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Techniques of Water-Resources Investigations
of the United States Geological Survey

Chapter A4

METHODS FOR COLLECTION AND ANALYSIS OF AQUATIC BIOLOGICAL AND MICROBIOLOGICAL SAMPLES

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This report supersedes TWRI 5A4, published in 1977, entitled "Methods for collection and analysis of aquatic biological and microbiological samples," edited by P.E. Greeson and others.

Revised 1987
Book 5

LABORATORY ANALYSIS

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

Presumptive test (B-0051-85)

Parameter and Code:
Coliform, fecal, EC broth at 44.5 °C (MPN): 31615

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. Positive cultures at 24 and 48 hours are transferred to EC broth, incubated at 44.5±0.2 °C for 24 hours, and examined for growth and gas production. The MPN of fecal coliform bacteria in the sample is determined from the distribution of gas-positive cultures among the inoculated EC broth culture tubes.

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.

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 *Bunsen burner*, for sterilizing inoculating loop.

4.5 *Crimper*, for attaching aluminum seals.

4.6 *Culture tubes and durham (fermentation) tubes*. The sizes of the culture tube and durham tube, used for the detection of gas production, should enable the durham tube to completely fill with medium and at least partly submerge in the culture tube. The specific choice of culture tubes and durham tubes depends on the volume of water to be tested and whether the test is to be done in the laboratory or onsite. The procedure described below specifies the use of culture tubes as culture vessels. Serum bottles may be more appropriate as culture vessels if samples are to be inoculated and incubated onsite. Apparatus needed for an onsite procedure is described in "Presumptive Onsite Test" (B-0040-85) subsection of the "Total Coliform Bacteria" section. The following combinations have been satisfactory:

4.6.1 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.

4.6.2 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.

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

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

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

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

4.11 *Incubator**, for operation at a temperature of 35±0.5 °C, or *water bath*, capable of maintaining 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.

4.12 *Incubator*, water bath, for operation at 44.5±0.2 °C. Precise, uniform temperature control is essential.

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

4.14 *Needle holder.*

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 *Rubber stoppers*, 13×20 mm.

4.19 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.

4.20 *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 *EC medium or broth.* Use premixed EC medium or broth, and prepare according to directions on bottle label. The medium also may be prepared according to American Public Health Association and others (1985) (Note 1).

Note 1: Because the number of positive lauryl tryptose cultures is unknown at the time of medium preparation, it is advisable to prepare a sufficient number of culture tubes of medium to enable inoculation of the maximum number of positives.

5.3.1 Place 10 mL of medium containing 37 g/L of EC medium or broth in a 20×150-mm culture tube for each culture tube or serum bottle of lauryl tryptose broth prepared in 5.4.

5.3.2 In each culture tube, place an inverted (mouth downward) 10×75-mm durham tube (fig. 3). Sterilize tubes 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 tube in which air bubbles are visible in the durham tube. Prepared medium may be retained at 4 °C for no longer than 96 hours.

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 20×150-mm culture tube for each 10-mL aliquot of sample to be tested.

5.4.2 Place 10 mL of medium containing 35.6 g/L of 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.

5.4.3 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

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 coliform organisms 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 2).

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 2: Use a sterile pipet or hypodermic syringe for each bottle. After each transfer, close and shake 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 Sterilize the inoculating loop by flaming in the burner. The long axis of the wire must be held parallel to the cone of the flame so the entire end of the wire and loop is heated to redness. Remove from flame and allow wire to cool for about 10 seconds. Do not allow the loop to contact any foreign surface during the cooling period.

6.6 Gently shake and uncap a positive culture tube of lauryl tryptose broth. Insert the inoculating loop beneath the liquid surface and carefully withdraw a loopful of culture. Uncap a tube of EC broth and insert the loop beneath the medium surface. Gently swirl the loop to disperse bacteria in the medium.

6.7 Recap both culture tubes. Flame the inoculating loop and inoculate additional tubes until all positive cultures have been transferred to EC broth. Sterilize the loop after each transfer. Place the culture-tube racks of inoculated EC tubes into a water-bath incubator and incubate at 44.5 ± 0.2 °C for 24 ± 2 hours. Place all inoculated EC tubes in the water bath as soon as possible and always within 30 minutes.

6.8 Return remaining gas-negative culture tubes of lauryl tryptose broth to the incubator and incubate at 35 ± 0.5 °C for an additional 24 ± 2 hours.

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 Remove culture tubes of EC broth and examine for gas production. Gas in any quantity indicates a positive test for fecal coliforms.

6.11 Remove remaining culture tubes of lauryl tryptose broth and examine for gas production. Transfer any positive cultures to EC broth and incubate as in 6.7 and 6.8.

6.12 Autoclave all gas-positive culture tubes of lauryl tryptose broth and EC broth before discarding as in 6.9.

6.13 Remove remaining culture tubes of EC broth incubated in 6.11 and examine for gas production.

6.14 Autoclave all culture tubes of EC broth before discarding as in 6.9.

7. Calculations

7.1 Record the number of gas-positive culture tubes of lauryl tryptose broth and EC broth at 24 and 48 hours for all sample volumes tested. Determine presumptive MPN of fecal coliform bacteria from the number of positive tubes of lauryl tryptose broth. Determine MPN of fecal coliform bacteria from the number of positive tubes of EC broth.

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.

Example	Decimal dilutions: 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

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 3).

Note 3: 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 3. If a series of decimal dilutions other than 1, 0.1, and 0.01 mL is used, the MPN value in table 3 needs to be corrected for the dilutions actually used. To do this, divide the value in table 3 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 3 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).

Table 3.—*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 3.—*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	---	---

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 3) is 1,100. Dividing by 10^{-6}, the MPN is computed to be 11×10^8 fecal coliform bacteria per 100 mL and 95-percent confidence limits of 3.1×10^8 and 25×10^8 fecal coliform bacteria per 100 mL.

8. Reporting of results

Report presumptive fecal coliform concentrations as MPN fecal 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 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 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 3.

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.

Fecal streptococcal bacteria (membrane-filter method)

Immediate incubation test (B-0055-85)

Parameter and Code:
Streptococci, fecal, MF, KF agar (colonies/100 mL): 31673

Fecal streptococci are increasingly used as indicators of substantial contamination of water because the normal habitat of these organisms is the intestines of man and animals. Fecal streptococcal data verify fecal pollution and may provide additional information concerning the recency and probable origin of pollution.

1. Applications

The method is applicable to most types of water.

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 fecal streptococci and to suppress the growth of other organisms. After incubation at 35±0.5 °C for 48 hours, the red or pink colonies are counted.

3. Interferences

3.1 Suspended materials may inhibit the filtration of sample volumes sufficient to produce significant results. Streptococcal colony formation on the filter may be inhibited by large numbers of nonstreptococcal 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 streptococcal 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 apparatus*, sterile, complete with membrane filter, 0.22-μm mean pore size, 25-mm diameter.

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

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

4.10 *Graduated cylinders*, 100-mL capacity.

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

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

4.13 *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.

4.14 *Membrane filters*, white, grid, sterile, 0.45-μm mean pore size, 47-mm diameter.

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

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

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

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

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

4.20 *Plastic syringe*, disposable, 20-mL capacity.

4.21 *Rubber stoppers*, 13×20 mm.

4.22 *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.

4.23 *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.

4.24 *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 *KF streptococcus agar.* Suspend 7.64 g KF streptococcus agar in 100 mL distilled water. Do not autoclave. Stir and heat to boiling in a water bath. Once boiling starts, heat an additional 5 minutes. Remove from heat and cool to 50 to 60 °C. Add 1 mL 1-percent TTC solution after the medium has cooled to less than 60 °C. If commercially prepared 1-percent sterile TTC solution is used, swab the rubber septum on the vial with 95-percent ethyl alcohol before use. Remove 1 mL using a sterile, disposable hypodermic syringe. When medium has cooled to approximately 50 °C, pour medium into 50×12-mm petri dishes to a depth of 4 mm (6–7 mL). When medium solidifies, store the prepared plates in a refrigerator. Discard after 2 weeks if sterile TTC was used and after 24 hours if unsterilized TTC was used.

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

5.6 *TTC solution.* Sterile 1-percent TTC solution is available from commercial sources. Alternatively, prepare a 1-percent sterile solution by dissolving 0.1 g triphenyltetrazolium chloride in 10 mL distilled water. Filter the solution aseptically through a 0.22-μm-membrane filter into a sterile, capped test tube. Store sterilized TTC solution at 2 to 8 °C in darkness and discard after container has been opened for 1 month or if contamination occurs, as indicated by color change or turbidity. As an expedient, substitute freshly prepared unsterilized TTC solution if the KF medium will be used promptly. TTC solution cannot be sterilized by heat.

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 100 fecal streptococcal colonies.

Fecal streptococci generally are present in fewer numbers than coliform bacteria; therefore, the filtered volume of sample needs to be larger than that used for other indicator bacterial determinations. When filtering water of unknown quality, the following sample volumes are suggested: 0.05, 0.25, 1, 5, 25, and 100 mL. This will include a range of 20 to 200,000 fecal streptococci per 100 mL using the criterion of 20 to 100 colonies on a filter as an ideal determination.

6.1 Pour agar medium at 45 to 50 °C into bottom (larger half) of each sterile plastic petri dish to a depth of about 4 mm (6–7 mL). Pads are not used. Replace petri dish tops.

6.2 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 the 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.

6.3 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.

6.4 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.

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

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

6.4.3 If the volume of original water sample is less than 1 mL, proceed as in 6.4.1 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.

6.5 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.

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

6.7 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.

6.8 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.

6.9 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.

6.10 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.

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

6.12 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 48 ± 2 hours. Filters need to be incubated within 20 minutes after placement on medium.

6.13 Count all red or pink colonies as fecal streptococci. The color plate in Millipore Corp. (1973, p. 42) may be helpful in identifying fecal streptococcal colonies. The counts are best made using 10× to 15× magnification. Illumination is not critical.

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 100, use the equation:

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

7.2 If all filters have counts less than the ideal of 20 colonies or greater than 100 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 streptococcal 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 100 fecal streptococcal 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):

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

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

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

8. Reporting of results

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

9. Precision

No numerical precision data are available.

10. Source of information

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

Fecal streptococcal bacteria (membrane-filter method)

Confirmation test (B-0060-85)

Parameter and Code: Not applicable

KF agar medium stimulates the growth of fecal streptococci. A few other types of bacteria, chiefly nonfecal streptococci, may appear occasionally on this medium. Colonies of nonfecal streptococci typically are very small but exhibit the characteristic red or pink coloration and could be counted as fecal streptococci in the membrane-filter method. Suspected colonies may be confirmed according to this test.

The fecal streptococcal bacteria are distinguished from other bacteria by the following three characteristics: (1) They lack the enzyme catalase; (2) they can grow at 45±0.5 °C; and (3) they grow in 40-percent bile. The confirmation test uses these three characteristics as criteria for identification. The procedure is similar to that in Bordner and others (1978) and the American Public Health Association and others (1985).

1. Applications

The confirmation test is applicable to fecal streptococcal colonies produced by the membrane-filter method. Confirmation must be made as soon as possible after completion of the membrane-filter method, but not later than 24 hours.

2. Summary of method

Cells from colonies to be tested are streaked on brain-heart infusion agar slants. Cells from the slants are tested for the presence of catalase and for the ability to grow at 45±0.5 °C and in the presence of 40-percent bile. Absence of catalase and growth at 45±0.5 °C and in 40-percent bile constitute a positive test for fecal streptococci. Presence of catalase or failure to grow at 45±0.5 °C or in 40-percent bile indicate that the original colony was not of the fecal streptococcal group.

3. Interferences

As far as is known, only fecal streptococci show the pattern of results described in this method.

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 *Bunsen burner*, for sterilizing inoculating loop.

4.2 *Culture tubes*, borosilicate glass, 16×150 mm, and *culture-tube caps*, 16 mm.

4.3 *Culture-tube rack*, galvanized, for 16-mm tubes.

4.4 *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.

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

4.6 *Microscope slides*, glass, 76×25 mm.

4.7 *Needle holder.*

4.8 *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 *Brain-heart infusion agar.* Add 52 g brain-heart infusion agar to 1 L distilled water. Heat in a water bath and vigorously stir until solution becomes clear. Remove from heat immediately on clearing. Place 5 mL of hot solution in each of twelve 16×150-mm culture tubes. CAUTION:—Do not allow solution to cool below 45 °C or it will solidify.

Cap each tube. Sterilize at 121 °C at 1.05 kg/cm^2 (15 psi)

for 15 minutes. Remove from sterilizer and set tubes of molten agar at an angle of about 20° from the horizontal (fig. 5). Allow to cool until the solution solidifies.

5.2 *Brain-heart infusion broth.* Add 37 g brain-heart infusion broth to 1 L distilled water. Stir until dissolved. Place 6 mL of broth in each of twelve 16×150-mm culture tubes. Cap each tube. Sterilize at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes.

5.3 *Brain-heart infusion-40-percent bile broth.* Add 37 g brain-heart infusion broth to 1 L distilled water. Stir until dissolved. Place 6 mL of brain-heart infusion broth in each of twelve 16×150-mm culture tubes. Cap each tube. Sterilize at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes.

Add 100 g oxgall to 1 L distilled water. Stir until dissolved. Place 4 mL of 10-percent oxgall solution in each of twelve 16×150-mm culture tubes. Cap each tube. Sterilize at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes.

Remove caps from a tube of sterile 10-percent oxgall solution and a tube of sterile brain-heart infusion broth. Quickly pour the oxgall solution into the brain-heart infusion-broth tube and recap.

5.4 *Distilled or deionized water.*

5.5 *Hydrogen peroxide solution,* 3 percent.

5.6 *Potassium iodide,* crystals.

6. Analysis

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

6.2 Select a colony or colonies to be confirmed for fecal streptococcal bacteria from the incubated membrane filter.

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 a single, well-isolated colony. Part of the colony material will adhere to the wire.

6.5 Uncap a culture tube of a brain-heart infusion-agar slant and hold it at an angle of about 45° with the flat surface of the slant upward (fig. 6). Insert the inoculating loop and colony material into the tube. Starting at the base of the slant, lightly rub the loop against the agar, moving toward the top, in a zigzag pattern (fig. 6).

6.6 Recap the culture tube. Flame the inoculating loop and inoculate additional tubes as in 6.4 and 6.5 until all colonies to be tested have been placed on agar in separate tubes. Place the inoculated tubes in the culture-tube rack and incubate at 35±0.5 °C for 24 to 48 hours.

6.7 Remove the culture tubes from the incubator and examine. Growth will be evident as a translucent, glistening film on the surface of the agar.

6.8 Test the potency of the hydrogen peroxide solution by placing a few milliliters in a test tube and adding a few

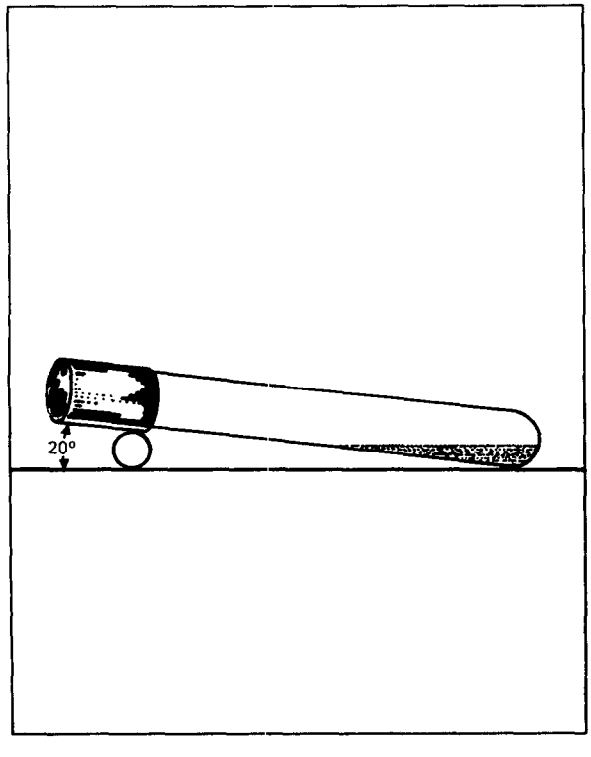

Figure 5.—Preparation of agar slant.

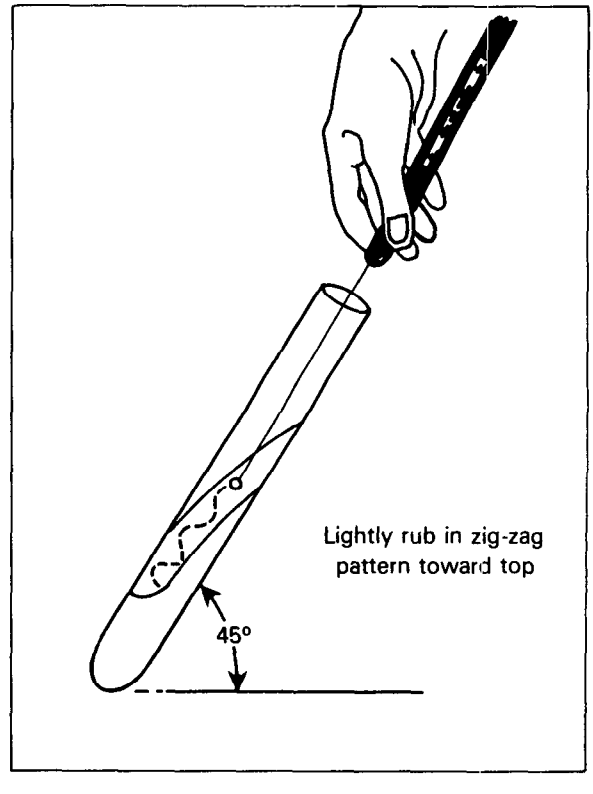

Figure 6.—Method of streaking on an agar slant.

potassium iodide crystals. A brown coloration and the appearance of bubbles in the solution indicate that the hydrogen peroxide solution is acceptable for use. If these reactions do not occur, discard and obtain a fresh hydrogen peroxide solution.

6.9 Flame the inoculating loop and allow to cool. Immediately uncap a culture tube of brain-heart infusion agar having growth. Remove a loopful of growth from the tube and smear on a clean glass slide. Add a few drops of freshly tested 3-percent hydrogen peroxide solution to the material on the slide. Immediately watch the slide for bubble formation. Observation of bubble formation may be facilitated by use of a low-power microscope. The absence of bubbles constitutes a negative catalase test indicating a probable fecal streptococcal culture, and the confirmation test should be continued. The presence of bubbles constitutes a positive catalase test indicating the presence of a nonstreptococcal bacteria, and the test may be terminated at this point.

6.10 Proceed as follows for all catalase-negative cultures. Uncap one culture tube each of brain-heart infusion broth and brain-heart infusion-40-percent bile broth. Using a flamed inoculating loop, transfer one loopful of material from the brain-heart infusion-agar slant to one of the tubes. Reflame the loop and transfer a loopful of material from the agar slant to the other tube. Recap the tubes.

6.11 Flame the inoculating loop and inoculate additional culture tubes as in 6.9 until all catalase-negative cultures have been placed in separate tubes of brain-heart infusion broth and brain-heart infusion-40-percent bile broth.

6.12 Place the inoculated culture tubes of brain-heart infusion broth in a culture-tube rack and incubate at 45 ± 0.5 °C for 48 ± 3 hours. Include tubes of uninoculated medium as controls.

6.13 Place the inoculated culture tubes of brain-heart infusion-40-percent bile broth in a culture-tube rack and incubate at 35 ± 0.5 °C for 72 ± 4 hours. Include tubes of uninoculated medium as controls.

6.14 Remove culture tubes from incubator and examine. Appearance of turbidity in the inoculated tubes, when compared to the controls, constitutes a positive test for growth.

Appearance of growth in the brain-heart infusion broth and the brain-heart infusion-40-percent bile broth constitutes a positive confirmation for the presence of fecal streptococci in the original colony. Absence of growth in either or both culture tubes indicates that the original colony was not of the fecal streptococcal group.

6.15 Autoclave all inoculated culture tubes and smeared slides at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 to 30 minutes before discarding.

7. Calculations

No calculations are necessary.

8. Reporting of results

Results of the fecal streptococcal confirmation test are included in the colony counts for fecal streptococcal 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.

Fecal streptococcal bacteria (most-probable-number, MPN, method)

Presumptive and confirmation test (B-0065-85)

Parameter and Code:
Streptococci, fecal (MPN): 31677

1. Applications

This method is not applicable to saline water. It is applicable to fresh water having large suspended-solids concentration and large populations of nonstreptococcal bacteria.

2. Summary of method

2.1 Decimal dilutions of multiple sample aliquots are inoculated into azide dextrose broth. The cultures are incubated at 35±0.5 °C and examined after 24 and 48 hours for evidence of growth. Positive cultures at 24 or 48 hours constitute a positive presumptive test for fecal streptococci.

2.2 Positive cultures at 24 and 48 hours are inoculated into ethyl violet azide broth and incubated at 35±0.5 °C and examined after 24 hours. Negative ethyl violet azide cultures after 24-hour incubation are reinoculated with original positive presumptive cultures of azide dextrose broth, incubated, and examined again after an additional 24 hours. Growth in ethyl violet azide after 24 or 48 hours constitutes a positive confirmation test for fecal streptococci.

3. Interferences

Certain members of the streptococcal group from soil, vegetative, and insect sources will test positive in this procedure; therefore, the test should be used concurrently with tests for other fecal indicators to substantiate the sanitary significance of the results (American Public Health Association and others, 1985). Differentiation of the streptococcal group requires additional taxonomic tests (Bordner and others, 1978, p. 144-153).

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 *Bunsen burner*, for sterilizing inoculating loop.

4.5 *Crimper*, for attaching aluminum seals.

4.6 *Culture tubes*. The size and the type of culture tube used depend on the volume of water to be tested and whether the test is to be done in the laboratory or onsite. The procedure described below specifies the use of test tubes as culture vessels. Serum bottles may be more appropriate as culture vessels if samples are to be inoculated and incubated onsite. Apparatus needed for an onsite procedure is described in the "Presumptive Onsite Test" (B-0040-85) subsection of the "Total Coliform Bacteria" section.

4.6.1 For testing 10-mL aliquots, use borosilicate glass culture tubes, 20×150 mm; tube caps, 20 mm.

4.6.2 For testing 1-mL or smaller aliquots, use borosilicate glass culture tubes, 16×125 mm; tube caps, 16 mm.

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

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

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

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

4.11 *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.

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

4.13 *Needle holder*.

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

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

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

4.17 *Rubber stoppers*, 13×20 mm.

4.18 *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.

4.19 *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 *Azide dextrose broth*. Use premixed azide dextrose broth, and prepare according to directions on bottle label. The medium also may be prepared according to American Public Health Association and others (1985).

5.1.1 Place 10 mL of medium containing 69.4 g/L azide dextrose broth in a 20×150-mm culture tube or a serum bottle for each 10-mL aliquot of sample to be tested.

5.1.2 Place 10 mL of medium containing 34.7 g/L azide dextrose broth in a 16×125-mm culture tube or a serum bottle for each 1-mL or smaller aliquot to be tested.

5.1.3 Sterilize capped culture tubes or serum bottles of media in upright position at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes.

5.2 *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.

5.3 *Distilled or deionized water*.

5.4 *Ethyl violet azide (EVA) broth*. Use premixed EVA broth, and prepare according to directions on bottle label (Note 1).

Note 1: Because the number of positive azide dextrose broth cultures is unknown at the time of medium preparation, prepare a sufficient number of culture tubes containing ethyl violet azide broth to enable inoculation of the maximum number of positives.

5.4.1 Place 10 mL of medium containing 35.8 g/L EVA broth in a 16×125-mm culture tube for each culture tube or serum bottle of azide dextrose broth prepared in 5.1.

5.4.2 Sterilize capped culture tubes or serum bottles of media in upright position at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes.

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. Order-of-magnitude estimates can be made using a one-tube series.

6.1 Set up five culture tubes of azide dextrose 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	1 milliliter of original sample	0.1 milliliter of original sample
1:100	1 milliliter of 1:10 dilution	1 milliliter of 1:100 dilution
1:1,000	1 milliliter of 1:100 dilution	0.1 milliliter of 1:100 dilution
1:10,000	1 milliliter of 1:1,000 dilution	1 milliliter of 1:10,000 dilution
1:100,000	1 milliliter of 1:10,000 dilution	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 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. Include a tube of uninoculated medium as a control.

6.4 Remove the inoculated culture tubes from the incubator and examine each tube for the presence of turbidity. Any quantity of turbidity in the inoculated tubes, when

compared to the control, constitutes a positive presumptive test for fecal streptococci.

6.5 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.

6.6 Remove from flame and allow wire to cool for about 10 seconds. Do not allow the inoculating loop to contact any foreign surface during the cooling period.

6.7 Gently shake and uncap a positive culture tube of azide dextrose broth. Insert the inoculating loop beneath the liquid surface and carefully withdraw a loopful of culture. Uncap a tube of EVA broth and insert the loop of culture beneath the liquid surface. Gently swirl the loop to disperse the bacteria. Repeat this procedure twice more, flaming the loop between inoculations, until three loopfuls of culture have been transferred to the tube containing the EVA broth.

6.8 Recap both culture tubes. Flame the inoculating loop and inoculate additional tubes as in 6.7, transferring three loopfuls of culture to each tube, until all positive cultures have been transferred to EVA broth.

6.9 Return all positive and negative culture tubes of azide dextrose broth and inoculated tubes of EVA broth to the incubator and incubate at 35 ± 0.5 °C for 24 ± 2 hours.

6.10 Remove all culture tubes from the incubator and examine. A positive culture on EVA broth is indicated by a purple button of growth at the bottom of the tube or occasionally by dense turbidity. Sterilize positive EVA broth tubes in the autoclave at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 to 30 minutes before discarding.

6.10.1 Reinoculate any negative EVA broth culture tubes using an additional three loopfuls of the original positive azide dextrose broth as in 6.7. Discard the original positive presumptive tubes after autoclaving.

6.10.2 Inoculate into EVA broth material from any additional culture tubes of azide dextrose broth that have become positive during the preceding 24 ± 2-hour incubation.

6.10.3 Return remaining positive azide dextrose broth culture tubes and remaining EVA broth tubes to the incubator and incubate as in 6.3.

6.11 Remove all culture tubes from the incubator and examine.

6.11.1 Discard after autoclaving any EVA broth culture tubes that remain negative after reinoculation in 6.10.1.

6.11.2 Reinoculate any negative EVA broth culture tubes from 6.10.2 with three loopfuls of original positive azide dextrose broth cultures.

6.11.3 Sterilize and discard all remaining culture tubes of azide dextrose broth cultures and all positive tubes of EVA broth.

6.11.4 Return remaining culture tubes of EVA broth to the incubator and incubate as in 6.3.

6.12 Remove the last EVA broth culture tubes and examine. Discard all remaining tubes after autoclaving.

7. Calculations

7.1 Record the number of positive culture tubes occurring for all sample volumes tested. Calculate presumptive fecal streptococci from the total number of positive tubes of azide dextrose broth. Use the number of positive tubes of EVA broth to determine the most probable number of confirmed fecal streptococci.

7.2 When more than three volumes are tested, use the 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 (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.

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 2).

Note 2: 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 4. If a series of decimal dilutions other than 1, 0.1, and 0.01 mL is used, the MPN value in table 4 needs to be corrected for the dilutions actually used. To do this, divide the value in table 4 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 4 by 0.1 mL. MPN tables for other combinations of sample volumes and number 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

Table 4.—*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 4.—*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	---	---

4) is 1,100. Dividing by 10^{-6}, the MPN is computed to be 11×10^8 streptococcal bacteria per 100 mL and 95-percent confidence limits of 3.1×10^8 and 25×10^8 streptococcal bacteria per 100 mL.

8. Reporting of results

Report fecal streptococcal concentration as MPN fecal streptococci 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 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 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 4.

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.

Nitrifying bacteria (most-probable-number, MPN, method)

(B-0420-85)

Parameter and Code:
Nitrifying bacteria (MPN): 31854

Nitrification is the biological oxidation of reduced nitrogen compounds to nitrite and nitrate. Most commonly, the initial substance is ammonium, and the final product is nitrate. The process has two distinct steps, each mediated by a specific group of bacteria. The *Nitrosomonas* group, which includes several genera of bacteria, oxidizes ammonium (NH_4^+) only to nitrite (NO_2^-) as shown:

$$NH_4^+ + 3/2 O_2 \rightarrow NO_2^- + 2H^+ + H_2O.$$

The *Nitrobacter* group of bacteria oxidizes NO_2^-, but not NH_4^+ or any other reduced nitrogen compound, to nitrate (NO_3^-) as shown:

$$NO_2^- + 1/2 O_2 \rightarrow NO_3^-.$$

Hydrogen ions produced by the oxidation of NH_4^+ to NO_3^- may be of some geochemical significance because the excess acid can dissolve minerals and can serve as the catalyst in exchange reactions on clays. Nitrification is important in soils because the process controls the supply of NO_3^- used by higher plants. In surface waters, nitrification contributes to oxygen demand.

The organisms, *Nitrosomonas* and *Nitrobacter*, are autotrophic bacteria; they obtain their energy from the inorganic oxidations indicated in the preceding paragraph and use carbon dioxide as a source of cellular carbon. Media used to isolate these bacteria are assumed to be free of organic carbon. This assumption is valid initially, and only nitrifiers will grow on the media; however, as these autotrophs grow, they release cell substances to the media, and heterotrophs may develop.

The medium for isolating *Nitrosomonas* contains NH_4^+. Appearance of NO_2^- in the inoculated cultures, but not in the control cultures, presumptively indicates the presence of *Nitrosomonas* in the sample. A negative test is not sufficient evidence to prove that *Nitrosomonas* is absent because NO_2^- produced by *Nitrosomonas* can be oxidized to NO_3^- by *Nitrobacter*. Therefore, a positive test for either NO_2^- or NO_3^- in the inoculated cultures indicates the presence of *Nitrosomonas*. The medium for isolating *Nitrobacter* contains NO_2^-; disappearance of NO_2^- from the inoculated cultures, but not from the control cultures, presumptively indicates the presence of *Nitrobacter*.

1. Applications

The method described is similar to that described by Alexander and Clark (1965) and is applicable to all types of soil and fresh and saline water.

2. Summary of method

Decimal dilutions of multiple sample aliquots are inoculated into organic-carbon-free media containing NH_4^+ ions for *Nitrosomonas* isolation or NO_2^- ions for *Nitrobacter* isolation. After incubation at 28±1 °C for 21 days, the inoculated cultures and control cultures are tested for the presence of NO_2^-. The most-probable-number (MPN) of each group of nitrifying bacteria is determined from the distribution of positive and negative tests among the inoculated tubes.

3. Interferences

No interferences are known for the procedure.

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.

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 caps*, borosilicate glass culture tubes, 16×125 mm; tube caps, 16 mm.

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

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

4.8 *Glass beads*, solid, 3 mm, may be necessary for soil samples.

4.9 *Hypodermic syringes*, sterile, 1-mL capacity, equipped with 26-gauge, 3/8-in. needles.

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

4.11 *Incubator*, for operation at a temperature of 28±1 °C, or *water bath* capable of maintaining a temperature of 28±1 °C.

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

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

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

4.15 *Rubber stoppers*, 13×20 mm.

4.16 *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.

4.17 *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 *Ammonium calcium carbonate medium* for MPN of *Nitrosomonas*. To 1 L distilled water, add 0.5 g ammonium sulfate [$(NH_4)_2SO_4$], 1 g potassium phosphate dibasic (K_2HPO_4), 0.03 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$), 0.3 g sodium chloride (NaCl), 0.3 g magnesium sulfate ($MgSO_4 \cdot 7H_2O$), and 7.5 g calcium carbonate ($CaCO_3$). Place 3 mL of medium in each culture tube; cap and autoclave at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes.

5.2 *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.

5.3 *Dilution water for soils*. For dilution blanks, place 95 mL distilled water and approximately three dozen, 3-mm diameter, glass beads in a milk dilution bottle. For each 95-mL dilution blank, also prepare 5 dilution blanks of 90 mL distilled water in milk dilution bottles. Omit the glass beads from the 90-mL dilution blanks. Autoclave 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.

5.4 *Distilled or deionized water.*

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

5.6 *Nitrite calcium carbonate medium* for MPN of *Nitrobacter*. To 1 L distilled water, add 0.006 g potassium nitrite (KNO_2), 1 g potassium phosphate dibasic (K_2HPO_4), 0.3 g sodium chloride (NaCl), 0.1 g magnesium sulfate ($MgSO_4 \cdot 7H_2O$), 1 g calcium carbonate ($CaCO_3$), and 0.3 g calcium chloride ($CaCl_2$). Place 3 mL of medium in each culture tube; cap and autoclave at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes.

5.7 *Nitrite-test reagent*. Add 200 mL concentrated phosphoric acid (specific gravity 1.69) and 20 g sulfanilamide to approximately 1.5 L demineralized water. Dissolve completely (warm if necessary). Add 1 g N-1 naphthylethylenediamine dihydrochloride and dissolve completely. Dilute to 2 L using demineralized water. Store in an amber bottle and refrigerate. The reagent must be at room temperature when it is used. The reagent is stable for approximately 1 month.

5.8 *Zinc copper manganese dioxide mixture*. Mix together 1 g powdered zinc metal (Zn), 0.1 g powdered copper (Cu), and 1 g powdered manganese dioxide (MnO_2).

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. For general use, the three-tube series is recommended and is described in this section. Order-of-magnitude estimates can be made using a one-tube series. The following test volumes are suggested:

1. For water samples, use volumes of 1, 0.1, 0.01, 0.001, and 0.0001 mL.
2. For soil samples, use dilutions of 10^{-2} to 10^{-6} mL.

It may be advisable to do an order-of-magnitude estimate prior to undertaking an extensive sampling program.

6.1 Before starting the analysis, clear an area of the laboratory bench and swab it using a bit of cotton moistened with 70-percent ethyl alcohol, undiluted isopropanol, or disinfectant.

6.2 Set out three culture tubes of ammonium calcium

carbonate medium and three tubes of nitrite calcium carbonate medium for each volume to be tested. For each dilution series, set aside one extra tube of each medium as an uninoculated control tube.

6.2.1 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). Carefully remove caps from sterile tubes to avoid contamination.

6.2.2 If the volume of the desired sample aliquot is less than 0.1 mL, proceed as in 6.2.1 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
1:100	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	
$1:10^6$	1 milliliter of 1:10,000 dilution	1 milliliter of $1:10^6$ dilution
$1:10^7$	0.1 milliliter of $1:10^6$ 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.

6.2.3 Dilution series of soil samples are prepared as follows: Transfer 10 g of soil to a dilution blank containing 95 mL water and glass beads. Cap the bottle and shake vigorously for 1 minute. Immediately transfer 10 mL from the center of the suspension to a 90-mL dilution blank and shake. Continue transferring 10-mL portions to 90-mL dilution blanks until the desired dilution is reached.

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

6.4 Place the inoculated culture tubes and control tubes in a culture-tube rack and incubate at 28 ± 1 °C for 21 days. Clearly defined results will occur only if the bacteria consume all the NO_2^- (or convert all NH_4^+ to NO_2^-). For this reason, incubation should always be for 21 days.

6.5 Test for the production of NO_2^-. After incubation, add 0.5 mL of the nitrite-test reagent to each inoculated culture tube and control tube. Observe the contents of each tube for the development within 5 minutes of a reddish color.

CAUTION.—Nitrite-test reagent contains acid and must be handled carefully.

6.6 Growth of *Nitrosomonas* usually is evidenced by a brick-red color at the bottom of a culture tube and a purplish-red coloration in the overlying liquid. Control tubes and inoculated tubes having no NO_2^- may turn faintly pink; thus, it is imperative that uninoculated control tubes be used in color comparison.

6.7 To all culture tubes of ammonium calcium carbonate medium (*Nitrosomonas*) that do not develop a purplish-red color within 5 minutes, add a small pinch of the zinc copper manganese dioxide mixture. If a reddish color develops, record the culture tube as positive for *Nitrosomonas* on the basis that the initial negative reading for NO_2^- indicated that the NO_2^- produced by *Nitrosomonas* was oxidized to NO_3^- by *Nitrobacter*.

6.8 Record as positive for *Nitrobacter* all culture tubes of nitrite calcium carbonate medium that do not develop the characteristic purplish-red color formed by the reaction of NO_2^- with the nitrite-test reagent.

6.9 A positive result in a control culture tube indicates a contamination of the medium and results of the test, therefore, are invalid.

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

7. Calculations

Record the number of positive inoculated culture tubes occurring for all sample volumes tested. 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 (American Public Health Association and others, 1985).

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

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

In example b, the 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 c, it needs to be placed in the result for the largest chosen dilution as in d (Note 2).

Note 2: The largest dilution has the smallest concentration of the sample; the largest dilution in the preceding table is 0.001.

7.3 The MPN for various combinations of positive and negative results, when three 1-, three 0.1-, and three 0.01-mL dilutions are used, are listed in table 5. If a series of decimal dilutions other than 1, 0.1, and 0.01 mL is used, the MPN value in table 5 needs to be corrected for the dilutions actually used. To do this, divide the value in table 5 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 5 by 0.1 mL. MPN tables for other combinations of sample volumes and

Table 5.—*Most-probable-number (MPN) index and 95-percent confidence limits for various combinations of positive and negative results when three 1-, three 0.1-, and three 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	
Three of 1 mL each	Three of 0.1 mL each	Three of 0.01 mL each		Lower	Upper
0	0	0	<30	---	---
0	0	1	30	<5	90
0	1	0	30	<5	130
1	0	0	40	<5	200
1	0	1	70	10	210
1	1	0	70	10	230
1	1	1	110	30	360
1	2	0	110	30	360
2	0	0	90	10	360
2	0	1	140	30	370
2	1	0	150	30	440
2	1	1	200	70	890
2	2	0	210	40	470
2	2	1	280	100	1,500
3	0	0	230	40	1,200
3	0	1	390	70	1,300
3	0	2	640	150	3,800
3	1	0	430	70	2,100
3	1	1	750	140	2,300
3	1	2	1,200	300	3,800
3	2	0	930	150	3,800
3	2	1	1,500	300	4,400
3	2	2	2,100	350	4,700
3	3	0	2,400	360	13,000
3	3	1	4,600	710	24,000
3	3	2	11,000	1,500	48,000
3	3	3	≥24,000	---	---

numbers of tubes at each level of inoculation are in American Public Health Association and others (1985).

7.4 If only one culture tube is inoculated at each decimal dilution level, record the smallest dilution showing a positive response compared to the largest dilution showing a negative response. Record the results as a range of numbers, for example 100 to 1,000 nitrifying bacteria per milliliter. If all tubes are positive, record the result as a number greater than that indicated by the value of the largest dilution of the series. For example, 1-, 0.1-, and 0.01-mL samples are tested, and all tubes are positive at the end of the test. Record the result as greater than 100 nitrifying bacteria per milliliter (greater than 10^4 nitrifying bacteria per 100 mL).

7.5 Examples of test results and calculations are listed below.

7.5.1 The following results were obtained with a three-tube series:

[−, negative; +, positive]

Volume (milliliters)	Culture tube number 1	2	3	Result
0.1	+	+	+	3/3
0.01	+	+	+	3/3
0.001	+	+	−	2/3
0.0001	−	−	−	0/3

Following the guideline given above and using 0.01-, 0.001-, and 0.0001-mL sample volumes, the test results indicate a sequence of 3-2-0. From this, an MPN of 930 is indicated (table 5). Dividing by 0.01 mL to correct for the effect of dilution, the MPN of the sample is 9.3×10^4

nitrifying bacteria per 100 mL. The 95-percent confidence limits are 1.5×10^4 and 38×10^4 nitrifying bacteria per 100 mL.

7.5.2 The following results were obtained with a three-tube series:

Volume (milliliters)	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Results	3/3	3/3	2/3	1/3	0/3.

Using 10^{-6}, 10^{-7}, and 10^{-8} mL sample volumes, the test results indicate a sequence of 3-2-1 for which the MPN (table 5) is 1,500. Dividing by 10^{-6}, the MPN is computed to be 15×10^8 nitrifying bacteria per 100 mL and 95-percent confidence limits of 3.0×10^8 and 44×10^8 nitrifying bacteria per 100 mL.

7.5.3 The following results were obtained with a three-tube series:

Volume (milliliters)	1	0.1	0.01	0.001
Results	0/3	1/3	0/3	0/3.

Use the sequence of 0-1-0 for which the MPN is 30 and 95-percent confidence limits of <5 and 130 (table 5).

7.6 The various combinations listed in table 5 represent those most likely to be obtained. Other combinations are statistically unlikely. If unlikely combinations are obtained, it is probable either that the multiple-tube technique is inapplicable or that errors of manipulation have occurred.

8. Reporting of results

Report concentration of nitrifying bacteria as MPN *Nitrosomonas* and MPN *Nitrobacter* per 100 mL for water samples or as MPN per 100 g for soil samples as follows: less than 10, whole numbers; 10 or more, two significant figures. Indicate the method of expressing unit weight (wet or dry) of soil samples.

9. Precision

9.1 Precision of the MPN method increases as the number of culture tubes is increased. Precision increases rapidly as 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 three 1-, three 0.1-, and three 0.01-mL dilutions are used, are listed in table 5.

10. Sources of information

Alexander, Martin, and Clark, F.E., 1965, Nitrifying bacteria, *in* Black, C. A., ed., Methods of soil analysis: Madison, Wis., American Society of Agronomy, Part 2, p. 1477-1483.

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