



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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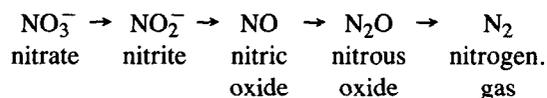
LABORATORY ANALYSIS

Denitrifying and nitrate-reducing bacteria (most-probable-number, MPN, method)

(B-0430-85)

Parameter and Code:
Denitrifying bacteria (MPN): 31856

Some bacteria reduce the nitrogen (N) atom of nitrate (NO_3^-). This occurs by a sequence of reactions that may stop at the level of nitrite (NO_2^-) or proceed to completion with the production of gaseous N compounds. The following pathway indicates the steps involved:



The bacteria that cause these reactions can be referred to collectively as nitrate-reducers or nitrate-respirers. Organisms that do only the first step produce NO_2^- and sometimes are called nitrite-accumulators. They also are commonly referred to by the more general terms nitrate-reducers or nitrate-respirers. The term denitrifiers is more specific and is used for those bacteria that remove N from the system by producing gaseous end products.

Regardless of the final product, the bacteria involved are using the N atom as a sink for the electrons generated during the oxidation of their energy source. Because these denitrifying bacteria also use oxygen as a terminal electron acceptor (aerobic respiration) and will do so as long as oxygen is available, NO_3^- and other oxidized N forms will not be reduced until oxygen has been depleted. Essentially, the bacteria continue respiration even though NO_3^- or NO_2^- has replaced oxygen in their metabolism.

A large and diverse group of bacteria causes NO_3^- reduction and denitrification. Typically, the number of nitrite-accumulators in an environment is greater than the number of denitrifiers. Species in the following genera are believed to be most significant in denitrification in soil: *Pseudomonas*, *Alcaligenes*, and *Flavobacterium* (Gamble and others, 1977). *Bacillus* and *Paracoccus* species may be significant in some environments.

Because of the diversity of the group of organisms responsible for NO_3^- reduction and denitrification, the environmental conditions necessary for the processes to occur are not too restrictive. Ranges reported for pH (5–9) and temperature (15–65 °C) are quite broad (Focht and Verstraete, 1977). Various types of soil, sediment, fresh and saline water, and sewage-treatment systems support NO_3^-

reduction and denitrification. There are two environmental factors that have an important effect on NO_3^- reduction: A suitable energy source (usually a carbon-containing compound) must be available, and oxygen must be absent because it will be used in preference to NO_3^- by denitrifying and nitrate-respiring bacteria. However, denitrification can take place in apparently well-aerated systems due to the existence of anaerobic microsites.

1. Applications

The method is for the determination of the most probable number (MPN) of nitrate-reducing and denitrifying bacteria. The method is applicable to all types of soil and fresh water.

2. Summary of method

2.1 Samples of soil or water and decimal dilutions thereof are inoculated into nutrient broth containing 0.1 percent potassium nitrate (KNO_3). The cultures are incubated at 28 ± 1 °C for 14 days and scored for gas production, production of NO_2^- , and loss of NO_3^- . The MPN of denitrifiers in the sample is determined by the distribution of culture tubes indicating gas production and loss of NO_3^- . Nitrate-reducers (nitrite-accumulators) in the sample may be isolated by the distribution of tubes containing NO_2^- .

2.2 The method is similar to that of Focht and Joseph (1973) and depends on trapping the gas produced and detecting any NO_2^- or NO_3^- remaining in the culture tube.

3. Interferences

Large concentrations of heavy metals or toxic chemicals in the soil or water sample to be tested may interfere.

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, screwcap culture tubes, 16×125 mm. Larger screwcap tubes may be used if larger volumes of water are analyzed. Screwcap tubes will slow diffusion of oxygen from the atmosphere and promote anaerobic conditions.

4.6 *Culture-tube rack*. Use any rack appropriate for culture tubes being used.

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

4.8 *Durham (fermentation) tubes*. The durham tube, used to detect gas production, must be completely filled with medium and at least partly submerged in the culture tube. For 16×125-mm culture tubes, use 6×50-mm durham tubes.

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

4.10 *Hypodermic syringes*, sterile, 1-mL capacity, equipped with 26-gauge, $\frac{3}{8}$ -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 28 ± 1 °C or *water bath* capable of maintaining a temperature of 28 ± 1 °C.

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

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

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

4.16 *Rubber stoppers*, 13×20 mm.

4.17 *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.

4.18 *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/cm² (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/cm² (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 *Dilution water for soil*. 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/cm² (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 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.

5.5 *Nitrate broth*. Use nitrate broth or nutrient broth, plus 0.1 percent KNO_3 . Prepare according to directions on bottle label. Place 9 mL medium in a 16×125-mm culture tube for each 1-mL or smaller aliquot of sample to be tested. In each culture tube, place an inverted (mouth downward) durham tube (fig. 3). Place caps on culture tubes. Sterilize tubes in upright position at 121 °C at 1.05 kg/cm² (15 psi) for 15 minutes as soon as possible after dispensing medium. Loosen screwcaps prior to sterilizing and tighten when tubes have cooled. 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.

5.6 *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.

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

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. Increased precision can be obtained using a five-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 sewage or heavily polluted water samples, use volumes of 10^{-2} to 10^{-6} mL.
3. 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 nitrate broth for each volume to be tested. For each dilution series, set aside one extra tube of 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
1:10	10 milliliters of original sample	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 original sample	0.1 milliliter of 1:100 dilution
1:10,000	0.01 milliliter of original sample	0.01 milliliter of 1:100 dilution
1:100,000	0.001 milliliter of original sample	0.001 milliliter of 1:100 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. This is a 1:10 dilution. 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. This is a 1:100 dilution. 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 tubes at 28 ± 1 °C for 14 days.

6.5 Examine the culture tubes after 14 days. Each tube will be examined for three characteristics in the following

order: gas formation, production of NO_2^- , and removal of NO_3^- . A flow diagram of the test procedure for each culture is shown in figure 7.

6.5.1 Gas production is determined by examining the Durham tube for gas bubbles (fig. 4). Any bubble is presumptive evidence for denitrification; however, a check for removal of NO_3^- is advised.

6.5.2 Test for the production of NO_2^- . Add 0.5 mL nitrite-test reagent to each inoculated culture tube and control tube. Tubes that show a red color are positive for NO_2^- .

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

6.5.3 Test for the presence of NO_3^- . To all culture tubes that remain colorless or have only a light pink color, add about 50 mg zinc copper manganese dioxide mixture. This mixture of metals reduces any NO_3^- remaining in the tube to NO_2^- . The NO_2^- reacts with the nitrite-test reagent already in the tube to give a deep red color. If the red color develops within 5 minutes, record the tube as positive for NO_3^- .

6.5.4 Examples of possible results for any given culture tube and interpretation:

[−, negative; +, positive]

Sample	Gas	Nitrite	Nitrate
A	−	−	+
B	+	−	−
C	−	+	not tested
D	−	+	+
E	+	+	−
F	+	−	+
G	−	−	−

Sample A: Negative for denitrification
Negative for nitrate reduction.

Sample B: Positive for denitrification.

Sample C: Negative for denitrification.
Positive for nitrate reduction.

Sample D: Negative for denitrification.
Positive for nitrate reduction.

Sample E: Positive for denitrification.
Positive for nitrate reduction.

Sample F: Inconclusive.

Sample G: NO_3^- has been removed, although there is no accumulation of NO_2^- and no apparent gas production. It is possible that nitrous oxide (N_2O), which is soluble in water, has been produced. It also is possible that the NO_3^- has been reduced to some other unknown compound.

6.6 Autoclave all cultures at 121 °C at 1.05 kg/cm² (15 psi) for 15 to 30 minutes before discarding.

7. Calculations

7.1 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).

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

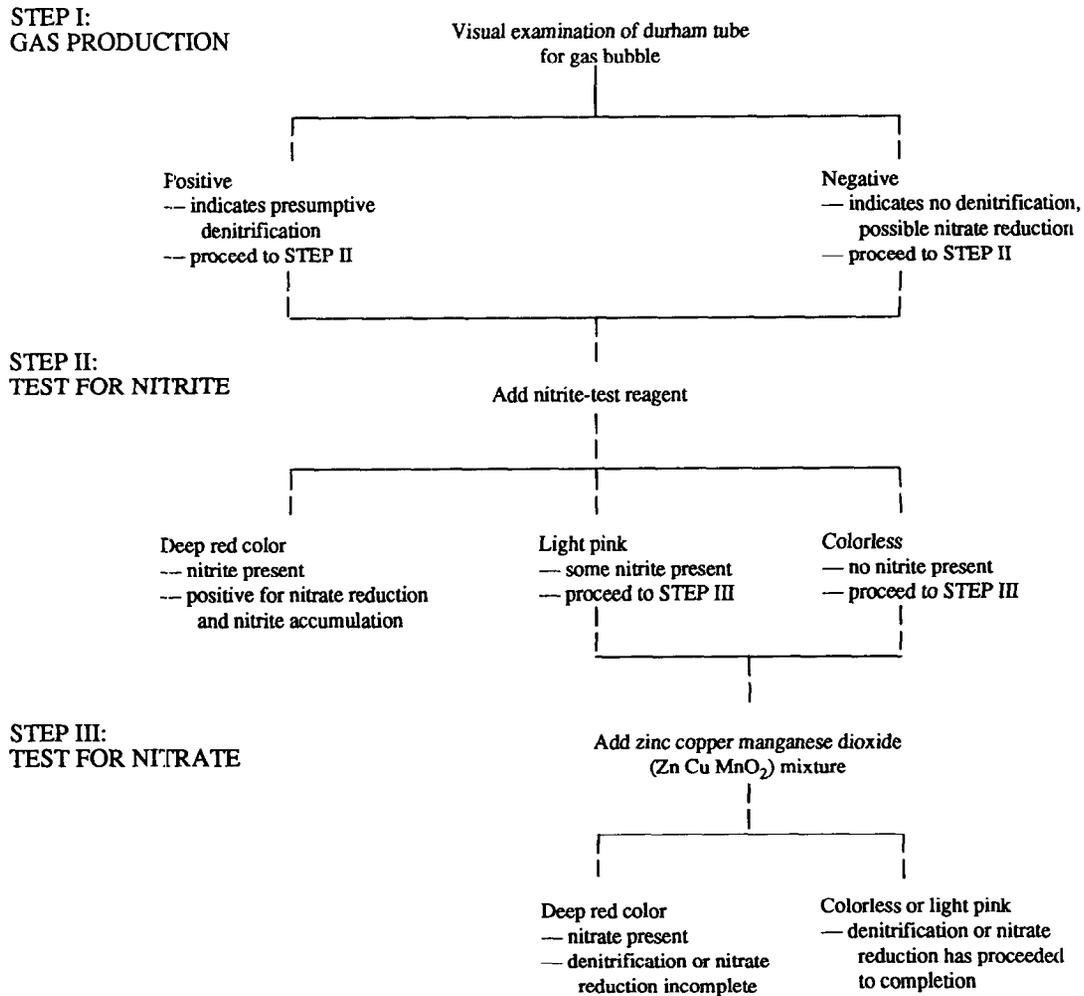


Figure 7.—Test procedure for each culture of denitrifying or nitrate-reducing bacteria.

Example	Decimal dilutions				Combination of positives
	1 milliliter	0.1 milliliter	0.01 milliliter	0.001 milliliter	
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 6. If a series of decimal dilutions other than 1, 0.1, and 0.01 mL is used, the MPN value in table 6 needs to be corrected for the dilutions actually used. To do this, divide the value in table 6 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 6 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).

7.4 If only one culture tube is inoculated at each decimal dilution level, record the smallest dilution indicating a positive response compared to the largest dilution indicating a negative response. Record the results as a range of numbers, for example 100 to 1,000 denitrifying 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 denitrifying bacteria per milliliter.

7.5 Examples of test results and calculations are listed below.

Table 6.—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 1 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	<0.3	---	---
0	0	1	.3	<0.05	0.9
0	1	0	.3	<.05	1.3
1	0	0	.4	<.05	2.0
1	0	1	.7	.1	2.1
1	1	0	.7	.1	2.3
1	1	1	1.1	.3	3.6
1	2	0	1.1	.3	3.6
2	0	0	.9	.1	3.6
2	0	1	1.4	.3	3.7
2	1	0	1.5	.3	4.4
2	1	1	2.0	.7	8.9
2	2	0	2.1	.4	4.7
2	2	1	2.8	1.0	15.0
3	0	0	2.3	.4	12.0
3	0	1	3.9	.7	13.0
3	0	2	6.4	1.5	38.0
3	1	0	4.3	.7	21.0
3	1	1	7.5	1.4	23.0
3	1	2	12.0	3.0	38.0
3	2	0	9.3	1.5	38.0
3	2	1	15.0	3.0	44.0
3	2	2	21.0	3.5	47.0
3	3	0	24.0	3.6	130.0
3	3	1	46.0	7.1	240.0
3	3	2	110.0	15.0	480.0
3	3	3	>240.0	---	---

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

[-, negative, +, positive]

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

Following the guideline in 7.3 and using 0.01-, 0.001-, and 0.0001-mL sample volumes, a sequence of 3-2-0 is indicated. From this, an MPN of 9.3 is indicated (table 6). Dividing by 0.01 mL to correct for the effect of dilution, the MPN of the sample is 930 denitrifying bacteria per milliliter. The 95-percent confidence limits are 150 and 3,800.

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 6) is 15.0. Dividing by 10⁻⁶, the MPN is computed to be 15×10⁶ denitrifying bacteria per milliliter and 95-percent confidence limits of 3.0×10⁶ and 44×10⁶ denitrifying bacteria per milliliter.

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 0.3 and 95-percent confidence limits of <0.05 and 1.3.

7.6 The various combinations listed in table 6 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 the concentration of denitrifying or nitrate-reducing bacteria, or both, as MPN per milliliter for water samples or as MPN per gram 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 6.

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.
- Focht, D.D., and Joseph, H., 1973, An improved method for the enumeration of denitrifying bacteria: Soil Science Society of America Proceedings, v. 37, p. 698-699.
- Focht, D.D., and Verstraete, W., 1977, Biochemical ecology of nitrification and denitrification, in Alexander, Martin, ed., Advances in microbiological ecology, v.1: New York, Plenum Press, p. 135-214.
- Gamble, T.N., Betlach, M.R., and Tiedje, J.M., 1977, Numerically dominant denitrifying bacteria from world soils: Applied and Environmental Microbiology, v. 33, p. 926-939.

Sulfate-reducing bacteria (most-probable-number, MPN, method)

(B-0400-85)

Parameter and Code:
Sulfate-reducing bacteria (MPN): 31855

Sulfate-reducing bacteria commonly are found in environments where reducing conditions prevail, such as ground water, the hypolimnion of stratified lakes, saturated soil, and mud from lake bottoms and stream bottoms. The geochemical implications of sulfate-reducing bacteria have been discussed by Kuznetsov and others (1963). Although many species of bacteria reduce sulfate during the synthesis of sulfur-containing amino acids, four genera of obligate anaerobic bacteria use sulfate reduction as a major energy-yielding reaction and produce large quantities of hydrogen sulfide. These are *Desulfovibrio*, *Desulfotomaculum*, *Desulfomonas*, and *Desulfobulbus*.

1. Applications

The method described in this section is similar to the sulfate-reducing bacteria test given in the American Petroleum Institute (1965). The method is applicable for all water, including brine with large salt concentrations.

2. Summary of method

2.1 Samples are collected and handled using techniques that minimize exposure to oxygen. The samples are incubated at 18 to 25 °C for 28 days, and results are recorded. The most probable number (MPN) of organisms in the sample is determined from the positive and negative responses among a number of inoculated serum bottles of suitable culture medium.

2.2 The sulfate-reducing bacteria are cultivated on a medium containing lactate as a carbon and energy source. Growth is enhanced in the presence of yeast extract. Ascorbic acid is present as a reducing agent. Hydrogen sulfide produced by the bacteria reacts with ferrous iron to produce an inky blackening of the culture medium. Blackening of the culture medium is a positive response and indicates the presence of sulfate-reducing bacteria.

3. Interferences

3.1 Other species of facultative and obligate anaerobic bacteria can grow in the lactate-yeast extract broth and produce a turbidity in the medium, but only sulfate reducers will produce the characteristic inky blackening.

3.2 According to Postgate (1959), the Eh of the culture medium must be less than -200 mV for initiation of growth of sulfate-reducing bacteria. The presence of traces of oxygen will render the medium unsuitable.

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 *Cotton balls*.

4.2 *Decapper*, for removing aluminum seals from spent serum bottles.

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

4.4 *Hypodermic syringes*, sterile, 10-mL capacity, equipped with 22-gauge, 1- to 1 1/2-in. needles.

4.5 *Rubber stoppers*, 13×20 mm.

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

4.7 *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 *Distilled or deionized water*.

5.2 *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.

5.3 *Sulfate API broth*. Ready-to-use presterilized medium packed in 10-mL serum bottles.

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. For general use, the three serum-bottle series is recommended and is described in this section. Order-of-magnitude estimates can be made using a one serum-bottle series. Increased precision can be obtained using a five serum-bottle series. The following test volumes are suggested: For water samples, use volumes of 1, 0.1, 0.01, 0.001, and 0.0001 mL. It may be advisable to do an order-of-magnitude estimate prior to undertaking an extensive sampling program.

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

6.2 Using a sterile syringe, withdraw 1 mL of sample.

6.3 Invert a serum bottle so 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 vigorously.

6.4 Using a new sterile syringe, withdraw 1 mL from the previously inoculated serum bottle and then inoculate a fresh bottle as in 6.3.

6.5 To conserve time and reagents, a scheme such as given in the following example is recommended. Suppose it is desired to test 0.1, 0.01, and 0.001 mL of a given water sample:

6.5.1 Set out 10 serum bottles of culture medium.

6.5.2 Prepare them as in 6.1.

6.5.3 Withdraw 1 mL of sample as in 6.2 and inoculate one serum bottle of medium as in 6.3.

6.5.4 Using the dilution prepared in 6.5.3, inoculate three fresh serum bottles of culture medium as in 6.4 to prepare the 0.1-mL dilutions.

6.5.5 Using one of the dilutions prepared in 6.5.4, inoculate three fresh serum bottles of culture medium as in 6.4 to prepare the 0.01-mL dilutions.

6.5.6 Using one of the dilutions prepared in 6.5.5, inoculate three fresh serum bottles of culture medium as in 6.4 to prepare the 0.001-mL dilutions.

Similar schemes can be established for other combinations using any number of bottles per dilution level.

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

6.7 Incubate serum bottles at room temperature (18 to 25 °C) for 28 days. Do not consider serum bottles that turn black within 2 hours as positive because this probably is due to the presence of sulfide ion in the sample. Subcultures of these false positives may be made after 1 week following the guidelines in 6.1 through 6.3.

6.8 Examine the serum bottles after 28 days. Record as positive all bottles that have substantial quantities of black precipitate. When shaken, the bottles should assume an inky black appearance. Record as negative all bottles in which the medium is turbid but only slightly grayish.

6.9 Autoclave all cultures at 121 °C at 1.05 kg/cm² (15 psi) for 15 to 30 minutes before discarding.

7. Calculations

7.1 Record the number of positive inoculated serum bottles 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).

7.2 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	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 1).

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

The MPN for various combinations of positive and negative results, when three and five 1-, 0.1-, and 0.01-mL dilutions are used, are listed in tables 7 and 8.

If a series of decimal dilutions other than 1, 0.1, and 0.01 mL is used, the MPN values in tables 7 and 8 need to be corrected for the dilutions actually used. To do this, divide the values in tables 7 and 8 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

Table 7.—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 serum bottles indicating positive reaction out of:			MPN index per 1 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	<0.3	---	---
0	0	1	.3	<0.05	0.9
0	1	0	.3	<0.05	1.3
1	0	0	.4	<.05	2.0
1	0	1	.7	.1	2.1
1	1	0	.7	.1	2.3
1	1	1	1.1	.3	3.6
1	2	0	1.1	.3	3.6
2	0	0	.9	.1	3.6
2	0	1	1.4	.3	3.7
2	1	0	1.5	.3	4.4
2	1	1	2.0	.7	8.9
2	2	0	2.1	.4	4.7
2	2	1	2.8	1.0	15.0
3	0	0	2.3	.4	12.0
3	0	1	3.9	.7	13.0
3	0	2	6.4	1.5	38.0
3	1	0	4.3	.7	21.0
3	1	1	7.5	1.4	23.0
3	1	2	12.0	3.0	38.0
3	2	0	9.3	1.5	38.0
3	2	1	15.0	3.0	44.0
3	2	2	21.0	3.5	47.0
3	3	0	24.0	3.6	130.0
3	3	1	46.0	7.1	240.0
3	3	2	110.0	15.0	480.0
3	3	3	>240.0	---	---

values in tables 7 and 8 by 0.1 mL. MPN tables for other combinations of sample volumes and number of serum bottles or culture tubes at each level of inoculation are in American Public Health Association and others (1985).

7.5 If only one serum bottle 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 sulfate-reducing bacteria per milliliter. If all bottles 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 sulfate-reducing bacteria per milliliter.

7.6 Examples of test results and calculations are listed below.

7.6.1 The following results were obtained with a three serum-bottle series:

[-, negative, +, positive]

Volume (milliliters)	Serum bottle number			Result
	1	2	3	
0.1 -----	+	+	+	3/3
0.01 -----	+	+	+	3/3
0.001 -----	+	+	-	2/3
0.0001 -----	-	-	-	0/3

Following the guideline in 7.3 and using 0.01-, 0.001-, and 0.0001-mL sample volumes, a sequence of 3-2-0 is indicated.

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Table 8.—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 serum bottles indicating positive reaction out of:			MPN index per 1 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	<0.2	---	---
0	0	1	.2	<0.05	0.7
0	1	0	.2	<0.05	.7
0	2	0	.4	<0.05	1.1
1	0	0	.2	<.05	.7
1	0	1	.4	<.05	1.1
1	1	0	.4	<.05	1.1
1	1	1	.6	<.05	1.5
1	2	0	.6	<.05	1.5
2	0	0	.5	<.05	1.3
2	0	1	.7	.1	1.7
2	1	0	.7	.1	1.7
2	1	1	.9	.2	2.1
2	2	0	.9	.2	2.1
2	3	0	1.2	.3	2.8
3	0	0	.8	.1	1.9
3	0	1	1.1	.2	2.5
3	1	0	1.1	.2	2.5
3	1	1	1.4	.4	3.4
3	2	0	1.4	.4	3.4
3	2	1	1.7	.5	4.6
4	0	0	1.3	.3	3.1
4	0	1	1.7	.5	4.6
4	1	0	1.7	.5	4.6
4	1	1	2.1	.7	6.3
4	1	2	2.6	.9	7.8
4	2	0	2.2	0.7	6.7
4	2	1	2.6	.9	7.8
4	3	0	2.7	.9	8.0
4	3	1	3.3	1.1	9.3
4	4	0	3.4	1.2	9.3
5	0	0	2.3	.7	7.0
5	0	1	3.1	1.1	8.9
5	0	2	4.3	1.5	11.0
5	1	0	3.3	1.1	9.3
5	1	1	4.6	1.6	12.0
5	1	2	6.3	2.1	15.0
5	2	0	4.9	1.7	13.0
5	2	1	7.0	2.3	17.0
5	2	2	9.4	2.8	22.0
5	3	0	7.9	2.5	19.0
5	3	1	11.0	3.1	25.0
5	3	2	14.0	3.7	34.0
5	3	3	18.0	4.4	50.0
5	4	0	13.0	3.5	30.0
5	4	1	17.0	4.3	49.0

Table 8.—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 serum bottles indicating positive reaction out of:			MPN index per 1 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	22.0	5.7	70.0
5	4	3	28.0	9.0	85.0
5	4	4	35.0	12.0	100.0
5	5	0	24.0	6.8	75.0
5	5	1	35.0	12.0	100.0
5	5	2	54.0	18.0	140.0
5	5	3	92.0	30.0	320.0
5	5	4	160.0	64.0	580.0
5	5	5	>240.0	---	---

From this, an MPN of 9.3 is indicated (table 7). Dividing by 0.01 mL to correct for the effect of dilution, the MPN of the sample is 930 sulfate-reducing bacteria per milliliter. The 95-percent confidence limits are 150 and 3,800.

7.6.2 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 8) is 11.0. Dividing by 10^{-6} , the MPN is computed to be 11×10^6 sulfate-reducing bacteria per milliliter and 95-percent confidence limits of 3.1×10^6 and 25×10^6 sulfate-reducing bacteria per milliliter.

7.6.3 The following results were obtained with a three serum-bottle 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 0.3 and 95-percent confidence limits of <0.05 and 1.3 (table 7).

8. Reporting of results

8.1 For one serum-bottle series, report the data as a range of numbers.

8.2 For a multiple serum-bottle series, report results as MPN of sulfate-reducing bacteria per milliliter 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. Precision increases rapidly as the number of bottles increases from 1 to 5, but then it increases at a slower rate, which makes the gain that is achieved by using 10 bottles instead of 5 much less than is achieved by using 5 bottles instead of 1. Variance as a function of the 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 three and five 1-, 0.1-, and 0.01-mL dilutions are used, are listed in tables 7 and 8.

10. Sources of information

- American Petroleum Institute, 1965, Recommended practice for biological analysis of subsurface injection waters (2d ed.): Dallas, Tex., American Petroleum Institute Division Production API RP 38, 6 p.
- 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.
- Kuznetsov, S.I., Ivanov, V.M., and Lyalikova, N.N., 1963, Introduction to geological microbiology: New York, McGraw-Hill, 252 p.
- Postgate, J.R., 1959, Sulfate reduction by bacteria, in Clifton, C.E., Raffel, Sidney, and Starr, M.P., eds., Annual review of microbiology: Palo Alto, Calif., Annual Reviews, Inc., p. 505-520.

Total bacteria (epifluorescence method)

(B-0005-85)

Parameter and Code:

Bacteria, total count, epifluorescence
(number/mL): 81803

Epifluorescent microscopy is one method for determining the bacterial density in water. It has the advantage of being more rapid than viable count methods (standard plate count, membrane filter, and most probable number). However, bacterial densities determined by epifluorescent microscopy are not directly comparable to viable cell counts or to other biomass measurements, such as adenosine triphosphate (ATP). Direct microscopic counts usually are greater than viable counts for two principal reasons. First, cells that are living as well as dead at the time of collection will be counted by direct microscopy. Second, only a fraction of the total bacteria is enumerated in a viable count.

1. Applications

The epifluorescence method is suitable for all water, except that having a large suspended-sediment concentration. It is similar to other published methods (Hobbie and others, 1977; Dutka, 1978).

2. Summary of method

A water sample is collected and preserved onsite using formaldehyde. In the laboratory, an aliquot of the sample is mixed with a fluorescent dye and filtered through a black membrane filter. The membrane filter is mounted on a microscope slide and viewed at 1,000 \times using epifluorescent microscopy. Bacteria and other life forms appear green, orange, or red against a black background. The number of bacteria per milliliter in the sample is calculated from the average bacterial density per microscopic field.

3. Interferences

Bacteria absorbed on particulate matter are difficult to isolate and the number may be underestimated. Fluorescence of nonbacterial matter, such as algae, protozoa, and fungi, also may cause enumeration errors. Some surfactants prevent the fluorescent dye from attaching to the bacteria or may remove dye from the membrane filter making analysis impossible. Excessive sediment on the filter makes it difficult to view underlying cells.

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 *Bottles*, milk dilution, screwcap.
- 4.2 *Cover slips*, 25-mm circles.
- 4.3 *Filter-holder assembly*, 25 mm.
- 4.4 *Filter-holder assembly*, 47 mm.
- 4.5 *Flasks*, 1 L, erlenmeyer (borosilicate glass).
- 4.6 *Laboratory film*, parafilm.
- 4.7 *Membrane filters*, cellulose, 0.45- μ m pore size, 25-mm diameter.
- 4.8 *Membrane filters*, polycarbonate, 0.2- μ m pore size, 25-mm diameter.
- 4.9 *Membrane filters*, white, grid, sterile, 0.45- μ m pore size, 47-mm diameter.
- 4.10 *Membrane forceps*.
- 4.11 *Microscope*, with lamp, heat filter, red attenuation filter, beam splitter, barrier filter, exciter filter, or equivalent apparatus.
- 4.12 *Microscope slides*, 25 \times 75 mm.
- 4.13 *Pipets*, 1-mL capacity, sterile.
- 4.14 *Pipets*, 10-mL capacity, sterile.
- 4.15 *Plastic petri dishes with covers*, disposable, sterile, 50 \times 12 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 *Stage micrometer*.
- 4.18 *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.

4.19 *Test tubes*, 16×100 mm, glass, disposable.

4.20 *Vacuum filtering flask*.

4.21 *Vacuum pump*.

5. Reagents

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

5.1 *Acridine orange, 0.1 percent*. Dissolve 0.1 g acridine orange in 97 mL distilled water, then add 3 mL 37-percent formaldehyde solution. Filter solution through a 0.45- μ m membrane filter to remove insoluble dye and store in an amber or black bottle in darkness. The acridine orange solution is stable for approximately 1 month at room temperature.

CAUTION.—Acridine orange resulted in mutagenic activity in the Ames test and needs to be treated with care.

5.2 *Distilled or deionized water*.

5.3 *Formaldehyde preservative, 37-percent formaldehyde solution*.

5.4 *Immersion oil*, low fluorescence.

5.5 *Irgalan black solution, 0.2 percent*. Dissolve 2 g irgalan black in 1 L distilled water containing 2 percent acetic acid.

5.6 *Particle-free sterile distilled or deionized water*. Filter distilled water through a 0.45- μ m membrane filter and transfer into a 1-L screwcap erlenmeyer flask. Sterilize by autoclaving at 121 °C at 1.05 kg/cm² (15 psi) for 20 minutes.

6. Analysis

6.1 Preserve the sample, immediately after collection, by the addition of formaldehyde solution (37 percent) at the rate of 5 mL of formaldehyde to 95 mL of sample. Record the volume of preservative added. Maintain the sample in a cool, dark location prior to analysis but prevent from freezing. Refrigeration is ideal but is not required. Sample analysis needs to be completed within 1 month of collection.

6.2 Soak the polycarbonate membrane filters in irgalan black solution for 8 to 24 hours. Rinse the filters in two successive sterile particle-free distilled water rinses and place in a sterile petri dish prior to use.

6.3 Shake the water sample vigorously for 10 seconds to distribute the contents evenly.

6.4 Using a sterile pipet, place 0.5 mL acridine orange solution into a 16×100-mm test tube. Place a 4.5-mL sample into the test tube or a 4.5-mL combination of sample plus particle-free distilled water. Cover the test tube with a small piece of parafilm and invert several times to mix. Let stand for 2 (or as much as 30) minutes.

6.5 Assemble the 25-mm filter-holder assembly with a cellulose membrane filter (0.45 μ m, 25-mm diameter) on the bottom and a polycarbonate filter (0.2 μ m, 25-mm diameter) on top. Attach vacuum pump to vacuum filtering flask.

6.6 Filter the acridine orange containing sample at 0.5 bar (15 in. of vacuum) until the filter just becomes dry. Rinse the test tube using about 5 mL of particle-free sterile distilled water and filter as before to rinse particulate matter from the inner surface of the filter-holder assembly.

6.7 When the polycarbonate filter just becomes dry, place it on a microscope slide. Allow to dry for an additional minute, place a drop of immersion oil on the filter, and add a cover slip.

6.8 Examine the preparation under epifluorescent microscopy at 100× following the manufacturer's instructions for the unit. When the filter surface is in focus, change to high dry (450×) and scan the filter looking for problems such as poor dispersion or excessive fluorescence. If the filter has no apparent problems, add a drop of immersion oil to the cover slip and change to 1,000× magnification. Count the bacteria either within the entire field or within the area enclosed by an ocular grid. Bacterial enumeration is easiest using a Whipple or similar ocular grid. Ideally, each microscopic field should have 5 to 50 bacteria. Generally, most bacteria fluoresce green, but a few also may fluoresce orange or red. Only objects having clearly discernible bacterial morphology should be counted. Count each field separately. Count at least 10 random fields until a total of 300 or more bacteria are counted. If the preparation is too concentrated or dilute, prepare another mount with a different sample volume.

7. Calculations

7.1 Calculate the number of bacteria per milliliter as follows:

$$\text{Bacteria/mL} = \frac{\frac{\text{Average count per field} \times \text{Effective filter area (square millimeters)}}{\text{Field area (square millimeters)}}}{\text{Sample volume filtered (milliliters)} \times \text{Dilution factor}}$$

The effective filter area is the area of filter exposed to the water sample. The 25-mm filter-holder assembly described in the "Apparatus" subsection has an effective filter diameter of 16 mm or an effective filter area of 201 mm². Other types of filter-holder assemblies may have different effective filter areas. The field area must be determined for each microscope using a stage micrometer and following the procedure described by the American Public Health Association and others (1985). The dilution factor corrects for the addition of preservative as follows:

$$\text{Dilution factor (Note 1)} = \frac{\text{Sample volume (milliliters)}}{\text{Sample volume (milliliters)} + \text{Preservative (milliliters)}}$$

Note 1: Addition of 5 mL of formaldehyde to 95 mL of sample will give a dilution factor of 0.95.

7.2 Example calculation:

95 mL of sample + 5 mL preservative = dilution factor 0.95

Sample volume=2 mL
 Effective filter area=201 mm²
 Field area of microscope=2.303 × 10⁻³ mm²
 Total bacteria per field=60

$$\begin{aligned} \text{Bacteria/mL} &= \frac{(60) \frac{201}{2.303 \times 10^{-3}}}{(2) (0.95)} \\ &= \frac{(60) (0.8727 \times 10^5)}{(2) (0.95)} \\ &= 2,756,130 \\ &= 2,760,000. \end{aligned}$$

8. Reporting of results

Report the bacterial density as bacteria per milliliter as follows: three significant figures.

9. Precision

The precision is dependent on the density of bacteria in the sample and the quantity of nonbacterial debris. For typical samples, the precision is approximately ± 10 percent.

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.

Dutka, B.J., ed., 1978, Methods for microbiological analysis of waters, wastewaters, and sediments: Burlington, Canada Centre for Inland Waters, 288 p.

Hobbie, J.E., Daley, R.J., and Jasper, S., 1977, Use of nucleopore filters for counting bacteria by fluorescence microscopy: Applied and Environmental Microbiology, v. 33, p. 1225-1228.

Salmonella* and *Shigella **(diatomaceous-earth and membrane-filter method)**

(B-0100-85)

Parameter and Code: Not applicable

Pathogenic bacteria of the genera *Salmonella* and *Shigella* may be isolated from water by similar methods. The genus *Salmonella* comprises more than 1,000 varieties, all of which are potentially pathogenic to humans. The more common diseases caused by *Salmonella* include typhoid and paratyphoid fever and salmonellosis. Because morphologically and physiologically similar *Salmonella* varieties can cause different diseases, *Salmonella* identification involves serology, which is specific for a particular type of *Salmonella*. The members of the genus *Shigella* are all potentially pathogenic and are similar to *Salmonella* in many aspects. *Shigella* causes acute bacillar dysentery, also known as shigellosis.

Salmonella and *Shigella* can inhabit the gastrointestinal tract of humans. The bacteria pass with the feces. These organisms share the same native environment and travel the water route along with fecal coliforms. The pathogens in water form an extremely small part of the total bacterial population because of excessive numbers of coliforms. Geldreich (1970) reported isolation of *Salmonella* in less than 27.6 percent of freshwater samples when the fecal coliform concentration was less than 200 colonies per 100 mL. *Salmonella* was isolated in 85.2 percent of water samples having fecal coliform concentrations between 200 and 2,000 colonies per 100 mL and was isolated in 98.1 percent of samples having fecal coliform concentrations exceeding 20,000 colonies per 100 mL.

Because of the small occurrence of pathogenic bacteria in most water, large volumes of sample must be filtered. In addition, selective enrichment culture is necessary to increase the population density of the pathogens so that detection is possible. Thus, the procedure is qualitative only. Quantification of pathogens in an original sample cannot be determined readily by this method.

This method is approved for use in the Water Resources Division by those individuals who have special training and knowledge in the handling of pathogenic organisms. Extreme care must be taken because the method provides for the reproduction and enhancement of growth of pathogenic bacteria. Following completion of tests, all cultures must be destroyed and all equipment sterilized by autoclaving at 121 °C at 1.05 kg/cm² (15 psi) for 30 minutes.

1. Applications

The method is applicable for all fresh and estuarine water. Very few reports of the occurrence of *Salmonella* and

Shigella in marine environments are available except to indicate that sediment may be an important source.

2. Summary of method

2.1 Samples are collected using sterile procedures to avoid contamination, while minimizing exposure of onsite personnel to possible pathogens. Several liters of water are filtered through either diatomaceous earth or a membrane filter. The bacteria-laden diatomaceous earth or membrane filter is divided into parts for inoculation into suitable enrichment media. Selenite and tetrathionate broth media are recommended for all *Salmonella* and most *Shigella* determinations.

2.2 Selective solid media plates are streaked at 24-hour intervals for as much as 5 days after incubation at 41.5 °C. Colonies that appear on the selective media having typical *Salmonella* or *Shigella* characteristics are purified and further classified by biochemical reactions. Several nonpathogenic organisms share some important biochemical characteristics with the *Salmonella* and *Shigella* groups. For this reason, many differential biochemical tests are necessary for presumptive identification of the pathogenic Enterobacteriaceae, of which *Salmonella* and *Shigella* are members. Identification cannot be done until the bacteria are verified serologically. A diagrammatic identification scheme is shown in figure 8.

3. Interferences

The membrane-filter method may not work with water having a large suspended-solids concentration. Additionally, many bacteria, other than *Salmonella* and *Shigella*, growing in the enrichment media make isolation and identification of the pathogenic Enterobacteriaceae difficult, even for experienced investigators. Cultures used for inoculation of media in biochemical tests must be pure; if not, false results will be obtained.

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 *Bacteriological transfer loops and needles.*
 4.2 *Bottles, milk dilution, screwcap.*
 4.3 *Diatomaceous earth.*
 4.4 *Durham tubes, flint glass, 6×50 mm.*
 4.5 *Filter-holder assembly** and syringe that has a *two-way valve** or *vacuum hand pump.*
 4.6 *Flasks, 125-mL, screwcap, erlenmeyer.*
 4.7 *Forceps**, stainless steel, smooth tips.
 4.8 *Hot plate, or kitchen stove.*
 4.9 *Incubator**, for operation at a temperature of 35 ± 0.5 °C and 41.5 °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.

- 4.10 *Laboratory balance, with sensitivity to 0.01 g.*
 4.11 *Membrane filters, white, grid, sterile, 0.45- μ m mean pore size, 47-mm diameter, and absorbent pads.*
 4.12 *Microscope slides, 25×75 mm.*
 4.13 *Plastic petri dishes with covers, disposable, sterile, 100×15 mm.*
 4.14 *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. Care when collecting the sample is advised to preclude the possibility of contamination of the sample or the collector. Sterile, disposable gloves are recommended. A minimum of 2 L of sample is necessary for filtration. Because this procedure will be used for qualitative determinations, samples

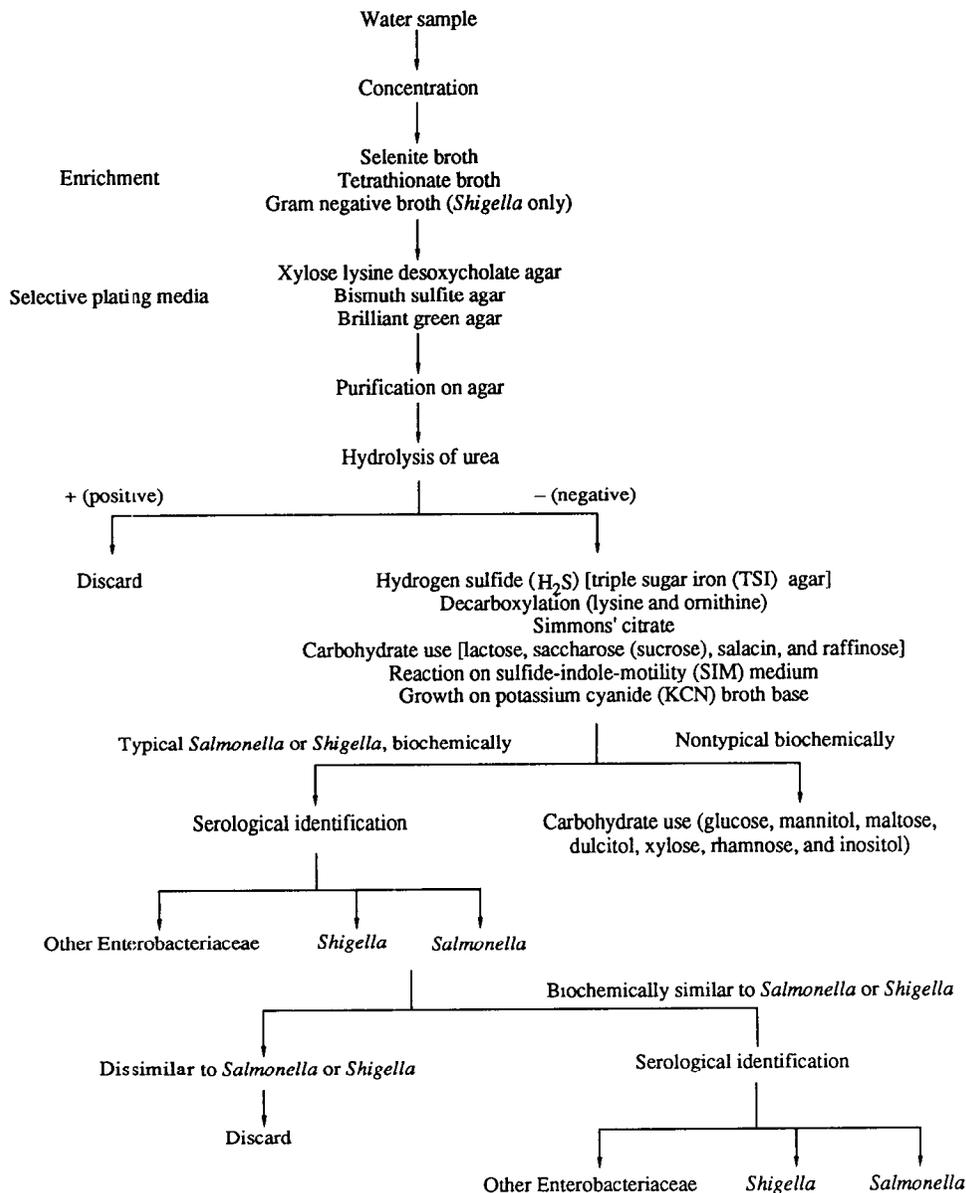


Figure 8.—Identification scheme for *Salmonella* and *Shigella*.

representative of mean flow of a stream generally are not required.

4.15 *Scissors*, autoclavable.

4.16 *Spatula*, laboratory, 120×20 mm.

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.

4.18 *Test tubes*, borosilicate glass, 16×150 mm, and tube caps, 16 mm.

5. Reagents

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

5.1 *Agar*.

5.2 *Bismuth sulfite agar*.

5.3 *Brilliant green agar*.

5.4 *Decarboxylase base Moeller*.

5.5 *Distilled or deionized water*.

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

5.7 *GN (gram negative) broth*.

5.8 *KCN (potassium cyanide) broth base*.

5.9 *Kligler iron agar*.

5.10 *Lactose*.

5.11 *L-lysine HCL*.

5.12 *L-ornithine HCL*.

5.13 *Potassium cyanide (KCN)*, powdered, reagent grade.

5.14 *Purple broth base*.

5.15 *Raffinose*.

5.16 *Saccharose*.

5.17 *Salicin*.

5.18 *Salmonella H Antiserum Kit*.

5.19 *Salmonella O Antiserum Kit*.

5.20 *Selenite broth*.

5.21 *SIM (sulfide-indole-motility) medium*.

5.22 *Simmons' citrate agar*.

5.23 *Sucrose*.

5.24 *Tetrathionate broth*.

5.25 *TSI (triple sugar iron) agar*.

5.26 *Urea agar base*.

5.27 *Veal infusion broth*.

5.28 *XLD (xylose lysine desoxycholate) agar*.

Note 1: It is important that manufacturer's instructions be followed closely in the preparation and storage of all media. If onsite inoculation is intended, discretion is advised in the final dispensing of selenite and tetrathionate broth. The container must allow room for diatomaceous earth and membrane filters and must fit in an onsite incubator.

6. Analysis

6.1 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/cm² (15 psi) for 15 minutes. Steam must contact all surfaces to ensure complete sterilization. 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.

6.2 Assemble the filter-holder assembly and, using flame-sterilized forceps (Note 3), place a sterile membrane filter over the porous plate of the assembly, grid side up, or a sterile absorbent pad in the funnel part of the filter-holder assembly (6.4). Carefully place funnel on filter to avoid tearing or creasing the membrane.

Note 3: 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.

6.4 Concentration: the sample must be concentrated before inoculation into selective media. Two procedures are available for concentration—diatomaceous-earth filtration and membrane filtration.

6.4.1 *Diatomaceous-earth filtration*: Place a sterile 47-mm diameter absorbent pad in the funnel part of the filter-holder assembly and fill the neck halfway with diatomaceous earth. Pour 2 L of sample slowly into the funnel and apply vacuum. When the sample has been completely filtered, transfer equal parts of the diatomaceous earth to the selective growth media (Note 4).

Note 4: Not all bacteria are retained; the filtrate will contain some bacteria and possibly pathogens.

6.4.2 *Membrane filtration*: Filter 2 L (minimum) of sample through a 0.45- μ m mean pore size membrane filter. Because of the small pore diameter, a 47-mm diameter membrane filter will clog quickly unless the water is relatively free of suspended material. Larger diameter filters, such as 100 or 150 mm, may be used if suitable filter-holder assemblies are available. When filtration is complete, remove the filter from the assembly, cut with sterile scissors, and transfer equal-sized pieces of the filter to selective growth media. Record volume of sample that was filtered.

6.5 If isolation of *Salmonella* is desired, transfer one-half of the diatomaceous earth or membrane filter(s) to previously prepared and prewarmed (41.5 °C) flasks of selenite and tetrathionate broth. Prepare flasks by placing 50-mL aliquots of appropriate broth medium in sterilized 125-mL screwcap erlenmeyer flasks. If only *Shigella* is desired, transfer one-half of the diatomaceous earth or membrane filter(s) to GN broth. GN broth cannot be used to isolate *Salmonella*.

6.6 Immediately place inoculated flasks into an incubator preset at 41.5 °C. No more than 24 hours may elapse between incubation and subsequent culture transfers (6.5).

6.7 After arrival at the laboratory, transfer primary culture flasks to a laboratory incubator prewarmed to 41.5 °C and prepare selective media. For *Salmonella*, use brilliant green agar, bismuth sulfite agar, and XLD agar. XLD agar also may be used for *Shigella*. One to four petri dishes of each medium will be needed for every primary (broth) culture.

6.8 After incubation periods indicated in this paragraph and using bacteriological needles, streak broth cultures having evidence of bacterial growth onto media prepared in 6.4. Selenite broth cultures displaying growth become turbid and develop orange-red coloration. Optimum recovery of *Salmonella* from selenite broth is obtained after incubation at 41.5 °C for 24 hours, but additional streaking after 48 and 72 hours may be needed to recover some slower growing strains. Incubate tetrathionate cultures for 48 hours before streaking. Repeated streaking from tetrathionate cultures may be necessary for as much as 5 days to recover all *Salmonella*. Streak the GN broth after 24-hour incubation only. Streak using care and precision so isolated colonies will grow in a discrete pattern (Note 5).

Note 5: The following streak pattern will give good results if care is taken to flame the needle after streaking each section:

(streak pattern)

6.9 Incubate inoculated petri dishes in an inverted (upside down) position at 41.5 °C. Incubate XLD agar petri dishes for 24 hours. Incubate all other petri dishes for 48 hours.

6.10 After incubation, inspect the petri dishes for *Salmonella* or *Shigella* colonies. The petri dishes usually have luxuriant bacterial growth, so care and discretion are necessary in the selection of possible colonies of pathogens. On brilliant green agar, *Salmonella* typically forms pinkish-white colonies having a red background (if well isolated). If the petri dish is overgrown with colonies, *Salmonella* may be indistinguishable from the usually more numerous nonpathogens. On bismuth sulfite agar, *Salmonella* develops black colonies that may or may not have a metallic sheen; sometimes a halo is produced around the colony. A few *Salmonella* strains develop a green, rather than black, coloration on bismuth sulfite agar. Therefore, isolate some green colonies. On XLD agar, *Shigella* forms red colonies, and *Salmonella* produces black-centered red colonies.

6.11 Carefully transfer all suspected *Salmonella* or *Shigella* colonies, using a sterile bacteriological loop, to fresh agar petri dishes. Incubate at 41.5 °C for 48 hours. Continue repeated examination, streaking, and incubation of suspected *Salmonella* and *Shigella* until pure cultures are obtained.

6.12 After the suspected *Salmonella* or *Shigella* colonies have been developed in pure culture, subject them to a series of biochemical tests. If cultures are still positive for *Salmonella* or *Shigella* following the biochemical testing, serological confirmation must be done. In some areas, State or local health departments may be able to perform the biochemical and serological testings. If not, use the scheme in figure 8.

Biochemical identification of large numbers of cultures is expensive and time consuming. It should not be attempted independently without previous training and experience in reading reactions and interpreting results. Additionally, care must be used in working with cultures if laboratory-acquired infections are to be avoided.

There are many published identification schemes for *Salmonella* and *Shigella*. Publications by Brezenski and Russomanno (1969), Claudon and others (1971), Presnell and Miescier (1971), and Edwards and Ewing (1972) describe various methods for the identification procedure. The manufacturers of bacteriological media also provide useful leaflets about certain testing procedures. Difco Laboratories publications (1968, 1969a, 1969b, 1971a, 1971b) are available on request to Difco Laboratories.

If local identification of a suspect culture is desired, first check for the production of urease. *Salmonella* and *Shigella* always are negative for urease production using the Christensen method (Difco Laboratories, 1969b). Screen urease negative cultures for biochemical action as follows: Lysine and ornithine decarboxylation using the Moeller method (Difco Laboratories, 1969a); citrate using the Simmons method (Difco Laboratories, 1953); hydrogen sulfide production on TSI; fermentation of lactose, saccharose (sucrose), salicin, and raffinose; growth in KCN broth; and action on SIM medium. Procedural details are listed in table 9.

If biochemical tests (table 10) indicate the isolated culture may be *Salmonella* or *Shigella*, identify serologically.

6.13 *Serological identification.* Serological identification of *Salmonella* or *Shigella* should be carried out as described by Edwards and Ewing (1972) and American Public Health Association and others (1985). Difco Laboratories (1971b) developed one procedure for the serological identification of *Salmonella*.

A brief description of the serological process may improve the nonserologist's understanding. If an organism is exposed to a foreign body, such as a bacterial cell, part of the organism's defense is the production of a specific protein, called an antibody, that renders the bacterium harmless or nonvirulent. Antibodies are found in the plasma fraction of

Table 9.—Biochemical test procedures for *Salmonella* and *Shigella*

[°C, degrees Celsius; mL, milliliters; H₂S, hydrogen sulfide; TSI, triple sugar iron; SIM, sulfide-indole-motility; KCN, potassium cyanide]

Test	Media requirements	Media preparation	Inoculation and incubation	Typical result
Urease.	Urea agar base or agar.	Prepare medium in slants with generous butts.	Make one streak along entire length. Do not inoculate butt. Incubate at 37 °C for 24 hours.	<i>Salmonella</i> and <i>Shigella</i> are negative (no color change). Others turn medium pink within 24 hours.
Decarboxylation of lysine and ornithine.	Decarboxylase base, L-lysine, and L-ornithine.	Use amino acids at 0.5 percent, added to base medium. Ornithine must be adjusted to pH 6.5 with 1 N sodium hydroxide. Dispense in 5-mL quantities in screwcap tubes.	Inoculate with a 24-hour agar slant culture. Screw caps on tightly and incubate at 37 °C for 24 hours.	Reddish violet, if positive; yellow, if negative. <i>Salmonella</i> , usually positive and <i>Shigella</i> , negative, on lysine; variable on ornithine (see table 10).
Citrate.	Simmons' citrate agar.	Prepare medium in slants with generous butts.	Make one streak along length and stab the butt using a needle. Incubate at 37 °C for 24 to 48 hours.	<i>Shigella</i> is negative (green color). Most <i>Salmonella</i> are positive (deep blue).
H ₂ S production.	TSI agar.	Prepare medium in slants with generous butts.	Streak slant heavily along entire length and stab the butt. Incubate at 37 °C for 24 hours.	<i>Salmonella</i> has red slant, yellow butt, positive for H ₂ S production (blackening), gas variable. <i>Shigella</i> has red slant, yellow butt, negative for H ₂ S production (no blackening).
Carbohydrate use.	Purple broth base, lactose, saccharose (sucrose), salicin, and raffinose.	Sterilize base and sugar separately, the latter by filter. Use 0.5 to 1 percent sugar. Add after sterilizing base in test tubes with Durham tubes.	Inoculate from 24-hour agar slant culture. Incubate at 37 °C. Examine daily for 7 days.	A positive reaction is production of acid (yellow color) with or without gas (bubbles in Durham tube). <i>Salmonella</i> is negative.

Table 9.—Biochemical test procedures for *Salmonella* and *Shigella*—Continued

Test	Media requirements	Media preparation	Inoculation and incubation	Typical result
SIM.	SIM medium and indole test strips.	Dispense in test tubes half full. Sterilize, allow to harden upright. Put a test strip in each tube.	Inoculate with needle from 24-hour agar slant culture. Stab in center to 1/2 depth. Incubate at 37 °C for 24 to 48 hours.	If indole is produced, paper turns pink. Medium blackens if H ₂ S is produced. <i>Salmonella</i> is negative for indole; may produce H ₂ S.
KCN.	KCN broth base KCN reagent, powder.	Sterilize base separately. Prepare 0.5 percent KCN solution and add 1.5 mL to 100 mL sterile base. Dispense 2 mL in test tubes and close with sterile paraflined stoppers.	Inoculate from 24-hour KCN broth culture without KCN. Incubate at 37 °C for 48 hours.	<i>Salmonella</i> and <i>Shigella</i> will not grow. Other Enterobacteriaceae may grow (see table 10).

the blood; hence, blood serum that contains antibodies against, for example, *Salmonella*, is called antiserum. Antiserum, if specific for a certain bacterium, will cause clumping of the bacteria. The clumping can be observed under 100× magnification. The serological process is so specific that more than 1,000 different *Salmonella* types (serotypes) have been identified.

A foreign body that stimulates the production of an antibody is called an antigen. *Salmonella* has two main types of antigens, the O (somatic or intracellular) antigens and H (flagellar) antigens. The O antigens are heat stable and provide basic differentiation into groups of bacteria. The H antigens are heat labile and are used for differentiation within a bacterial group. Occasionally another somatic antigen, termed Vi, is observed. The Vi antigen can block activity of an O antigen and must be inactivated by heat during the serological grouping tests.

The serological procedure for the identification of *Shigella* is similar to that of *Salmonella*; therefore, only the *Salmonella* serology is further detailed. A simplified scheme devised by Spicer and Edwards (Difco Laboratories, 1971b) can be used for tentative serological identification of *Salmonella* using minimal effort. The O antigen is identified first using *Salmonella* O antiserum. If clumping occurs, the results are positive, and the culture is one of the genus *Salmonella*. If only verification that the culture is a *Salmonella* is needed, the O antigen analysis is sufficient.

If further identification is desired, the H antigen needs to be determined using *Salmonella* H antiserum. In this step, most *Salmonella* can be classified into a specific serotype. A diagrammatic serological scheme for *Salmonella* is shown in figure 9.

All cultures not retained for serological testing should be autoclaved at 121 °C at 1.05 kg/cm² (15 psi) for 15 to 30 minutes before discarding.

If Difco reagents are used for serological identification, the procedure is as follows:

6.13.1 Somatic O Antigen Analysis (Difco Laboratories, 1971b).

1. Only micro-organisms that give typical *Salmonella* reactions culturally and biochemically should be tested.
2. Colonies growing on TSI agar or Kligler iron agar are satisfactory.
3. Prepare a dense suspension of the organisms to be tested by suspending the growth from an 18-hour TSI agar slant in 0.5 mL of 0.85 percent sodium chloride solution. This should produce a dense homogeneous suspension approximating 50 times that of a McFarland barium sulfate standard. Care must be taken to ensure an even suspension.
4. Using a wax pencil, mark a microscope slide or glass plate into sections about 1 cm square.
5. Place a drop (0.05 mL) of the appropriate *Salmonella* O antiserum poly on the ruled section of slide or plate as shown.

Table 10.—Differentiation of Enterobacteriaceae by biochemical tests

[+, 90 percent or more cultures positive within 1- or 2-day incubation; - or +, majority of cultures negative; -, 90 percent or more cultures negative; d, different biochemical reactions (+, (+), -); + or -, majority of cultures positive; d^w, different biochemical reactions (+, (+), -), weak reaction; (+), delayed positive reaction; - or (+), majority of cultures negative (delayed reaction of 3 days or more); (+) or +, majority of reactions delayed, some occur within 1 or 2 days; + or (+), 90 percent or more positive (delayed reaction of 3 days or more); °C, degrees Celsius; from Edwards and Ewing, 1972]

Test or substrate	Escherichiaceae		Edwardsiella	Salmonelleae	
	Escherichia	Shigella		Salmonella	Arizona
Indol-----	+	- or +	+	-	-
Methyl red-----	+	+	+	+	+
Voges-proskauer-----	-	-	-	-	-
Simmons' citrate-----	-	-	-	d	+
Hydrogen sulfide (TSI)-----	-	-	+	+	+ ^w or -
Urease-----	-	-	-	-	d
KCN-----	-	-	-	-	+
Motility-----	+ or -	-	+	+	+
Gelatin (22 °C)-----	-	-	-	-	-
Lysine decarboxylase---	d	-	+	(+)	-
Arginine dihydrolase---	d	- or (+)	-	+	-
Ornithine decarboxylase-----	d	d ⁽¹⁾	+	+	d
Phenylalanine deaminase-----	-	-	-	-	-
Malonate-----	-	-	-	+	d
Gas from glucose-----	+	-(¹)	+	+	+
Lactose-----	+	-(¹)	-	d	d
Saccharose (Sucrose)---	d	-(¹)	-	-	d
Mannitol-----	+	+ or -	-	+	+
Dulcitol-----	d	d	-	d ⁽²⁾	d
Salicin-----	d	-	-	-	d
Adonitol-----	-	-	-	-	-
Inositol-----	-	-	-	-	-
Sorbitol-----	+	d	-	d	+
Arabinose-----	+	d	-	+ ⁽²⁾	+
Raffinose-----	d	d	-	-	d
Rhamnose-----	d	d	-	+	+

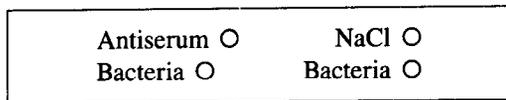
Table 10.—Differentiation of Enterobacteriaceae by biochemical tests—Continued

Test or substrate	Proteaceae					
	Proteus			Providencia		
	Vulgaris	Mirabilis	Morganii	Rettgeri	Alcalifaciens	Stuartii
Indol-----	+	-	+	+	+	+
Methyl red-----	+	+	+	+	+	+
Voges-proskauer-----	-	- or +	-	-	-	-
Simmons' citrate-----	d	+ or (+)	-	+	+	+
Hydrogen sulfide (TSI)-----	+	+	-	-	-	-
Urease-----	+	+	+	+	-	-
KCN-----	+	+	+	+	+	+
Motility-----	+	+	+	+	+	+
Gelatin (22 °C)-----	+ or (+)	+	-	-	-	-
Lysine decarboxylase-----	-	-	-	-	-	-
Arginine dihydrolase-----	-	-	-	-	-	-
Ornithine decarboxylase-----	-	+	+	-	-	-
Phenylalanine deaminase-----	+	+	+	+	+	+
Malonate-----	-	-	-	-	-	-
Gas from glucose-----	+ or -	+	d	- or +	+ or -	-
Lactose-----	-	-	-	-	-	-
Saccharose (Sucrose)-----	+	d	-	d	d	d
Mannitol-----	-	-	-	+ or -	-	d
Dulcitol-----	-	-	-	-	-	-
Salicin-----	d	d	-	d	-	-
Adonitol-----	-	-	-	d	+	-
Inositol-----	-	-	-	+	-	+
Sorbitol-----	-	-	-	d	-	d
Arabinose-----	-	-	-	-	-	-
Raffinose-----	-	-	-	-	-	-
Rhamnose-----	-	-	-	+ or -	-	-

(¹) Certain biotypes of *S. flexneri* produce gas; *S. sonnei* cultures ferment lactose and sucrose slowly and decarboxylate ornithine.

(²) *S. typhi*, *S. cholerae-suis*, *S. enteritidis* bioserv. *S. enteritidis* bioserv. Paratyphi A and Pollorum, and a few others ordinarily do not ferment dulcitol promptly. *S. cholerae-suis* does not ferment arabinose.

(³) Gas volumes produced by cultures of *Serratia*, *Proteus*, and *Providencia* are small.



6. Place one drop of 0.85 percent sodium chloride solution in the square adjacent to the one containing the antiserum. This will serve as a negative control of the bacterial suspension.
7. Using a clean bacteriological loop, transfer a loopful (0.05 mL) of the bacterial suspension (step 3) to the square containing sodium chloride solution. Mix bacterial and sodium chloride solutions thoroughly to obtain an even mixture.
8. Transfer a second loopful of bacterial suspension (step 3) to the square containing the antiserum. Mix bacterial solutions and antiserum thoroughly to obtain an even mixture.
9. Positive agglutination will be completed within 1 to 2 minutes. A delayed or partial agglutination should be considered negative.
10. If positive agglutination occurs, identify the group to which the bacterium belongs by using the desired individual *Salmonella* O antisera groups in the same manner as described in steps 5 through 9 for the *Salmonella* O antiserum poly.

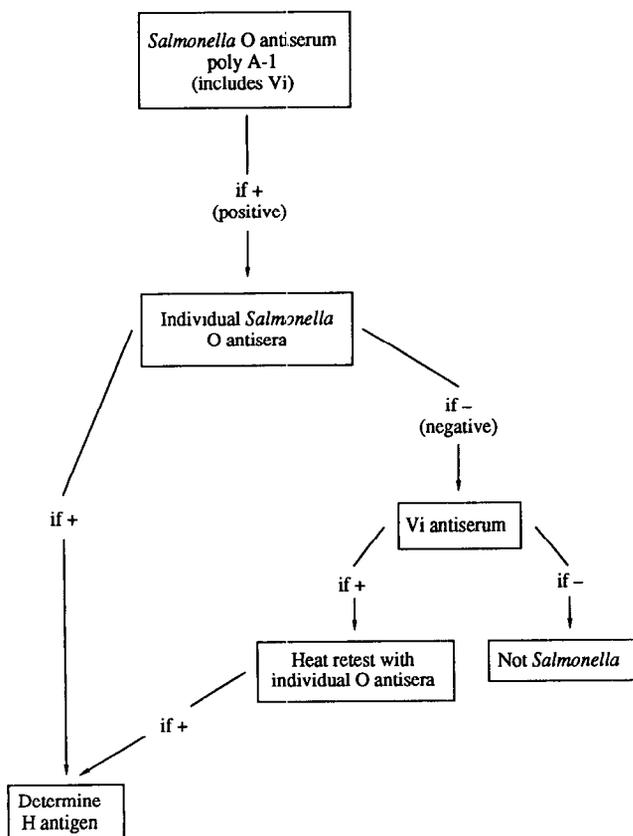


Figure 9.—*Salmonella* serology (from Difco Laboratories, 1971b).

11. If the culture reacts with *Salmonella* O antiserum poly A-1, step 10, but does not react with the specific *Salmonella* O antisera groups, it should be checked using *Salmonella* Vi antiserum by the method described in steps 5 through 9. If there is no agglutination caused by *Salmonella* Vi antiserum at this point, the culture may be regarded as not of the *Salmonella* genus. If the culture reacts with the *Salmonella* Vi antiserum, the culture suspension should be heated in a boiling water bath for 10 minutes and cooled. After cooling, the heated culture should be retested using the desired individual *Salmonella* O antisera groups and the *Salmonella* Vi antiserum. If the culture does not react with the Vi antiserum after heating but reacts with the *Salmonella* O antiserum group D, factor 9, it is most likely *Salmonella typhi* and should be confirmed using *Salmonella* H antiserum d and an unheated culture.
12. If the heated culture in step 11 continues to react with the Vi antiserum and does not react with any of the *Salmonella* O antisera, the culture may be classified as a member of the *Citrobacter* (*Citrobacter freundii*) group. Edwards and Ewing (1972) recommended resubmitting for further biochemical tests all cultures having a typical reaction with *Salmonella* Vi antiserum and *Salmonella* O antiserum (poly or individual groups). They recommend using lysine decarboxylase broth and KCN broth. This step will aid in the elimination of the *Citrobacter* group (*Bethesda-Ballerup*) of bacteria.
13. Cultures having positive agglutination with *Salmonella* O antiserum groups may be analyzed further for their H antigens using the appropriate *Salmonella* H antisera, if necessary.

6.13.2 Flagellar H Antigen Analysis (Difco Laboratories, 1971b). For final identification of the *Salmonella* serotypes within a group, as determined by the *Salmonella* O antisera, it is necessary to determine the H antigens and the phase of the bacterium. Use tube-test procedure developed by Edwards and Bruner (1947). It is necessary to have a motile bacterium when testing for H antigens. Usually TSI broth cultures of fresh isolates are satisfactory for use as antigens. Occasionally, it is necessary to increase the motility of the test bacteria by making several consecutive transfers in SIM medium. This is a semi-solid medium that permits visual determination of bacterial movement. If the bacterium grows well on SIM medium, the biochemical test procedure is described in table 9. Inoculate the test tubes slightly below the surface of the medium by the stab method. Incubate the tubes at 41.5 °C for 18 to 20 hours. Transfer only those bacteria that have migrated to the bottom of the tube when making successive cultures. After several transfers, if the bacteria in

the culture travel 50 to 60 mm through the medium in 18 to 20 hours, it is ready for use.

1. Inoculate a veal infusion broth using the motile bacteria from the last transfer (in motility medium) and incubate at 41.5 °C overnight.
2. Inactivate the culture using equal volumes of culture and 0.6 percent physiological saline solution (6 mL of 40 percent formaldehyde solution plus 8.5 g sodium chloride in 1 L distilled water).
3. Dilutions containing *Salmonella* H antisera depend on which sera are to be used. In general, use a 1:1,000 dilution with the majority of the H sera. This is done by diluting the rehydrated antiserum in a ratio of 0.1 mL antiserum to 33 mL 0.85-percent sodium chloride solution. A few of the specific single-factor sera must be used at a 1:500 dilution because extensive absorption is necessary to render them specific. The 1:500 dilution is recommended when *Salmonella* H antisera s, z13, z15, and z28 are used. To prepare a 1:500 dilution, add 0.1 mL rehydrated antiserum to 16 mL 0.85-percent sodium chloride solution. When using *Salmonella* H antiserum poly a-z, use a dilution of 1:100. To obtain this dilution, add 1 mL rehydrated polyvalent antiserum to 33 mL 0.85-percent sodium chloride solution. *Salmonella* H antisera poly A, B, C, D, E, and F, however, are used at a 1:1,000 dilution as prepared above. Prepare only the quantity of diluted *Salmonella* H sera that can be used in any given day. Discard all excess.
4. Add 0.5 mL of the appropriate serum dilution to Kahn-type serological tubes.
5. Add 0.5 mL of the antigen and incubate in a water bath at 50 °C for 1 hour.
6. Observe the agglutination and record. Autoclave all cultures at 121 °C at 1.05 kg/cm² (15 psi) for 15 to 30 minutes before discarding.

7. Calculations

Not applicable.

8. Reporting of results

Report results only as positive or negative for *Salmonella* or *Shigella* in the sample. Record the sample volume if it is known.

9. Precision

No precision data are available.

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Pseudomonas aeruginosa (membrane-filter method)

(B-0105-85)

Parameter and Code: *Pseudomonas aeruginosa*
MF (colonies/100 mL): 71220

The occurrence of *Pseudomonas aeruginosa* is of increasing concern because it is frequently the causative agent of skin, ear, eye, nose, and throat infections among those engaged in water-contact sports. *P. aeruginosa* also has often been implicated as the cause of some hospital-acquired infections. *P. aeruginosa* is a natural inhabitant of soil, surface water, and vegetation. The majority of the strains identified as *P. aeruginosa* are nonpathogenic to humans. However, the appearance and biochemical characteristics of pathogenic strains are indistinguishable from nonpathogenic *P. aeruginosa* (in the method reported here) so that caution should be observed while handling all *Pseudomonas* cultures. *P. aeruginosa* is a gram-negative, rod-shaped bacterium, motile by monotrichous polar flagella. Most strains produce a variety of pigments, some of which are used for identification in this method. A fluorescent greenish-blue pigment and pyocyanin, a blue pigment, are the most common, but some strains also produce pyorubin, a brownish-red pigment. An incubation temperature of 41.5 ± 0.5 °C is used because other fluorescent pseudomonads, such as *P. fluorescens*, will not grow at, or above, 41 °C.

The presence of *P. aeruginosa* in water used for swimming has caused health concern. Presently, insufficient work has been done to indicate safe limits of *P. aeruginosa* in bathing waters. Brodsky and Nixon (1974) reported that 43 percent of the swimming pools studied had greater than 18 *P. aeruginosa* per 100 mL and 77 percent had a count of greater than 160 *P. aeruginosa* per 100 mL. The occurrence and pathogenicity of *P. aeruginosa* in surface water is not well known except that *P. aeruginosa* is widely distributed in all water.

1. Applications

The method is applicable to all water that does not have large suspended-solids concentration.

2. Summary of method

A water sample is filtered through a 0.45- μ m pore size membrane filter (0.7- μ m filters would allow passage of the pseudomonads). The membrane filter is placed on m-PA agar and incubated at 41.5 ± 0.5 °C for 48 ± 2 hours. After incubation, colonies having typical diffuse brown pigment are counted. Typical colonies may be verified by reaction on skim milk agar.

3. Interferences

Suspended materials may inhibit the filtration of sufficiently large sample volumes to produce statistically valid results. In addition, some suspended material is toxic to bacteria and inhibits their growth. If suspended material is a problem, the multiple-tube method described by the American Public Health Association and others (1985) may be used to estimate *P. aeruginosa* numbers.

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 *Analytical balance*, with sensitivity of 0.1 mg.

4.4 *Bacteriological transfer needle*.

4.5 *Bottles*, milk dilution, screwcap.

4.6 *Bottles*, serum.

4.7 *Crimper*, for attaching aluminum seals.

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

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

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

4.11 *Graduated cylinders*, 100-mL capacity.

4.12 *Hypodermic syringes*, sterile, 1-mL capacity, equipped with 26-gauge, $\frac{3}{8}$ -in. needles.

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

4.14 *Incubator**, for operation at a temperature of 41.5 ± 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. A water bath capable of maintaining a temperature of 41.5 ± 0.5 °C also is satisfactory.

4.15 *Membrane filters*, white, grid, sterile, 0.45- μ m pore size, 47-mm diameter.

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

4.17 *pH meter*.

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

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

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

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

4.22 *Plastic petri dishes with covers*, disposable, sterile, 100×15 mm.

4.23 *Rubber stoppers*, 13×20 mm.

4.24 *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.

4.25 *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.

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

4.27 *Whirl-Pak*, 18 oz.

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/cm² (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/cm² (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 *m-PA agar*. This agar medium is not available in dehydrated form and requires preparation from the basic ingredients. The composition of m-PA agar is listed in table 11. To prepare m-PA agar, combine all ingredients, except antibiotics, and adjust to pH 6.5 using 1 *N* NaOH. Sterilize at 121 °C at 1.05 kg/cm² (15 psi) for 15 minutes. Cool to 55 to 60 °C and aseptically readjust to pH 7.1 ± 0.1 . This is done by removing small aliquots of medium to check the pH after adding 1 *N* NaOH. If the quantities in table 11 are followed, approximately 1.1 mL of 1 *N* NaOH is needed at this point to attain pH 7.1. After the pH of 7.1 has been achieved, add the antibiotics listed in table 11, using a gentle swirling motion. Pour the medium into 50-mm diameter petri dishes to a depth of 4 mm (6–8 mL) when the melted medium has cooled to 50 °C or less.

5.5 *Skim milk agar*

Solution A:

Skim milk	-----	100 g
Distilled water	-----	500 mL

Solution B:

Nutrient broth	-----	12.5 g
Sodium chloride (NaCl)	-----	2.5 g
Agar	-----	15 g
Distilled water	-----	500 mL

Heat solutions separately to boiling and dispense in convenient volumes (such as 75 mL in 160-mL milk dilution bottles). Sterilize at 121 °C at 1.05 kg/cm² (15 psi) for 15 minutes. Cool to approximately 60 °C, then combine equal volumes of solutions A and B, and pour into 100-mm petri dishes to a depth of 4 mm (15 mL). After solidification occurs, store the petri dishes in a plastic bag at 2 to 5 °C (refrigerated) for not more than 2 weeks. Sterile skim milk agar (solutions uncombined) also may be refrigerated for 2 weeks and can be melted and combined as needed.

6. Analysis

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/cm² (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 for all sites.

Table 11.—Composition of *m*-PA agar

Ingredients	Quantity
L-lysine HCl	2.5 grams
Sodium chloride	2.5 grams
Yeast extract	1.0 gram
Xylose	1.25 grams
Sucrose	.62 gram
Lactose	.62 gram
Phenol red	.04 gram
Ferric ammonium citrate	.40 gram
Sodium thiophosphate	3.40 grams
Agar	7.5 grams
Distilled water	500 milliliters
Antibiotics:	
Sulfapyridine	88 milligrams
Kanamycin	4.25 milligrams
Naladixic acid	18.5 milligrams
Actidione	75 milligrams

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. For most surface water, sample volumes of 10, 40, 100, and 200 mL are suggested. Filtration volumes more than 100 mL need to be split between two or more filters.

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	1 milliliter 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 bacteria 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 are present under the filter (indicated by bulges), 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 petri dishes with filters in an inverted position (agar and filter at the top) at 41.5 ± 0.5 °C for 48 ± 2 hours. Filters need to be incubated within 20 minutes after placement on medium. If a water-bath incubator is used, the petri dishes should either be taped to prevent water entry or the dishes put into Whirl-Pak, or equivalent plastic bags. The dishes must be incubated below the water surface.

6.12 After incubation, remove petri dish lids and count typical colonies at $15\times$ magnification. Angle of illumination is not critical. *P. aeruginosa* colonies are dark brown, have an irregular margin, and are almost flat. A light-brown pigment diffusing radially away from the colony is usually visible. Petri dishes having between 20 and 80 *P. aeruginosa*

colonies are considered to be ideal for counting purposes and should be used for calculation, if possible.

6.13 Some of the colonies counted as *P. aeruginosa* should be confirmed by determining growth on skim milk agar. Sterilize the bacteriological transfer needle (sterile round toothpicks also are suitable) by flaming in the burner. The long axis of the needle needs to be held parallel to the cone of the flame so that the entire length of the needle is heated to redness. Remove from flame and allow the needle to cool for about 10 seconds. Do not allow the needle to contact any foreign surface during the cooling period. When cool, remove a small portion of a colony using the sterilized needle and lightly streak the skim milk agar surface. Several such transfers may be made to each petri dish [multiple (24) well petri dishes are useful], sterilizing the needle between each inoculation. Every inoculation should have appropriate notation to identify the source.

6.14 Invert and incubate each inoculated petri dish at 20 to 35 °C for 24 to 48 hours. *P. aeruginosa* causes casein hydrolysis (clearing of the agar) where growth occurs. A yellow-green diffusible pigment should be visible when the petri dish is viewed from the side.

6.15 Autoclave all cultures at 121 °C at 1.05 kg/cm² (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:

P. aeruginosa (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 equation in 7.5 for only those filters having at least one colony and not having colonies too numerous to count. Report results as the number per 100 mL, followed by the statement, "Estimated count based on nonideal colony count."

7.3 If no filters develop characteristic *P. aeruginosa* 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 *P. aeruginosa* 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 on all such filters. The method for calculating and averaging is as follows (Note 5):

$$\begin{array}{r} \text{Volume filter 1} \\ + \text{Volume filter 2} \\ \hline \text{Volume sum} \end{array} \qquad \begin{array}{r} \text{Colony count filter 1} \\ + \text{Colony count filter 2} \\ \hline \text{Colony count sum} \end{array}$$

P. aeruginosa colonies/100 mL =

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

Note 5: Do not calculate the *P. aeruginosa* colonies per 100 mL for each volume filtered and then average the results. If a large filtered volume was divided between several filters, make the count using the equations as in 7.5. Such counts are considered to be in the ideal range if the sum of the colonies is between 20 and 80 colonies.

8. Reporting of results

Report *P. aeruginosa* concentration as *P. aeruginosa* colonies per 100 mL as follows: less than 10 colonies, whole numbers; 10 or more colonies, two significant figures.

9. Precision

Carson and others (1975) reported a mean recovery of 95 percent of naturally occurring *P. aeruginosa* using m-PA agar.

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.
- Brodsky, M.H., and Nixon, M.C., 1974, Membrane filter: method for the isolation and enumeration of *Pseudomonas aeruginosa* from swimming pools: Applied Microbiology, v. 27, no. 5, p. 938-943.
- Carson, L.A., Peterson, N.J., Favero, M.S., Doto, I.L., Collins, D.E., and Levin, M.A., 1975, Factors influencing detection and enumeration of *Pseudomonas aeruginosa* by most-probable-number and membrane filtration techniques: Applied Microbiology, v. 30, no. 6, p. 935-942.