CAUTION.—If vertical autoclaves or pressure cookers are used, they need to be equipped with an accurate pressure gauge, a thermometer with the bulb 2.5 cm above the water level, automatic thermostatic control, metal air-release tubing for quick exhaust of air in the sterilizer, metal-to-metal-seal eliminating gaskets, automatic pressure-release valve, and clamping locks preventing removal of lid while pressure exists. These features are necessary in maintaining sterilization conditions and decreasing safety hazards.

To obtain adequate sterilization, do not overload sterilizer. Use a sterilization indicator to ensure that the correct combination of time, temperature, and saturated steam has been obtained.

4.22 *Thermometer*, having a temperature range of at least 40 to 100 °C.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 *Buffered dilution water*. Dissolve 34 g potassium dihydrogen phosphate (KH_2PO_4) in 500 mL distilled water. Adjust to pH 7.2 using 1 *N* sodium hydroxide (NaOH). Dilute to 1 L using distilled water. Sterilize in dilution bottles at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Add 1.25 mL KH_2PO_4 solution to 1 L distilled water containing 0.1 percent peptone. (Do not store KH_2PO_4 solutions for more than 3 months.) Dispense in milk dilution or serum bottles (capped with rubber stoppers and crimped with aluminum seals) in quantities that will provide 99±2 mL after autoclaving at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Allow enough space between bottles for steam to circulate during autoclaving. Loosen caps prior to sterilizing and tighten when bottles have cooled.

5.2 *Cyclohexamide*. Dissolve 500 mg of cyclohexamide in 10 mL distilled water. The cyclohexamide solution needs to be refrigerated; storage should not exceed 6 months.

CAUTION.—Cyclohexamide is a powerful skin irritant and needs to be handled according to the manufacturer's directions. Add 1 mL of cyclohexamide solution to 100 mL of M-Endo preservative medium described in 5.6.

5.3 *Distilled or deonized water*.

5.4 *Ethyl alcohol*, 95-percent denatured or absolute ethyl alcohol for sterilizing equipment. Absolute methyl alcohol also may be used for sterilization.

5.5 *M-Endo agar*. Add 4.8 g of M-Endo broth MF to 100 mL 2 percent nondenatured ethyl alcohol, then add 1.5 g agar. Stir well and place the beaker containing the medium in a boiling water bath and heat the medium to 96 °C, stirring constantly. Do not autoclave the medium. When the medium begins to boil, promptly remove from heat and cool to 45 to 50 °C. Pour to a depth of 4 mm (6–7 mL) in 50-mm petri dish bottoms. When the medium solidifies, store the prepared petri dishes at 2 to 10 °C for a maximum period of 4 to 5 days.

5.6 *M-Endo preservative medium*. Add 4.8 g M-Endo broth MF to 100 mL 2 percent nondenatured ethyl alcohol in a beaker and stir for 3 minutes. Place the beaker on a hot plate and heat to boiling, stirring constantly. (Prevent scorching or boiling over of the medium.) When the medium reaches the boiling point, promptly remove from heat and cool to less than 45 °C. Do not sterilize by autoclaving. To 100 mL of M-Endo broth, add 3.2 mL 12 percent sodium benzoate solution. Store the finished medium in the dark at 2 to 10 °C for a maximum period of 4 to 5 days.

5.7 *Methyl alcohol*, absolute, for sterilizing filter-holder assembly.

5.8 *Sodium benzoate solution, 12 percent*. Dissolve 12 g sodium benzoate ($C_7H_5NaO_2$) in sufficient distilled water to make 100 mL. Sterilize by filtration through a 0.45-μm pore-size membrane filter or autoclave at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes. Discard unused solution after 6 months.

6. Analysis

The volumes of sample to be filtered depend on the expected bacterial density of the water being tested, but the volumes should be enough that, after incubation, at least one of the membrane filters will contain from 20 to 80 total coliform colonies and not more than 200 of all types (total coliform plus noncoliform colonies). It is extremely important that the limitation on total coliform colonies be observed, otherwise the medium used in the method may not support development of the characteristic metallic sheen. If the upper limit of 80 total coliform colonies per membrane filter is exceeded, interferences from crowding, deposits of extraneous material, and other factors will give questionable results.

The lower limit of 20 total coliform colonies per membrane filter is arbitrarily set as a number below which statistical validity becomes questionable. However, even with a bacterial population of 200 or fewer colonies (coliform plus noncoliform) per 100 mL of sample, fewer than 20 total coliform colonies will be present on the membrane filter of some samples.

The following sample volumes are suggested for filtration:

1. Unpolluted raw surface water: 0.1-, 0.4-, 1.5-, 6-, 25-, and 100-mL samples will include a range of 20 to 80,000 total coliform colonies per 100 mL using the criterion of 20 to 80 total coliform colonies on a filter as an ideal determination.
2. Polluted raw surface water: 0.002-, 0.006-, 0.025-, 0.1-, 0.4-, and 1.6-mL samples will include a range of 1,200 to 4,000,000 total coliform colonies per 100 mL.

6.1 Place a sterile absorbent pad in the bottom (larger half) of each sterile plastic petri dish using flame-sterilized forceps (Note 1).

Note 1: Flame-sterilized forceps—Dip forceps in ethyl or methyl alcohol, pass through flame to ignite alcohol, and allow to burn out. Do not hold forceps in flame.

6.2 Saturate each pad with about 2 mL M-Endo preservative medium and tilt the petri dish to expel excess liquid. Replace petri dish tops (not tightly to prevent excessive condensation).

6.3 Sterilize filter-holder assembly (Note 2). In the laboratory, wrap the funnel and filter base parts of the assembly separately in kraft paper or polypropylene bags and sterilize in the autoclave at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes. Cool to room temperature before use.

Note 2: Onsite sterilization of filter-holder assembly needs to be in accordance with the manufacturer's instructions but usually involves application and ignition of methyl alcohol to produce formaldehyde. Autoclave sterilization in the laboratory prior to the trip to the sampling site is preferred. Sterilization must be performed at all sites.

6.4 Assemble the filter holder and, using flame-sterilized forceps, place a sterile membrane filter over the porous plate of the assembly, grid side up. Carefully place funnel on filter to avoid tearing or creasing the membrane.

6.5 Shake the sample vigorously about 25 times to obtain an equal distribution of bacteria throughout the sample before transferring a measured portion of the sample to the filter-holder assembly.

6.5.1 If the volume of sample to be filtered is 10 mL or more, transfer the measured sample directly onto the dry membrane.

6.5.2 If the volume of sample is between 1 and 10 mL, pour about 20 mL sterilized buffered dilution water into the funnel before transferring the measured sample onto the membrane. This facilitates distribution of bacteria.

6.5.3 If the volume of original water sample is less than 1 mL, proceed as in 6.5.1 after preparing appropriate dilutions by adding the sample to buffered dilution water in a sterile milk dilution bottle (Note 3) in the following volumes:

Dilution	Volume of sample added to 99-milliliter milk dilution bottle	Filter this volume
1:10	11 milliliters of original sample	1 milliliter of 1:10 dilution
1:100	1 milliliter of original sample	1 milliliter of 1:100 dilution
1:1,000	1 milliliter of 1:10 dilution	1 milliliter of 1:1,000 dilution
1:10,000	1 milliliter of 1:100 dilution	1 milliliter of 1:10,000 dilution

Note 3: Use a sterile pipet or hypodermic syringe for each bottle. After each transfer, close and shake the bottle vigorously at least 25 times to maintain distribution of the organisms in the sample. Diluted samples need to be filtered within 20 minutes after preparation.

6.6 Apply vacuum and filter the sample. When vacuum is applied using a syringe fitted with a two-way valve, proceed as follows: Attach the filter-holder assembly to the inlet of the two-way valve with plastic tubing. Draw the syringe plunger very slowly on the initial stroke to avoid the danger of air lock before the assembly fills with water. Push the plunger forward to expel air from the syringe. Continue until the entire sample has been filtered. If the filter balloons or develops bubbles during sample filtration, disassemble the two-way valve and lubricate the rubber valve plugs lightly with stopcock grease. If a vacuum hand pump is used, do not exceed a pressure of 25 cm to avoid damage to bacteria.

6.7 Rinse sides of funnel twice with 20 to 30 mL of sterile buffered dilution water while applying vacuum.

6.8 Maintaining the vacuum, remove the funnel from the base of the filter-holder assembly and, using flame-sterilized forceps, remove the membrane filter from the base and place it on the broth-soaked absorbent pad in the plastic petri dish, grid side up, using a rolling action at one edge. Use care to avoid trapping air bubbles under the membrane (Note 4).

Note 4: Hold the funnel while removing the membrane filter and place it back on the base of the assembly when the membrane filter has been removed. Placement of the funnel on anything but the base of the assembly may result in contamination of the funnel.

6.9 Place top on petri dish and proceed with filtration of the next volume of water. Filter in order of increasing sample volume, rinsing with sterile buffered dilution water between filtrations.

6.10 Clearly mark the lid of each plastic petri dish indicating location, time of collection, time of incubation, sample number, and sample volume. Use a waterproof felt-tip marker or grease pencil.

6.11 Inspect the membrane in each petri dish for uniform contact with the saturated pad. If air bubbles are present under the filter (indicated by bulges), remove the filter using sterile forceps and roll onto the absorbent pad again.

6.12 Close the plastic petri dish by firmly pressing down on the top.

6.13 Place the petri dish containing the membrane filter in an insulated shipping container and mail. The container needs to arrive in the laboratory within 72 hours. Limited bacterial growth sometimes occurs on the preservative medium when high temperatures are encountered.

6.14 In the laboratory, transfer the membrane from the petri dish in which it was shipped to a fresh sterile petri dish containing M-Endo agar. Use sterile forceps and ensure a good contact between the filter and medium.

6.15 Incubate the filters in the tightly closed petri dishes in an inverted position (agar and filter at the top) at 35 ± 0.5 °C for 20 to 22 hours. Filters need to be incubated within 20 minutes after placement on medium.

6.16 Using forceps, remove the filters and allow to dry for at least 1 minute on an absorbent surface. Membranes that have colonies having poor sheen production can be allowed to dry completely. This will enhance sheen production.

6.17 Count the number of coliform sheen colonies, that is, dark colonies having a golden-green metallic sheen. The sheen may cover the entire colony or appear only in a central area or on the periphery. The color plate in Millipore Corp. (1973, p. 42) may be helpful in identifying total coliform colonies. The counts are best made using 10× to

15× magnification. Place the illuminator (fluorescent) as directly above the filter as possible.

6.18 Autoclave all cultures at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 to 30 minutes before discarding.

7. Calculations

7.1 If only one filter has a colony count between the ideal of 20 and 80, use the equation:

$$\text{Total coliform colonies/100 mL} = \frac{\text{Number of colonies counted} \times 100}{\text{Volume of original sample filtered (milliliters)}}.$$

7.2 If all filters have counts less than the ideal of 20 colonies or greater than 80 colonies, calculate using the equations in 7.5 for only those filters having at least one colony and not having colonies too numerous to count. Report results as number per 100 mL, followed by the statement, "Estimated count based on nonideal colony count."

7.3 If no filters develop characteristic total coliform colonies, report a maximum estimated value. Assume a count of one colony for the largest sample volume filtered, then calculate using the equation in 7.1. Report the results as less than (<) calculated value per 100 mL.

7.4 If all filters have colonies too numerous to count, report a minimum estimated value. Assume a count of 80 total coliform colonies for the smallest sample volume filtered, then calculate using the equation in 7.1. Report the results as greater than (>) the calculated value per 100 mL.

7.5 Sometimes two or more filters of a series will produce colony counts within the ideal counting range. Make colony counts for all such filters. The method for calculating and averaging is as follows (Note 5):

$$\frac{\text{Volume filter 1} + \text{Volume filter 2}}{\text{Volume sum}} \qquad \frac{\text{Colony count filter 1} + \text{Colony count filter 2}}{\text{Colony count sum}}$$

$$\text{Total coliform colonies/100 mL} = \frac{\text{Colony count sum} \times 100}{\text{Volume sum (milliliters)}}.$$

Note 5: Do not calculate the total coliform colonies per 100 mL for each volume filtered and then average the results.

8. Reporting of results

Report the total coliform concentration as total coliform colonies per 100 mL, M-Endo delayed incubation at 35 °C as follows: less than 10 colonies, whole numbers; 10 or more colonies, two significant figures.

9. Precision

No numerical precision data are available.

10. Sources of information

Millipore Corp., 1973, Biological analysis of water and wastewater: Bedford, Mass., Application Manual AM302, 84 p.

Total coliform bacteria (most-probable-number, MPN, method)

Presumptive test (B-0035-85)

Parameter and Code:
Coliform, presumptive (MPN): 31507

1. Applications

This method is applicable to fresh and saline water, water having large suspended-solids concentration, and water having large populations of noncoliform bacteria.

2. Summary of method

Decimal dilutions of multiple sample aliquots are inoculated into lauryl tryptose broth. The cultures are incubated at 35±0.5 °C and examined after 24 and 48 hours for evidence of growth and gas production. The most probable number (MPN) of coliform organisms in the sample is determined from the distribution of gas-positive cultures among the inoculated tubes or serum bottles. Do not use the presumptive test unless the confirmation test (B-0045-85) also is done.

3. Interferences

Large concentrations of heavy metals or toxic chemicals may interfere when large volumes of sample are added to small volumes of concentrated lauryl tryptose broth. Certain noncoliform organisms can ferment lactose with gas formation.

4. Apparatus

All materials used in microbiological testing need to be free of agents that inhibit bacterial growth. Most of the materials and apparatus listed in this section are available from scientific supply companies.

The following apparatus list assumes the use of an onsite kit for microbiological water tests, such as the portable water laboratory (Millipore, or equivalent). If other means of sample filtration are used, refer to the manufacturer's instructions for proper operation of the equipment. Items marked with an asterisk (*) in the list are included in the portable water laboratory (fig. 1).

4.1 *Aluminum seals*, one piece, 20 mm.

4.2 *Bottles*, milk dilution, screwcap.

4.3 *Bottles*, serum.

4.4 *Crimper*, for attaching aluminum seals.

4.5 *Culture tubes and durham (fermentation) tubes.* Two combinations of culture tubes and durham (fermentation) tubes may be used. The choice will depend on the volume of water to be tested. The durham tube, used to detect gas production, must be completely filled with medium and at least partly submerged in the culture tube. The following combinations have been satisfactory:

4.5.1 For testing 10-mL aliquots, use borosilicate glass culture tubes, 20×150 mm; tube caps, 20 mm; and use borosilicate glass culture tubes, 10×75 mm, as durham tubes.

4.5.2 For testing 1-mL or small aliquots, use borosilicate glass culture tubes, 16×125 mm; tube caps, 16 mm; and use flint glass culture tubes, 6×50 mm, as durham tubes.

4.6 *Culture-tube rack*, galvanized, for 16- and 20-mm culture tubes.

4.7 *Decapper*, for removing aluminum seals from spent tubes.

4.8 *Hypodermic syringes*, sterile, 1-mL capacity, equipped with 26-gauge, ⅜-in. needles.

4.9 *Hypodermic syringes*, sterile, 10-mL capacity, equipped with 22-gauge, 1- to 1½-in. needles.

4.10 *Incubator**, for operation at a temperature of 35±0.5 °C. A portable incubator as provided in the portable water laboratory, or *heaterblock* (fig. 2), which operates on either 115 V ac or 12 V dc, is convenient for onsite use. A larger incubator, having more precise temperature regulation, is satisfactory for laboratory use.

4.11 *Pipets*, 1-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.12 *Pipets*, 10-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.13 *Pipettor*, or *pi-pump*, for use with 1- and 10-mL pipets.

4.14 *Rubber stoppers*, 13×20 mm.

4.15 *Sample-collection apparatus.* Use an appropriate device for collecting a representative sample from the environment to be tested, following guidelines in the "Collection" subsection of the "Bacteria" section.

4.16 *Sterilizer*, horizontal steam autoclave, or vertical steam autoclave.

CAUTION.—If vertical autoclaves or pressure cookers are used, they need to be equipped with an accurate pressure gauge, a thermometer with the bulb 2.5 cm above the water level, automatic thermostatic control, metal air-release tubing for quick exhaust of air in the sterilizer, metal-to-metal-seal eliminating gaskets, automatic pressure-release valve, and clamping locks preventing removal of lid while pressure

exists. These features are necessary in maintaining sterilization conditions and decreasing safety hazards.

To obtain adequate sterilization, do not overload sterilizer. Use a sterilization indicator to ensure that the correct combination of time, temperature, and saturated steam has been obtained.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 *Buffered dilution water.* Dissolve 34 g potassium dihydrogen phosphate (KH_2PO_4) in 500 mL distilled water. Adjust to pH 7.2 using 1 *N* sodium hydroxide (NaOH). Dilute to 1 L using distilled water. Sterilize in dilution bottles at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Add 1.25 mL KH_2PO_4 solution to 1 L distilled water containing 0.1 percent peptone. (Do not store KH_2PO_4 solutions for more than 3 months.) Dispense in milk dilution or serum bottles (capped with rubber stoppers and crimped with aluminum seals) in quantities that will provide 99±2 mL after autoclaving at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Allow enough space between bottles for steam to circulate during autoclaving. Loosen caps prior to sterilizing and tighten when bottles have cooled.

5.2 *Distilled or deionized water.*

5.3 *Lauryl tryptose broth.* Use premixed lauryl tryptose broth or lauryl sulfate broth, and prepare according to directions on bottle label. The medium also may be prepared according to American Public Health Association and others (1985).

5.3.1 Place 10 mL of medium containing 71.2 g/L lauryl tryptose broth or lauryl sulfate broth in a 20×150-mm culture tube for each 10-mL aliquot of sample to be tested.

5.3.2 Place 10 mL of medium containing 35.6 g/L lauryl tryptose broth or lauryl sulfate broth in a 16×125-mm culture tube for each 1-mL or smaller aliquot of sample to be tested.

5.3.3 In each culture tube, place an inverted (mouth downward) durham tube (fig. 3). Sterilize culture tubes in upright position at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes as soon as possible after dispensing medium. Air will be expelled from the inverted durham tubes during heating; each will fill completely with medium during cooling. Discard any culture tubes in which air bubbles are visible in the durham tubes.

6. Analysis

Two questions must be answered when planning a multiple-tube test:

1. What volumes of water need to be tested?
2. How many culture tubes of each volume need to be tested?

Choose a range of volumes so positive and negative results are obtained throughout the range tested. The method fails if only positive or only negative results are obtained when all volumes are tested. The number of culture tubes used per sample volume depends on the precision required. The greater the number of tubes inoculated with each volume, the greater the precision, but the effort involved and expense also are increased. A five-tube series is described below. The following sample volumes are suggested:

1. Unpolluted raw surface water: 0.1-, 1-, and 10-mL samples will include an MPN range of <2 to ≥2,400 coliforms per 100 mL.
2. Polluted raw surface water: 0.001-, 0.01-, 0.1-, and 1-mL samples will include an MPN range of 20 to 240,000 coliforms per 100 mL.

6.1 Set up five culture tubes of lauryl tryptose broth for each sample volume to be tested.

6.1.1 If the volume to be tested is 0.1 mL or more, transfer the measured samples directly to the culture tubes using sterile pipets (Note 1).

6.1.2 If the volume of original water sample is less than 0.1 mL, proceed as in 6.1.1 after preparing appropriate dilutions by adding the sample to buffered dilution water in a sterile milk dilution bottle in the following volumes:

Dilution	Volume of sample added to 99-milliliter milk dilution bottle	Size of inoculum
1:10		0.1 milliliter of original sample
1:100	1 milliliter of original sample	1 milliliter of 1 100 dilution
1 1,000		0 1 milliliter of 1 100 dilution
1.10,000	1 milliliter of 1 100 dilution	1 milliliter of 1 10,000 dilution
1:100,000		0 1 milliliter of 1:10,000 dilution

Note 1: Use a sterile pipet or hypodermic syringe for each bottle. After each transfer, close and shake the bottle vigorously at least 25 times to maintain distribution of the organisms in the sample. Diluted samples need to be inoculated within 20 minutes after preparation.

6.2 Clearly mark each set of culture tubes indicating location, time of collection, sample number, and sample volume. Code each tube for easy identification.

6.3 Place the inoculated culture tubes in the culture-tube rack and incubate at 35±0.5 °C for 24±2 hours. Tubes must be maintained in an upright position.

6.4 Remove culture tubes from incubator and examine. Gas in any quantity in the durham tube, even a pinhead-sized bubble, constitutes a positive test (fig. 4). The appearance of an air bubble must not be confused with actual gas production. If the gas is formed as a result of fermentation, the broth medium will become cloudy. Active fermentation may be indicated by the continued appearance of small bubbles of gas in the medium outside the durham tube when the culture tube is shaken gently (Bordner and others, 1978; American Public Health Association and others, 1985).

6.5 After submitting all gas-positive culture tubes to the confirmation test (B-0045-85), autoclave at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 to 30 minutes before discarding.

6.6 Return all gas-negative culture tubes to incubator and incubate at 35±0.5 °C for an additional 24±2 hours.

6.7 Remove culture tubes from incubator and examine for gas formation. Autoclave all remaining tubes of lauryl tryptose broth as in 6.5 before discarding.

7. Calculations

7.1 Record the number of gas-positive culture tubes at 24 and 48 hours occurring for all sample volumes tested.

7.2 When more than three volumes are tested, use results from only three of them when computing the MPN. To select the three dilutions for the MPN index, use as the first, the smallest sample volume in which all tests are positive (no larger sample volume having any negative results) and the next two succeeding smaller sample volumes (Bordner and others, 1978; American Public Health Association and others, 1985).

7.3 In the examples listed below, the number in the numerator represents positive culture tubes; the denominator represents the total number of tubes inoculated.

	Decimal dilutions				
Example	1 milliliter	0.1 milliliter	0.01 milliliter	0.001 milliliter	Combination of positives
a	5/5	5/5	2/5	0/5	5-2-0
b	5/5	4/5	2/5	0/5	5-4-2
c	0/5	1/5	0/5	0/5	0-1-0
d	5/5	3/5	1/5	1/5	5-3-2
e	5/5	3/5	2/5	0/5	5-3-2

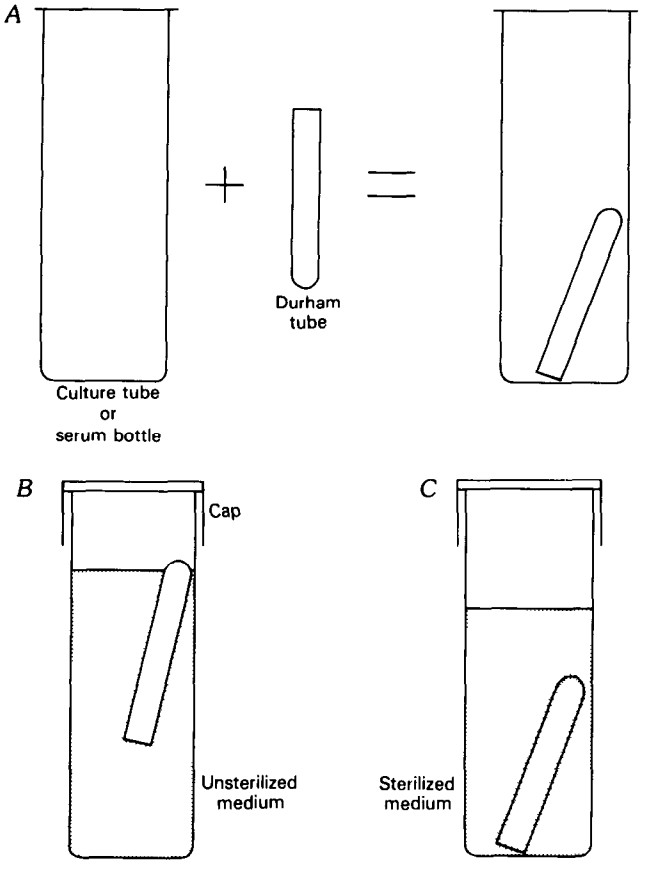

Figure 3.—Preparation of culture tube or serum bottle: (*A*) Invert durham tube inside culture tube or serum bottle; (*B*) add unsterilized medium and cap; (C) durham tube fills with medium following sterilization.

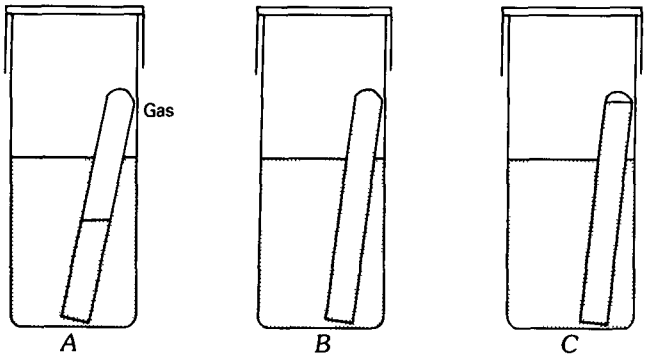

Figure 4.—Examination for gas formation: (*A*) Positive; (*B*) negative; (C) positive.

In example c, the first three dilutions need to be taken to place the positive results in the middle dilution. When a positive result occurs in a dilution larger than the three chosen according to the guideline, as in d, it needs to be placed in the result for the largest chosen dilution as in e (Note 1).

Note 1: The largest dilution has the smallest concentration of the sample; the largest dilution in the preceding table is 0.001.

7.4 The MPN for various combinations of positive and negative results, when five 1-, five 0.1-, and five 0.01-mL dilutions are used, are listed in table 1. If a series of decimal dilutions other than 1, 0.1, and 0.01 mL is used, the MPN value in table 1 needs to be corrected for the dilutions actually used. To do this, divide the value in table 1 by the dilution factor of the first number in the three-number sequence (the culture tubes having the largest concentration of the sample). For example, if dilutions of 0.1, 0.01, and 0.001 mL are used, divide the value in table 1 by 0.1 mL. MPN tables for other combinations of sample volumes and numbers of tubes at each level of inoculation are in American Public Health Association and others (1985).

7.5 Example: The following results were obtained with a five-tube series:

Volume (milliliters)	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Results	5/5	5/5	3/5	1/5	0/5.

Using 10^{-6}, 10^{-7}, and 10^{-8} mL sample volumes, the test results indicate a sequence of 5-3-1 for which the MPN (table 1) is 1,100. Dividing by 10^{-6}, the MPN is computed to be 11×10^8 total coliform bacteria per 100 mL and 95-percent confidence limits of 3.1×10^8 and 25×10^8 total coliform bacteria per 100 mL.

8. Reporting of results

Report total coliform concentrations as MPN total coliforms per 100 mL as follows: less than 10, whole numbers; 10 or more, two significant figures.

9. Precision

9.1 Precision of the MPN method increases as the number of culture tubes is increased. Precision increases rapidly as

Table 1.—*Most-probable-number (MPN) index and 95-percent confidence limits for various combinations of positive and negative results when five 1-, five 0.1-, and five 0.01-milliliter dilutions are used*

[mL, milliliters; MPN, most probable number; ---, not applicable; modified from American Public Health Association and others, 1985]

Number of culture tubes indicating positive reaction out of:			MPN index per 100 mL	95-percent confidence limits	
Five of 1 mL each	Five of 0.1 mL each	Five of 0.01 mL each		Lower	Upper
0	0	0	<20	---	---
0	0	1	20	<5	70
0	1	0	20	<5	70
0	2	0	40	<5	11
1	0	0	20	<5	70
1	0	1	40	<5	110
1	1	0	40	<5	110
1	1	1	60	<5	150
1	2	0	60	<5	150
2	0	0	50	<5	130
2	0	1	70	10	170
2	1	0	70	10	170
2	1	1	90	20	210
2	2	0	90	20	210
2	3	0	120	30	280
3	0	0	80	10	190
3	0	1	110	20	250
3	1	0	110	20	250
3	1	1	140	40	340
3	2	0	140	40	340
3	2	1	170	50	460
4	0	0	130	30	310
4	0	1	170	50	460
4	1	0	170	50	460
4	1	1	210	70	630
4	1	2	260	90	780
4	2	0	220	70	670
4	2	1	260	90	780
4	3	0	270	90	800
4	3	1	330	110	930
4	4	0	340	120	930
5	0	0	230	70	700
5	0	1	310	110	890
5	0	2	430	150	1,100
5	1	0	330	110	930
5	1	1	460	160	1,200
5	1	2	630	210	1,500
5	2	0	490	170	1,300
5	2	1	700	230	1,700
5	2	2	940	280	2,200
5	3	0	790	250	1,900
5	3	1	1,100	310	2,500
5	3	2	1,400	370	3,400
5	3	3	1,800	440	5,000
5	4	0	1,300	350	3,000
5	4	1	1,700	430	4,900

Table 1.—*Most-probable-number (MPN) index and 95-percent confidence limits for various combinations of positive and negative results when five 1-, five 0.1-, and five 0.01-milliliter dilutions are used*—Continued

Number of culture tubes indicating positive reaction out of:			MPN index per 100 mL	95-percent confidence limits	
Five of 1 mL each	Five of 0.1 mL each	Five of 0.01 mL each		Lower	Upper
5	4	2	2,200	570	7,000
5	4	3	2,800	900	8,500
5	4	4	3,500	1,200	10,000
5	5	0	2,400	680	7,500
5	5	1	3,500	1,200	10,000
5	5	2	5,400	1,800	14,000
5	5	3	9,200	3,000	32,000
5	5	4	16,000	6,400	58,000
5	5	5	≥24,000	---	---

the number of tubes increases from 1 to 5, but then it increases at a slower rate, which makes the gain that is achieved by using 10 tubes instead of 5 much less than is achieved by using 5 tubes instead of 1. Variance as a function of the number of tubes inoculated from a tenfold dilution series is listed below:

Number of culture tubes at each dilution	Variance for tenfold dilution series
1	0.580
3	335
5	259
10	.183

9.2 The 95-percent confidence limits for various combinations of positive and negative results, when five 1-, five 0.1-, and five 0.01-mL dilutions are used, are listed in table 1.

10. Sources of information

American Public Health Association, American Water Works Association, and Water Pollution Control Federation, 1985, Standard methods for the examination of water and wastewater (16th ed.): Washington, D.C., American Public Health Association, 1,268 p.

Bordner, R.H., Winter, J.A., and Scarpino, Pasquale, eds., 1978, Microbiological methods for monitoring the environment, water and wastes: Cincinnati, Ohio, U.S. Environmental Protection Agency, EPA-600/8-78-017, 338 p.

Total coliform bacteria (most-probable-number, MPN, method)

Presumptive onsite test (B-0040-85)

Parameter and Code:
Coliform, presumptive (MPN): 31507

1. Applications

This method is applicable to fresh and saline water, water having large suspended-solids concentration, and water having large populations of noncoliform bacteria. It is suitable for application at the sampling site to eliminate sample transport and storage.

2. Summary of method

Decimal dilutions of multiple sample aliquots are inoculated into lauryl tryptose broth. The cultures are incubated at 35±0.5 °C and examined after 24 and 48 hours for evidence of growth and gas production. The most probable number (MPN) of coliform organisms in the sample is determined from the distribution of gas-positive cultures among the inoculated serum bottles. The method described in this section is similar to the total coliform MPN method (presumptive test, B-0035-85) except provision is made for the incubation of samples onsite. Do not use the presumptive onsite test unless the confirmed test (B-0045-85) also is done.

3. Interferences

Large concentrations of heavy metals or toxic chemicals may interfere when large volumes of sample are added to small volumes of concentrated lauryl tryptose broth. Certain noncoliform organisms can ferment lactose during gas formation.

4. Apparatus

All materials used in microbiological testing need to be free of agents that inhibit bacterial growth. Most of the materials and apparatus listed in this section are available from scientific supply companies.

The following apparatus list assumes the use of an onsite kit for microbiological water tests, such as the portable water laboratory (Millipore, or equivalent). If other means of sample filtration are used, refer to the manufacturer's instructions for proper operation of the equipment. Items marked with an asterisk (*) in the list are included in the portable water laboratory (fig. 1).

4.1 *Aluminum seals*, one piece, 20 mm.

4.2 *Bottles*, milk dilution, screwcap.

4.3 *Bottles*, serum.

4.4 *Crimper*, for attaching aluminum seals.

4.5 *Decapper*, for removing aluminum seals from spent tubes.

4.6 *Hypodermic syringes*, sterile, 1-mL capacity, equipped with 26-gauge, ⅜-in. needles.

4.7 *Hypodermic syringes*, sterile, 10-mL capacity, equipped with 22-gauge, 1- to 1½-in. needles.

4.8 *Incubator** for operation at a temperature of 35±0.5 °C. A portable incubator as provided in the portable water laboratory, or *heaterblock* (fig. 2), which operates on either 115 V ac or 12 V dc, is convenient for onsite use. A larger incubator, having more precise temperature regulation, is satisfactory for laboratory use.

4.9 *Pipets*, 1-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.10 *Pipets*, 10-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.11 *Pipettor*, or *pi-pump*, for use with 1- and 10-mL pipets.

4.12 *Rubber stoppers*, 13×20 mm.

4.13 *Sample-collection apparatus*. Use an appropriate device for collecting a representative sample from the environment to be tested, following guidelines in the "Collection" subsection of the "Bacteria" section.

4.14 *Serum bottles and durham (fermentation) tubes*. Two combinations of serum bottles and durham (fermentation) tubes may be used. The choice will depend on the volume of water to be tested. The durham tube, 6×25 mm test tubes, used to detect gas production, must be completely filled with medium and at least partly submerged in the serum bottle. The following combinations have been satisfactory:

4.14.1 For testing 10-mL aliquots, use borosilicate glass serum bottles, 20-mL capacity.

4.14.2 For testing 1-mL or smaller aliquots, use borosilicate glass serum bottles, 10-mL capacity.

4.15 *Sterilizer*, horizontal steam autoclave, or vertical steam autoclave.

CAUTION.—If vertical autoclaves or pressure cookers are used, they need to be equipped with an accurate pressure gauge, a thermometer with the bulb 2.5 cm above the water level, automatic thermostatic control, metal air-release tubing for quick exhaust of air in the sterilizer, metal-to-metal-seal eliminating gaskets, automatic pressure-release valve, and clamping locks preventing removal of lid while pressure exists. These features are necessary in maintaining sterilization conditions and decreasing safety hazards.

To obtain adequate sterilization, do not overload sterilizer. Use a sterilization indicator to ensure that the correct combination of time, temperature, and saturated steam has been obtained.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 *Buffered dilution water.* Dissolve 34 g potassium dihydrogen phosphate (KH_2PO_4) in 500 mL distilled water. Adjust to pH 7.2 using 1 *N* sodium hydroxide (NaOH). Dilute to 1 L using distilled water. Sterilize in dilution bottles at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Add 1.25 mL KH_2PO_4 solution to 1 L distilled water containing 0.1 percent peptone. (Do not store KH_2PO_4 solutions for more than 3 months). Dispense in milk dilution or serum bottles (capped with rubber stoppers and crimped with aluminum seals) in quantities that will provide 99±2 mL after autoclaving at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Allow enough space between bottles for steam to circulate during autoclaving. Loosen caps prior to sterilizing and tighten when bottles have cooled.

5.2 *Distilled or deionized water.*

5.3 *Ethyl alcohol, 70 percent.* Dilute 74 mL 95-percent ethyl alcohol to 100 mL using distilled water. Undiluted isopropanol (ordinary rubbing alcohol) may be used instead of 70-percent ethyl alcohol.

5.4 *Lauryl tryptose broth.* Use premixed lauryl tryptose broth or lauryl sulfate broth, and prepare according to directions on bottle label. The medium also may be prepared according to American Public Health Association and others (1985).

5.4.1 Place 10 mL of medium containing 71.2 g/L lauryl tryptose broth or lauryl sulfate broth in a 20-mL serum bottle for each 10-mL aliquot of sample to be tested.

5.4.2 Place 10 mL of medium containing 35.6 g/L lauryl tryptose broth or lauryl sulfate broth in each 10-mL serum bottle for each 1-mL or smaller aliquot of sample to be tested.

5.4.3 In each serum bottle, place an inverted (mouth downward) durham tube (fig. 3). Place rubber stopper in mouth of bottle and attach aluminum seal using crimper. Sterilize bottles in upright position at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes as soon as possible after dispensing medium. Air will be expelled from the inverted durham tubes during heating; each will fill completely with medium during cooling. Discard any bottle in which air bubbles are visible in the durham tube.

6. Analysis

Two questions must be answered when planning a multiple serum-bottle test:

1. What volumes of water need to be tested?
2. How many serum bottles of each volume need to be tested?

Choose a range of volumes so positive and negative results are obtained throughout the range tested. The method fails if only positive or only negative results are obtained when all volumes are tested. The number of serum bottles used per sample volume depends on the precision required. The greater the number of bottles inoculated with each volume, the greater the precision, but the effort involved and expense also are increased. A five serum-bottle series is described below. The following sample volumes are suggested:

1. Unpolluted raw surface water: 0.1-, 1-, and 10-mL samples will include an MPN range of <2 to ≥2,400 coliforms per 100 mL.
2. Polluted raw surface water: 0.001-, 0.01-, 0.1-, and 1-mL samples will include an MPN range of 20 to 240,000 coliforms per 100 mL.

6.1 Set up five serum bottles of lauryl tryptose broth or lauryl sulfate broth for each sample volume to be tested.

6.1.1 If the volume to be tested is 0.1 mL or more, transfer the measured samples directly to the serum bottles using presterilized disposable hypodermic syringes (Note 1).

6.1.2 If the volume of original water sample is less than 0.1 mL, proceed as in 6.1.1 after preparing appropriate dilutions by adding the sample to buffered dilution water in a sterile milk dilution bottle in the following volumes:

Dilution	Volume of sample added to 99-milliliter milk dilution bottle	Size of inoculum
1:10		0.1 milliliter of original sample
1:100	1 milliliter of original sample	1 milliliter of 1:100 dilution
1:1,000		0.1 milliliter of 1:100 dilution
1:10,000	1 milliliter of 1:100 dilution	1 milliliter of 1:10,000 dilution
1:100,000		0.1 milliliter of 1:10,000 dilution

Note 1: Use a sterile hypodermic syringe for each serum bottle. After each transfer, close and shake the bottle vigorously at least 25 times to maintain distribution of the organisms in the sample. Diluted samples need to be inoculated within 20 minutes after preparation.

6.2 When using serum bottles with rubber septums, proceed as follows:

6.2.1 Remove the inserts from the metal caps and swab the exposed area of the rubber septum using a bit of cotton saturated with 70-percent ethyl alcohol, undiluted isopropanol, or disinfectant.

6.2.2 Carefully invert a serum bottle so that the rubber septum is at the bottom. Inoculate the medium by carefully puncturing the septum with the sterile hypodermic syringe and insert the needle until only the beveled tip is inside the bottle. Discharge the contents of the syringe into the bottle and withdraw the needle. Agitate the bottle gently to mix the contents.

6.2.3 Carefully return serum bottle to the normal, upright position with septum at top. Make sure that the inverted durham tube is completely filled with medium and no residual bubbles remain in the durham tube.

6.3 Clearly mark each set of serum bottles indicating location, time of collection, sample number, and sample volume. Code each bottle for easy identification.

6.4 Place the inoculated serum bottles in the incubator and incubate at 35±0.5 °C for 24±2 hours. Bottles must be maintained in an upright position.

6.5 Remove serum bottles from incubator and examine. Gas in any quantity in the durham tube, even a pinhead-sized bubble, constitutes a positive test (fig. 4). The appearance of an air bubble must not be confused with actual gas production. If the gas is formed as a result of fermentation, the broth medium will become cloudy. Active fermentation may be indicated by the continued appearance of small bubbles of gas in the medium outside the durham tube when the bottle is shaken gently (Bordner and others, 1978; American Public Health Association and others, 1985).

6.6 After submitting all gas-positive serum bottles to the confirmation test (B-0045-85), autoclave at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 to 30 minutes before discarding.

6.7 Return all gas-negative serum bottles to incubator and incubate at 35±0.5 °C for an additional 24±2 hours.

6.8 Remove serum bottles from incubator and examine for gas formation. Autoclave all remaining bottles of lauryl tryptose broth as in 6.6 before discarding.

7. Calculations

7.1 Record the number of gas-positive serum bottles occurring for all sample volumes tested.

7.2 When more than three volumes are tested, use results from only three of them when computing the MPN. To select the three dilutions for the MPN index, use as the first, the smallest sample volume in which all tests are positive (no larger sample volume having any negative results) and the next two succeeding smaller sample volumes (Bordner and others, 1978; American Public Health Association and others, 1985).

7.3 In the examples listed below, the number in the numerator represents positive serum bottles; the denominator represents the total number of bottles inoculated.

Example	Decimal dilutions				Combination of positives
	1 milliliter	0.1 milliliter	0.01 milliliter	0.001 milliliter	
a	5/5	5/5	2/5	0/5	5-2-0
b	5/5	4/5	2/5	0/5	5-4-2
c	0/5	1/5	0/5	0/5	0-1-0
d	5/5	3/5	1/5	1/5	5-3-2
e	5/5	3/5	2/5	0/5	5-3-2

In example c, the first three dilutions need to be taken to place the positive results in the middle dilution. When a positive result occurs in a dilution larger than the three chosen according to the guideline, as in d, it needs to be placed in the result for the largest chosen dilution as in e (Note 1).

Note 1: The largest dilution has the smallest concentration of the sample; the largest dilution in the preceding table is 0.001.

7.4 The MPN for various combinations of positive and negative results, when five 1-, five 0.1-, and five 0.01-mL dilutions are used, are listed in table 2. If a series of decimal dilutions other than 1, 0.1, and 0.01 mL is used, the MPN value from table 2 needs to be corrected for the dilutions actually used. To do this, divide the value from table 2 by the dilution factor of the first number in the three-number sequence (the serum bottles having the largest concentration of the sample). For example, if dilutions of 0.1, 0.01, and 0.001 mL are used, divide the value in table 2 by 0.1 mL. MPN tables for other combinations of sample volumes and numbers of bottles at each level of inoculation are in the American Public Health Association and others (1985).

7.5 Example: The following results were obtained with a five serum-bottle series:

Volume (milliliters)	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Results	5/5	5/5	3/5	1/5	0/5.

Using 10^{-6}, 10^{-7}, and 10^{-8} mL sample volumes, the test results indicate a sequence of 5-3-1 for which the MPN (table 2) is 1,100. Dividing by 10^{-6}, the MPN is computed to be 11×10^8 total coliform bacteria per 100 mL and 95-percent confidence limits of 3.1×10^8 and 25×10^8 total coliform bacteria per 100 mL.

8. Reporting of results

Report total coliform concentrations as MPN total coliforms per 100 mL as follows: less than 10, whole numbers; 10 or more, two significant figures.

9. Precision

9.1 Precision of the MPN method increases as the number of serum bottles is increased. It increases rapidly as the number of bottles increases from 1 to 5, but then it increases at a slower rate making the gain, when using 10 bottles instead of 5, much less than is achieved by increasing the number of bottles from 1 to 5. Variance as a function of number of bottles inoculated from a tenfold dilution series is listed below:

Number of serum bottles at each dilution	Variance for tenfold dilution series
1	0.580
3	335
5	259
10	183

9.2 The 95-percent confidence limits for various combinations of positive and negative results, when five 1-, five 0.1-, and five 0.01-mL dilutions are used, are listed in table 2.

10. Sources of information

American Public Health Association, American Water Works Association, and Water Pollution Control Federation, 1985, Standard methods for the examination of water and wastewater (16th ed.): Washington, D.C., American Public Health Association, 1,268 p.

Bordner, R.H., Winter, J.A., and Scarpino, Pasquale, eds., 1978, Microbiological methods for monitoring the environment, water and wastes: Cincinnati, Ohio, U.S. Environmental Protection Agency, EPA-600/8-78-017, 338 p.

Table 2.—*Most-probable-number (MPN) index and 95-percent confidence limits for various combinations of positive and negative results when five 1-, five 0.1-, and five 0.01-milliliter dilutions are used*

[mL, milliliters; MPN, most probable number; ---, not applicable; modified from American Public Health Association and others, 1985]

Number of culture tubes indicating positive reaction out of:			MPN index per 100 mL	95-percent confidence limits	
Five of 1 mL each	Five of 0.1 mL each	Five of 0.01 mL each		Lower	Upper
0	0	0	<20	---	---
0	0	1	20	<5	70
0	1	0	20	<5	70
0	2	0	40	<5	11
1	0	0	20	<5	70
1	0	1	40	<5	110
1	1	0	40	<5	110
1	1	1	60	<5	150
1	2	0	60	<5	150
2	0	0	50	<5	130
2	0	1	70	10	170
2	1	0	70	10	170
2	1	1	90	20	210
2	2	0	90	20	210
2	3	0	120	30	280
3	0	0	80	10	190
3	0	1	110	20	250
3	1	0	110	20	250
3	1	1	140	40	340
3	2	0	140	40	340
3	2	1	170	50	460
4	0	0	130	30	310
4	0	1	170	50	460
4	1	0	170	50	460
4	1	1	210	70	630
4	1	2	260	90	780
4	2	0	220	70	670
4	2	1	260	90	780
4	3	0	270	90	800
4	3	1	330	110	930
4	4	0	340	120	930
5	0	0	230	70	700
5	0	1	310	110	890
5	0	2	430	150	1,100
5	1	0	330	110	930
5	1	1	460	160	1,200
5	1	2	630	210	1,500
5	2	0	490	170	1,300
5	2	1	700	230	1,700
5	2	2	940	280	2,200
5	3	0	790	250	1,900
5	3	1	1,100	310	2,500
5	3	2	1,400	370	3,400
5	3	3	1,800	440	5,000
5	4	0	1,300	350	3,000
5	4	1	1,700	430	4,900

Table 2.—*Most-probable-number (MPN) index and 95-percent confidence limits for various combinations of positive and negative results when five 1-, five 0.1-, and five 0.01-milliliter dilutions are used*—Continued

Number of culture tubes indicating positive reaction out of:			MPN index per 100 mL	95-percent confidence limits	
Five of 1 mL each	Five of 0.1 mL each	Five of 0.01 mL each		Lower	Upper
5	4	2	2,200	570	7,000
5	4	3	2,800	900	8,500
5	4	4	3,500	1,200	10,000
5	5	0	2,400	680	7,500
5	5	1	3,500	1,200	10,000
5	5	2	5,400	1,800	14,000
5	5	3	9,200	3,000	32,000
5	5	4	16,000	6,400	58,000
5	5	5	>24,000	---	---

Total coliform bacteria (most-probable-number, MPN, method)

Confirmation test (B-0045-85)

Parameter and Code:
Coliform, confirmed (MPN): 31505

All gas-positive cultures from the presumptive test (B-0035-85 or B-0040-85) need to be verified by the confirmation test. When the membrane-filter method is used, some members of the coliform group may react atypically and not produce the characteristic colonies on M-Endo medium. Thus, the identity of suspected coliform colonies need to be verified. Geldreich and others (1967) discussed verification and other aspects of the membrane-filter method.

Because coliform organisms are defined on the basis of their ability to ferment lactose with gas formation at 35±0.5 °C within 48 hours, verification is readily accomplished by using the lactose fermentation-tube method described in this section. Only a minimum of special equipment is needed. Ready-to-use sterile media are commercially available.

1. Applications

The confirmation test is applicable to all gas-positive cultures from the presumptive test and to coliform colonies produced by the membrane-filter method. Initiation of the confirmation test needs to be made immediately for gas-positive cultures from the presumptive test and as soon as possible after completion of the membrane-filter method, but not later than 24 hours.

2. Summary of method

2.1 Material from selected colonies on the membrane filters is placed in tubes of sterile lactose broth and incubated at 35±0.5 °C for 48 hours. Material from these tubes indicating gas formation within 48 hours or gas-positive cultures from the presumptive test are placed in tubes of sterile, brilliant green lactose bile broth. Gas production in the brilliant green lactose bile broth at 35±0.5 °C within 48 hours confirms the presence of coliform bacteria.

2.2 The confirmation test is compatible with the procedure described by Bordner and others (1978) and the American Public Health Association and others (1985).

3. Interferences

Certain noncoliform organisms can ferment lactose with gas formation, but their presence in this double enrichment method is unlikely.

4. Apparatus

All materials used in microbiological testing need to be free of agents that inhibit bacterial growth. Most of the materials and apparatus listed in this section are available from scientific supply companies.

The following apparatus list assumes the use of an onsite kit for microbiological water tests, such as the portable water laboratory (Millipore, or equivalent). If other means of sample filtration are used, refer to the manufacturer's instructions for proper operation of the equipment. Items marked with an asterisk (*) in the list are included in the portable water laboratory (fig. 1).

4.1 *Aluminum seals*, one piece, 20 mm.

4.2 *Bunsen burner*, for sterilizing inoculating loop.

4.3 *Crimper*, for attaching aluminum seals.

4.4 *Culture tubes and durham (fermentation) tubes*. Two combinations of culture tubes and durham (fermentation) tubes may be used. The choice will depend on the volume of water to be tested. The durham tube, used to detect gas production, must be completely filled with medium and at least partly submerged in the culture tube. The following combinations have been satisfactory:

4.4.1 For testing 10-mL aliquots, use borosilicate glass culture tubes, 20×150 mm; tube caps, 20 mm; and use borosilicate glass culture tubes, 10×75 mm, as durham tubes.

4.4.2 For testing 1-mL or smaller aliquots, use borosilicate glass culture tubes, 16×125 mm; tube caps, 16 mm; and use flint glass culture tubes, 6×50 mm, as durham tubes.

4.5 *Culture-tube rack*, galvanized, for 16- and 20-mm culture tubes.

4.6 *Decapper*, for removing aluminum seals from spent tubes.

4.7 *Hypodermic syringes*, sterile, 1-mL capacity, equipped with 26-gauge, ⅜-in. needles.

4.8 *Hypodermic syringes*, sterile, 10-mL capacity, equipped with 22-gauge, 1- to 1½-in. needles.

4.9 *Incubator**, for operation at a temperature of 35±0.5 °C. A portable incubator as provided in the portable water laboratory, or *heaterblock* (fig. 2), which operates on either 115 V ac or 12 V dc, is convenient for onsite use. A larger incubator, having a more precise temperature regulation, is satisfactory for laboratory use.

4.10 *Inoculating loop*, platinum-iridium wire, 3 mm, Brown and Sharpe gauge 26.

4.11 *Needle holder.*

4.12 *Rubber stoppers*, 13×20 mm.

4.13 *Sterilizer*, horizontal steam autoclave, or vertical steam autoclave.

CAUTION.—If vertical autoclaves or pressure cookers are used, they need to be equipped with an accurate pressure gauge, a thermometer with the bulb 2.5 cm above the water level, automatic thermostatic control, metal air-release tubing for quick exhaust of air in the sterilizer, metal-to-metal-seal eliminating gaskets, automatic pressure-release valve, and clamping locks preventing removal of lid while pressure exists. These features are necessary in maintaining sterilization conditions and decreasing safety hazards.

To obtain adequate sterilization, do not overload sterilizer. Use a sterilization indicator to ensure that the correct combination of time, temperature, and saturated steam has been obtained.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 *Brilliant green lactose broth*, prepackaged brilliant green lactose broth in 16×125-mm tubes and fermentation shell. The medium also may be prepared according to American Public Health Association and others (1985). Use brilliant green bile, 2 percent, or brilliant green bile broth, 2 percent, and prepare according to directions on bottle label. Place 10 mL of medium in a culture tube for each colony to be tested. In each culture tube, place an inverted (mouth downward) durham tube (fig. 3). Sterilize culture tubes in upright position at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes as soon as possible after dispensing medium. Air will be expelled from the inverted durham tube during heating; each will fill completely with medium during cooling. Discard any culture tube in which air bubbles are visible in the durham tube.

5.2 *Lauryl tryptose broth*, prepackaged lauryl tryptose broth in 16×125-mm tubes and fermentation shell, or use premixed lauryl tryptose broth or lauryl sulfate broth, and prepare according to directions on bottle label. The medium also may be prepared according to American Public Health Association and others (1985).

5.2.1 Place 10 mL of medium in a culture tube for each colony to be tested.

5.2.2 In each culture tube, place an inverted (mouth downward) durham tube (fig. 3). Sterilize tubes in upright position at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes as soon as possible after dispensing medium. Air will be expelled from the inverted durham tube during heating; each will fill completely with medium during cooling. Discard any tube in which air bubbles are visible in the durham tube.

6. Analysis

6.1 Complete the membrane-filter method for total coliform bacteria according to procedures described in this chapter.

6.2 Select a colony or colonies to be confirmed for total coliform bacteria from the incubated membrane filters.

6.3 Sterilize the inoculating loop by flaming in the burner. The long axis of the wire needs to be held parallel to the cone of the flame so the entire end of the wire and loop is heated to redness.

6.4 Remove from flame and allow the wire to cool for about 10 seconds. Do not allow the inoculating loop to contact any foreign surface during the cooling period. When cool, touch the loop lightly to the colony. Part of the colony material will adhere to the wire.

6.5 Uncap a culture tube containing lauryl tryptose broth and hold it at an angle of about 45°. Insert the inoculating loop and colony material into the tube. Rub the wire loop and attached bacteria against the side of tube at the liquid meniscus to disperse the bacteria in the liquid.

6.6 Recap the culture tube. Flame the inoculating loop and inoculate additional tubes as in 6.5 until all colonies to be tested have been placed into broth in separate tubes. Place the inoculated tubes in the culture-tube rack and incubate at 35±0.5 °C for 24±2 hours.

6.7 Remove culture tubes from incubator and examine. Gas in any quantity in the durham tube constitutes a positive test (fig. 4). Return all gas-negative tubes to incubator and incubate at 35±0.5 °C for an additional 24±2 hours.

6.8 Using a sterile inoculating loop, transfer one loopful of broth from each culture tube indicating gas to a culture tube of sterile brilliant green lactose broth. Sterilize the loop after each transfer.

6.9 Autoclave all gas-positive culture tubes of lauryl tryptose broth at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 to 30 minutes before discarding.

6.10 Incubate the culture tubes of brilliant green lactose broth at 35±0.5 °C for 48±3 hours.

6.11 Examine the remaining culture tubes of lauryl tryptose broth. Transfer one loopful of material from each tube producing gas to a culture tube of brilliant green lactose broth as in 6.8 and continue as in 6.10. If no gas appears in the tube of lauryl tryptose broth within 48±3 hours, the original colony was not of the coliform group. Autoclave all tubes of lauryl tryptose broth as in 6.9 before discarding.

6.12 Examine culture tubes of brilliant green lactose broth after 24±2 and 48±3 hours. The formation of gas in any quantity in the durham tube constitutes a positive confirmation for the presence of total coliform bacteria. If no gas appears in the tube of brilliant green lactose broth within 48±3 hours, the original colony was not of the coliform group, even though gas was produced in the tube of lauryl tryptose broth.

6.13 Culture tubes of brilliant green lactose broth need to be autoclaved as in 6.9 before discarding.

7. Calculations

No calculations are necessary.

8. Reporting of results

Results of the total coliform confirmation test are included in the results of the membrane-filter and presumptive tests for total coliform bacteria.

9. Precision

No precision data are available.

10. Sources of information

American Public Health Association, American Water Works Association, and Water Pollution Control Federation, 1985, Standard methods for the examination of water and wastewater (16th ed.): Washington, D.C., American Public Health Association, 1,268 p.

Bordner, R.H., Winter, J.A., and Scarpino, Pasquale, eds., 1978, Microbiological methods for monitoring the environment, water and wastes: Cincinnati, Ohio, U.S. Environmental Protection Agency, EPA-600/8-78-017, 338 p.

Geldreich, E.E., Jeter, H.L., and Winter, J.A., 1967, Technical considerations in applying the membrane filter procedure: Health Lab Science, v. 4, p. 113-125.

Fecal coliform bacteria (membrane-filter method)

Immediate incubation test (B-0050-85)

Parameter and Code:
Coliform, fecal, 0.7-μm, M-FC media at 44.5 °C (colonies/100 mL): 31625

Fecal coliforms are those organisms of the coliform group that are present in the intestines and feces of warm-blooded animals. They are capable of producing gas from lactose in a suitable culture medium at 44.5 °C. Bacterial organisms from other sources generally cannot produce gas when subjected to the same conditions (Bordner and others, 1978; American Public Health Association and others, 1985).

For the purpose of the method described in this section, the fecal coliform group is defined as all organisms that produce blue colonies when incubated at 44.5±0.2 °C within 24 hours on M-FC medium. The nonfecal coliform colonies are gray to cream colored.

1. Applications

The method is applicable to fresh and saline waters.

2. Summary of method

The sample is filtered onsite immediately after collection, and the filter is placed on a nutrient medium containing a pH-sensitive color indicator. Filters are incubated at a temperature of 44.5±0.2 °C for 24 hours in an incubator to suppress growth of nonfecal coliform bacteria, thereby selectively favoring growth of fecal coliforms.

3. Interferences

3.1 Suspended materials may inhibit the filtration of sample volumes sufficient to produce significant results. Fecal coliform colony formation on the filter may be inhibited by large numbers of noncoliform colonies, by the presence of algal filaments and detritus, or by toxic substances.

3.2 Water samples having a large suspended-solids concentration may be divided between two or more membrane filters. The multiple-tube method, which is described in this chapter, will give the most reliable results when suspended-solids concentrations are large and fecal coliform counts are small.

4. Apparatus

All materials used in microbiological testing need to be free of agents that inhibit bacterial growth. Most of the materials and apparatus listed in this section are available from scientific supply companies.

The following apparatus list assumes the use of an onsite kit for microbiological water tests, such as the portable water laboratory (Millipore, or equivalent). If other means of sample filtration are used, refer to the manufacturer's instructions for proper operation of the equipment. Items marked with an asterisk (*) in the list are included in the portable water laboratory (fig. 1).

4.1 *Alcohol burner*, glass or metal, containing ethyl alcohol for flame sterilizing of forceps.

4.2 *Aluminum seals*, one piece, 20 mm.

4.3 *Bottles*, milk dilution, screwcap.

4.4 *Bottles*, serum.

4.5 *Crimper*, for attaching aluminum seals.

4.6 *Decapper*, for removing aluminum seals from spent tubes.

4.7 *Filter-holder assembly** and *syringe* that has a *two-way valve** or *vacuum hand pump.*

4.8 *Forceps**, stainless steel, smooth tips.

4.9 *Graduated cylinders*, 100-mL capacity.

4.10 *Hypodermic syringes*, sterile, 1-mL capacity, equipped with 26-gauge, ⅜-in. needles.

4.11 *Hypodermic syringes*, sterile, 10-mL capacity, equipped with 22-gauge, 1- to 1½-in. needles.

4.12 *Incubator**, for operation at a temperature of 35±0.5 °C. A portable incubator as provided in the portable water laboratory, or *heaterblock* (fig. 2), which operates on either 115 V ac or 12 V dc, is convenient for onsite use. A larger incubator, having more precise temperature regulation, is satisfactory for laboratory use.

4.13 *Membrane filters*, white, grid, sterile, 0.7-μm pore size, 47-mm diameter.

4.14 *Microscope*, binocular wide-field dissecting-type, and *fluorescent lamp*.

4.15 *Pipets*, 1-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.16 *Pipets*, 10-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.17 *Pipettor*, or *pi-pump*, for use with 1- and 10-mL pipets.

4.18 *Plastic petri dishes with covers*, disposable, sterile, 50×12 mm.

4.19 *Rubber stoppers*, 13×20 mm.

4.20 *Sample-collection apparatus*. Use an appropriate device for collecting a representative sample from the environment to be tested, following guidelines in the "Collection" subsection of the "Bacteria" section.

4.21 *Sterilizer*, horizontal steam autoclave, or vertical steam autoclave.

CAUTION.—If vertical autoclaves or pressure cookers are used, they need to be equipped with an accurate pressure gauge, a thermometer with the bulb 2.5 cm above the water level, automatic thermostatic control, metal air-release tubing for quick exhaust of air in the sterilizer, metal-to-metal-seal eliminating gaskets, automatic pressure-release valve, and clamping locks preventing removal of lid while pressure exists. These features are necessary in maintaining sterilization conditions and decreasing safety hazards.

To obtain adequate sterilization, do not overload sterilizer. Use a sterilization indicator to ensure that the correct combination of time, temperature, and saturated steam has been obtained.

4.22 *Thermometer*, having a temperature range of at least 40 to 100 °C.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 *Buffered dilution water.* Dissolve 34 g potassium dihydrogen phosphate (KH_2PO_4) in 500 mL distilled water. Adjust to pH 7.2 using 1 *N* sodium hydroxide (NaOH). Dilute to 1 L using distilled water. Sterilize in dilution bottles at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Add 1.25 mL KH_2PO_4 solution to 1 L distilled water containing 0.1 percent peptone. (Do not store KH_2PO_4 solutions for more than 3 months.) Dispense in milk dilution or serum bottles (capped with rubber stoppers and crimped with aluminum seals) in quantities that will provide 99 ± 2 mL after autoclaving at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Allow enough space between bottles for steam to circulate during autoclaving. Loosen caps prior to sterilizing and tighten when bottles have cooled.

5.2 *Distilled or deionized water.*

5.3 *Ethyl alcohol*, 95-percent denatured or absolute ethyl alcohol for sterilizing equipment. Absolute methyl alcohol also may be used for sterilization.

5.4 *Methyl alcohol*, absolute, for sterilizing filter-holder assembly.

5.5 *M-FC agar.* Add 5.2 g M-FC agar to 100 mL distilled water. Do not autoclave. Heat to 90 °C in a water bath stirring occasionally, then add 1 mL rosolic acid solution. Continue heating for a maximum of 1 minute, then remove from heat and allow to cool to 50 °C. Pour to a depth of 4 mm (6–7 mL) in 50-mm petri dish bottoms. Replace petri dish tops loosely until medium solidifies, then close tightly and store the prepared petri dishes at 2 to 10 °C for a maximum of 72 hours; preferably the medium should not be stored for more than 24 hours.

5.6 *Rosolic acid solution.* Add 10 mL 0.2 *N* NaOH to 0.10 g rosolic acid crystals. Stir vigorously to dissolve. Do not heat. Store in the dark at room temperature for a maximum of 2 to 3 weeks. Discard if color changes from deep red to orange.

6. Analysis

The volumes of the sample to be filtered depend on the expected bacterial density of the water being tested, but the volumes should be enough that after incubation, at least one of the membrane filters will contain from 20 to 60 fecal coliform colonies.

The following sample volumes are suggested for filtration:

1. Unpolluted raw surface water: 0.1-, 0.3-, 1-, 3-, 10-, 30-, and 100-mL samples will include a range of 20 to 60,000 fecal coliforms per 100 mL using the criterion of 20 to 60 coliform colonies on a filter as an ideal determination.
2. Polluted raw surface water: 0.01-, 0.03-, 0.1-, 0.3-, 1-, and 3-mL samples will include a range of 670 to 600,000 fecal coliforms per 100 mL.

6.1 Sterilize filter-holder assembly (Note 1). In the laboratory, wrap the funnel and filter base parts of the assembly separately in kraft paper or polypropylene bags and sterilize in the autoclave at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes. Steam must contact all surfaces to ensure complete sterilization. Cool to room temperature before use.

Note 1: Onsite sterilization of filter-holder assembly needs to be in accordance with the manufacturer's instructions but usually involves application and ignition of methyl alcohol to produce formaldehyde. Autoclave sterilization in the laboratory prior to the trip to the sampling site is preferred. Sterilization must be performed at all sites.

6.2 Assemble the filter holder and, using flame-sterilized forceps (Note 2), place a sterile membrane filter over the porous plate of the assembly, grid side up. Carefully place funnel on filter to avoid tearing or creasing the membrane.

Note 2: Flame-sterilized forceps—Dip forceps in ethyl or methyl alcohol, pass through flame to ignite alcohol, and allow to burn out. Do not hold forceps in flame.

6.3 Shake the sample vigorously about 25 times to obtain an equal distribution of bacteria throughout the sample before transferring a measured portion of the sample to the filter-holder assembly.

6.3.1 If the volume of sample to be filtered is 10 mL or more, transfer the measured sample directly onto the dry membrane.

6.3.2 If the volume of sample is between 1 and 10 mL, pour about 20 mL sterilized buffered dilution water into the funnel before transferring the measured sample onto the membrane. This facilitates distribution of bacteria.

6.3.3 If the volume of original water sample is less than 1 mL, proceed as in 6.3.1 after preparing appropriate dilutions by adding the sample to buffered dilution water in a sterile milk dilution bottle (Note 3) in the following volumes:

Dilution	Volume of sample added to 99-milliliter milk dilution bottle	Filter this volume
1:10	11 milliliters of original sample	1 milliliter of 1:10 dilution
1:100	1 milliliter of original sample	1 milliliter of 1:100 dilution
1:1,000	1 milliliter of 1:10 dilution	1 milliliter of 1:1,000 dilution
1:10,000	1 milliliter of 1:100 dilution	1 milliliter of 1 10,000 dilution

Note 3: Use a sterile pipet or hypodermic syringe for each bottle. After each transfer, close and shake the bottle vigorously at least 25 times to maintain distribution of the organisms in the sample. Diluted samples need to be filtered within 20 minutes after preparation.

6.4 Apply vacuum and filter the sample. When vacuum is applied using a syringe fitted with a two-way valve, proceed as follows: Attach the filter-holder assembly to the inlet of the two-way valve with plastic tubing. Draw the syringe plunger very slowly on the initial stroke to avoid the danger of air lock before the assembly fills with water. Push the plunger forward to expel air from the syringe. Continue until the entire sample has been filtered. If the filter balloons or develops bubbles during sample filtration, disassemble the two-way valve and lubricate the rubber valve plugs lightly with stopcock grease. If a vacuum hand pump is used, do not exceed a pressure of 25 cm to avoid damage to bacteria.

6.5 Rinse sides of funnel twice with 20 to 30 mL of sterile buffered dilution water while applying vacuum.

6.6 Maintaining the vacuum, remove the funnel from the base of the filter-holder assembly and, using flame-sterilized forceps, remove the membrane filter from the base and place it on the agar surface in the plastic petri dish, grid side up, using a rolling action at one edge. Use care to avoid trapping air bubbles under the membrane (Note 4).

Note 4: Hold the funnel while removing the membrane filter and place it back on the base of the assembly when the membrane filter has been removed. Placement of the funnel on anything but the base of the assembly may result in contamination of the funnel.

6.7 Place top on petri dish and proceed with filtration of the next volume of water. Filter in order of increasing sample volume, rinsing with sterile buffered dilution water between filtrations.

6.8 Clearly mark the lid of each plastic petri dish indicating location, time of collection, time of incubation, sample number, and sample volume. Use a waterproof felt-tip marker or grease pencil.

6.9 Inspect the membrane in each petri dish for uniform contact with the agar. If air bubbles (indicated by bulges) are present under the filter, remove the filter using sterile forceps and roll onto the agar again.

6.10 Close the plastic petri dish by firmly pressing down on the top.

6.11 If using a water-bath incubator, place each petri dish in a waterproof plastic bag or seal the dish with waterproof plastic tape.

6.12 Incubate the filters in the tightly closed petri dishes in an inverted position (agar and filter at the top) at 44.5 ± 0.2 °C for 24 ± 2 hours. Filters need to be incubated within 20 minutes after placement on medium.

6.13 Count the fecal coliform colonies (blue color) within 20 minutes after the dishes have been removed from the incubator. M-FC medium is very selective, and growth of colonies other than fecal coliform is inhibited. Colonies that are not fecal coliform will be gray to cream colored. The color plate in Millipore Corp. (1973, p. 42) may be helpful in identifying fecal coliform colonies. The counts are best made using 10× to 15× magnification.

6.14 Autoclave all cultures at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 to 30 minutes before discarding.

7. Calculations

7.1 If only one filter has a colony count between the ideal of 20 and 60, use the equation:

$$\text{Fecal coliform colonies/100 mL} = \frac{\text{Number of colonies counted} \times 100}{\text{Volume of original sample filtered (milliliters)}}.$$

7.2 If all filters have counts less than the ideal of 20 colonies or greater than 60 colonies, calculate using the equations in 7.5 for only those filters having at least one colony and not having colonies too numerous to count. Report results as number per 100 mL, followed by the statement, "Estimated count based on nonideal colony count."

7.3 If no filters develop characteristic fecal coliform colonies, report a maximum estimated value. Assume a count of one colony for the largest sample volume filtered, then calculate using the equation in 7.1. Report the results as less than (<) the calculated value per 100 mL.

7.4 If all filters have colonies too numerous to count, report a minimum estimated value. Assume a count of 60 coliform colonies for the smallest sample volume filtered, then calculate using the equation in 7.1. Report the results as greater than (>) the calculated value per 100 mL.

7.5 Sometimes two or more filters of a series will produce colony counts within the ideal counting range. Make colony counts for all such filters. The method for calculating and averaging is as follows (Note 5):

$$\frac{\text{Volume filter 1} + \text{Volume filter 2}}{\text{Volume sum}} \qquad \frac{\text{Colony count filter 1} + \text{Colony count filter 2}}{\text{Colony count sum}}$$

$$\text{Fecal coliform colonies/100 mL} = \frac{\text{Colony count sum} \times 100}{\text{Volume sum (milliliters)}}.$$

Note 5: Do not calculate the fecal coliform colonies per 100 mL for each volume filtered and then average the results.

8. Reporting of results

Report fecal coliform concentration as fecal coliform colonies per 100 mL as follows: less than 10 colonies, report whole numbers; 10 or more colonies, two significant figures.

9. Precision

No numerical precision data are available. However, the method gives 93-percent accuracy for differentiating between fecal coliforms and coliforms from other sources (American Public Health Association and others, 1985).

10. Sources of information

American Public Health Association, American Water Works Association, and Water Pollution Control Federation, 1985, Standard methods for the examination of water and wastewater (16th ed.): Washington, D.C., American Public Health Association, 1,268 p.

Bordner, R.H., Winter, J.A., and Scarpino, Pasquale, eds., 1978, Microbiological methods for monitoring the environment, water and wastes: Cincinnati, Ohio, U.S. Environmental Protection Agency, EPA-600/8-78-017, 338 p.

Millipore Corp., 1973, Biological analysis of water and wastewater: Bedford, Mass., Application Manual AM302, 84 p.