U.S. Geological Survey
Techniques of Water-Resources Investigations

Book 9
Handbooks for Water-Resources Investigations

National Field Manual for the Collection of Water-Quality Data

Chapter A7.

BIOLOGICAL INDICATORS

Third Edition

Edited by **D.N. Myers and F.D. Wilde**

USGS
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Foreword

The mission of the Water Resources Discipline of the U.S. Geological Survey (USGS) is to provide the information and understanding needed for wise management of the Nation's water resources. Inherent in this mission is the responsibility to collect data that accurately describe the physical, chemical, and biological attributes of water systems. These data are used for environmental and resource assessments by the USGS, other government agencies and scientific organizations, and the general public. Reliable and quality-assured data are essential to the credibility and impartiality of the water-resources appraisals carried out by the USGS.

The development and use of a *National Field Manual* is necessary to achieve consistency in the scientific methods and procedures used, to document those methods and procedures, and to maintain technical expertise. USGS field personnel use this manual to ensure that the data collected are of the quality required to fulfill our mission.

(signed)

Robert M. Hirsch
Associate Director for Water

Book 9
Handbooks for Water-Resources Investigations

Chapters of Section A, *National Field Manual for the Collection of Water-Quality Data*

National Field Manual for the Collection of Water-Quality Data

Chapter A7.

BIOLOGICAL INDICATORS

Notes:

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Citations for chapter sections are listed at the end of the table of contents for each section.

Chapter A7. BIOLOGICAL INDICATORS

Third Edition
***Edited by* D.N. Myers and F.D. Wilde**

ABSTRACT

The *National Field Manual for the Collection of Water-Quality Data* (*National Field Manual*) provides guidelines and standard procedures for U.S. Geological Survey (USGS) personnel who collect data used to assess the quality of the Nation's surface-water and ground-water resources. This chapter of the manual includes procedures for the (1) determination of biochemical oxygen demand using a 5-day bioassay test; (2) collection, identification, and enumeration of fecal indicator bacteria; (3) collection of samples and information on two laboratory methods for fecal indicator viruses (coliphages); and (4) collection of samples for protozoan pathogens.

Each chapter of the *National Field Manual* is published separately and revised periodically. Newly published and revised chapters are posted on the World Wide Web on the USGS page "National Field Manual for the Collection of Water-Quality Data." The URL for this page is http://pubs.water.usgs.gov/twri9A/ (accessed November 25, 2003).

INTRODUCTION

As part of its mission, the U.S. Geological Survey (USGS) collects the data needed to determine the quality of our Nation's water resources. A high degree of reliability and standardization of these data are paramount to fulfilling this mission. Documentation of nationally accepted methods used by USGS personnel serves to maintain consistency and technical quality in data-collection activities. *The National Field Manual for the Collection of Water-Quality Data*

(National Field Manual) describes protocols (required and recommended procedures) and provides guidelines for USGS personnel who collect those data on surface-water and ground-water resources. Chapter A7 includes procedures for the (1) determination of biochemical oxygen demand using a 5-day bioassay test; (2) collection, identification, and enumeration of fecal indicator bacteria; (3) collection of samples and information on two laboratory methods for fecal indicator viruses (coliphages); and (4) collection of samples for protozoan pathogens. Many of the equipment-sterilization and sample-collection procedures are identical for fecal indicator bacteria, viruses, and protozoans; these nevertheless appear in each section for the convenience of the user.

The *National Field Manual* is Section A of Book 9 of the USGS publication series "Techniques of Water-Resources Investigations" (TWRI) and consists of individually published chapters designed to be used in conjunction with each other. Chapter numbers are preceded by an "A" to indicate that the report is part of the *National Field Manual*. Other chapters of the *National Field Manual* are referred to in the text by the abbreviation "NFM" and the specific chapter number (or chapter and section number). For example, NFM 6 refers to chapter A6 entitled *Field Measurements,* and NFM 6.4 refers to the section in Chapter A6 on field measurement of pH.

PURPOSE AND SCOPE

The *National Field Manual* is targeted specifically toward field personnel in order to (1) establish and communicate scientifically sound methods and procedures, (2) encourage consistency in the use of field methods for the purpose of producing nationally comparable data, (3) provide methods that minimize data bias and, when properly applied, result in data that are reproducible within acceptable limits of variability, and (4) provide citable documentation for USGS water-quality data-collection protocols.

Data collectors must have formal training and field apprenticeship in order to correctly implement the procedures described in this chapter. The *National Field Manual* is meant to complement such training. A description of the determination for ultimate carbonaceous biochemical oxygen demand is beyond the scope of Section 7.0 (Five-Day Biochemical Oxygen Demand), but is provided in Stamer and others (1979, 1983). The information

provided in Section 7.1 (Fecal Indicator Bacteria) and in Section 7.2 (Fecal Indicator Viruses) is to be used in conjunction with *Methods for Collection and Analysis of Aquatic Biological and Microbiological Samples* edited by L.J. Britton and P.E. Greeson (TWRI, Book 5, Chapter A4, 1989), the 20th edition of Standard Methods for the Examination of Water and Wastewater, and with the other chapters of this *National Field Manual* series.

It is impractical to provide guidance that would encompass the entire spectrum of data-collection objectives, site characteristics, environmental conditions, and technological advances related to water-quality studies. The fundamental responsibility of data collectors is to select methods that are compatible with the scientific objective for the field work and to use procedures that are consistent with USGS standard procedures to the extent possible. Whenever a standard procedure is modified or not used, a description of the procedure used and supporting quality-assurance information are to be reported with the data.

REQUIREMENTS AND RECOMMENDATIONS

As used in the *National Field Manual,* the terms **required** and **recommended** have the following USGS-specific meanings.

Required (require, required, or requirements) pertains to USGS protocols and indicates that USGS Office of Water Quality policy has been established on the basis of research and (or) consensus of the technical staff and has been reviewed by water-quality specialists and selected District[1] or other professional personnel, as appropriate. Technical memorandums or other documents that define the policy pertinent to such requirements are referenced in this manual. Personnel are instructed to use required equipment or procedures as described herein. Departure from or modifications to the stipulated requirements that might be necessary to accomplishing specific data-quality requirements or study objectives must be based on referenced research and good field judgment, and be quality assured and documented in permanent and readily accessible records.

[1]"District" refers to an organizational unit of the USGS in any of the States or Territories of the United States.

Recommended (recommend, recommended, recommendation) pertains to USGS protocols and indicates that USGS Office of Water Quality policy recognizes that one or several alternatives to a given procedure or equipment selection are acceptable on the basis of research and (or) consensus. References to technical memorandums and selected publications pertinent to such recommendations are cited in this chapter to the extent that such documents are available. Specific data-quality requirements, study objectives, or other constraints affect the choice of recommended equipment or procedures. Selection from among the recommended alternatives should be based on referenced research and good field judgment, and reasons for the selection must be documented. Departure from or modifications to recommended procedures must be quality assured and documented in permanent and readily accessible records.

FIELD MANUAL REVIEW AND REVISION

This is the third edition of Chapter A7, "Biological Indicators," dated November 2003; this edition updates and expands upon the second edition dated March 2003. As chapters of the *National Field Manual* are reviewed and revised to correct any errors, incorporate technical advances, and address additional topics, dates of revisions appear in the footer of the report. Refer to "Comments and Errata" on the *National Field Manual's* Home page (http://pubs.water.usgs.gov/twri9A/) for each chapter's revision history.

Comments on the *National Field Manual*, and suggestions for updates or revisions, should be sent to nfm-owq@usgs.gov. Newly revised and reissued chapters or chapter sections will be posted on the World Wide Web on the USGS page "National Field Manual for the Collection of Water-Quality Data." The URL for this page is http://pubs.water.usgs.gov/twri9A/ (accessed November 25, 2003). This page also contains a link to the NFM "Comments and Errata" page that chronicles revisions to each chapter.

ACKNOWLEDGMENTS

The information included in this *National Field Manual* is based on existing manuals, a variety of reference documents, and a broad spectrum of colleague expertise. In addition to the references provided, important source materials included unpublished USGS training and field manuals and technical memorandums. The authors wish to acknowledge the work of M.A. Sylvester, who was instrumental in developing the original version of Section 7.1, Fecal Indicator Bacteria.

Technical critique and contributions that improved the section on *Five-Day Biochemical Oxygen Demand* were provided by C.R. Demas, D.N. Myers, G.B. Ozuna, F.A. Rinella, J.K. Stamer, W.E. Webb, and W.G. Wilber.

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Special thanks go to T.L. Miller and S.K. Sorenson, whose support of this project continues to be instrumental to its achievement.

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FIVE-DAY BIOCHEMICAL OXYGEN DEMAND 7.0

by G.C. Delzer and S.W. McKenzie

Illustrations

Tables

Notes:

References for section 7.0, Five-day biochemical oxygen demand, are located at the end of Chapter A7 in the "Selected References and Documents" section, which begins on page REF-1.

See Appendix A7-A, Table 1, for information on the parameter code for biochemical oxygen demand that is used in the National Water Information System (NWIS) of the U.S. Geological Survey.

The citation for this section (7.0) of NFM 7 is as follows:

Delzer, G.C., and McKenzie, S.W., November 2003, Five-day biochemical oxygen demand: U.S. Geological Survey Techniques of Water-Resources Investigations, book 9, chap. A7 (3d ed.), section 7.0, accessed ___*date*___, from http://pubs.water.usgs.gov/twri9A/.

7.0 FIVE-DAY BIOCHEMICAL OXYGEN DEMAND

The presence of a sufficient concentration of dissolved oxygen is critical to maintaining the aquatic life and aesthetic quality of streams and lakes. Determining how organic matter affects the concentration of dissolved oxygen (DO) in a stream or lake is integral to water-quality management. The decay of organic matter in water is measured as biochemical or chemical oxygen demand. Oxygen demand is a measure of the amount of oxidizable substances in a water sample that can lower DO concentrations (Nemerow, 1974; Tchobanoglous and Schroeder, 1985).

Biochemical oxygen demand represents the amount of oxygen consumed by bacteria and other microorganisms while they decompose organic matter under aerobic conditions at a specified temperature.

The test for biochemical oxygen demand (BOD) is a bioassay procedure that measures the oxygen consumed by bacteria from the decomposition of organic matter (Sawyer and McCarty, 1978). The change in DO concentration is measured over a given period of time in water samples at a specified temperature. Procedures used to determine DO concentration are described in NFM 6.2. It is important to be familiar with the correct procedures for determining DO concentrations before making BOD measurements. BOD is measured in a laboratory environment, generally at a local or USGS laboratory.

Accurate measurement of BOD requires an accurate determination of DO.

There are two stages of decomposition in the BOD test: a carbonaceous stage and a nitrogenous stage (fig. 7.0-1).

- The carbonaceous stage, or first stage, represents that portion of oxygen demand involved in the conversion of organic carbon to carbon dioxide.
- The nitrogenous stage, or second stage, represents a combined carbonaceous plus nitrogeneous demand, when organic nitrogen, ammonia, and nitrite are converted to nitrate. Nitrogenous oxygen demand generally begins after about 6 days. For some sewage, especially discharge from wastewater treatment plants utilizing biological treatment processes, nitrification can occur in less than 5 days if ammonia, nitrite, and nitrifying bacteria are present. In this case, a chemical compound that prevents nitrification should be added to the sample if the intent is to measure only the carbonaceous demand. The results are reported as carbonaceous BOD (CBOD), or as $CBOD_5$ when a nitrification inhibitor is used.

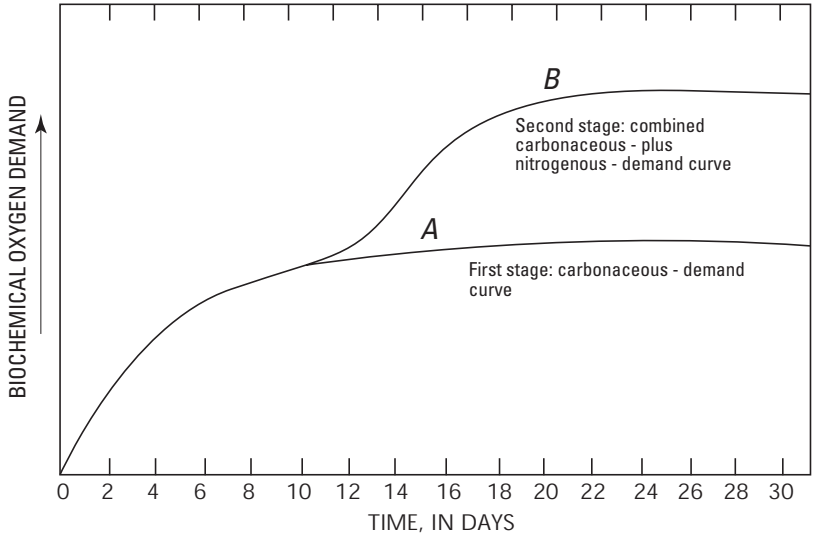

Figure 7.0-1. Biochemical oxygen demand curves: (*A*) typical carbonaceous-demand curve showing the oxidation of organic matter, and (*B*) typical carbonaceous- plus nitrogeneous-demand curve showing the oxidation of ammonia and nitrite. (Modified from Sawyer and McCarty, 1978.)

The standard oxidation (or incubation) test period for BOD is 5 days at 20 degrees Celsius (°C) (BOD_5). The BOD_5 value has been used and reported for many applications, most commonly to indicate the effects of sewage and other organic wastes on dissolved oxygen in surface waters (see TECHNICAL NOTE). The 5-day value, however, represents only a portion of the total biochemical oxygen demand. Twenty days is considered, by convention, adequate time for a complete biochemical oxidation of organic matter in a water sample, but a 20-day test often is impractical when data are needed to address an immediate concern.

- The BOD_5 and $CBOD_5$ tests have limited value by themselves in the assessment of stream pollution and do not provide all of the relevant information to satisfy every study objective (Nemerow, 1974; Stamer and others, 1983; Veltz, 1984). Additional analyses of water samples for chemical oxygen demand, fecal bacteria, and nutrients can aid in the interpretation of BOD_5.
- An ultimate carbonaceous BOD ($CBOD_u$) test is needed to obtain additional BOD information, and can be used for modeling DO regimes in rivers and estuaries (Hines and others, 1978; Stamer and others, 1983). Guidelines for the $CBOD_u$ determination are described in Stamer and others (1979, 1983).
- Note that BOD results represent approximate stream oxygen demands because the laboratory environment does not reproduce ambient stream conditions such as temperature, sunlight, biological populations, and water movement.

TECHNICAL NOTE: A 5-day duration for BOD determination has no theoretical grounding but is based on historical convention. Tchobanoglous and Schroeder (1985) provide the following background: "In a report prepared by the Royal Commission on Sewage Disposal in the United Kingdom at the beginning of the century, it was recommended that a 5-day, 18.3°C, BOD value be used as a reference in Great Britain. These values were selected because British rivers do not have a flow time to the open sea greater than 5 days and average long-term summer temperatures do not exceed 18.3°C. The temperature has been rounded upward to 20°C, but the 5-day time period has become the universal scientific and legal reference."

7.0.1 EQUIPMENT AND SUPPLIES

Table 7.0-1 lists equipment and supplies commonly used in the BOD_5 test using amperometric determination of DO. For more detailed guidance on equipment, supplies, maintenance, and calibration of the DO instrument, refer to NFM 6.2. If the iodometric (Winkler) method of DO determination is to be used, refer to table 6.2-3 in NFM 6.2 for a list of equipment and supplies. Equipment used for BOD sampling must be thoroughly cleaned with nonphosphate detergent and rinsed with tap water and deionized water, as described in NFM 3.

CAUTION: Before handling chemical reagents, refer to Material Safety Data Sheets. Wear safety glasses, gloves, and protective clothing.

Table 7.0-1. Equipment, supplies, chemical reagents, and preparation of dilution water and chemical solutions used in the procedure for determination of 5-day biochemical oxygen demand

[±, plus or minus; °C, degrees Celsius; BOD, biochemical oxygen demand; mL, milliliter; mm, millimeter; NFM, *National Field Manual for the Collection of Water-Quality Data*; L, liter; g, gram; KH_2PO_4, potassium dihydrogen phosphate; $KHPO_4$, potassium monohydrogen phosphate; Na_2HPO_4, sodium monohydrogen phosphate; NH_4Cl, ammonium chloride; *N*, normality; DO, dissolved oxygen; KCl, potassium chloride; $CoCl_3$, cobalt chloride]

Item	Description
Equipment and supplies	
Constant-temperature chamber or water bath	Thermostatically controlled to maintain 20 ± 1°C. During incubation, exclude all light to prevent the possibility of photosynthetic production of oxygen.
Aquarium pump, plastic air tubing, and air diffusion stones	Wash tubing and air diffusion stone thoroughly with a 0.2-percent nonphosphate detergent solution and rinse thoroughly 3 to 5 times with deionized or distilled water before use.
BOD bottles	300 mL, ground glass stoppered. Wash bottles thoroughly with a 0.2-percent nonphosphate detergent solution and rinse with deionized or distilled water before each test. Label bottles appropriately for sample identification.
Glass beads	Borosilicate, solid spherical; 5-mm diameter. Wash thoroughly with a 0.2-percent nonphosphate detergent solution and rinse with deionized or distilled water before use.
Graduated cylinder	Borosilicate, 50- to 250-mL capacity, depending on the volume of sample to be tested.
Overcap	Paper or plastic cup, or aluminum foil, to be placed over BOD stoppers to prevent evaporation of the water seal.
Pipet	Bacteriological, large bore, borosilicate, volume ranging from 1 to 50 mL, depending on the volume of sample to be tested.
Thermometer	Calibrated within temperature range of approximately 5 to 40°C with 0.5°C graduations (NFM 6.1).
Sample container(s)	Wide mouth, screwtop lid, polyethylene, polypropylene, or borosilicate glass. Containers of 1-L capacity are sufficient for most samples.
Waste disposal container(s)	Capped, and of appropriate material to contain specified sample and chemical wastes.
Chemical reagents[1] and preparation of dilution water	
Calcium chloride ($CaCl_2$) solution[2]	Dissolve 27.5 g of $CaCl_2$ in deionized water and dilute to 1 L.
Dilution water	Deionized water of high quality; must be free from toxic substances such as chlorine or toxic metals.
Ferric chloride ($FeCl_3$) solution[2]	Dissolve 0.25 g of $FeCl_3 \cdot 6H_2O$ in deionized water and dilute to 1 L.
Magnesium sulfate ($MgSO_4$) solution[2]	Dissolve 22.5 g of $MgSO_4 \cdot 7H_2O$ in deionized water and dilute to 1 L.
Phosphate buffer solution[2]	Dissolve 8.5 g of KH_2PO_4, 21.8 g of $KHPO_4$, 33.4 g of $Na_2HPO_4 \cdot 7H_2O$, and 1.7 g of NH_4Cl in about 500 mL of deionized water. Dilute to 1 L.

(Table 7.0-1 continues on the next page.)

Table 7.0-1. Equipment, supplies, chemical reagents, and preparation of dilution water and chemical solutions used in the procedure for determination of 5-day biochemical oxygen demand—*Continued*

Item	Description
Chemical reagents for sample pretreatment and preparation of chemical solutions	
Sodium hydroxide (NaOH) for caustic acidity pretreatment	Add 40 g of NaOH to about 900 mL of deionized water. Mix and dilute to 1 L (1 *N* NaOH). Store in a plastic container.
Sodium sulfite (Na_2SO_3) or sodium thiosulfate ($Na_2S_2O_3$) for residual chlorine pretreatment solution	Dissolve 1.575 g of Na_2SO_3 or NaS_2O_3 in 1 L of deionized water. This solution is not stable and should be prepared daily to weekly, as needed. Store refrigerated in a dark bottle.
Sulfuric acid (H_2SO_4) for caustic alkalinity pretreatment	Slowly and while stirring add 28 mL of concentrated H_2SO_4 to about 900 mL of deionized water. Mix and dilute acid solution to 1 L (1 *N* H_2SO_4).
DO equipment and supplies (refer to NFM 6.2)	
Calibration chamber	Follow manufacturer's recommendations.
DO instrument system	Temperature and pressure compensated.
Stirrer attachment for DO sensor	Must fit in 300-mL BOD bottle.
Pocket altimeter-barometer	Calibrated, Thommen™ model 2000 or equivalent.
DO sensor membrane replacement kit	Membranes, O-rings, KCl filling solution.
Oxygen solubility table	Refer to table 6.2-6 in NFM 6.2.
Zero DO calibration solution	Dissolve 1 g Na_2SO_3 and a few crystals of $CoCl_3$ in 1 L water. Prepare fresh zero DO solution before each use.

[1] Properly discard chemical reagents if there is any sign of biological growth or if past the expiration date.
[2] Can be purchased from the HACH™ Instrument Company in the form of nutrient buffer pillows ready for immediate use.

SAMPLE COLLECTION AND STORAGE 7.0.2

Samples can degrade significantly during extended storage. To minimize sample degradation, and thus avoid negative bias in the measurement of BOD_5, analyze samples promptly or store chilled without freezing (maintain a temperature from 1 to 4°C). Chilling the sample is not necessary if the analysis begins within 2 hours of collection (American Public Health Association and others, 1995).

- If a sample is refrigerated prior to analysis, allow the sample to warm to 20°C before starting the test. A sample may be removed from an ice chest or refrigerator during transit to allow it to warm to 20°C before analysis begins.
- It is optimum to start the BOD_5 analysis immediately after sample collection to minimize changes in bacterial concentration.
- **The maximum holding time of a sample to be analyzed for BOD is 24 hours.**

Do not freeze samples.

Bacteria are commonly associated with suspended sediment, which can vary spatially and temporally along a stream cross section (Britton and Greeson, 1989). Like suspended sediment, the oxygen-demanding compounds may not be equally distributed along a cross section. Where possible, use the equal-width-increment or equal-discharge-increment procedures described in NFM 4 to collect a BOD sample representative of the stream cross section.

When using cross-sectional, depth-integrating, or discharge-weighted methods:

1. Use a DH-81 or D-77 sampler in most situations (NFM 2). If stream depths exceed 5m (meters) (16.4 feet), use the bag version of the D-77 sampler.
2. Clean all equipment thoroughly and rinse with sample water before use (NFM 3).
3. Collect samples using appropriate procedures and pour sample water into a compositing device (NFM 4; Edwards and Glysson, 1999).
4. Withdraw a composite sample from the sample-compositing device into a clean container of sufficient capacity to perform the desired BOD tests. The volume of sample depends on the number of BOD tests to be completed and any prior knowledge of BOD for the water of interest. Generally, a 1-liter (L) sample is sufficient.
5. Cap the container securely and protect the sample from light during transport to the laboratory for analysis.
6. Store the sample on ice if not processed and analyzed within 2 hours of collection.

If depth-width-integrated or discharge-weighted methods cannot be used, collect a grab sample by the hand-dip method. A grab sample can be collected directly from the stream using a clean container of sufficient capacity (American Public Health Association and others, 1995).

When collecting a hand-dipped sample:

1. Grasp the sample container near the base on the downstream side of the bottle.
2. Plunge the bottle opening downward below the water surface. **Avoid contact with the streambed during this process.**
3. Allow the sample container to fill with the opening pointed slightly upward into the current.
4. Cap the container securely and protect the sample from light during transport to the laboratory for analysis.

FIVE-DAY TEST FOR BIOCHEMICAL OXYGEN DEMAND 7.0.3

The BOD_5 test procedure is based on DO concentration and requires an accurate DO determination. Follow procedures described in NFM 6.2 to determine DO concentration. Iodometric titration or amperometric (DO meter) methods used to measure DO are used for the BOD_5 test procedure (American Public Health Association and others, 1995). The procedures presented below incorporate the amperometric method for determining DO concentration. Refer to section 6.2.1.B in NFM 6.2 if the iodometric method will be used to determine DO.

> **TECHNICAL NOTE:** If using the iodometric titration method to measure DO concentration, double the sample volume, number of dilutions, and number of bottles to account for determining an initial DO and a final DO.

SAMPLE PREPARATION 7.0.3.A

Most relatively unpolluted streams have a BOD_5 that ranges from 1 to 8 mg/L (milligrams per liter) (Nemerow, 1974). If the BOD_5 value of a sample is less than 7 mg/L, sample dilution is not needed. A BOD_5 value greater than 7 mg/L requires sample dilution. Dilution is necessary when the amount of DO consumed by microorganisms is greater than the amount of DO available in the air-saturated BOD_5 sample (American Public Health Association and others, 1995). The BOD_5 analyst is responsible for determining the dilution(s) that will be needed. Table 7.0-2 provides general dilutions based on anticipated ranges of BOD_5 (Sawyer and McCarty, 1978).

BOD_5 values are acceptable only if the following criteria are met:

- The DO concentration after 5 days must be at least 1 mg/L and at least 2 mg/L lower in concentration than the initial DO (American Public Health Association and others, 1995).

- At least three different dilutions are set per sample to cover the anticipated range of BOD. The three sample volumes used are selected to provide an overlapping range in expected BOD concentrations. For example, if the BOD_5 is known to range from 3 to 28 mg/L for a particular stream, then the sample volumes used for the test would be 50 mL, 100 mL, and 300 mL (no dilution). If there is no prior knowledge of the BOD_5 of the stream water, use a minimum of four volumes to accommodate a range of BOD_5 from 0 to 210 mg/L.

When less than a 300-mL sample is to be analyzed, sample volumes are added to a standard solution of dilution water to bring the total sample volume to 300 mL. Because bacteria need nutrients and micronutrients to survive, these compounds are added to the dilution water. Similarly, the pH of the dilution water needs to be maintained in a range suitable for bacterial growth (6.5 to 7.5). Consequently, sulfuric acid or sodium hydroxide may need to be added to the dilution water to lower or raise the pH, respectively.

Some types of sewage, such as untreated industrial wastes, disinfected wastes, and wastes that have been heated to a high temperature contain too few bacteria to perform the test. Thus, the samples must be seeded with a population of microorganisms to produce an oxygen demand. Discussion of the seeding procedure is beyond the scope of this chapter. Most natural waters contain an adequate amount of microorganisms. For guidance on seeding procedures, including the BOD_5 equation when dilution water is seeded, refer to American Public Health Association and others (1995).

Table 7.0-2. Recommended sample volumes for the 5-day biochemical oxygen demand test

[Adapted from Sawyer and McCarty, 1978. BOD_5, 5-day biochemical oxygen demand]

Anticipated range of BOD_5 (in milligrams per liter)	Milliliters of sample	Milliliters of dilution water
0–7	300	0
6–21	100	200
12–42	50	250
30–105	20	280
60–210	10	290
120–420	5	295
300–1,050	2	298
600–2,100	1	299

INTERFERENCES 7.0.3.B

Certain constituents present in a water sample can inhibit biochemical oxidation and interfere with the BOD analysis. Interferences in the BOD analysis include caustic alkalinity or acidity; the presence of residual chlorine; or the presence of toxic elements, including trace elements such as copper, lead, chromium, mercury, and arsenic, or compounds such as cyanide. Procedures for pretreating samples for some common interferences are described in this chapter. Refer to American Public Health Association and others (1995) for further guidance on sample seeding and pretreatment.

The following preparations are needed before implementing the BOD_5 test procedure:

1. Prepare dilution water 3 to 5 days before initiating BOD_5 tests to ensure that the BOD of the dilution water is less than 0.2 mg/L. **Discard dilution water if there is any sign of biological growth.**
2. Determine sample pH. Adjust sample to a pH between 6.5 and 7.5, if necessary, using sulfuric acid (H_2SO_4) for samples with pH greater than 7.5 or sodium hydroxide (NaOH) for samples with pH less than 6.5 (American Public Health Association and others, 1995).
3. Add sodium sulfite (Na_2SO_3) to remove residual chlorine, if necessary. Samples containing toxic metals, arsenic, or cyanide often require special study and pretreatment (American Public Health Association and others, 1995). Samples must be seeded after pretreatment.

7.0.3.C BOD_5 TEST PROCEDURE

Use the following procedure for the BOD_5 test (troubleshooting suggestions are provided in section 7.0.5,* table 7.0-3*):

1. Determine the amount of sample to be analyzed; if available, use the historical results of a previous test of BOD_5 for a particular sampling site, and refer to table 7.0-2.
2. Place a clean, calibrated thermometer into the constant temperature chamber. (See NFM 6.1 for thermometer care and calibration.)
3. Turn on the constant temperature chamber to allow the controlled temperature to stabilize at 20°C ±1°C.
4. Turn on the DO instrument, but not the stirring attachment. Some DO instruments need to be turned on 30 to 60 minutes before calibration—check the manufacturer's instruction manual.
5. Aerate dilution water before adding nutrient solutions.

6. After aeration,

   a. Add to dilution water

      - 1 mL each of the potassium phosphate, magnesium sulfate, calcium chloride, and ferric chloride solutions per 1 L of dilution water, or
      - Hach Company nutrient buffer pillows to a selected volume of dilution water per the manufacturer's recommendation.

   b. Shake the container of dilution water for about 1 minute to dissolve the slurry and to saturate the water with oxygen.

   c. Place the dilution water in the constant temperature chamber to maintain a temperature of 20°C until sample dilutions and analyses begin.

   d. The initial and final (after 5 days ± 4 hours) DO tests of the dilution water is determined and recorded simultaneously with each batch of environmental samples.

7. Check the temperature of the air incubator or water bath using a laboratory thermometer to ensure that the temperature has been maintained at 20° ± 1°C. A minimum/maximum recording thermometer can be used to audit the temperature during times when checks cannot be made.

8. Place the sample container in the constant-temperature chamber or water bath to begin warming the sample to 20°C ± 1°C. While the sample is warming, insert the air diffusion stone into the container and aerate the sample for about 15 minutes. After removing the air diffusion stone, allow several minutes for excess air bubbles to dissipate. The initial DO of the BOD sample needs to be at or slightly below saturation.

9. **Prepare dilutions as required—**Measure the appropriate amounts of sample necessary for the analysis. BOD_5 dilutions should result in a DO residual of at least 1 mg/L and a DO depletion of at least 2 mg/L after a 5-day incubation to produce the most reliable results. Prepare the dilutions to obtain a DO uptake in this range using the dilution water prepared earlier.

   a. For each subsample, mix thoroughly by inverting 20 times.

      - Use a large-bore pipet for sample volumes less than 50 mL. Withdraw a subsample that is representative of all the particle sizes present.

- Use a graduated cylinder for sample volumes greater than or equal to 50 mL.

b. Dilute two additional samples to bracket the appropriate dilution by a factor of two to three. Prepare at least three samples diluted according to volumes specified in table 7.0-2.

c. Pour the sample from the pipet or graduated cylinder into a clean BOD bottle.

- Agitate the dilution water and fill the remaining portion of the BOD bottle with dilution water.
- Prepare three samples containing only dilution water. These samples serve as blanks for quality control. If two of the three samples meet the blank-water criterion, accept the data.

10. Calibrate the DO instrument in accordance with the procedures outlined in NFM 6.2.

11. After bringing the samples to saturation and preparing the dilutions (steps 8 and 9 above), measure the initial DO concentration (D_1) of each sample and each dilution blank.

    a. Carefully insert the self-stirring sensor into the BOD bottle, avoiding air entrapment.

    b. Turn on the stirrer and allow 1 to 2 minutes for the DO and temperature readings to stabilize.

12. Record the bottle number, date, time, and D_1 on a form similar to that shown in figure 7.0-2.

13. Turn off the stirrer and remove the sensor from the BOD bottle. Rinse the sensor and stirrer with deionized water from a wash bottle. Discard rinse water into a waste container.

14. Add glass beads to the BOD bottle, if necessary, to displace the sample up to the neck of the bottle so that inserting a glass stopper will displace all air, leaving no bubbles.

15. Carefully cap the BOD bottle with the ground-glass stopper. Tip the bottle to one side and check for an air bubble.

    - If an air bubble is present, add glass beads to the bottle until the bubble is removed. Cap the bottle and check again for an air bubble. Repeat if necessary.
    - If no bubble is present in the sample, create a water seal by adding distilled or deionized water to the top of the BOD bottle around the glass stopper. Then place the overcap over the stopper on the BOD bottle to minimize evaporation from the water seal.

16. Place the sealed BOD sample in the air incubator or water bath and incubate the sample at 20°C ± 1°C for 5 days.

17. At the end of 5 days ± 4 hours, remove the BOD bottles from the incubator, remove the overcap, pour off the water seal, remove the ground-glass stopper, and measure the final DO concentration (D_2).

    - The DO uptake ($DO_{0\ days}$ - $DO_{5\ days}$) in the dilution water should not be greater than 0.2 mg/L and preferably not more than 0.1 mg/L. **Exceeding the 0.2-mg/L criterion could be grounds for rejecting results of the BOD analysis of the environmental sample**.
    - Dilution water of poor quality will cause an oxygen demand and appear as sample BOD. Improve purification or get the dilution water from another source if DO uptake exceeds 0.2 mg/L (see section 7.0.5, Troubleshooting).

18. Complete the field form by recording the date, time, and D_2 for each respective sample bottle (fig. 7.0-2).

Quality control. The BOD_5 test can be quite variable. Collect sufficient field and split replicates (10 to 20 percent) to provide an estimate of method variability.

5-Day Biochemical Oxygen Demand (BOD_5) Worksheet

Site/station:_____ **Collection date and time:**_____
Project:_____ **Personnel:**_____

Dilution-water blanks

Bottle number	Initial DO reading (D_1)	Date/time of reading	Final DO reading (D_2)	Date/time of reading	BOD (D_1-D_2)	BOD average (<0.2 mg/L)

Environmental sample

Bottle number	Sample size (mL)	Initial DO reading (D_1)	Date/ time of reading	Final DO reading (D_2)	Date /time of reading	BOD $\frac{D_1 - D_2}{P}$	BOD average (mg/L)

If dilution-water demand is <0.2 milligrams per liter (mg/L), use

$$BOD_5\ (mg/L) = \frac{D_1 - D_2}{P}$$

where

D_1 = initial sample dissolved-oxygen (DO) concentration (in mg/L)
D_2 = sample DO (in mg/L) after 5 days
P = decimal volumetric fraction of sample used

Figure 7.0-2. Example of a 5-day biochemical oxygen demand worksheet.

CALCULATIONS 7.0.4

The general equation for the determination of a BOD_5 value is:

$$BOD_5\,(mg/L) = \frac{D_1 - D_2}{P}$$

where D_1 = initial DO of the sample,
D_2 = final DO of the sample after 5 days, and
P = decimal volumetric fraction of sample used.

If 100 mL of sample are diluted to 300 mL, then $P = \frac{100}{300} = 0.33$. Notice that if no dilution was necessary, $P = 1.0$ and the BOD_5 is determined by $D_1 - D_2$.

If more than one dilution of the sample results in residual DO of at least 1 mg/L and a DO depletion of at least 2 mg/L, and there is no evidence of toxicity at higher sample concentrations or the existence of an obvious anomaly, average the results that are in the acceptable range (American Public Health Association and others, 1995).

7.0.5 TROUBLESHOOTING

The troubleshooting suggestions in table 7.0-3 are not all-inclusive. Refer to the troubleshooting suggestions for DO instruments (table 6.2-4 in NFM 6.2). Remember that faulty batteries can cause erratic readings.

Table 7.0-3. Troubleshooting guide for the 5-day biochemical oxygen demand test

[DO, dissolved oxygen; BOD_5, 5-day biochemical oxygen demand; mg/L, milligrams per liter; HCl, hydrochloric acid]

Symptom	Possible cause and corrective action
DO readings drift downward	• Weak batteries for stirring unit result in inadequate flow across membrane—replace batteries.
BOD_5 demand in dilution water is greater than the acceptable 0.2 mg/L	• Deionized water contains ammonia or volatile organic compounds—increase purity of dilution water or obtain from another source. Age water for 5-10 days before use. • Deionized water contains semivolatile organic compounds leached from the resin bed—increase purity of dilution water or obtain from another source. Age water for 5-10 days before use. • Bacterial growth in reagents and poorly cleaned glassware—more vigorous cleaning of glassware, including washing followed by a 5- to 10-percent HCl rinse followed by 3-5 rinses with deionized water. Discard reagents properly.
Sample BOD values are unusually low in the diluted sample (BOD_5 dilution water is within the acceptable range)	• Dilution water contains interferences inhibiting the biochemical oxidation process—increase purity of dilution water or obtain from another source. • Use deionized water that has been passed through mixed-bed resin columns. **Never use copper-lined stills.** Distilled water may be contaminated by using copper-lined stills or copper fittings—obtain from another source.

REPORTING 7.0.6

When reporting results of a BOD_5 test, be sure to use the correct parameter code (Appendix A7-A, table 1).

- Report BOD_5 values less than 2 mg/L as <2 mg/L rather than as 2.0 mg/L.
- Report BOD_5 values less than 10 mg/L to the nearest 0.1 mg/L.
- Report BOD_5 values greater than or equal to 10 mg/L to two significant figures.
- Report the results of replicate samples and dilution blanks with the BOD_5 results.

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FECAL INDICATOR BACTERIA 7.1

By **Donna N. Myers, Donald M. Stoeckel, Rebecca N. Bushon, Donna S. Francy, and Amie M.G. Brady**

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Illustrations

Tables

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FECAL INDICATOR BACTERIA 7.1

Fecal indicator bacteria are used to assess the microbiological quality of water. Although these bacteria are not typically disease causing, they are associated with fecal contamination and the possible presence of waterborne pathogens. The density of indicator bacteria[1] is a measure of water safety for body-contact recreation or for consumption.

Fecal indicator bacteria: bacteria used to measure the sanitary quality of water

Fecal material from warm-blooded animals may contain a variety of intestinal microorganisms (viruses, bacteria, and protozoa) that are pathogenic to humans. For example, bacterial pathogens of the genera *Salmonella*, *Shigella*, and *Vibrio* can result in several types of illness and diseases in humans, including gastroenteritis and bacillary dysentery, typhoid fever, and cholera.

Bacteriological tests for specific indicator bacteria are used to assess the sanitary quality of water and sediments and the potential public health risk from gastrointestinal pathogens carried by water. The suitability of indicator organisms for these purposes is ranked according to a specific set of criteria, described below.

Criteria for selecting an indicator of fecal contamination in water

The preferred fecal indicator:

- Can be tested for easily
- Is of human or other animal origin
- Survives as long as, or longer than, pathogens
- Is present at densities correlated with fecal contamination
- Can be used as a surrogate for many different pathogens
- Is appropriate for fresh and saline aqueous environments

[1]The term "indicator bacteria" is used synonymously with fecal indicator bacteria in this section.

This section describes tests that can be completed in the field for identifying and enumerating five types of fecal indicator bacteria: total coliform bacteria, fecal coliform bacteria, *Escherichia coli* (*E. coli*), fecal streptococci, and enterococci (Britton and Greeson, 1989; U.S. Environmental Protection Agency, 1985, 1991a, 1996, 2000, 2002a, b, c, d). Two methods can be used to test for indicator bacteria in the field: (1) the membrane-filtration method (section 7.1.3.C) and (2) the liquid broth method, using the presence-absence format (section 7.1.3.D) or the most-probable-number (MPN) format (section 7.1.3.E). Also included is guidance on how to collect samples to be analyzed for *Clostridium perfringens* (*C. perfringens);* these samples are shipped to a microbiological laboratory for analysis (U.S. Environmental Protection Agency, 1996).

- The presence in water of *E. coli*, and often enterococci, is direct evidence of fecal contamination from warm-blooded animals. Their presence indicates the possible presence of pathogens (Dufour, 1977; Wade and others, 2003). A few strains of *E. coli* are pathogenic, such as *E. coli* O157:H7, but most strains are not.
- Densities of other indicator bacteria (total coliforms, fecal coliforms, and fecal streptococci) can be, but are not necessarily, associated with fecal contamination. Despite this limitation, total coliforms are used to indicate ground-water susceptibility to fecal contamination. Fecal coliforms also are used to assess the sanitary quality of shellfish-growing waters and, in some States, for attainment of recreational-water-quality standards. The use of fecal streptococci generally has been discontinued by the U.S. Geological Survey for water-quality monitoring.
- The presence of *C. perfringens* in water, as spores and (or) vegetative cells, indicates contamination of water with treated or untreated sewage or similar wastes (Bisson and Cabelli, 1980; Fujioka and Shizumura, 1985). *C. perfringens* is used as an alternative indicator of fecal contamination in tropical and subtropical waters because other indicator bacteria may regrow in these environments.

The indicator bacteria used to assess fecal contamination depend on regulations associated with the type of water being tested, which is classified according to its use, as shown below.

Type of water	Description of water type and its use	Federally required indicator bacteria
Ambient water	Any water body encountered in the environment, regardless of use designation.	(Depends on use.)
Recreational water	Water bodies where people engage in, or are likely to engage in, activities that could lead to ingestion of the water or immersion in the water. Recreational water is designated as such in State and Tribal water-quality standards.	Enterococci and *E. coli* — required for ocean and Great Lakes beaches (coastal waters). Requirements for inland beaches are subject to State regulations.
Shellfish-growing water	Any site that supports or could support the propagation and harvesting of shellstock (molluscan shellfish, such as oysters, clams, mussels, and scallops) in the natural environment or at fish farms.	Total coliform and fecal coliform.
Potable (drinking) water	A water supply that meets the requirements of the Safe Drinking Water Act, as administered by the U.S. Environmental Protection Agency and any applicable State or local jurisdictions.	Total coliform. Detection requires follow-up testing for fecal coliform and *E. coli*.
Treated drinking water	Potable water from a public water supply that has been treated by physical or chemical means to improve water quality.	The U.S. Environmental Protection Agency Ground Water Rule for public supply systems includes testing for total coliform, *E. coli*, enterococci, and coliphage viruses.
Public water system	A water system that serves 25 or more people or that has 15 or more service connections and operates at least 60 days per year.	

Water-quality criteria have been developed for densities of indicator bacteria in recreational and ambient waters with designated uses (U.S. Environmental Protection Agency, 1986).

- **Recreational waters.** The U.S. Environmental Protection Agency (USEPA) criteria for indicator bacteria, used to classify the sanitary quality of recreational waters, are shown in table 7.1–1 and are used to develop State standards. ***E. coli* and enterococci are the indicators of sanitary quality most commonly used for recreational waters because both are predictors of swimming-associated gastroenteritis.** In 1986 they replaced total and fecal coliforms and fecal streptococci as the recommended indicator bacteria, as the latter have not been shown to be predictive of swimming-associated gastroenteritis (U.S. Environmental Protection Agency, 1986 and 2000; Cabelli, 1977; Dufour and Cabelli, 1984; Wade and others, 2003). The Beaches Environmental Assessment and Coastal Health Act of 2000 (Public Law 106-284) requires the use of *E. coli* and (or) enterococci to assess water quality of coastal and Great Lakes beaches in all bathing-beach monitoring programs; this became effective May 2004, based on the USEPA criteria of 1986 (table 7.1–1).

  — Enterococci are the preferred indicator bacteria in marine waters because of their salt tolerance.

  — Either *E. coli* or enterococci are recommended for monitoring fresh water (U.S. Environmental Protection Agency, 2004).

Table 7.1–1. Recreational water criteria under the Beaches Environmental Assessment and Coastal Health Act of 2000 (U.S. Environmental Protection Agency, 2004).

[mL, milliliters]

Indicator	Geometric mean: 5 samples (density per 100 mL)	Single-sample maximum: criterion may be exceeded in no more than 10 percent of samples (density per 100 mL)			
		Designated beach area[1]	Moderate use, full-body contact[2]	Light use, full-body contact[2]	Infrequent use, full-body contact[2]
	Fresh water				
Escherichia coli	126	235	298	410	576
Enterococci	33	62	78	107	151
Marine water					
Enterococci	35	104	158	276	501

[1]Designated beach areas are frequently lifeguard protected, provide parking and other public access, and are heavily used by the public (U.S. Environmental Protection Agency, 1986, p. 7).
[2]Other recreational uses, which involve various levels of full-body contact, are designated by individual State water-quality standards (U.S. Environmental Protection Agency, 1986, p. 7).

- **Shellfish-growing area.** Water-quality criteria for shellfish-growing areas have been developed by the U.S. Food and Drug Administration under the National Shellfish Sanitation Program. The 2005 guide for the control of molluscan shellfish (U.S. Food and Drug Administration, 2005) specifies criteria, based on total coliform and fecal coliform densities, to indicate the sanitary quality of water in shellfish-growing areas.

- **Potable water supplies: treated, untreated, private, and public.** Water-quality criteria for drinking water, based on total coliform density, are specified in the Safe Drinking Water Act, as amended in 1986 (U.S. Environmental Protection Agency, 1986).

  — Under the provisions of the Safe Drinking Water Act, all public water supply systems must disinfect their water unless criteria are met that ensure equivalent protection.

  — Under the Total Coliform Rule (U.S. Environmental Protection Agency, 2001), public water supply systems also must monitor distribution systems for contamination. When total coliforms are detected, follow-up tests for fecal coliforms or *E. coli* are required and a more intensive monitoring schedule may be required.

  — Water-quality criteria for ground water are specified in the Ground Water Rule, which was passed by Congress in October 2006. The Rule covers public water systems, which are defined as those that serve 25 or more people or have 15 or more service connections and operate at least 60 days per year. In addition to total coliforms and *E. coli* as indicators of sanitary quality in ground water, the Rule includes enterococci and coliphage viruses (U.S. Environmental Protection Agency, 2006). Ground water typically contains substantially lower densities of indicator bacteria compared to bodies of surface water.

The USEPA maintains a listing of approved methods for microbiological monitoring at http://www.epa.gov/safewater/methods/rules_micro.html (accessed January 21, 2007).

7.1.1 SAMPLING EQUIPMENT AND EQUIPMENT STERILIZATION PROCEDURES

Sterile technique must be followed and documented when collecting and processing samples for fecal indicator bacteria. Specific equipment and supplies are needed for collection of samples and analysis for indicator bacteria by use of sterile technique. The equipment and supplies listed in table 7.1–2 should be sufficient to begin membrane-filtration, presence-absence, or most-probable-number analysis of fecal indicator bacteria in water and sediment. Table 7.1–3 describes equipment cleaning and sterilization procedures.

- **Equipment for the collection and analysis of bacterial samples must first be cleaned and then sterilized** (table 7.1–3). Sterilize the filtration unit and sampling equipment before traveling between sites or before each sample collected at the same site at different times. There are several sterilization methods, but autoclaving is preferred.
- **Quality assurance and quality control of sterilization procedures must be documented.** Keep a logbook of autoclave operation or other sterilization procedure(s) used. In the log, include a brief description of the quality-assurance procedures used and quality-control tests run; note the date, the test results, and the name of the autoclave operator and (or) analyst.
- **If sample water contains residual chlorine or other halogens:** Add sodium thiosulfate ($Na_2S_2O_3$) to the sample bottles before the bottles are autoclaved. Residual chlorine commonly is found in samples collected from sources such as treated drinking water (withdrawn from taps), wastewater effluents, and in the mixing zones directly downstream from wastewater-treatment plants, or from the residue of sodium hypochlorite used to sterilize nonautoclavable sampling equipment (section 7.1.1.D).

Table 7.1–2. Equipment and supplies used for membrane-filtration and liquid broth analyses (presence-absence or most-probable-number format) for fecal indicator bacteria in water or sediment samples.

[TD, to deliver; NIST, National Institute of Standards and Technology; UV, ultraviolet; mENDO, total coliform medium; mTEC, *Escherichia coli* medium; mFC, fecal coliform medium; NFM, *National Field Manual for the Collection of Water-Quality Data;* MPN, most probable number; °C, degrees Celsius; mL, milliliters; nm, nanometers; mm, millimeters; µm, micrometer; psi, pounds per square inch; cm, centimeters]

✓	Item	Description
General equipment and supplies needed for microbiology		
	Autoclave	For sterilization, capable of maintaining 121°C
	Balance	For measuring weight, sensitive to 0.01 gram
	Buffered water	Sterile phosphate-buffered water with magnesium chloride
	Distilled or deionized water	Sterile distilled or deionized water, unbuffered, for use when diluting samples for defined-substrate tests
	Graduated cylinders	Borosilicate glass or plastic, 25 and 100 mL, covered and sterilized
	Incubator	Aluminum heat sink (heater block), or forced-air, or water bath incubator; capable of maintaining specified temperature ranges during incubation (temperature is test-specific)
	Pipets	Sterile, TD, bacteriological or Mohr, glass or plastic with cotton plugs; 1, 10, and 25 mL
	Pipettor or pipet bulb	For drawing liquids into pipets
	Thermometer	Range 30-110°C, glass-alcohol or digital, calibrated in 0.2°C increments, checked against a NIST-certified thermometer
	Ultraviolet lamp, long wave	For use with various tests that result in UV-fluorescent colonies or wells, 366-nm, 6-watt
	Ultraviolet view box	To help with viewing UV-fluorescent test results
	Wrapping for equipment	Kraft paper, aluminum foil, autoclavable plastic bags
Equipment and supplies needed for membrane-filtration analyses		
	Absorbent pads	For use with total coliform test on mENDO medium and with *Escherichia coli* on mTEC medium for urease test
	Alcohol burner	Glass or metal, containing ethanol, for flame sterilizing forceps
	Alcohol bottle	Wide mouth, 100 mL, containing 70 percent ethanol for forceps sterilization
	Cultivation media and amendments	Liquid or solid media and reagents specific to the test method, prepared in advance (NFM 7.1.3.A)
	Counter	Handheld, for counting bacterial colonies
	Dilution bottles	Plastic or glass, 100-mL capacity or greater, with autoclavable screwcaps, filled with 90 or 99-mL buffered water, sterilized and labeled with volume and date
	Filtration assembly	Filter funnel, filter base, and stainless steel, glass, or plastic filter holder; wrapped in aluminum foil, autoclavable bag, or kraft paper; sterile; autoclavable

Table 7.1–2. Equipment and supplies used for membrane-filtration and liquid broth analysis (presence-absence or most-probable-number format) for fecal indicator bacteria in water or sediment samples—*Continued*

✓	Item	Description
	Forceps	Stainless steel, smooth tips
	Hot plate	With magnetic stirrer or boiling water bath for media preparation
	Magnifier	Wide-field type dissecting scope with 5 to 15 magnifications, or equivalent, with fluorescent lamp
	Membrane filters	47-mm, sterile, white, gridded, mixed cellulose ester, cellulose acetate, or cellulose nitrate, 0.45-µm pore size, 0.65-µm may be used with mFC agar
	Culture plates	Sterile, plastic, disposable top and bottom plates, 50 by 12 mm in size
	Vacuum source	Hand pump with gage, electric vacuum, or peristaltic pump; vacuum not to exceed 5 psi or 25 cm mercury
Equipment and supplies needed for liquid broth analyses (presence-absence format)		
	Comparator bottle	Required to evaluate the threshold for a positive reaction when using Colilert-based tests
	Cultivation bottles	Cultivation bottles, greater than 100 mL capacity, with autoclavable screwcaps for cultivation of water samples
	Defined-substrate reagent packs	Single-use snap packs containing defined-substate broth reagents, such as Colilert and Enterolert
Equipment and supplies needed for liquid broth analyses (MPN format)		
	Comparator tray	Required to evaluate the threshold for a positive reaction when using Colilert-based tests
	Defined-substrate reagent packs	Single-use snap packs containing liquid broth reagents, such as Colilert and Enterolert
	Dilution bottles	Glass or plastic, 100-mL capacity or greater, with autoclaveable screwcaps, filled with 90 or 99-mL distilled or deionized water, sterilized
	Quanti-Trays	Quanti-Tray 200 or 2000, depending on target concentration
	Quanti-Tray sealer	Needed to seal sample into Quanti-Trays
Equipment and supplies needed for analyses of indicator bacteria eluted from sediment samples		
	Bottles	Sterile plastic, used to mix sediment with buffer water during elution of bacteria from sediment into buffered water
	Drying dish	Heat-tolerant glass or metal dish used for determination of proportion dry-weight sediment
	Drying oven	Oven capable of maintaining 105°C to measure proportion dry-weight sediment
	Jars	Wide mouth, sterile plastic; used to composite sample in laboratory (if necessary)
	Spatula	Stainless steel, sterile or flame sterilized
	Wrist-action shaker	Used to shake samples during elution of bacteria from sediment into buffered water

- **Equipment that has been decontaminated using a methanol rinse can affect the viability of the microbial population** for which analyses will be performed. Ensure the removal of methanol residue from sampling equipment before samples are collected for bacteria analysis.

  — Allow the methanol to evaporate completely from the interior and exterior surfaces of equipment. In an office setting, filtered argon or nitrogen gas under pressure can be forced through equipment to help evaporate the methanol from interior spaces that cannot be exposed adequately to the atmosphere.

  — After evaporating the methanol, rinse the equipment with pesticide grade blank water (PBW) or volatile/pesticide grade blank water (VPBW) and autoclave the equipment. For nonautoclavable equipment, rinse thoroughly with PBW or VPBW that has been autoclaved. **Methanol-tainted water must be collected and disposed of as a hazardous substance: follow local and (or) State and Federal regulations.**

  — Collection and analysis of a blank sample for volatile organic compound analysis can help document the absence (or presence) of methanol in the sample.

  — Collect bacteria samples last.

  — As an alternative to the procedures described above, use completely separate sampling equipment that is dedicated for microbial sample collection.

- **When using a pump system to collect ground-water samples for both chemical and bacteria analysis,** it is recommended that individual lengths of tubing be dedicated to, and prepared for, each well at which samples will be collected.

  — To clean the tubing, (a) follow the office cleaning procedures described in NFM 3, removing the methanol by pushing it out with at least two tubing volumes of PBW or VPBW or (and) by forcing clean gas through the tubing, as described above; and (b) autoclave the tubing. Be certain, first, that the tubing can be autoclaved. Sterilization by autoclave is the preferred method. If the tubing cannot be autoclaved, use autoclaved PBW or VPBW to push methanol from, and subsequently rinse, the tubing.

— Tubing should be dry if it will be stored and transported under warm conditions, to prevent microbial growth. Forced gas can be used to dry the tubing; however, the tubing should be autoclaved afterwards.

— Between sites, clean the pump using the field procedures described in NFM 3, taking extra care to remove methanol residue from the pump interior either by using a forced gas method or rinsing copiously with autoclaved VPBW or PBW.

Store and transport sterile equipment in a sterile container.

Table 7.1–3. Equipment cleaning and sterilization procedures

[DIW, distilled or deionized water; mL, milliliter; $Na_2S_2O_3$, sodium thiosulfate; L, liter; EDTA, ethylenediaminetetraacetic acid; UV, ultraviolet light; psi, pounds per square inch; °C, degrees Celsius]

Equipment	Procedures
Cleaning	
All equipment (this includes water-level tape measure, all sample-collection and sample-processing equipment used in the field and laboratory)	Wash equipment thoroughly with a dilute, nonphosphate laboratory-grade detergent. Rinse three times with tap water. Rinse again three to five times with DIW Wipe the wetted portion of water-level tapes with disinfectant (0.005 percent bleach solution or 70-percent methyl or ethyl alcohol) and rinse thoroughly with sterile water.
Sterilization (Refer to table 7.1–4 for recommended times for autoclaving glassware, liquids, and other media and materials.)	
Magnetic plastic filtration field units, glass, plastic, and Teflon bottles and containers, volumetric flasks, pipets and pipettors, and other autoclavable materials	If sample may contain residual chlorine or other halogens, add 1 mL of 10-percent $Na_2S_2O_3$ solution per liter volume of sample. This can be added to the sample bottle before autoclaving. If sample may contain toxic trace metals, add 3 mL of a sterile 15-percent EDTA stock solution per 1 L of sample. This can be added to the sample bottle before autoclaving. Wrap equipment in kraft paper, aluminum foil, or place into autoclavable bags. Autoclave at 121°C, 15 psi, for 15 minutes. **NOTE:** If an autoclave is not available, refer to sections 7.1.1.B, 7.1.1.C, and 7.1.1.D for alternative sterilization techniques.

Table 7.1–3. Equipment cleaning and sterilization procedures—*Continued*

Equipment	Procedures
Stainless steel filtration field units	Autoclave at 121°C, 15 psi, for 15 minutes, flame sterilize with methanol (Millipore Hydrosol® units only, section 7.1.1.B), or use ultraviolet irradiation for 15 minutes (section 7.1.1.C).
Portable submersible pumps and pump tubing	**Autoclavable equipment** (preferred): Wrap components in kraft paper, aluminum foil, or place into autoclavable bags. Autoclave at 121°C, 15 psi, for 15 minutes. **Non-autoclavable equipment:** (1) Submerge sampling system in pH-neutral sodium hypochlorite solution prepared from household laundry bleach (section 7.1.1.D). (2) Circulate solution through pump and tubing for 30 minutes. (3) Follow step 2 by thoroughly rinsing, inside and out, with a working solution of 1 mL 10-percent sterile $Na_2S_2O_3$ per liter of water, and circulate solution for 5 minutes. (4) Pump $Na_2S_2O_3$ to waste, then circulate sterile DIW through pump, followed by sample water pumped from the well. Dispose of waste solutions according to regulatory requirements. **CAUTION:** Prolonged or repeated use of a hypochlorite solution on interior or exterior metallic surfaces of a pump can cause corrosion or other damage to the pump and compromise the quality of samples collected for a trace-element or organic-compound analysis.

To prepare a 10-percent stock solution of sodium thiosulfate ($Na_2S_2O_3$) for treatment of samples:

1. Dissolve 100 grams (g) of $Na_2S_2O_3$ into 500 milliliters (mL) of deionized or distilled water; stir until dissolved, and fill a flask to 1,000 mL (Bordner and Winter, 1978, p. 6; American Public Health Association and others, 1998, p. 9-19). Sterilize by autoclaving (table 7.1-3).

2. Dispense 1 milliliter (mL) of 10-percent $Na_2S_2O_3$ stock solution for every liter of sample (final concentration is 0.01 percent).

3. Store the $Na_2S_2O_3$ stock solution at room temperature in a tightly capped bottle that is labeled with its contents and expiration date. Discard after 6 months and prepare a fresh solution.

- **If sample water contains toxic trace metals:** Add ethylenediaminetetraacetic acid (EDTA) to sample bottles when water to be collected contains toxic concentrations of trace metals. EDTA can be combined with the $Na_2S_2O_3$ solution in the sample bottle before sterilization.

  Although thresholds for toxic concentrations vary somewhat in the literature, trace metals such as copper, nickel, or zinc that are present at concentrations greater than 10 to 1,000 micrograms per liter (µg/L) are generally toxic to bacteria (Britton and Greeson, 1989, p. 5; Bordner and Winter, 1978, p. 6; American Public Health Association and others, 1998, p. 9-19). Toxic concentrations may be found in urban runoff samples or industrial effluents. When in doubt, add EDTA to sterilized sample bottles before adding the water sample.

To prepare a 15-percent stock solution of EDTA or treatment of sample bottles:

1. Dissolve 100 g of EDTA in 90 mL of deionized or distilled water; stir until dissolved, and fill a flask to 100 mL. Adjust to pH 6.5 and sterilize by autoclaving (table 7.1-3).
2. Dispense 3 mL of the 15-percent EDTA stock solution per 1 liter (L) of sample (American Public Health Association and others, 1998, p. 9-19).
3. Store the EDTA stock solution at room temperature in a tightly capped bottle that is labeled with its contents and expiration date. Discard after 6 months and prepare a fresh solution.

7.1.1.A AUTOCLAVING

Autoclaves that have temperature, pressure, and dry-utensil-cycle controls are recommended. In addition, a liquid-cycle control is needed for autoclaving liquids. Steam sterilizers, vertical autoclaves, and pressure cookers without temperature controls are not recommended.

- Take care to ensure that materials to be autoclaved, such as tubing and containers, are thermally stable. Plastic polymers that can be autoclaved include polycarbonate, polypropylene, polyallomer, polymethylpentene, Teflon® and Tefzel®. Each material type has different thermal characteristics and tolerances to repeated autoclaving.

- Before autoclaving, wrap clean equipment in Kraft paper, autoclavable plastic bags, or aluminum foil. Wrap loosely to allow steam to penetrate the wrapping. Cap tubing ends with aluminum foil.
  - Sterilize and store the equipment in a clean area.
  - Resterilize equipment if the foil, bag, or Kraft paper is torn.
- Consult table 7.1–4 for recommended times for autoclave sterilization of various media and materials.
  - Liquids must be exposed to 121°C at 15 psi (pounds per square inch (lbs/in^2)) for the specified time for effective sterilization—larger volumes of liquid take longer to reach 121°C.
  - If the autoclave does not reach the specified temperature and pressure or fails a quality-control test, service the autoclave and then resterilize all materials (American Public Health Association and others, 1998, p. 9-2 to 9-14).
- In addition to the guidance listed above, it is necessary to:
  - Use sterilization indicator tape with each load.
  - Use commercially available biological indicators at least quarterly to test autoclave performance. Biological indicators are composed of endospores—living cells that are resistant to heat but are killed by effective autoclaving.
  - Drain the autoclave daily. Clean with mild soap and water once per week during periods of daily use. Record cleaning procedures in the logbook.
  - Avoid overloading the autoclave with equipment or materials; overloading will result in incomplete sterilization.

Table 7.1–4. Recommended times to autoclave media and materials.
[°C, degrees Celsius; mL, milliliters]

Media or material	Autoclave time[1]
Glassware and other dry materials	15 minutes
Liquid, 250 mL	15 minutes
Liquid, 500 to 2,000 mL	30 minutes
Liquid, 2,000 to 6,000 mL	15 minutes per 1,000 mL
Liquid, greater than 6,000 mL	90 minutes
Carbohydrate-containing media	15 minutes
Contaminated materials and discarded cultures	45 minutes

[1]Timing should begin after the autoclave reaches operating temperature (121°C).

Autoclaving is the preferred method for sterilizing equipment that is used to test for fecal indicator bacteria.

7.1.1.B FLAME STERILIZATION OF THE HYDROSOL® FIELD FILTRATION UNIT

The Millipore Hydrosol® field filtration units are designed to be flame sterilized with methanol. Formaldehyde gas, a by-product of methanol combustion, kills all bacteria in the unit. However, the use of autoclavable units is preferred over flame-sterilized units because of safety concerns. If an autoclave is not available, presterilized disposable funnels are a safe alternative.

The following sterilization procedure is acceptable for the Hydrosol unit (fig. 7.1–1) in field situations where other sterilization techniques are not practicable (Millipore, 1973, p. 48–49).**When following these procedures, work in a ventilated area and wear appropriate protective equipment such as safety glasses, face mask, and gloves. Avoid breathing noxious fumes.**

CAUTION:
When flame sterilizing, have proper safety equipment such as a fire extinguisher on hand, and implement procedures carefully.

To flame sterilize the Hydrosol unit, carefully:

1. Remove the clean, dry stainless steel flask from the base of the filter-holder assembly.
2. Saturate the asbestos ring (wick) around the base assembly with methanol dispensed from a squeeze bottle or with an eye dropper.
3. Ignite the methanol on the asbestos wick and allow the wick to burn for 30 seconds.

4. Invert the stainless steel flask over the funnel and the burning asbestos ring, and seat the flask on the base of the filter-holder assembly. Leave the flask in place for 15 minutes. Before filtering the next sample, rinse the flask and funnel thoroughly with sterile buffered water to remove all residues of formaldehyde.
5. Repeat the sterilization procedure before processing the next sample.

Figure 7.1–1. Procedure to flame sterlize the Millipore Hydrosol® field filtration unit.

7.1.1.C STERILIZATION OF EQUIPMENT BY ULTRAVIOLET IRRADIATION

Ultraviolet (UV) germicidal irradiation makes use of short-wave UV light (specifically, 254 nanometers) to disinfect equipment (table 7.1–2) and should not be confused with the long-wave UV light (366 nanometers) used to detect positive reactions in various analytical methods (see table 7.1–2, and table 7.1–9 in section 7.1.3.C). Several commercial units are specifically designed to field-sterilize stainless-steel filtration units. Manufacturers' recommendations should be followed when using these sterilization units and equipment should be tested for sterility following treatment. **Since UV light does not penetrate most materials (even most clear plastic and glass) only surfaces that are directly exposed to UV light are properly sterilized.**

7.1.1.D STERILIZATION OF EQUIPMENT WITH SODIUM HYPOCHLORITE

A solution of sodium hypochlorite (bleach) is used to sterilize equipment that is non-autoclavable or to sterilize equipment in the field when an autoclave is not readily available. Sodium thiosulfate is used to remove residual chlorine after sterilization.

1. Prepare a working solution of 50 mg/L (0.005 percent) sodium hypochlorite from household bleach by adding 1 mL of fresh household bleach per liter of distilled or deionized water. Most household bleach is 5 to 7 percent sodium hypochlorite (50,000 to 70,000 mg/L), **but household bleach that has been opened for more than 60 days before use may not be full strength.** Prepare fresh working solutions with each use, because the concentration will diminish with time. (U.S. Environmental Protection Agency, 1982, p. 253 and 1996, p. VIII-41).
2. Adjust the pH of the working solution from pH 6 to pH 7 with 1 *Normal* hydrochloric acid (1 *N* HCL). The 1 *N* HCL can be purchased from a commercial supplier of scientific products. The unadjusted pH of bleach is approximately 12, a pH at which the hypochlorite ion has limited germicidal activity (U.S. Environmental Protection Agency, 1996).

3. Clean the equipment and submerge it in the sodium hypochlorite solution, or completely fill the equipment with the sodium hypochlorite solution. Maintain contact for 30 minutes.
4. Remove or drain the equipment.
5. Thoroughly rinse the equipment, inside and out, with sterile $Na_2S_2O_3$ solution (prepared as 1 mL of 10-percent stock per liter of water) to remove residual chlorine. Maintain contact for 5 minutes.
6. Remove or drain the equipment.
7. Rinse the equipment thoroughly with sterile deionized or distilled water.
8. If adding EDTA to the sample bottle, use a sterile pipet and sterile EDTA.

CAUTION:
Prolonged or repeated use of a sodium hypochlorite solution on interior or exterior metallic surfaces of equipment can cause corrosion or other damage and compromise the quality of samples collected for a trace-element or organic-compound analysis.

7.1.2 SAMPLE COLLECTION, PRESERVATION, STORAGE, AND HOLDING TIMES

Sterile conditions must be maintained during collection, preservation, storage, and analysis of indicator bacteria samples. Specific procedures have been developed that must be strictly followed; these vary with types of equipment and sample source (surface water, ground water, treated water, or wastewater) (table 7.1–5).

Methanol residue (from decontamination of equipment used for sampling organic compounds) can kill bacteria. If sampling with equipment that has been exposed to methanol, take extra care or use special procedures to ensure that the methanol has completely evaporated from all exterior and interior surfaces of the equipment (see section 7.1.1). Collect the bacteria sample after collecting samples for other analyses.

Table 7.1–5. Summaries of equipment for sample collection, procedures for sample preservation, and holding times for indicator bacteria

[EWI, equal-width increment; EDI, equal-discharge increment; L, liter; mL, milliliter; EDTA, ethylenediaminetetraacetic acid; °C, degrees Celsius; *E. coli*, *Escherichia coli*; *C. perfringens*, *Clostridium perfringens*]

Equipment for sample collection
To collect EWI or EDI surface-water samples: US D-95, US DH-95, or US DH-81 with sterile, 1-L wide-mouth bottle, with sterile caps and nozzles. US D-96 with sterile autoclavable bag.
To collect surface-water and ground-water samples using a pump, point samplers from a tap, or by the hand-dipped method: a sterile container, 125-, 250-, 500-, or 1,000-mL capacity, depending on the number of tests and samples.
All containers must be composed of sterilizable materials such as borosilicate glass, polypropylene, stainless steel, or Teflon®.
Procedures for sample preservation
If necessary, add 1 mL of a 10-percent sodium thiosulfate solution per 1 L of sample for halogen neutralization (see section 7.1.1).
If necessary, add 3 mL of a 15-percent EDTA stock solution per 1 L of sample for chelation of trace elements (see section 7.1.1).
Chill all samples at 1 to 4°C before analysis.
Maximum holding times for indicator bacteria
A 30-hour holding time after sample collection for total coliform bacteria, fecal coliform bacteria, and *E. coli* collected from **drinking-water sources** (Bordner and Winter, 1978 p. 30).
A 6-hour holding time for *E. coli*, fecal coliform bacteria, total coliform bacteria, and enterococci in **nonpotable water for compliance purposes** (American Public Health Association and others, 1998, p. 9.21).
A 24-hour holding time for *E. coli*, fecal coliform bacteria, total coliform bacteria, enterococci, and fecal streptococci in **water for noncompliance purposes** (American Public Health Association and others, 1998, p. 9.21).
A 24-hour holding time for *C. perfringens*. A 6-hour maximum holding time after sample collection for *C. perfringens* is recommended if comparisons between *C. perfringens* and other fecal-indicator bacteria collected at the same time are planned (U.S. Environmental Protection Agency, 1996, p. XI-8).
A 24-hour holding time between **bed sediment** collection and initiation of analysis of fecal-indicator bacteria. Do not exceed the recommended 24-hour holding time.

7.1.2.A SURFACE-WATER SAMPLE COLLECTION

The areal and temporal distribution of bacteria in surface water can be as variable as the distribution of suspended sediment because bacteria commonly are associated with solid particles. To obtain representative data for bacteria analysis, follow the same methods used to collect surface-water samples for suspended sediment analysis (NFM 4.1 and table 7.1–4). Sample bottles are not to be field rinsed with native water but should be autoclaved or otherwise sterilized before use.

- **Flowing water**—use depth- and width-integrating sampling methods (NFM 4.1.3.A).[2]
- **Still water** (lakes or other surface-water conditions for which depth- and width-integrating methods may not be applicable)—use the hand-dip method or a sterile point sampler (NFM 4.1.3.C). It may be necessary to collect multiple samples across the depth or area of the targeted lake volume to accomplish data-quality objectives.

Wear laboratory-type gloves and avoid sample contact with eyes, nose, mouth, and skin when working in and with contaminated waters.

[2]Sample-collection methods may be modified to ensure consistency with study objectives and as appropriate for site conditions.

Depth- and width-integrating methods

Depth- and width-integrating sampling methods (the equal-discharge increment (EDI) method or the equal-width increment (EWI) method) are the standard U.S. Geological Survey (USGS) methods used when sampling flowing waters and are required unless study objectives or site characteristics dictate otherwise (NFM 4.1.3.A and table 7.1–5).

- The EDI method is preferred to the EWI method for sites where there is some knowledge of the distribution of streamflow in the cross section; for example, at a gaging station with a long period of discharge record (Edwards and Glysson, 1999).
- Select the appropriate sampler and equipment (recommended sampling devices may change as a result of technological advances or other considerations—check for updates in NFM 2.1 and 4.1). **Sampling equipment that comes in contact with the sample water must be sterile,** including the collection bottle, nozzle, and cap (or bag for the bag sampler) (table 7.1–3).
  - For streams with depths of 5 meters (m) or less, use a US D-95, US DH-95, or a US DH-81 sampler (NFM 2.1.1).
  - For stream sections where depths exceed 5 m, use the US D-96, with either autoclavable Teflon® bags or autoclavable cooking bags. Thermotolerant polymers are described in 7.1.1, "Sampling Equipment and Equipment Sterilization Procedures."
  - For wide channels, several samples—each composed of subsamples composited into a sterile large-volume container—may be needed. A sterile 3-L or larger bottle may be used to composite subsamples.
  - For narrow channels, collect subsamples at 5 to 10 or more vertical locations in the cross section without overfilling the bottle.
  - Use the proper nozzle size and transit rate for the velocity conditions in the section to ensure isokinetic collection of the sample (NFM 2.1 and 4.1).

Point-sampling methods

If the stream depth and (or) velocity is not sufficient to use a depth- and width-integrating method to collect a sample, use the hand-dip method (table 7.1–5). Sampling at depth in lakes, reservoirs, estuaries, and oceans often requires a sterile point sampler. Niskin, ZoBell, and Wheaton point samplers, for example, hold a sterile bottle or bag.

To collect a hand-dipped sample:

1. Grasp the bottle near the base with hand and arm on the downstream side of the bottle.
2. One of two methods may be used to avoid collecting surface scum: (a) submerge the bottle with cap on and remove the cap underwater to collect the sample, or (b) plunge the open bottle mouth quickly downward below the water surface. Lower the bottle in a manner that avoids contact with or disturbance of the streambed.
3. Allow the bottle to fill with the opening pointed slightly upward into the current.
4. Remove the bottle with the opening pointed upward toward the water surface and tightly cap it, allowing about 2.5 to 5 centimeters (cm) of headspace for proper mixing (American Public Health Association and others, 1998, p. 9-19; Bordner and Winter, 1978, p. 8). Another option would be to cap the bottle underwater. When the bottle is out of the water, uncap it and pour off enough water to allow adequate headspace for mixing. Then recap the bottle.

CAUTION:
Do not sample in or near a water body without wearing a correctly fitted personal flotation device (PFD).

Special considerations for beach-water sampling. The steps below will aid in collecting samples for use in support of beach closure or posting decisions for swimming or other full-body-contact recreation. Sampling procedures for other purposes are based on project objectives.

1. Collect samples in the area used for swimming at 0.7- to 1-m water depths, maintaining consistency in water depth throughout the sampling period. The sample typically is taken 15 to 30 cm below the water surface using the hand-dip method. Position the bottle to collect the sample from any incoming current (U.S. Environmental Protection Agency, 2002e). Avoid contaminating the water sample with bottom material kicked up from the bottom while sampling.
2. At some beaches, multiple samples may be needed to adequately represent overall water-quality conditions. Producing a composite from multiple samples on an equal-volume basis may provide results that are as accurate as those obtained by averaging analyses from multiple points.
3. **A Chain-of-Custody record is recommended for beach sampling done in support of beach closures or posting of warnings to swimmers** (U.S. Environmental Protection Agency, 2002e, Appendix J).

Quality control in surface-water sampling. Depending on the data-quality requirements of the study and site conditions, quality-control samples will include field blanks, equipment and procedure blanks, field replicates, and positive and negative control samples (controls). Quality-control terms (shown below in bold type) are defined at the beginning of NFM 7, in "Conversion Factors, Selected Terms, Symbols, Chemical Formulas, and Abbreviations."

- **Field blanks—**Collect and analyze field blanks at a frequency of one blank for every 10 to 20 samples, or as required by the data-collection objectives of the study, to document that the sampling and analysis equipment have not been contaminated. If sampling for compliance with beach regulations, at a minimum collect a field blank at the beginning, middle, and end of the sampling season.
    1. Pass sterile buffered water (for membrane filtration) or sterile distilled or deionized water (for liquid broth tests) through sterile sampling equipment and into a sterile sampling container.
    2. Analyze field blanks for fecal indicator bacteria. If no growth is observed, then the sample was collected by use of sufficiently sterile procedures.

- **Analytical blanks—**Process **filter blanks** and **procedure blanks** (for membrane filtration) or **method blanks** (for liquid broth tests) during sample processing to document that equipment and rinse or dilution water were sterile.
  - — A filter blank is processed for each sample before the sample is filtered.
  - — A procedure blank is processed through the filtration unit after the sample has been filtered, at a frequency of one blank for every 10 to 20 samples.
  - — Method blanks are processed at a frequency of one blank for every 10 to 20 samples.
- **Field concurrent replicates—**Collect and analyze one field replicate for every 10 to 20 samples. A split concurrent replicate is recommended. Two samples are collected and each sample is analyzed in duplicate by membrane filtration. Replicate data are used to quantify the uncertainty in density estimates (see Francy and Darner, 1998, for an example).
- **Positive and negative controls—**These types of quality-control samples are required if media are prepared from basic ingredients by field or laboratory personnel, and are recommended if media are purchased from a commercial supplier. The frequency of analysis depends on project objectives and the type of medium. Positive controls test the medium's ability to recover target bacteria; negative controls are used to ensure that nontarget bacteria are inhibited or recognized. Refer to section 7.1.3.A for details.

7.1.2.B GROUND-WATER SAMPLE COLLECTION

As with surface water, most bacteria in ground water are associated with solid particles. Collecting a 100-mL ground-water sample for bacteria analysis is standard procedure because ambient ground water flowing through aquifers typically contains much fewer particulates, and bacteria density is expected to be low. Applying the protocols for purging wells before collecting water-quality samples (NFM 4.2) is necessary to ensure that the particulate content and bacteria density sampled represent ambient aquifer conditions.

- When using the same sampling equipment for chemical and bacteria analyses, give special consideration to the effect of equipment-preparation procedures on sample integrity (section 7.1.1).
  - Sampling equipment that has been sterilized for microbial sample collection using chlorinating and dechlorinating agents can affect the chemistry of samples collected for analyses of some inorganic analytes.
  - Equipment subjected to a methanol rinse for decontamination for organic-compound sample collection can affect the viability of the microbial population for which analyses will be performed.
  - **Recommendation:** Prepare separate tubing lengths that are designated for the sole use of sampling at a specified well. Clean the tubing at the office or office laboratory. Tubing should be autoclaved after routine cleaning, if possible (section 7.1.1).
- Collect bacteria samples last.
- If a different sampler will be used for bacteria sampling, remove at least one well volume of well water and compare the turbidity and dissolved-oxygen measurements with those recorded after purging the well with the first sampler, to ensure collection of a sample that represents ambient ground-water quality.

Supply wells

Selection of a sampling strategy for supply wells (NFM 4.2) depends, in part, on the objectives of the study. For all objectives, select a tap (spigot) that supplies water from a service pipe connected directly to the main: **do not use a tap that leaks or one that is attached to a pipe served by a cistern or storage tank** (American Public Health Association and others, 1998, p. 9-19 to 9-20; Britton and Greeson, 1989, p. 5; Bordner and Winter, 1978, p. 5-16).

- For aquifer-monitoring studies, locate the point of ground-water withdrawal upgradient of (before the water reaches) a chlorination or other treatment system (unless study objectives dictate otherwise).
- For drinking-water studies, sample the ambient water in the well regardless of the history of treatment. Dechlorination with $Na_2S_2O_3$ is required if the sample is collected after the water has passed through a chlorination unit (section 7.1.1).

To sample a supply well for indicator bacteria:

1. Before collecting the sample, remove screens, filters, or other devices from the tap.
2. Swab or spray the inside and outside rim of the tap with ethanol. If possible, flame sterilize the tap and allow it to dry and cool. Rinse the tap with sterile deionized or distilled water.
3. Supply wells commonly are equipped with permanently installed pumps.
   - If the well is pumped daily, then purge the tap water for a minimum of 5 minutes, discarding the purged water appropriately. Monitor field measurements and record stabilized values (NFM 6.0).
   - If the well is used infrequently, then purge the tap until a minimum of three well volumes are purged and stable field measurements are obtained in sequential measurements (NFM 4.2 and 6.0).
4. Collect a sample directly from the tap into a sterile bottle without splashing or allowing the sample bottle to touch the tap.

Monitoring wells

If a well used to monitor ground-water quality does not have an in-place pump, then obtain samples by using a portable sampler, such as a submersible pump or a bailer (U.S. Environmental Protection Agency, 1982). If possible, autoclave or disinfect the sampling devices and the sample line (table 7.1–3). If disinfected with a sodium hypochlorite solution, then the sampler and sample line must be dechlorinated and rinsed with sterile deionized or distilled water. In either case, finish by flushing the sampler and sample line with native ground water before samples are collected into sterile bottles.

- ▶ Use autoclavable samplers, if possible. After flushing the sterilized pump lines with sample water, collect the sample directly into the sterile sample bottles.
- ▶ Check data-quality objectives before using a disinfectant. Disinfectants are corrosive; they can damage the metal parts of a pump, and can render the pump inadequate for sampling trace-element and other constituents.

- Some sampling equipment does not require chlorine disinfection. If the water level in a well is less than 7 to 10 m (roughly 20 to 30 ft) below land surface, then a sample can be collected without contamination and without chlorine disinfection by use of a peristaltic or vacuum pump, as long as the tubing is sterile.
- If sampling equipment has been in contact with methanol, implement the methanol removal techniques described in section 7.1.1.

To disinfect a pump with a sodium hypochlorite solution:

1. Follow the instructions for cleaning equipment with a hypochlorite solution (bleach) (section 7.1.1.D and table 7.1–3)
2. Lower the pump carefully into the well. Purge the residual chlorine and $Na_2S_2O_3$ from the system by pumping three tubing volumes of well water through the system; contain or appropriately discard this waste water. Take care not to contaminate samples for chemical analysis with residual chlorine or $Na_2S_2O_3$. **The pump must have a backflow check valve (an antibacksiphon device) to prevent residual chlorine from flowing back into the well.**

To use a pump that cannot be disinfected:

1. Clean equipment as thoroughly as possible (section 7.1.1).
2. Handle the pump and tubing carefully to avoid contamination. If the pump is a downhole dedicated pump, skip to step 4.
3. Collect field blanks through the sampling equipment.
4. Lower the pump in the well to the desired intake location.
5. Purge the well with the pump to thoroughly flush the pump and tubing with aquifer water before sampling (NFM 4.2 and 6.0).
6. An alternative to sampling with the pump is to remove the pump after purging the well. Complete the collection of other samples, and then collect the bacteria sample using a sterile bailer (U.S. Environmental Protection Agency, 1982, p. 252–253). When using the bailer method, the potential for bias exists from stirring up particulates to which bacteria may adhere during pump removal and bailing that would not otherwise be included in the sample.

Sample-preparation activities, such as purging, must be carried out in such a way as to avoid contaminating the well, the equipment, or the samples. Avoid collecting surface film from the well water in the sample, and ensure that the sampler intake is within that portion of the screened interval targeted for study.

- Select a point sampler, such as a bailer with a double-check valve.
- Use only bailers that can be appropriately sterilized; preferably autoclaved.

Be aware that the type of well, its use, construction, composition, and condition can lead to alteration or contamination of the ambient aquifer water that enters the well. For example, a poor surface seal around the well opening can allow contaminants to move quickly from the land surface to the well water.

Exercise the following precautions when collecting samples from monitoring wells:

1. Avoid collecting samples from wells with casings made of galvanized materials; such casings can contain bactericidal metals. If samples must be collected from these types of wells, add 3 mL of EDTA solution per 1 L of sample to the sample bottle prior to autoclaving (section 7.1.1). Collect the sample directly into the bottle.
2. Purge the well (NFM 4.2.3) while monitoring field measurements. Measurements of turbidity and dissolved oxygen are especially relevant. For wells in which field measurements do not stabilize after increasing the total number of measurements, record the problem and the measurements and proceed with sampling.

Quality control for ground-water sample collection. Depending on the data-quality requirements of the study, quality-control samples include pump, filter, procedure, and method blanks; field replicates; and positive and negative controls. Quality-control terms (shown below in bold type) are defined at the beginning of NFM 7 in "Conversion Factors, Selected Terms, Symbols, Chemical Formulas, and Abbreviations."

- **Pump blanks—**This type of blank should be collected ahead of sampling so that results can be evaluated before field sampling. Thereafter, collect pump blanks with ground-water samples at a frequency of one blank for every 10 to 20 samples, or as required by the data-quality objectives of the study. Collect pump blanks by passing sterile buffered water (for membrane filtration) or sterile distilled or deionized water (for liquid broth tests) through the sampling equipment and into a sterile sampling container. A standpipe may be used to collect a pump blank, but it first must be cleaned and disinfected. Analyze pump blanks for fecal indicator bacteria and record results. If no growth is observed, the use of sufficiently sterile procedures is confirmed and documented.

- **Analytical blanks—**Process **filter blanks** and **procedure blanks** (for membrane filtration) or **method blanks** (for liquid broth tests) during sample processing to document that the equipment and the rinse or dilution water were sterile. A filter blank is processed for each sample before the sample is filtered. A procedure blank is processed through the filtration unit after the sample has been filtered, at a frequency of one blank for every 10 to 20 samples. Method blanks also are processed at a frequency of one blank for every 10 to 20 samples.

- **Field sequential replicates**—Because few ground-water samples test positive for indicator bacteria, it may be necessary to collect field sequential replicates for every sample. A lower frequency may be used if a large percentage of wells are positive or study objectives do not require quantification of variability.

- **Positive and negative controls—**These types of quality-control samples are required if media are prepared from basic ingredients, and is recommended if the medium is purchased from a commercial supplier. The frequency of analysis depends on project objectives and the type of medium. Positive controls test the medium's ability to recover target bacteria; negative controls are used to ensure that nontarget bacteria are inhibited or recognized. Refer to section 7.1.3.A and table 7.1–6 for details.

7.1.2.C BED-SEDIMENT SAMPLE COLLECTION

Due to the spatial heterogeneity of bacteria in sediments, three bed-sediment samples should be collected from each site at the same depth and composited (Francy and Darner, 1998). One of two sampling methods can be used, depending on the depth of the water: (1) sampling by wading, or (2) using a sampler for deep-water sites.

To sample by wading. Use three sterile, plastic, wide-mouthed, 125-mL or 250-mL jars are used for sample collection.

1. Secure the lid on a sterile jar and plunge to the bottom.
2. Upon reaching the bottom, open the jar and scoop the bed sediments into the jar.
3. To minimize contamination by overlying water, secure the lid before surfacing.
4. Repeat for the remaining two jars.
5. Immediately place the jars on ice in a cooler and keep them chilled until the samples are processed.

To use a sampler for deep-water sites. Select the grab sampler that is most appropriate for the site to be sampled (for example, Ponar, and Petite Ponar, Van Veen, and Ekman samplers). These heavy devices collect sediment samples by biting down into bottom materials and closing tightly to hold the sample. Collect and composite three grab samples into one sterile jar in the field as follows:

1. The sampler needs to be sterilized before collecting samples for bacterial analysis. Because of the dimensions of these samplers, autoclaving generally is not practical. **If more than one site is to be sampled, the sampler needs to be resterilized in the field at each of the sites.** To field-sterilize the sampler:
    a. Put on laboratory gloves.
    b. Wash and scrub the sampler in dilute nonphosphate, laboratory-grade detergent and rinse with tap water and then deionized or distilled water.
    c. Soak the sampler in a 0.005-percent sodium hypochlorite solution for 15 minutes (section 7.1.1.D)
    d. Soak in a sterile 0.01-percent $Na_2S_2O_3$ solution for 5 minutes (section 7.1.1).

2. Lower the sampler through the water column and collect the sediment sample according to the manufacturer's instructions.
3. Drain off excess water. Deposit the sediment into a clean, sterile washtub.
    a. Sterilize the washtub by (1) autoclaving, if possible, or (2) following procedures for sterilization with sodium hypochlorite (section 7.1.1.D).
    b. Once sterilized, store washtubs individually in new, clean plastic bags (such as garbage bags) until ready for use.
4. Collect two more grab samples from the same site and deposit in the same washtub. Since samples will be composited, the sampler does not need to be resterilized between collection of each of the three subsamples.
5. Use a sterile spatula to mix the three samples thoroughly and then deposit a portion into a sterile jar. For indicator-bacteria analysis, collect at least 200 g of sediment.
6. Immediately refrigerate or place the samples on ice in a cooler until the samples can be processed. See section 7.1.2.D for sample-preservation and holding-time requirements.
7. Sterilize the sampler before using it at another site (section 7.1.1.D).

Quality control for bed-sediment sample collection. Depending on the data-quality requirements of the study, quality-control samples include field blanks, filter and procedure blanks or method blanks, field replicates, and positive and negative controls. Quality-control terms (shown below in bold type) are defined at the beginning of NFM 7 in "Conversion Factors, Selected Terms, Symbols, Chemical Formulas, and Abbreviations."

- **Field blanks**—Collect and analyze field blanks when using a sampler to collect bed-sediment samples at a frequency of one blank for every 10 to 20 samples, or as required by study objectives, to document that sampling and analysis equipment have not been contaminated. Process field blanks before sample collection if the sampler does not need to be sterilized in the field. If the sampler does need to be sterilized in the field, process field blanks after collection of a sample and resterilization of the sampler. This will demonstrate that the field-sterilization procedure is working appropriately.

    1. Pass sterile buffered water (for membrane filtration) or sterile distilled or deionized water (for liquid broth tests) over the sterile sampler and into a sterile washtub. Collect the field blank into a sterile bottle or jar.
    2. Analyze field blanks for fecal indicator bacteria. If no growth is observed, then the sample was collected by use of sufficiently sterile procedures.

- **Analytical blanks—**Process **filter blanks** and **procedure blanks** (for membrane filtration) or **method blanks** (for liquid broth tests) during sample processing to document that the equipment and the rinse or dilution water were sterile. A filter blank is processed for each sample before the sample is filtered. A procedure blank is processed through the filtration unit after the sample has been filtered, at a frequency of one blank for every 10 to 20 samples, or as otherwise required. Method blanks also are processed at a frequency of one blank for every 10 to 20 samples.

- **Field replicates**—Collect and analyze one field replicate for every 10 to 20 samples, or as otherwise required by study objectives. A split sequential replicate is recommended. For samples collected from wading sites, an additional three jars of sediment are collected and treated as a separate sample. For samples collected using a sampler, the sampler is resterilized before collection of the replicate; the sediment is deposited into a new, sterile washtub, composited in the field, and treated as a separate sample. In the laboratory, each sequential replicate is analyzed twice to produce a total of four split sequential replicate samples.

- **Positive and negative control samples—**These types of quality-control samples are required if media are prepared from basic ingredients by field or laboratory personnel and recommended if media are purchased from a commercial supplier. The frequency of analysis depends on project objectives and the type of medium. Positive control samples test the medium's ability to recover target bacteria; negative control samples are used to ensure that nontarget bacteria are inhibited or recognized. Refer to section 7.1.3.A for details.

SAMPLE PRESERVATION, STORAGE, AND HOLDING TIMES 7.1.2.D

After collection, immediately chill samples in an ice chest or refrigerator at 1 to 4°C. **Do not freeze samples. Process water samples as quickly as possible; store on ice if not analyzed within 1 hour of collection** (American Public Health Association and others, 1998, p. 9-21). Adhering to holding times minimizes changes in the density of indicator bacteria; however, for non-compliance ambient monitoring a longer holding time may be used as long as it is consistently maintained and documented (Pope and others, 2003). Holding times for indicator bacteria are summarized in table 7.1–5.

- For treated drinking water, do not exceed 30 hours before initiation of analysis.
- For nonpotable water for compliance purposes, analyze samples within 6 hours of collection.
- For other types of water for noncompliance purposes, samples should be analyzed within 24 hours of collection.
- *C. perfringens* spores can survive for extended periods of time, and a 24-hour holding time is acceptable if a relation between *C. perfringens* and other fecal indicator bacteria is not part of the planned study; otherwise, observe the same holding time as for the other indicators (U.S. Environmental Protection Agency, 1996). An acceptable holding time for bed-sediment samples is 24 hours.
  - *C. perfringens* is analyzed at the laboratory, and not in the field. Information on analysis of *C. perfringens* is available at http://oh.water.usgs.gov/micro/clos.html (accessed January 16, 2007).
  - Ship samples for analysis of *C. perfringens* to the laboratory in a double-bagged sample container separate from any bagged ice in the ice chest. Include a chain-of-custody form with sample identification and relevant information for use by the laboratory.

Chill samples from 1 to 4°C and store samples in the dark until analysis.

7.1.3 IDENTIFICATION AND ENUMERATION METHODS

Membrane-filtration (MF) and liquid broth tests (presence-absence and most-probable-number (MPN) formats) are used for identification and enumeration of indicator bacteria. Procedures to analyze water samples using the MF method and a liquid broth method (enzyme substrate test in presence-absence or MPN format) are described below in sections 7.1.3.C, D, and E. Procedures to elute bacteria from sediments as a preliminary step to analysis by MF or liquid broth methods are described in section 7.1.3.B. For general enumeration of indicator bacteria, either the MF or enzyme substrate test in MPN format may be used.

Fecal indicator bacteria are operationally defined by the method employed for identification and enumeration, as shown in table 7.1–6. Enumeration is done based on observation of reactions typical of the target bacteria on the test medium. Detailed confirmation and identification of these bacteria require additional culturing and biochemical testing, the details of which are beyond the scope of this manual. Additional confirmation methods may be needed under certain circumstances, such as use of the data in support of environmental regulation and enforcement (U.S. Environmental Protection Agency, 2000). Methods should be selected that are appropriate for the sample and project objectives. For example, methods for analyzing total coliform and *E. coli* in ground water and drinking water are different from those recommended for surface water and recreational water (table 7.1–6).

Table 7.1–6. Fecal-indicator test media, typical applications, incubation times and temperatures, and types of rinse or dilution water.

[mENDO, total coliform medium; ±, plus or minus; °C, degrees Celsius; MI, total coliform and *Escherichia coli* medium; $MgCl_2$, magnesium chloride; NA-MUG, *E. coli* medium; mTEC, *E. coli* medium; mFC, fecal coliform medium; KF, fecal streptococci medium; mEI, enterococci medium; mCP, *Clostridium perfringens* medium.]

Test (medium)	Typical application	Incubation time and temperature	Type of rinse and (or) dilution water[1]
Total coliform bacteria (mENDO)	Drinking water and ground water	24 ± 2 hours at 35.0 ± 0.5°C	Phosphate-buffered water with $MgCl_2$
Total coliform bacteria (MI)	Drinking water and ground water	24 ± 2 hours at 35.0 ± 0.5°C	Phosphate-buffered water with $MgCl_2$
Total coliform bacteria (Colilert or Colilert-18)	Drinking water and ground water	24 ± 2 hours at 35.0 ± 0.5°C (Colilert) 20 ± 2 hours at 35.0 ± 0.5°C (Colilert-18)	Distilled or deionized water
Escherichia coli (NA-MUG)	Drinking water and ground water	4 hours at 35 ± 0.5°C after primary culture on mENDO medium	(See mENDO)
Escherichia coli (MI)	Drinking water and ground water	24 ± 2 hours at 35.0 ± 0.5°C	Phosphate-buffered water with $MgCl_2$
Escherichia coli (modified mTEC)	Fresh waters—recreational and other surface water	Resuscitate, 2 hours, 35.0 ± 0.5°C Incubate, 22 to 24 hours, 44.5 ± 0.2°C	Phosphate-buffered water with $MgCl_2$
Escherichia coli (on urea substrate broth after primary culture on mTEC)	Fresh waters—recreational and other surface water	Resuscitate, 2 hours, 35.0 ± 0.5°C Incubate, 22 to 24 hours, 44.5 ± 0.2°C Transfer filter to urea substrate broth, 15 to 20 minutes, before counting	Phosphate-buffered water with $MgCl_2$
Escherichia coli (Colilert or Colilert-18)	Fresh waters—recreational and other surface water, drinking water and ground water	24 ± 2 hours at 35.0 ± 0.5°C (Colilert) 20 ± 2 hours at 35.0 ± 0.5°C (Colilert-18)	Distilled or deionized water
Fecal coliform bacteria (mFC)	Recreational water, shellfish-harvesting water	24 ± 2 hours at 44.5 ± 0.2°C	Phosphate-buffered water with $MgCl_2$
Fecal streptococci (KF)	Recreational water	48 ± 2 hours at 35.0 ± 0.5°C	Phosphate-buffered water with $MgCl_2$
Enterococci (mEI)	Fresh and saline recreational waters, proposed for ground water	24 hours at 41.0°C ± 0.5°C	Phosphate-buffered water with $MgCl_2$
Enterococci (Enterolert)	Fresh and saline recreational waters, proposed for ground water	24 hours at 41.0°C ± 0.5°C	Distilled or deionized water
Clostridium perfringens (mCP)	All waters	24 ± 2 hours at 44.5 ± 0.2°C	Phosphate-buffered water with $MgCl_2$

[1]Buffered water type for all tests changed as of November 2004.

7.1.3.A CULTURE MEDIA AND REAGENTS

Analyses for indicator bacteria require several types of culture media and reagents specific to the indicator bacteria and method being used. Detailed information about sources of media and preparation protocols are described in Office of Water Quality Technical Memorandum 2005.02 (U.S. Geological Survey, 2005) and on the Ohio Water Microbiology Laboratory Web page http://oh.water.usgs.gov/micro/qcmanual/manual.html (accessed January 17, 2007). The necessary media and reagents include sterile buffered water, sterile distilled or deionized water, agar- or liquid broth-based selective and differential growth media, and media and reagents for additional biochemical tests (as needed). **The preparation of selective and differential culture media for indicator bacteria is an important part of analysis. Adhering to and documenting proper preparation, storage, and holding-time requirements will help ensure data quality.**

- Sterile phosphate-buffered water amended with magnesium chloride (U.S. Environmental Protection Agency, 2000) is used to dilute samples and to rinse the filtration unit and utensils.
  - Sterile buffered water can be obtained in 99-mL dilution bottles and in 500-mL volumes.
  - **Do not use sterile buffered water that exceeds the expiration date indicated on the label.**
  - Obtain buffered water from a commercial vendor that provides quality-control documentation. Buffered water also can be prepared according to the instructions found at http://oh.water.usgs.gov/micro/qcmanual/appendm.pdf (accessed January 17, 2007). Buffered water prepared according to these instructions must be autoclaved and checked for sterility before use.
- Culture media (including dehydrated media) for enumeration of fecal indicator bacteria for USGS studies are obtained commercially. Instructions for preparation are printed on the labels of dehydrated media bottles and should be followed carefully. For studies that require small amounts of media, or that require media with complex preparation steps (such as mEI and MI agars), the use of pre-poured plates is recommended. Sources of dehydrated and pre-poured media are listed in Office of Water Quality Technical Memorandum 2005.02 (U.S. Geological Survey, 2005). Updated, detailed information about media and reagent preparation also can be found at http://oh.water.usgs.gov/micro/qcmanual/manual.html (accessed January 16, 2007).

- Enzyme substrate tests in presence-absence or MPN format can be done in the field by use of commercially produced media that commonly come in the form of single-use dry reagent packs (such as Colilert® and Enterolert™).

To store media and reagents:

1. Refer to the manufacturer's instructions for the storage of dehydrated media. Store reagents in a dust-free laboratory cabinet (not in a field vehicle) or in a laboratory desiccator.
2. Label all media with the date received, date opened, and analyst's initials. Discard media and reagents that have an expired shelf life.
3. Refrigerate reagents when required.
4. Label all prepared plates to identify the media type, the preparation date, and the analyst.
5. Store prepared plates upside down in a sealed plastic bag in a refrigerator.

Do not use sterile buffered water beyond its expiration date—discard it.

Quality control for culture media and reagents. Each batch of media that is prepared from basic ingredients or dehydrated media by the analyst must be quality-control tested. Pre-poured plates are already quality-control tested by the manufacturer; however, some testing is still required.

- If sterile buffered water is prepared by the user, it should be prepared under laboratory conditions and must be quality-control tested.
- Buffered water obtained from a commercial vendor already has been quality-control tested and does not require further testing.
- Use the quality-control procedures applicable to microbiological testing found in the 20th edition of "Standard Methods" (American Public Health Association and others, 1998, p. 9-18) and Office of Water Quality Technical Memorandum 2005.02 (U.S. Geological Survey, 2005).

For each batch of media prepared from **basic ingredients or dehydrated media**, it is recommended to analyze a filter blank, and positive and negative control samples (quality-control terms are described below). It is also recommended to analyze a filter blank and positive and negative control samples on **pre-poured plates**. These plates should be tested at least at the beginning and middle of the sampling period, and when the lot number of the plates has changed.

To test the sterility of the **buffered water**, analyze a filter blank each time the buffer is prepared in the laboratory.

- **Positive control—**Positive controls test the ability of the medium and reagents to support growth of the target microorganism. Refer to table 7.1–7 for guidance on which organism to use for specific media. Refer to the distributor's instructions for preparation and processing of positive control samples.
- **Negative control—**Negative controls are used to ensure that the medium does not support the growth of nontarget organisms. Refer to table 7.1–7 for guidance on which organism to use for specific media. Refer to the distributor's instructions for preparation and processing of negative control samples.
- **Filter blanks—**Filter blanks document that buffered water and equipment are sterile. A 50- to 100-mL sample of sterile buffered water is passed through the filtration unit onto a sterile membrane filter. Growth on the filter after incubation indicates contamination.

Table 7.1–7. Positive- and negative-control test organisms for specific media types

[TC, total coliform; NC, non-coliform; FS, fecal streptococci; KF, fecal streptococcus medium; mEI, enterococci medium; mENDO, total coliform medium; NA-MUG, *Escherichia coli* medium; mFC, fecal coliform medium; FC, fecal coliform; MI, total coliform and *Escherichia coli* medium; mTEC, *Escherichia coli* medium]

Media type	Positive control organism	Negative control organism
Colilert and Colilert-18	*Escherichia coli* and *Enterobacter cloacae* (TC)	*Pseudomonas aeruginosa* (NC)
Enterolert	*Enterococcus faecalis* (FS)	*Enterobacter cloacae* (TC)
KF	*Enterococcus faecalis* (FS)	*Enterobacter cloacae* (TC)
mEI	*Enterococcus faecalis* (FS)	*Enterobacter cloacae* (TC)
mENDO and mENDO/NA-MUG	*Escherichia coli* and *Enterobacter cloacae* (TC)	*Pseudomonas aeruginosa* (NC)
mFC	*Escherichia coli* (FC)	*Enterobacter cloacae* (TC)
MI	*Escherichia coli* and *Enterobacter cloacae* (TC)	*Pseudomonas aeruginosa* (NC)
modified mTEC	*Escherichia coli*	*Enterobacter cloacae* (TC)
mTEC	*Escherichia coli*	*Enterobacter cloacae* (TC)

PROCESSING BED SEDIMENTS 7.1.3.B

Standard methods for processing bed sediments for analysis of fecal indicator bacteria are not documented by the American Public Health Association or by the U.S. Environmental Protection Agency. The following method is recommended for general use.

Samples are processed in a laboratory environment to elute fecal indicator bacteria from bed sediments. Once eluted, the supernatant is analyzed for fecal indicator bacteria by use of membrane-filtration or enzyme substrate methods. The proportional dry weight of the bed sediment is also determined.

To process bed sediments:

1. Prepare for processing by labeling the following items with site identifiers and date and time of sample collection: sterile jar for compositing (if done in the laboratory), a 500-mL sterile bottle for eluting, a 500-mL sterile bottle for collection of supernatant, and a dish for proportional dry-weight analysis.
2. Samples collected at deep-water sites with a sampler are composited in the field. If the sample was collected from a wading site, prepare a composite in the laboratory, as follows:
    a. Measure the tare weight of a clean, sterile, wide-mouthed jar.
    b. Using a sterile spatula, remove 50 g of bed sediment from each of the three replicate sample jars and place into the wide-mouthed composite jar.
    c. Mix the 150 g of sediment thoroughly.
3. Prepare an aliquot of composite bed sediment for proportional dry weight of sediment.
    a. Weigh a clean, dry, heat-tolerant glass or metal dish and record as "tare weight."
    b. Add approximately 25 g of composited sediment and record as "weight before drying."
    c. Place in an oven at 105^{o}C. If an oven is not available, dry in a desiccator until a constant weight is obtained.

4. Elute bacteria from the sediment as soon as possible after compositing the sample.

    a. Place 20 g of the sediment composite into a bottle containing 200 mL of phosphate buffered water with magnesium chloride (U.S. Environmental Protection Agency, 2000). NOTE: If preparing a sample for split replicate analysis, increase the amount of sediment and buffered water appropriately. For example, place 30 g of the sediment composite into a bottle containing 300 mL of buffered water.

    b. Label the lid of this bottle with the time the bottle should be removed from the shaker (the bottle will be shaken for 45 minutes).

    c. Place the bottle on a wrist-action shaker.

    d. After 45 minutes, remove the bottle from the shaker and let it stand for 30 seconds undisturbed. Pour off the supernatant into a new, labeled sterile bottle.

5. Analyze the supernatant using the membrane-filtration method (section 7.1.3.C) or by the enzyme substrate MPN method (section 7.1.3.E). Autoclave the sediment and supernatant and discard.

> **TECHNICAL NOTE:** Supernatants commonly carry high concentrations of suspended sediments. In cases when the sediments in the supernatant clog membrane filters, the enzyme substrate in MPN format is recommended.

6. Remove the dish for proportional dry weight of sediment after 24 hours or until a constant weight is obtained.

    a. Record the constant weight obtained as "weight after drying."

    b. Use the following equation to calculate the proportional dry weight:

    Proportional dry weight = $(W_{dry} - W_{tare}) / (W_{wet} - W_{tare})$

    where,

    W_{tare} = Tare weight of empty dish,

    W_{wet} = Weight of dish with wet bed sediment before drying, and

    W_{dry} = Weight of dish with bed sediment after drying.

MEMBRANE FILTRATION 7.1.3.C

Before beginning to process the sample, select the appropriate sample volumes and assemble and label plates with the station number (or other site identifiers), the volume of sample filtered, and the date and time of sample collection. Select several sample volumes that are anticipated to yield one or two filters with counts in the ideal range (tables 7.1–8 and 7.1–9).

> **TECHNICAL NOTE:** It is useful to review the historical data for each site to help determine the number of sample volumes to be filtered. Where past analyses of samples from a site have shown a small variation in the number of fecal indicator bacteria, the filtration of as few as three or four sample volumes may suffice. However, where past analyses have shown the variation to be large or where the variation is not known, filtering a series of volumes in half-log-scale intervals is recommended.

Always wear laboratory gloves when processing samples for analysis of fecal indicator bacteria.

To prepare to filter samples:

1. If possible, process samples inside the field vehicle or a building, and out of direct sunlight and wind.
2. Before and after processing the samples, clean counter tops with an antibacterial cleaning solution, such as 70-percent isopropyl or ethyl alcohol, or 0.005-percent sodium hypochlorite.
3. Preheat incubators for at least 2 hours at temperatures specified for each test (table 7.1–6). Portable heater-block incubators must not be left in closed, unventilated vehicles when the outside air temperature is less than 15°C (60°F) or greater than 37°C (98°F).

To filter samples:

1. Select several sample volumes (table 7.1–8, fig. 7.1–2) that are expected to yield one or two filters with counts in the ideal range. The ideal range and number of sample volumes to filter depend on the test and the expected bacterial densities (table 7.1–9).
2. Record the site name, date, time of sample collection, and sample volume on the plate and on the record sheet or field form. Label filter and procedure blanks and other quality-control samples. Record the time of sample processing on the record sheet or field form.

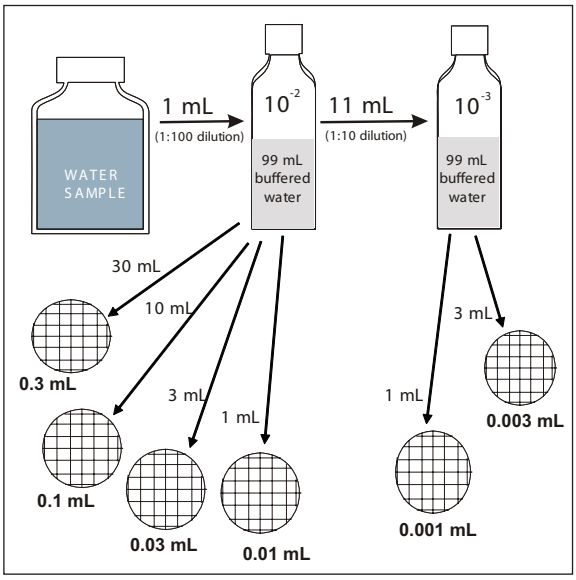


Figure 7.1–2. Preparation of sample volumes by dilution.

Table 7.1–8. Detection ranges achieved by analyzing various sample-water volumes by membrane filtration

[mL, milliliter; CFU, colony-forming units]

Detection limits for various volumes plated in membrane filtration analysis		
Sample volume (mL)[1]	**Volume of sample added (in mL)[2]**	**Detection limits (for ideal count of 20 to 80 colonies)**
100	100	<1 to 80
30	30	60 to 270
10	10	200 to 800
3.0	3.0	600 to 2,700
1.0	1.0	2,000 to 8,000
.3	3.0 of a 1:10 dilution[2] or 30 of a 1:100 dilution	6,000 to 27,000
.1	1.0 of a 1:10 dilution or 10 of a 1:100 dilution	20,000 to 80,000
.03	3.0 of a 1:100 dilution	60,000 to 270,000
.01	1.0 of a 1:100 dilution	200,000 to 800,000
.003	3.0 of a 1:1,000 dilution, prepared by diluting 11 mL of a 1:100 in 99 mL	600,000 to 27,000,000

[1]Sample volumes smaller than those indicated may be needed when bacterial concentrations are greater than those listed.

[2]All sample volumes less than 1.0 mL require dilution in sterile buffered water.

Table 7.1–9. Test (medium type), ideal colony count range, and typical colony color, size, and morphology for indicator bacteria colonies

[m-ENDO, total coliform medium; mm, millimeters; MI, total coliform and *Escherichia coli* medium; nm, nanometer; NA-MUG, *Escherichia coli* medium; mTEC, *E. coli* medium; mFC, fecal coliform medium; KF, fecal streptococcus medium; mE, enterococcus medium; EIA, enterococcus confirmation medium; mEI, enterococci medium; mCP, *Clostridium perfringens* medium]

Test (medium type)	Ideal colony count range (colonies per filter)	Typical colony color, size, and morphology
Total coliform bacteria (mENDO)	20 to 80	Colonies are round, raised, and smooth; red with a golden-green metallic sheen.
Escherichia coli After primary culture as total coliform colonies on mENDO (NA-MUG)	Not applicable[1]	Colonies are cultured on m-ENDO media as total coliform colonies. After incubation on NA-MUG, colonies have blue fluorescent halos with a dark center. Count under a long-wave ultraviolet lamp at 366 nm in a completely darkened room or viewing box.
Total coliform bacteria (MI)	20 to 80	Colonies fluoresce blue-white or blue-green or have a blue-green fluorescent halo[2] under long-wave ultraviolet light (366 nm); blue colonies that do not fluoresce are also total coliforms. Count in a completely darkened room or viewing box.
Escherichia coli (MI)	Not applicable[1]	Colonies are blue under ambient light, and blue green with or without fluorescent edges under long-wave ultraviolet light (366 nm).
Escherichia coli (mTEC)	20 to 80	Colonies are round, raised, and smooth; colonies remain yellow, yellow-green, or yellow brown after urease test; may have darker raised centers.
Escherichia coli (modified mTEC)	20 to 80	Colonies are round, raised, and smooth; deep pink to magenta.
Fecal coliform bacteria (mFC)	20 to 60	Colonies are round, raised, and smooth with even to lobate margins; light to dark blue in whole or part. Some may have brown or cream-colored centers.
Fecal streptococci (KF)	20 to 100	Colonies are small, raised, and spherical; glossy pink or red.
Enterococci (mEI)	20 to 60	Colonies have blue halos regardless of colony color. Count under a fluorescent lamp with 2 to 5 times magnification.
Clostridium perfringens (mCP)	20 to 80[3]	Colonies are round and straw yellow before exposure to ammonium hydroxide, dark pink to magenta afterward.

[1] mENDO/NA-MUG and MI media are used to detect concentration of total coliforms and presence of *Escherichia coli* in ground or drinking water.

[2]Be aware that non-target colonies grow and fluoresce paler orange or green on MI agar. It sometimes is difficult to distinguish target from non-target growth on MI agar.

[3]*C. perfringens* colonies often bubble on mCP agar, making it difficult to achieve the recommended ideal colony count upper limit of 80 colonies (U.S. Environmental Protection Agency, 1996).

3. If the sample volume to be plated is less than 1 mL, prepare dilutions with sterile buffered water in 99-mL dilution bottles (fig. 7.1–2 and table 7.1–8).

    - Transferring 11 mL of sample to a 99-mL dilution bottle creates a 1 to 10 dilution. Transferring 1 mL of sample to a 99- mL dilution bottle creates a 1 to 100 dilution.
    - These can be diluted in series, as needed. For example, transferring 1 mL of the 1 to 100 dilution to another 99-mL dilution bottle creates a 1 to 10,000 dilution.
    - When preparing a dilution series, use a sterile pipet to measure each sample volume. After each sample-volume transfer, close and shake the dilution bottle vigorously at least 25 times.
    - **Filter the diluted samples within 20 minutes after preparation.** Keep dilution bottles out of sunlight and do not transfer less-concentrated sample volumes with pipets that were used to transfer more-concentrated sample volumes.

4. Assemble the filtration unit by inserting the base of the sterile filter-holder assembly into a side-arm flask or manifold (fig. 7.1–3). Connect the filtration unit to a hand-held pump, vacuum pump, or peristaltic pump.

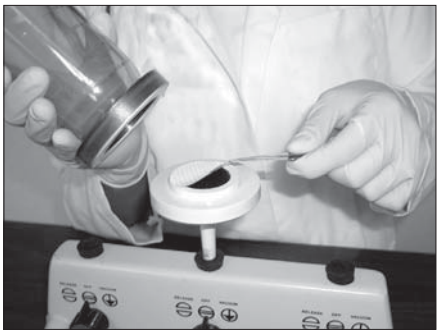
5. If flame sterilization is used (Hydrosol units), rinse the inside of the filtration unit with sterile buffered water to remove any residue of formaldehyde.

6. Sterilize stainless steel forceps:

    a. Immerse tips in a small bottle or flask containing 70- or 90-percent ethanol.
    b. Pass forceps through the open flame of an alcohol burner. Allow the alcohol to burn out and allow the forceps to cool to avoid scorching the membrane filter.

7. Remove the filter from its sleeve. Remove the sterilized funnel from the base. Always hold the funnel in one hand while placing or removing the membrane filter. Placing the funnel on anything other than the filter unit base might result in contamination of the funnel.

8. Using the sterile forceps, place a sterile, gridded membrane filter (47 mm) on top of the filter base, gridded side up (fig. 7.1–3). Carefully replace and secure the filter funnel on the filter base. Avoid tearing or creasing the membrane filter.



A

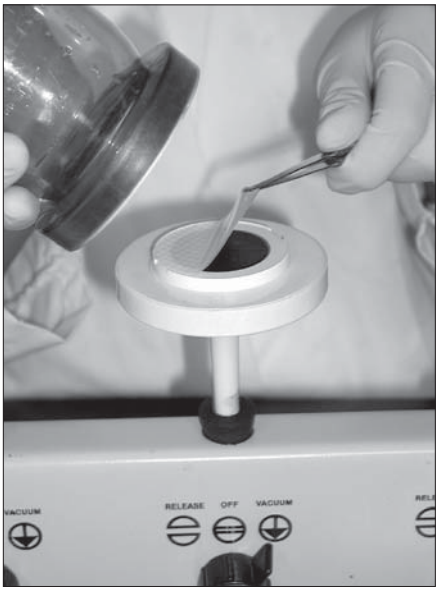
B

C

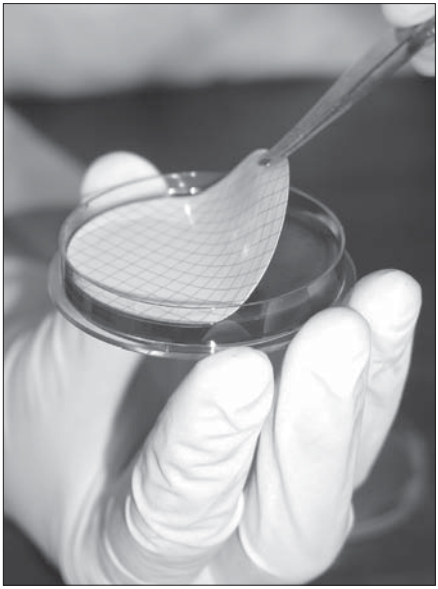
PROCEDURE

1. Preheat the incubator, prepare work areas.
2. Select sample volumes. If needed prepare dilutions for filtration of sample volumes less than 1.0 mL (tables 7.1–6 and 7.1–8; and figure 7.1–2).
3. Label plates.
4. Assemble sterile filtration apparatus.
5. Place sterile filter on filtration apparatus using sterile forceps (A).
6. Shake sample 25 times and deliver to filtration apparatus by use of graduated cylinder (B) or pipet (C). Add 20 mL sterile buffered water to filtration apparatus before filtering sample volumes less than 10 mL.
7. Apply vacuum; afterwards, rinse filtration apparatus and cylinder twice with sterile buffered water.

Figure 7.1–3. Steps in membrane-filter procedure.



D



E

PROCEDURE

8. Sterilize forceps and remove filter (D). Replace funnel on filtration apparatus.

9. Roll filter onto media in plate (E). Place inverted plate in incubator.

10. Repeat steps 4–9 for each sample volume on order of the smallest to the largest volume. A filter blank is processed before each sample. Filter a procedure blank after every 10 to 20 samples or once per day at each site, according to study objectives.

11. Filter a replicate sample after every 10 to 20 samples or at each site, according to study objectives.

Figure 7.1-3. Steps in membrane-filter procedure—*Continued*

9. Return forceps to the alcohol container between transfers. **Do not set forceps on the countertop.**

   Quality control. Rinse the funnel with about 100 mL of sterile buffered water before filtering sample volumes to obtain a filter blank. Place the filter on the plate labeled "filter blank."

10. Filter the sample in order of smallest to largest sample volume. Resterilize forceps before each use.

11. Shake the sample vigorously at least 25 times before each sample volume is withdrawn in order to break up particles and to ensure an even distribution of indicator bacteria in the sample container.

Always shake the sample before removing a volume for plating to make sure the bacteria are evenly distributed in the sample.

12. Remove the required volume by pipet or by pouring into a graduated cylinder (>10 mL) within 5 seconds of shaking the sample. If pipetting, place the pipet tip in the center of the sample volume and use a pipettor or pipet bulb with a valve for volume control. It is acceptable to use the upper and lower graduations to measure the volume (line-to-line) or simply draw up the selected volume.

13. Pour or pipet the measured volume of sample into the filter funnel (fig. 7.1-3*B* or *C*).

    a. **If the volume of sample to be filtered is from 1 to 10 mL,** pour about 20 mL of sterile buffered water into the funnel before pipetting the sample to allow even distribution of bacteria on the membrane filter.

    b. **If the volume of sample to be filtered is more than 10 mL,** transfer the sample with a sterile pipet or graduated cylinder directly into the funnel.

14. Allow the pipet to drain, touching the pipet to the inside of the funnel to remove any remaining sample (fig. 7.1–3*C*). However, if a serological pipet is used, a small amount of liquid will remain in the tip after the liquid is dispensed. Gently force out the remaining liquid using a pipettor or pipet bulb, taking care not to produce an aerosol by blowing out the pipet too forcefully.

CAUTION: Do not pipet by mouth.

15. Apply a vacuum. **To avoid damage to bacteria, do not exceed a pressure of about 5 lb/in^2 (25 cm of mercury).**
16. Rinse the inside of the funnel twice with 20 mL to 30 mL of sterile buffered water while applying a vacuum. If a graduated cylinder is used, rinse the cylinder with sterile buffered water and deliver rinse water to the filtration unit.
17. Remove the funnel and hold it in one hand—do not set the funnel on the counter top.
18. Remove the membrane filter using sterile forceps (fig. 7.1–3*D*).

Do not exceed 5 psi of pressure when filtering the sample.

19. Replace the funnel on the filter base and release the vacuum. (Releasing the vacuum after removing the filter prevents backflow of sample water onto the filter. Unnecessarily wet filters promote confluent growth of colonies and poor results.)
20. Open a labeled plate and place the membrane filter on the medium, grid side up, and starting at one edge by use of a rolling action (fig. 7.1–3*E*). Avoid trapping air bubbles under the membrane filter. If air is trapped, use sterile forceps to remove the membrane filter and roll it onto the medium again. **Do not expose prepared plates to direct sunlight.**
21. Close the plate by pressing the top firmly onto the bottom. Invert the plate. Incubate within 20 minutes to avoid growth of interfering microorganisms.
22. Continue to filter the other sample volumes in order, from smallest to largest volume. Record on field forms the volumes filtered and the time of processing. For USGS personnel, the microbiology section of the Personal Computer Field Form (PCFF) version 5.2.1 and above is a tool to help record and maintain analytical data and perform key calculations.

Quality control. After filtrations are complete, place a sterile, gridded-membrane filter onto the filtration unit base, replace the funnel, and rinse with about 100 mL of sterile buffered water to obtain a procedure blank. Procedure blanks are analyzed at a frequency of one blank for every 10 to 20 samples.

23. Place the inverted plates into a preheated aluminum heater-block or into water-tight plastic bags and then into a water-bath incubator. Incubate at the prescribed times and temperatures (table 7.1–6).

24. Wash the counter top between each sample with an antibacterial cleaning solution (see "To prepare to filter samples" at the beginning of section 7.1.3.C). Wash and sterilize the filter apparatus before the next use.

Quality control. Verify the incubator temperature on a regular schedule against a National Institute of Standards and Technology (NIST) thermometer or a thermometer certified to a NIST thermometer. Record results in a logbook with the date and analyst's name. Do not use incubators that fail to meet temperature criteria until they are repaired or the problem is corrected.

To count colonies and calculate results:

1. After the prescribed length of incubation, remove the plates from the incubator. For each sample volume filtered, count and record on the field forms the number of target colonies (table 7.1–9). **Recount the colonies until results agree within 5 percent, and record the results.** Recounting is accomplished by turning the plate 90 degrees to obtain a different view. Count by use of a preset plan (a side-to-side pattern along grid lines is suggested; fig. 7.1–4). Count the colonies with the aid of 5 to 15 magnification and a fluorescent illuminator or other light source placed directly above the filter.

   Quality control. A second analyst should recount the colonies and record the results for at least one in every 20 samples. Table 7.1–9 and figure 7.1–5 contain further information on colony identification.

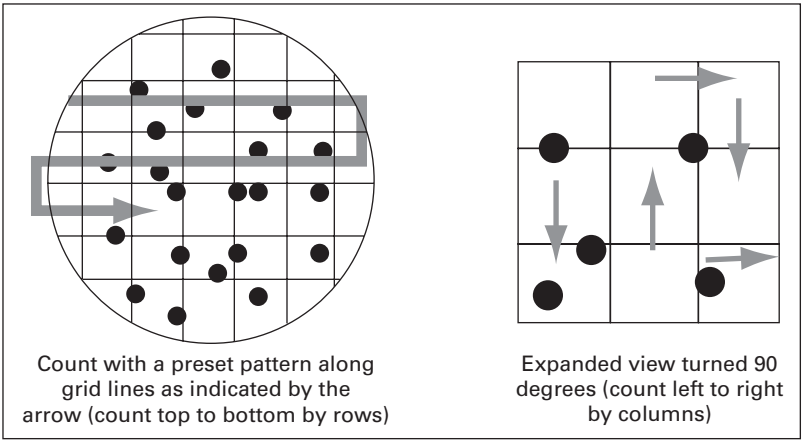


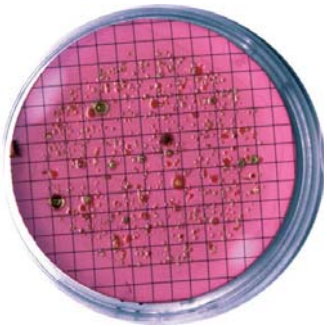
Figure 7.1–4. Method for counting colonies on gridded membrane filters.

Media-specific guidance for making colony counts:

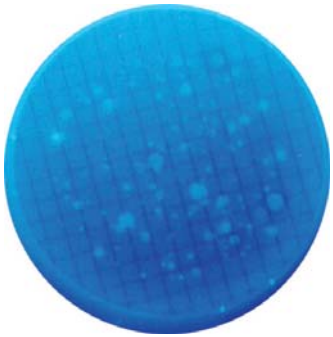
- For total coliform colonies on mENDO medium, count pink to dark red colonies with a golden-green metallic sheen. Enhance sheen production by removing filters from media and placing them on absorbent pads to dry for at least 1 minute before counting (fig. 7.1–5*A*).
- If the NA-MUG test is done for *E. coli*, transfer the mENDO total coliform filter onto NA-MUG plates and incubate for 4 hours at 35°C. Afterwards, count colonies with a dark center and bright blue fluorescent halo under a long-wave ultraviolet light in a completely darkened room (U.S. Environmental Protection Agency, 1991b) or in a viewing box (fig. 7.1–5*D*).
- For total coliforms on MI medium, count colonies that fluoresce blue-white or blue-green or have a blue-green-fluorescent halo under a long-wave ultraviolet light in a completely darkened room (U.S. Environmental Protection Agency, 2002d) or in a viewing box (fig. 7.1–5*B*). Blue colonies that do not fluoresce are also counted as total coliforms. Be aware that non-target colonies may have pale orange- or green-colored fluorescence under long-wave ultraviolet light.
- For *E. coli* on MI medium, count blue colonies under natural light (U.S. Environmental Protection Agency, 2002d; fig. 7.1–5*C*).

- For *E. coli* on mTEC medium, transfer the filter to a filter pad saturated with urea-phenol reagent; count only colonies that are yellow, yellow-green, or yellow-brown after 15 to 20 minutes at room temperature (U.S. Environmental Protection Agency, 2002a; fig. 7.1–5*E*).
- For *E. coli* on modified mTEC medium, count colonies that are red to magenta under natural light (U.S. Environmental Protection Agency, 2002c; fig. 7.1–5*F*).
- For fecal coliforms on mFC medium, count colonies that are light to dark blue, in whole or in part, under natural light (fig 7.1–5*G*).
- For fecal streptococci on KF medium, count colonies, using magnification, that are glossy pink or red under natural light (fig. 7.1–5*H*).
- For enterococci on mEI medium, count colonies of any color that have a blue halo under magnification with a small fluorescent lamp. (U.S. Environmental Protection Agency, 2002b; fig. 7.1–5*J*). Always use 2 to 5 times magnification when counting colonies on mEI agar.
- For *C. perfringens* on mCP medium, count colonies that are straw yellow, turning dark pink to magenta under natural light when exposed to ammonium hydroxide in a laboratory fume hood (U.S. Environmental Protection Agency, 1996; fig. 7.1–5*K*).

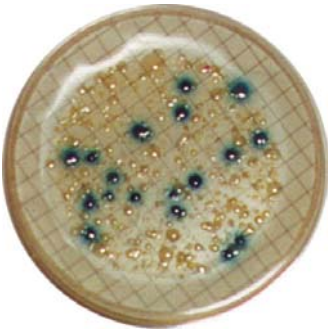
2. Check quality-control blanks for colony growth, and report results on the field forms. The presence of colonies on blanks indicates that results of the bacterial analyses are suspect and should not be reported or the results should be clearly qualified. It is not valid to subtract colony counts on blanks from results calculated for samples.
   - One or more colonies on the field or filter blank indicates inadequate sterilization of either the equipment or the buffered water, or contamination during the sampling and analysis process.
   - One or more colonies on the procedure blank indicates either inadequate rinsing or contamination of the equipment or the buffered water during sample processing.
3. Calculate the number of colonies per 100 mL of sample as described in section 7.1.4.
4. Put all plates to be discarded into an autoclavable bag and **autoclave at 121°C for 45 minutes before discarding in the trash**. If plates cannot be autoclaved immediately, they may be held in a freezer or refrigerator for up to a week before being autoclaved. Other contaminated, disposable supplies should also be placed in autoclavable bags for autoclaving. Reusable equipment that contains contaminated sample water, including sample bottles and dilution bottles, should be autoclaved before disposing of the water.



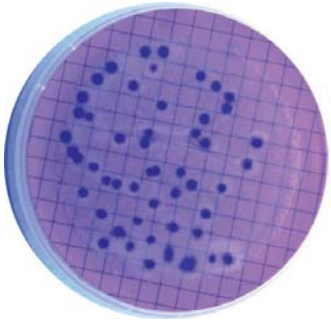
(a) Total coliform bacteria on mENDO medium



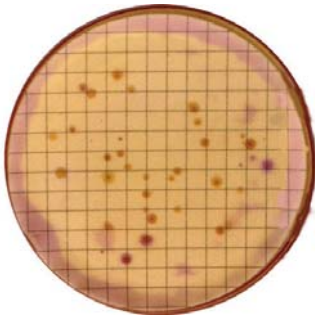
(b) Total coliform bacteria on MI medium



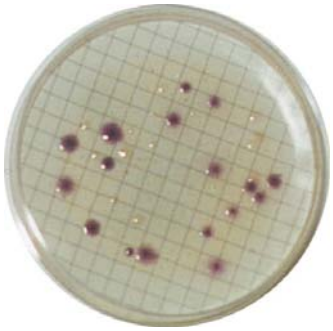
(c) *Escherichia coli* on MI medium



(d) *Escherichia coli* on NA-MUG medium

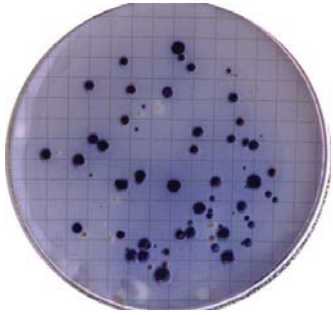


(e) *Escherichia coli* on mTEC medium

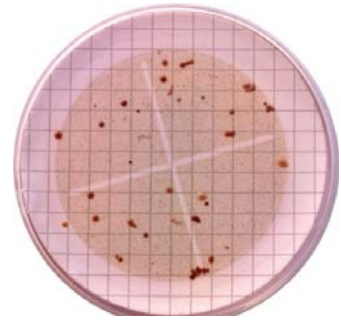


(f) *Escherichia coli* on modified mTEC medium

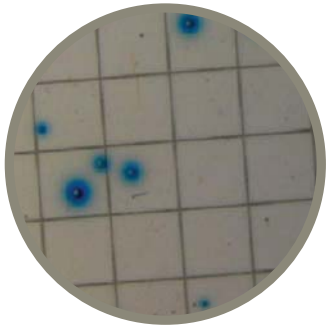
Figure 7.1–5. Photographs of typical colonies of fecal indicator bacteria on culture media.



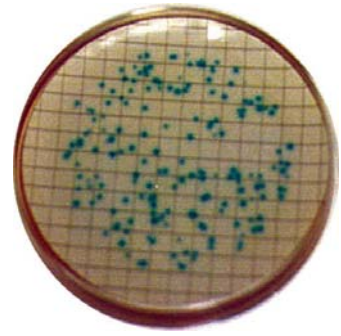
(g) Fecal coliform bacteria on mFC medium



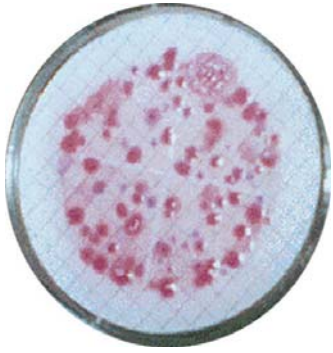
(h) Fecal streptococci bacteria on KF medium



(i) Enterocci bacteria on mEI medium under 2-5X magnification



(j) Enterocci bacteria on mEI medium



(k) *Clostridium perfringens* on mCP medium

Figure 7.1–5. Photographs of typical colonies of fecal indicator bacteria on culture media—*continued*

7.1.3.D ENZYME SUBSTRATE TESTS IN THE PRESENCE-ABSENCE FORMAT

Potable surface water or ground water used as a drinking-water source typically is tested for fecal indicator bacteria by use of enzyme substrate tests in the presence-absence format. The volume of sample tested is usually 100 mL. Commercially available, USEPA-approved enzyme substrate media include Colilert and Enterolert (IDEXX, Westbrook, Maine), Readycult® (EMD Chemicals, Gibbstown, N.J.), E*colite™ (Charm Sciences, Lawrence, Mass.), and Colitag™ (CPI International, Santa Rosa, Calif.). These media measure fecal indicator bacteria in a presence-absence format. For these tests, the manufacturers supply single-use reagent packs and culture bottles. The Colilert (for total coliforms and *E. coli*) and Enterolert (for enterococci) tests are described herein as commonly used examples.

To prepare to process samples:

1. If possible, process samples inside the field vehicle or a building, and out of direct sunlight and wind.
2. Before and after processing the samples, clean counter tops with an antibacterial cleaning solution, such as 70-percent isopropyl or ethyl alcohol, or 0.005-percent bleach. Turn on incubators or waterbaths with sufficient time to reach operating temperature.

Q**uality control.** For each day's samples, run at least one method blank consisting of 100 mL sterile distilled or deionized water with a reagent pack added to test for equipment cleanliness and sterility.

To process samples and read results:

1. For analysis of potable water by use of Colilert-18, pre-warm the sample in a 35°C water bath for 20 minutes or in a 44.5°C water bath for 7-10 minutes.

   Quality control. Verify the incubator temperature before beginning the analysis.
2. Record the site name, date, time of sample collection, and time of sample processing on the culture bottle and on the record sheet or field form.
3. Shake the sample at least 25 times.

4. Measure 100 mL of sample by use of a sterile graduated cylinder and pour into the culture bottle.
5. Holding the reagent packet with the foil toward you, snap the packet open. There may be a puff of powdered reagents that should be directed away from yourself and other people.
6. Pour the contents of the reagent packet into the culture bottle.
7. Mix well by shaking at least 25 times.
8. Incubate for 24 to 28 hours (Colilert and Enterolert) or for the portion of 18 to 22 hours remaining after the pre-warming step (Colilert-18).
9. Read total coliform positive (yellow) or negative (colorless) and *E. coli* or enterococci positive (fluoresces under ultraviolet light) or negative (does not fluoresce).

   Quality control. Use a comparator (available from the manufacturer) to evaluate whether lightly colored or dimly fluorescing Colilert results are above the threshold of positive reactions.
10. Sterilize culture bottles by autoclaving before disposal.

ENZYME SUBSTRATE TESTS IN THE MOST-PROBABLE-NUMBER FORMAT 7.1.3.E

The enzyme substrate MPN test uses a multi-well disposable tray into which the sample is poured and mixed with medium. A sealer is used to seal the tray and distribute the sample among the wells. The incubator must be large enough to accommodate the trays; several trays may be stacked in the incubator. For these tests, the manufacturers supply single-use trays, reagent packs, and mixing bottles. The Quanti-Tray and Quanti-Tray2000 (IDEXX Laboratories, Inc., Westbrook, Maine) are commercially produced products in the enzyme substrate MPN format. Colilert (for total coliforms and *E. coli*) and Enterolert (for enterococci) are enzyme-substrate media produced by IDEXX and are described herein as commonly used examples.

The sample volume tested is typically 100 mL, as this will provide results for densities ranging from less than 1 to 200 or 2,000 MPN/100 mL for Quanti-Tray or Quanti-Tray2000, respectively. If greater densities are expected, samples may be diluted. The enzyme substrate MPN test is recommended when water is too turbid to give accurate results by membrane filtration.

TECHNICAL NOTE: Data summary for densities measured by enzyme substrate MPN tests includes both the uncertainty in the MPN estimate and analytical variability. As a result, the 95-percent confidence interval around the geometric mean tends to be broad.

To prepare to process samples:

1. If possible, process samples inside the field vehicle or a building, and out of direct sunlight and wind.
2. Before and after processing the samples, clean counter tops with an antibacterial cleaning solution, such as 70-percent isopropyl or ethyl alcohol, or 0.005-percent bleach. Turn on incubators with sufficient time to reach operating temperature.
3. Pre-warm the sealer and ensure that the sealer is level to allow even distribution of sample among the wells.

Q**uality control.** For each day's samples, run at least one method blank consisting of 100 mL sterile distilled or deionized water with a reagent pack added to test for equipment cleanliness and sterility.

To process samples and read results:

1. For analysis of potable water by use of Colilert-18, pre-warm the sample in a 35°C water bath for 20 minutes or in a 44.5°C water bath for 7-10 minutes.

   Quality control. Verify the incubator temperature before beginning the analysis.
2. Label the back of a tray with the station (or other site identifier) and the date and time of sample collection, along with the dilution factor.
3. If needed, prepare a 1:10 dilution by mixing 10 mL of sample with 90 mL of sterile distilled or deionized water. Prepare a 1:100 dilution by mixing 1 mL of sample with 99 mL of sterile distilled or deionized water. Dilutions must be made with sterile distilled or deionized water because the reagent packs contain all necessary buffers.
   - All marine waters must be diluted at least 1:10 when using Colilert or Enterolert products.
   - Prepare a dilution for any water type when fecal indicator bacteria densities are expected to be high.

It is critical to mix the sample gently but thoroughly in order to distribute bacteria as evenly as possible without causing foaming.

4. Shake the sample at least 25 times.
5. Measure 100 mL of sample by use of a sterile graduated cylinder and pour into the sterile mixing bottle.
6. Holding the reagent packet with the foil toward you, snap the packet open. There may be a puff of powdered reagents that should be directed away from yourself and other people.
7. Pour the contents of the reagent packet into the mixing bottle.
8. Mix well by shaking at least 25 times.
9. Add mixture to the multi-well tray. With well-side down, hold at an angle and tap lower wells to release air bubbles.
10. Place loaded tray into rubber sealer mat and seal.
11. Incubate for 24 to 28 hours (Colilert and Enterolert) or for 18 to 22 hours (Colilert-18).
12. Count wells that are total coliform positive (yellow) and *E. coli* or enterococci positive (fluoresces under ultraviolet light).
    - Use a comparator tray provided by the manufacturer to determine positive wells, if available.
    - The IDEXX Quanti-Tray has 51 wells and the IDEXX Quanti-Tray2000 has 49 large wells and 48 small wells; results for large and small wells must be recorded separately for the Quanti-Tray2000.
13. Record results and obtain MPN density by use of the tables provided by the manufacturer (IDEXX), or an electronic database such as the one written into PCFF versions 5.2.1 and later.
14. Before being disposed of, the tray(s) must be autoclaved or otherwise sterilized.

7.1.4 CALCULATING AND REPORTING FECAL INDICATOR BACTERIA DENSITIES

The range of ideal colony counts depends on the fecal indicator group to be enumerated (table 7.1–9). Crowding and competition for nutrients to support full development of colonies can result if the bacterial density on the filter exceeds the upper limit of the ideal range. As the number of colonies fall below the lower limit of the ideal range, statistical validity becomes questionable (Britton and Greeson, 1989, p. 14). For potable waters, results are routinely less than 20 colonies per filter. Consult table 7.1–9 and figure 7.1–5 for information on typical colony color, size, and shape. Density per 100 mL is calculated by dividing the colony count for the sample by the volume filtered, then multiplying by 100.

The MPN result is based on the number of wells in the well tray that test positive, the sample volume analyzed, and the total number of wells tested. The MPN can be determined by calculation or, more simply, by using a table provided by the manufacturer. If more than one dilution for a sample is analyzed, the most reliable estimate should be reported; this can be determined as the result having the smallest 95-percent confidence interval. Analyses with many or only a few positive wells have wide confidence intervals compared with analyses with an intermediate number of positive wells.[3] The MPN statistics require that each well has an equal probability of holding each indicator bacteria cell, so insufficient mixing is an important potential source of error and variability in this method.

For bed-sediment analyses, the ideal count and 95-percent confidence rules for membrane filtration and enzyme substrate MPN tests, respectively, should be used.

- Enumeration results for membrane-filtration methods in water are expressed as a density in units of colony-forming units per 100 mL (CFU/100 mL).

[3]For USGS personnel, use version 5.2.1 and later of the personal computer field form (PCFF). USGS personnel can find the correct parameter codes to report fecal indicator bacteria data in the USGS National Water Information System by accessing the QWDATA component of NWIS.

- Results for the presence-absence methods in water are expressed as presence or absence per 100 mL.
- Enumeration results for MPN methods in water are expressed as most probable number per 100 mL (MPN/100 mL).
- Enumeration results for density in bed sediment are expressed as CFU or MPN per gram dry-weight sediment (CFU/g_{DW} or MPN/g_{DW}), depending on the analytical method used.
- Whole numbers are reported for results less than 10, and two significant figures are reported for results greater than or equal to 10.

For calculations based on colony count for water samples: Scenarios that are commonly experienced when counting colonies are presented in the following six cases.[4]

Case 1. Colony counts in the ideal range.

Case 2. Colony counts outside the ideal range but not zero or too numerous to count.

Case 3. No typical colonies on any of the filters.

Case 4. Less than the ideal range, including some zero counts but no filters with colonies that are too numerous to count.

Case 5. Colony counts on all filters exceed the ideal count but a credible count is possible (fewer than approximately 200 colonies).

Case 6. Colony counts on all filters exceed the ideal count and a credible count is not possible (confluent growth) (too numerous to count).

[4]For USGS personnel, the appropriate calculations have been coded into the PCFF version 5.2.1 and later software to assist in data reporting.

Case 1: Colony counts in the ideal range.

Example 1: Ideal colony count on one filter

Sample volume	Colony count
3	7 (do not use)
10	21
30	101 (do not use)
Sum 10	21

Density = (21 x 100)/10 = 210 CFU/100 mL

Example 2: Ideal colony counts on two or more filters

Sample volume	Colony count
3	7 (do not use)
10	21
30	58
Sum 40	79

Density = (79 x 100)/40 = 200 CFU/100 mL

Example 3: Ideal colony count on one filter with a sample volume less than 1 mL:

Sample volume	Colony count
0.1*	50
0.3	TNTC (do not use)
1.0	TNTC (do not use)
Sum 0.1	50

Density = (50 x 100)/0.1 = 50,000 CFU/100 mL

*0.1 mL is obtained by filtering 10 mL of a 1:100 dilution.

CFU, colony-forming unit
TNTC, too numerous to count

Case 2: Colony counts outside the ideal range but not zero or too numerous to count

Example 1: Less than ideal range on all filters

Sample volume	Colony count
3	2
10	6
30	18
Sum 43	26

Density = (26 x 100)/43 = 60 CFU/100 mL

Qualify the reported density as an estimate because of non-ideal colony count.

Example 2: Both less than and greater than the ideal range

Sample volume	Colony count
3	18
10	82
30	TNTC (do not use)
Sum 13	100

Density = (100 x 100)/13 = 770 CFU/100 mL

Qualify the reported density as an estimate because of non-ideal colony count.

CFU, colony-forming unit
TNTC, too numerous to count

Case 3: No typical colonies on any of the filters

Sample volume	Colony count
10	0 (do not use)
30	0 (do not use)
100	0 assume 1
Sum 100	1

Density = (1 x 100)/100 < 1 CFU/100 mL

Qualify the reported density as less than 1 CFU/100 mL.

CFU, colony-forming unit

Case 4: Less than the ideal range, including some zero counts* but no filters with colonies that are too numerous to count

Example 1: Only one filter has colonies

Sample volume	Colony count
3	0 (do not use)
10*	0 (do not use)
30	5
Sum 30	5

Density = (5 x 100)/30 = 17 CFU/100 mL

Qualify the reported density as an estimate because of non-ideal colony count.

Example 2: More than one filter has colonies

Sample volume	Colony count
3	1
10*	0
30	5
Sum 43	6

Density = (6 x 100)/43 = 14 CFU/100 mL

Qualify the reported density as an estimate because of non-ideal colony count.

*Zero values are used in the calculation if bracketed by plates with colony growth.

CFU, colony-forming unit

Case 5: Colony counts on all filters exceed the ideal count but a credible count is possible

Sample volume	Colony count
10	112
30	TNTC (do not use)
100	TNTC (do not use)
Sum 10	112

Density = (112 x 100)/10 = 1,100 CFU/100 mL

Qualify the reported density as an estimate because of non-ideal colony count.

TNTC, too numerous to count
CFU, colony-forming unit

Case 6: Colony counts on all filters are too numerous to count

Sample volume	Colony count
10	TNTC (assume 80*)
30	TNTC (do not use)
100	TNTC (do not use)
Sum 10	80

Density = (80 x 100)/10 > 800 CFU/100 mL

Qualify the reported density as greater than 800 CFU/100 mL.

*Assume upper ideal count on the filter with the smallest volume filtered.

TNTC, too numerous to count
CFU, colony-forming unit

For MPN estimation based on enzyme substrate media reactions for water samples: Enzyme substrate results are obtained by consulting an MPN table or by entering the results in an MPN calculator (available from IDEXX Laboratories or, for USGS personnel, the Microbiology Field Form within PCFF versions 5.2.1 and later). Enumeration results for enzyme substrate MPN methods in water are expressed as most probable number per 100 mL (MPN/100 mL).

For calculations based on colony count or enzyme substrate MPN tests for sediment samples: Densities of bacteria in sediment are reported as colony-forming units per gram of dry-weight sediment (CFU/g_{DW}) or most-probable number per gram of dry-weight sediment (MPN/g_{DW}).

- ▶ Measure and calculate the sediment dilution factor.
  - Sediment dilution factors are site specific and are determined by performing several displacement experiments of representative sediments.
  - Example: based on beach sediments from Lake Erie (Francy and Darner, 1998), 20 g of dry or wet sediment displaced approximately 10 mL of water, so the total volume of the sediment/buffer mixture was 210 mL. The dilution factor for the sediment samples in this study was, therefore, 10.5 mL/g (210 mL/20 g).
- ▶ To calculate CFU/g_{DW} for membrane filtration results:

  CFU/g_{DW} = (density x dilution factor) / (proportional DW),

  where density is the result in CFU/100 mL, dilution factor is the site specific dilution factor in mL/g, and DW is proportional dry weight of sediment (see section 7.1.3.B).

- ▶ To calculate MPN/g_{DW} for enzyme substrate MPN results:

  MPN/g_{DW} = (density x dilution factor) / (proportional DW),

  where density is the result in MPN/100 mL, dilution factor is the site specific dilution factor in mL/g, and DW is the proportional dry weight of sediment.

Two scenarios are provided to illustrate these calculations:

Case 7. Calculation of results in terms of CFU per gram of dry-weight sediment.

Case 8. Calculation of results in terms of MPN per gram of dry-weight sediment.

Case 7: Calculation of results in terms of CFU per gram dry weight

Calculate proportional dry weight

Tare weight of empty dish (W_{tare})	1.86 g
Weight of dish with wet bed sediment before drying (W_{wet})	27.4 g
Weight of dish with bed sediment before drying (Wdry)	13.6 g

Proportional dry weight 0.46
(13.6 g - 1.86 g) / (27.4 g - 1.86 g)

Calculate density in supernatant

Sample volume	Colony count
3	7 (do not use)
10	21
30	101 (do not use)
Sum 10	21

Density in supernatant = (21 CFU / 10 mL) X 100 = 210 CFU/100 mL

Calculate densitiy in sediment

Sediment dilution factor (calculated from site specific displacement experiments)	10.5 mL/g
Density in supernatant	210 CFU/100 mL
Proportional dry weight	0.46

Density in sediment ***48 CFU/g $_{DW}$***
(210 CFU/100 mL x 10.5 mL/g) / 0.46

CFU, colony-forming unit

Case 8: Calculation of results in terms of MPN per gram dry weight

Calculate proportional dry weight

Tare weight of empty dish (W_{tare})	1.86 g
Weight of dish with wet bed sediment before drying (W_{wet})	27.4 g
Weight of dish with bed sediment after drying (W_{dry})	13.6 g

Proportional dry weight 0.46

(13.6 g - 1.86 g) / (27.4 g - 1.86 g)

Calculate density in supernatant

Positive large wells	46
Positive small wells	10

Density in supernatant 150 MPN/100 mL

from MPN table

Calculate density in sediment

Sediment dilution factor (calculated from site specific displacement experiments)	10.5 mL/g_{DW}
Density in supernatant	150 MPN/100 mL
Proportional dry weight	0.46

Density in sediment ***34 MPN/g_{DW}***

(150 MPN/100 mL x 10.5 mL/g) / (0.46)

MPN, most probable number

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7.1.6 Acknowledgments

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FECAL INDICATOR VIRUSES 7.2

By **R.N. Bushon**

Tables

Notes:

References for section 7.2, Fecal indicator viruses, are located at the end of Chapter A7 in the "Selected References and Documents" section, which begins on page REF-1.

See Appendix A7-A, Table 3, for parameter codes for somatic and F-specific coliphages that are used in the National Water Information System (NWIS) of the U.S. Geological Survey.

The citation for this section (7.2) of NFM 7 is as follows:

Bushon, R.N., November 2003, Fecal indicator viruses: U.S. Geological Survey Techniques of Water-Resources Investigations, book 9, chap. A7 (3d ed.), section 7.2, accessed ___*date*___, from http://pubs.water.usgs.gov/twri9A/

FECAL INDICATOR VIRUSES 7.2

More than 100 types of human pathogenic viruses may be present in fecal-contaminated waters, but only a small number of them can be detected by currently available methods (Havelaar and others, 1993). Coliphages are used as indicators of fecal contamination and of the microbiological quality of the water.[5] Coliphages are viruses that infect and replicate in coliform bacteria and are not pathogenic to humans; coliphages have been suggested as potential indicators of enteric viruses because of their similar structure, transport, and persistence in the environment (Gerba, 1987).

Two main groups of coliphages are used as viral indicators:

Coliphages: Viruses that infect and replicate in coliform bacteria. Coliphages are used as indicators of fecal contamination in water.

- **Somatic coliphages** infect coliform bacteria by attaching to the outer cell membrane or cell wall. They are widely distributed in both fecal-contaminated and uncontaminated waters.
- **F-specific coliphages** attach only to hairlike projections (called F pili) of coliform bacteria that carry an extrachromosomal genetic element called the F plasmid; F pili are produced only by bacteria grown at higher temperatures. F-specific coliphages presumably come from warm-blooded animals or sewage (Handzel and others, 1993).

[5]The term "fecal indicator viruses" is used synonymously with coliphages in this report, as coliphage analysis currently is the only standard viral method used by the U.S. Geological Survey for indicating fecal contamination.

Somatic and F-specific coliphages are found in high numbers in sewage and are thought to be reliable indicators of sewage contamination of waters (International Association of Water Pollution Research and Control Study Group on Health Related Microbiology, 1991). Raw sewage typically contains somatic and F-specific coliphage concentrations of about 1,000 plaque-forming units per milliliter (Sobsey and others, 1995).

Two methods are commonly used to analyze samples for somatic and F-specific coliphages:

- The single-agar layer (SAL) method is recommended for use with surface-water samples. It is a quantitative, plaque assay method that can analyze sample volumes of 100 mL (milliliters).
- The two-step enrichment method is recommended for use with ground-water samples. It is a presence/absence method that can analyze sample volumes of either 100 mL, 1 L (liter), or 4 L.

Coliphage methods of analysis must be performed in the laboratory by a trained microbiologist.

The type of coliphage detected by these methods depends on the bacterial host strain used. Two host strains commonly used for the detection of somatic coliphages are *Escherichia coli* (*E. coli)* C and *E. coli* CN-13. Both hosts are equivalent in coliphage detection; however, *E. coli* CN-13 is resistant to nalidixic acid and is preferable for analyzing samples with a high background or unknown level of indigenous bacteria (Sobsey and others, 1995). Antibiotics such as nalidixic acid are used to minimize overgrowth of indigenous bacteria in environmental samples; this overgrowth may mask the detection of coliphage. Three host strains commonly used for the detection of F-specific coliphages are *E. coli* F-amp, *E. coli* C3000, and *Salmonella typhimurium* WG49. The *E. coli* F-amp strain appears to be the most reliable host for detecting only F-specific coliphages; the F-amp strain is resistant to ampicillin and streptomycin, so it is less susceptible to bacterial contamination in water samples (Sobsey and others, 1995).

SAMPLING EQUIPMENT AND EQUIPMENT STERILIZATION PROCEDURES 7.2.1

Sterile techniques must be followed and documented when collecting and processing samples for fecal indicator viruses. **The specific equipment and supplies that are needed to collect and analyze samples for fecal indicator viruses must be kept clean and sterile** (tables 7.2-1, 7.2-2). The equipment and procedures described in the following paragraphs are applicable to fecal indicator viruses and to fecal indicator bacteria (NFM 7.1). Equipment to be autoclaved must first be wrapped in aluminum foil, autoclavable bags, or kraft paper. Non-autoclavable equipment must be cleaned and, if possible, sterilized and then similarly wrapped for storage and transport.

- Sterilize and store the equipment in a clean area.
- Resterilize equipment if foil, bag, or kraft paper is torn.

Add sodium thiosulfate ($Na_2S_2O_3$) to sample bottles before sterilization if the water to be collected is suspected to contain residual chlorine or other halogens. $Na_2S_2O_3$ also may be added to the sample bottle immediately after sample collection. Residual chlorine commonly is found in treated (disinfected) potable water (for example, public water systems), and in sources such as wastewater effluents or mixing zones directly downstream from wastewater-treatment plants. Most taps or wells (for example, small private water systems) do not contain residual chlorine.

Autoclaving is the preferred method for sterilizing equipment.

Table 7.2-1. Equipment cleaning and sterilization procedures

[NFM, *National Field Manual for the Collection of Water-Quality Data*; DIW, distilled or deionized water; mL, milliliter; $Na_2S_2O_3$, sodium thiosulfate; °C, degrees Celsius; mg/L, milligrams per liter]

Equipment	Cleaning and sterilization procedures
All equipment (this includes water-level tape measure, all sample-collection and sample-processing equipment used in the field and laboratory)	Wash equipment thoroughly with a dilute nonphosphate, laboratory-grade detergent (NFM3). Rinse three times with hot tap water. Rinse again three to five times with DIW. Wipe down the wetted portion of water-level tapes with disinfectant (0.005-percent bleach solution or methyl or ethyl alcohol) and rinse thoroughly with DIW.
Autoclavable glass, plastic, and Teflon bottles	If sample will contain residual chlorine or other halogens, add 0.5 mL of a 10-percent $Na_2S_2O_3$ solution per liter of sample to the sample bottles. Wrap all autoclavable equipment in aluminum foil, kraft paper, or place into autoclavable bags.[1] Autoclave at 121°C for 15 minutes.
Portable submersible pumps and pump tubing	**Autoclavable equipment** (preferred): Wrap components in aluminum foil, kraft paper, or place into autoclavable bags. Autoclave at 121°C for 15 minutes. **Non-autoclavable equipment: (1)** Submerge sampling system into a 50-mg/L (0.005 percent) sodium hypochlorite solution prepared from household laundry bleach. **(2)** Circulate solution through pump and tubing for 30 minutes. **(3)** Follow step (2) by thoroughly rinsing, inside and out, with 0.5 mL of a 10-percent sterile $Na_2S_2O_3$ solution per liter of water and circulate solution for 5 minutes; **(4)** pump $Na_2S_2O_3$, discarding this waste appropriately; pump sterile DIW through the pump, followed by pumping three tubing volumes of well water to waste (discard appropriately) before collecting the sample. **CAUTION:** Prolonged or repeated use of a hypochlorite solution on interior or exterior surfaces of a pump can cause corrosion or other damage to the pump and compromise the quality of samples collected for trace-element or organic-compound analysis.

[1]Equipment to be wrapped in aluminum foil, kraft paper, or placed into autoclavable bags includes, for example, bottles, tubing, flasks, bailers, pump components. The $Na_2S_2O_3$ solution also is autoclaved.

To prepare for collecting a halogenated sample:

1. Prepare a 10-percent solution of $Na_2S_2O_3$ as follows:
   a. In a volumetric flask, dissolve 100 g of $Na_2S_2O_3$ into 500 mL of deionized or distilled water (DIW).
   b. Stir until dissolved.
   c. Fill the flask to 1,000 mL (Bordner and Winter, 1978, p. 6; American Public Health Association and others, 1998, p. 9-19). Autoclave at 121°C for 30 minutes (U.S. Environmental Protection Agency, 1996, p. VIII–II).
   d. Store the $Na_2S_2O_3$ solution at room temperature or under refrigeration. After 6 months prepare a fresh solution.
2. Before collecting the sample, pipet into the sample bottle 0.5 mL of 10-percent $Na_2S_2O_3$ solution for every 1 L of sample. If the sterile $Na_2S_2O_3$ is used, be sure to use only sterile pipets and sterile sample bottles. If the $Na_2S_2O_3$ is not sterile, dispense the required volume of $Na_2S_2O_3$ into the sample bottle and autoclave at 121°C for 15 minutes.

$Na_2S_2O_3$ solution has a 6-month shelf life. Discard unused solution that has expired, prepare fresh solution, and label bottle with date of preparation.

Clean and sterilize sampling equipment (table 7.2-1). All equipment, including tubing and containers, must be cleaned and sterilized between sites or for each sample collected at the same site at different times. Autoclaving is the preferred method of sterilization.

- Use only autoclaves that have temperature, pressure, and liquid- and dry-utensil-cycle controls. Steam sterilizers, vertical autoclaves, and pressure cookers without temperature controls are not recommended.
- Take care to ensure that materials to be autoclaved are thermally stable. Plastics (such as polycarbonate, polypropylene, polyallomer, and polymethylpentene) and Teflons and Tefzel® (such as perfluoroalkyoxypolymers or PFA, ethylenetetrafluoroethylene or ETFE, fluorinated ethylene propylene or FEP, and polytetrafluoroethylene polymers or PTFE) can be autoclaved. **Note that each of these materials has different thermal characteristics and tolerances to repeated autoclaving.**
- When autoclaving, be sure to:
  - Use sterilization indicator tape with each load.
  - Use commercially available biological indicators at least quarterly to test autoclave performance. Biological indicators are composed of endospores—living cells that are resistant to heat, but are destroyed by autoclaving.
  - Drain the autoclave at the end of each period of use. Clean with mild soap and water once per week during periods of daily use. Record cleaning dates in the logbook.
  - Autoclave cultures of microorganisms and all media plates for at least 30 minutes before disposal.
  - Wrap silicone tubing in kraft paper or aluminum foil before autoclaving.
  - Avoid overloading the autoclave with equipment or materials; overloading will result in incomplete sterilization.

- The 20th edition of "Standard Methods for the Examination of Water and Wastewater" (American Public Health Association and others, 1998, p. 9-2 to 9-14) contains specifications for the length of time, temperature, and pressure for autoclave sterilization of various media and materials.

Quality control in sterilization procedures is mandatory. Keep a logbook of autoclave operation. Enter into the logbook the quality-assurance and quality-control procedures used, noting the date, the test results, and the name of the autoclave operator and (or) analyst. Record the autoclave temperature, pressure, date, and time of each autoclave run. If the autoclave does not reach the specified temperature and pressure or fails a quality-control test, then the autoclave should be serviced and all materials resterilized (American Public Health Association and others, 1998, p. 9-2 to 9-14).

SAMPLE COLLECTION, PRESERVATION, TRANSPORT, AND HOLDING TIMES 7.2.2

Sterile conditions must be maintained during collection, preservation, transport, and analysis of fecal indicator virus samples. Specific procedures have been developed that must be strictly followed. These procedures vary with types of sampling equipment and sources of sample (surface water, ground water, treated water, or wastewater).

A summary of requirements for sample-collection containers and procedures for sample preservation is given in table 7.2-2.

Table 7.2-2. Summary of equipment for sample collection and procedures for sample preservation of fecal indicator viruses

[EWI, equal-width-increment; EDI, equal-discharge increment; L, liter; NFM, *National Field Manual for the Collection of Water-Quality Data*; mL, milliliter; $Na_2S_2O_3$, sodium thiosulfate; °C, degrees Celsius]

Equipment for sample collection (All containers must be composed of sterilizable materials such as borosilicate glass, polypropylene, stainless steel, or Teflon®)
To collect EWI or EDI surface-water samples: US D-95, US DH-95, or US DH-81 with sterile 1-L wide-mouth bottle, caps, and nozzles. US D-96 with sterile autoclavable bag (NFM 2.1.1).
To collect surface-water and ground-water samples using point samplers from a tap, or hand-dipped method: a sterile, narrow-mouth container, 500 mL to 1 L capacity, or a sterile 3-L container if both types of coliphages are to be analyzed.
To collect pumped samples: Use sterile tubing, clean and sterile pump components (autoclaved, if possible; see text).
Procedures for sample preservation
Before sample collection, if halogen neutralization is necessary, add 0.5 mL of a 10-percent $Na_2S_2O_3$ solution per 1 L of sample. – If sterile $Na_2S_2O_3$ is used, dispense with sterile pipet into sterile bottle. – If $Na_2S_2O_3$ is not sterile, dispense with pipet into sample bottle and autoclave (table 7.2-1). **Chill all samples at 1-4°C before analysis.**

7.2.2.A SURFACE-WATER SAMPLE COLLECTION

The areal and temporal distribution of fecal indicator viruses in surface water can be as variable as the distribution of suspended sediment because viruses commonly are associated with solid particles. To obtain representative data for fecal indicator virus analysis, follow the same methods used to collect surface-water samples for suspended sediment analysis (Edwards and Glysson, 1999; NFM 4.1 and table 7.2-2).

- **Flowing water**—use depth-and-width-integrating sampling methods[6] (NFM 4.1.1.A).
- **Still water** (lakes or reservoirs, or other surface-water conditions for which depth-and-width-integrating methods are not applicable)—use the hand-dip method or a sterile point sampler (NFM 4.1.1.B).

[6]Sample-collection methods may be modified to ensure consistency with study objectives and as appropriate for site conditions. It is necessary to describe any methods modifications in a report of the results of the study.

- **Beach water**—use a hand-dip method in shallow wadable water and a sterile point sampler for deeper water. Collect samples by the hand-dip method at knee depth, a depth of approximately 15 to 30 cm (6 to 12 in.) below the water surface.
  - — Collect samples near known or suspected pollution sources, in areas of concentrated activity (for example, near lifeguard chairs), or for approximately every 500 m (every quarter mile) of beach length (U.S. Environmental Protection Agency, 2002).
  - — Position the sampler downstream from any water currents to collect the sample from the incoming flow (U.S. Environmental Protection Agency, 2002) and record sampling location. Avoid contaminating the water sample with bottom material dislodged by disturbing the bottom while sampling.
  - — A Chain of Custody record is recommended for beach sampling done in support of beach closures or posting of warnings to swimmers (U.S. Environmental Protection Agency, 2002, Appendix J).

Always wear laboratory gloves when handling sampling equipment and samples. Take care to prevent contaminated water from contacting skin, mouth, nose, or eye areas (NFM 9.7).

Depth-and-width-integrating methods

Depth-and-width-integrating sampling methods (the equal-discharge-increment (EDI) method or the equal-width-increment (EWI) method) are the standard USGS methods used when sampling flowing waters, and are recommended unless study objectives or site characteristics dictate otherwise (NFM 4.1.1.A and table 7.1-4).

Select the EDI or EWI method:

1. The EDI method is preferred to the EWI method for sites where the velocity distribution across a stream section is well established or at a section where the depth varies; for example, at a gaging station (Edwards and Glysson, 1999).

2. Select the appropriate sampler and equipment. **Sampling equipment must be sterile,** including the collection bottle, nozzle, and cap (or bags for the bag sampler) (table 7.2-1).

   - For streams with depths of 5 m (16.4 ft) or less, use a US D-95, US DH-95, or a US DH-81 sampler (NFM 2.1.1).
   - For stream sections where depths exceed 5 m (16.4 ft), use the US D-96, with either autoclavable Teflon® bags or autoclavable cooking bags. Thermotolerant polymers are described in more detail in section 7.2.1 under "Sampling Equipment and Equipment Sterilization Procedures."
   - For compositing subsamples, use a sterile 3-L or larger bottle.
     — For wide channels, several samples, each composed of subsamples composited into a sterile large-volume container, may be needed.
     — For narrow channels, collect subsamples at 5 to 10 or more vertical locations in the cross section without overfilling the bottle.
   - Use the proper nozzle size and transit rate for the velocity conditions in the section to ensure isokinetic collection of the sample.

Hand-dip method

If the stream depth and (or) velocity is not sufficient to use a depth-and-width-integrating method, collect a sample using a hand-dip method (table 7.1-4). Sampling still water or sampling at depth in lakes, reservoirs, estuaries, and oceans requires a sterile point sampler. Niskin, ZoBell, and Wheaton point samplers hold a sterilizable bottle or bag.

Wearing laboratory gloves, collect a hand-dipped sample as follows:

1. Open a sterile, narrow-mouth borosilicate glass or plastic bottle; grasp the bottle near the base, with hand and arm on the downstream side of the bottle.
2. Without rinsing, plunge the bottle opening downward, below the water surface. Allow the bottle to fill with the opening pointed slightly upward into the current.

3. Remove the bottle with the opening pointed upward from the water surface and tightly cap it, allowing about 2.5 to 5 cm of headspace (American Public Health Association and others, 1998, p. 9-19; Bordner and Winter, 1978, p. 8). This procedure minimizes collection of surface film and prevents contact with the streambed.

Do not sample in or near an open water body without wearing a correctly fitted personal flotation device (PFD).

Quality control in surface-water sampling. Depending on the data-quality requirements of the study and site conditions, quality-control (QC) samples (field blanks, field replicates, and matrix spikes) generally constitute from 5 to 20 percent or more of the total number of samples collected over a given period of time. See "Selected Terms and Symbols" in the Conversion Factors section at the end of this chapter, which contains definitions of the quality-control terms shown below in bold type.

- **Field blanks**—Collect field blanks at a frequency of 1 in every 10 to 20 samples to document that sampling equipment has not been contaminated.

  Process field blanks before collecting the water sample as follows:

  1. Pass sterile DIW through sterile sampling equipment and into a sterile sample container.
  2. Analyze sterile DIW for fecal indicator viruses. If no viruses are observed, then the sample was collected by use of sterile procedures.

- **Field replicates**—Collect one field replicate for every 10 to 20 samples.

- **Matrix spikes**—Collect a set of matrix spike samples for each coliphage type when samples are first received from a water source. Once received from a water source, collect a set of matrix spike samples after every 20th sample from that source. The matrix spike samples are spiked with known amounts of coliphage by the analyzing laboratory.

7.2.2.B GROUND-WATER SAMPLE COLLECTION

As with surface water, most viruses in ground water are associated with solid particles. Stable values of field measurements (turbidity, temperature, dissolved-oxygen concentration, pH, and specific conductance), especially turbidity and dissolved oxygen, are important criteria for judging whether a well has been sufficiently purged for the collection of a representative ground-water sample for fecal indicator virus analysis (NFM 4.2 and 6.0.3.A). Sampling equipment that has been subjected to chlorinating and dechlorinating agents can affect the chemistry of samples collected for non-microbial analysis; therefore, collect blank samples to be analyzed for chloride, sulfate, and other constituents, as appropriate, to document that chemical sample quality has not been compromised.

- If using the same equipment for chemical-analysis and virus-analysis samples, clean the equipment by first using standard procedures (NFM 3), followed by disinfecting and rinsing procedures described in section 7.2.1. Purge the well as described in NFM 4.2 before collecting samples.
- If different equipment will be deployed in a well for virus sampling, first check for stable turbidity and dissolved-oxygen readings to ensure collection of a representative sample.

Supply wells

If samples are to be collected from a water-supply well (see definition in NFM 4.2), select a tap (spigot) that supplies water from a service pipe connected directly to the main; do not use a tap on a pipe served by a cistern or storage tank (American Public Health Association and others, 1998, p. 9-19 to 9-20; Britton and Greeson, 1989, p. 5; Bordner and Winter, 1978, p. 5-16). Avoid sampling after downhole chlorination. Dechlorination with $Na_2S_2O_3$ is required if you cannot avoid collecting the sample before the water has passed through the treatment unit.

Do not sample from leaking taps.

To sample a supply well for fecal indicator viruses:

1. Before collecting the sample, remove screens, filters, or other devices from the tap.
2. Before sampling, swab the inside and outside rim of the tap with ethanol. Flame sterilize the tap and allow it to dry and cool. Rinse the tap with sterile DIW.
3. Collect a sample directly from the tap into a sterile bottle without splashing or allowing the sample bottle to touch the tap.
    - Supply wells commonly are equipped with permanently installed pumps. If the well is pumped daily, then (a) purge the tap water for a minimum of 5 minutes, discarding the purged water appropriately; (b) monitor field measurements and record stabilized values (NFM 6); and (c) collect the sample directly from the tap into a sterile container (described in table 7.2-2).
    - If the well is used infrequently, then purge the tap or well of water until a minimum of three borehole volumes are purged and stable field measurements are obtained in sequential measurements (NFM 4.2 and 6.0.3.A).

Monitoring wells

If a monitoring well does not have an in-place pump, then obtain samples by using a portable sampler, such as a submersible pump or a bailer (U.S. Environmental Protection Agency, 1982). Samplers and sample lines must be sterilized or disinfected (table 7.2-1). If disinfected, then the sampler and sample line must be dechlorinated and rinsed with sterile DIW. In either case, finish by flushing the sampler and sample line with native ground water before samples are collected into sterile bottles.

- ▶ Use autoclavable samplers, if possible. After flushing the sterilized pump lines with sample, collect the sample directly into the sterile sample bottles.
- ▶ Check data-collection objectives before using a disinfectant. Disinfectants are corrosive; repeated use can result in damage to the metal and plastic parts of a pump, thus rendering the pump inadequate for sampling trace elements and other constituents.

To disinfect a pump:

1. Submerge the pump and pump tubing in a 0.005 percent (50 mg/L) sodium hypochlorite solution prepared from household laundry bleach.

    - Most bleach is about 5 to 7 percent sodium hypochlorite (50,000 to 70,000 mg/L), but bleach in a container left open for more than 60 days may not be full strength.
    - Prepare solutions fresh with each use, because they will diminish in concentration with time. Add 1 mL of household laundry bleach to 900 mL of water and bring to a volume of 1,000 mL for a 0.005 percent disinfectant solution (U.S. Environmental Agency, 1982, p. 253 and 1996, p. VIII–41). This concentration is sufficient for waters with pH between 6 and 8 and temperatures greater than 20°C. Outside these ranges, a more concentrated disinfectant solution, up to 0.02 percent (200 mg/L), should be used (U.S. Environmental Protection Agency, 1982, p. 253).

2. Circulate the disinfectant through the pump and tubing for 30 minutes.

3. Next, rinse the pump thoroughly with a sterile $Na_2S_2O_3$ solution. The $Na_2S_2O_3$ solution is prepared by adding 0.5 mL of a 10-percent sterile solution to every 1 L of sterile DIW. Recirculate for 5 minutes and rinse with sterile DIW.

4. Lower the pump carefully into the well. Pump some well water to waste to remove any residual chlorine and $Na_2S_2O_3$. Take care not to contaminate samples for chemical analysis with residual disinfectant or $Na_2S_2O_3$. **The pump must have a backflow check valve (an antibacksiphon device) to prevent residual disinfectant from flowing back into the well.**

If the pump cannot be disinfected:

1. Handle the pump and tubing carefully to avoid contamination. If the pump is a downhole dedicated pump, proceed to step 3.
2. Collect field blanks through the sampling equipment. Lower the pump in the well to the desired intake location.
3. Purge the well with the pump used for sampling to allow the pump and tubing to be thoroughly flushed with aquifer water before sampling (NFM 4.2 and 6.0.3.A).
4. An alternative to sampling with the pump is to remove the pump after completion of purging and collection of other samples, and then to collect the coliphage sample using a sterile bailer (U.S. Environmental Protection Agency, 1982, p. 252–253). Evaluate the potential for bias from stirring up particulates during pump removal and bailing that otherwise would not be included in the sample.

Sample-preparation activities, such as purging, must be carried out in such a way as to avoid contaminating the well, the equipment, or the samples. Avoid collecting surface film from the well water in the sample and ensure that the sampler intake is within the screened interval targeted for study. Select a point-source sampler, such as a bailer with a double-check valve. Do not use a bailer unless the bailer can be sterilized.

The type of well, its use, construction, composition, and condition could lead to alteration or contamination of samples. For example, a poor surface seal around the well opening can allow contaminants to move quickly from the land surface into the well water.

- If the water level is less than 7 to 10 m (roughly 20 to 30 ft) below land surface, a sample can be collected without contamination and without chlorine disinfection by use of a surface peristaltic or vacuum pump, a sterile vacuum flask, and two lengths of sterile tubing (U.S. Environmental Protection Agency, 1982).

- Purge the well (see NFM 4.2 and 6.0.3.A) while monitoring field measurements, especially measures of turbidity and dissolved oxygen. For wells in which field measurements do not stabilize after increasing the total number of measurements, record the final measurements and proceed with sampling.

Be vigilant in avoiding contamination. The detection of even one coliphage in ground water is cause for concern because it indicates the possible presence of pathogens.

Quality control for ground-water sample collection. Depending on the data-quality requirements of the study, quality-control samples (field blanks and matrix spikes) generally constitute from 5 to 20 percent or more of the total number of samples collected over a given time period. See "Selected Terms and Symbols" in the Conversion Factors section at the end of this chapter, which contains definitions of the quality-control terms shown below in bold type.

- **Field blanks—**Collect field blanks at a frequency of 1 in every 10 to 20 samples if required by data-quality objectives. **Process field blanks before collecting the water sample.** Pass sterile DIW through the sampling equipment and into a sterile sample container. Analyze the field blank for fecal indicator viruses and record the results. If no viruses are observed, the use of sterile procedures is confirmed and documented.

> **TECHNICAL NOTE:** The field blank discussed herein is equivalent to the "pump blank" described in NFM 4.3.1. Refer to NFM 4.3.1 for more information on collecting a field blank for ground-water sampling. A standpipe may be used to collect a field blank, but first must be cleaned and then disinfected. **This type of blank should be collected a week or more ahead of time so that results can be evaluated before field sampling.**

- **Field replicates** (sequentially collected samples)—Field replicates for ground-water samples are optional and their use depends on study objectives and site conditions. Ground-water samples typically are negative for coliphage.
- **Matrix spikes—**Collect a set of matrix spike samples for each coliphage type when samples are first received from a water source or aquifer type. Once received from a water source or aquifer type, collect a set of matrix spike samples after every 20th sample from that source or type. The matrix spike samples are spiked with known amounts of coliphage by the analyzing laboratory.

SAMPLE PRESERVATION, TRANSPORT, AND HOLDING TIMES 7.2.2.C

After collection, immediately chill samples in an ice chest or refrigerator at 1 to 4°C. **Do not freeze samples.** To ship samples to the laboratory, double bag the sample containers before placing them into the bagged ice in the ice chest. Seal the analytical services request form and chain-of-custody form in double plastic bags and tape this to the inside lid of the ice chest being shipped to the laboratory. Check that the sample identification and relevant information for use by the laboratory have been recorded correctly. **The laboratory must begin the analysis of samples within 48 hours of sample collection.**

The holding time for fecal indicator virus samples is 48 hours from the time of sample collection.

7.2.3 LABORATORY METHODS

Two methods described in this manual for the detection of fecal indicator viruses are the single-agar layer (SAL) method and the two-step enrichment method. The host bacteria recommended for use by these methods are *E. coli* CN-13 for the detection of somatic coliphage and *E. coli* F-amp for the detection of F-specific coliphage. Analytical protocols are available in more detail from the USGS Ohio District Microbiology Laboratory (U.S. Geological Survey, website: http://oh.water.usgs.gov/micro/lab.html#am) (accessed November 25, 2003).

7.2.3.A SINGLE-AGAR LAYER METHOD

The SAL method detects and enumerates somatic and F-specific coliphages in water. It is a plaque assay method that is recommended for use with surface-water samples.

USEPA Method 1602 (U.S. Environmental Protection Agency, 2001b) is a SAL method that requires the addition of host bacteria, magnesium chloride, appropriate antibiotics, and double-strength molten agar to the sample, followed by pouring the total volume of the mixture into plates. After an overnight incubation, the plates from a sample are examined for plaque formation (zones of bacterial host lawn clearing). The plaques are counted and summed for all plates from a single sample. The quantity of coliphage in a sample is expressed as plaques per 100 milliliters. This method requires one overnight incubation; therefore, results are available 24 hours after the beginning of the analysis.

Quality Control. Each laboratory and analyst that uses USEPA Method 1602 must fulfill the following general quality-control requirements, as described in the method:

- **Initial Precision and Recovery (IPR)**—The laboratory and analyst must demonstrate the ability to generate acceptable results by performing an IPR test before analyzing any environmental samples.
- **Method Blanks**—The laboratory must analyze reagent water samples containing no coliphage to demonstrate freedom from contamination. Method blanks should be run with each batch of samples. A batch is defined as all samples analyzed during a single day, up to a maximum of 20 samples per coliphage type.
- **Ongoing Precision and Recovery (OPR)**—The laboratory must demonstrate that the method is in control on an ongoing basis through analysis of OPR samples. OPR samples are reagent-water samples spiked with known amounts of coliphage and analyzed exactly like environmental samples. The laboratory must analyze one OPR sample for each batch of samples. A batch is defined as all samples analyzed during a single day, up to a maximum of 20 samples per coliphage type. The OPR serves as the positive control for Method 1602.

TWO-STEP ENRICHMENT METHOD 7.2.3.B

The two-step enrichment method determines the presence or absence of somatic and F-specific coliphages in water. **This method is recommended for use with ground-water samples.**

USEPA Method 1601 (U.S. Environmental Protection Agency, 2001a) is a two-step enrichment method that requires the enrichment of coliphage in tryptic soy broth supplemented with magnesium chloride, appropriate antibiotics, and host bacteria. After an overnight incubation, samples are spotted onto a lawn of host bacteria specific for each type of coliphage. The spot plates are incubated and examined for lysis zone formation in the lawn. Lysis zone formation indicates the presence of coliphages in the sample. This method requires two overnight incubations; therefore, results are available 48 hours after the beginning of the analysis.

Quality control. Each laboratory and analyst that uses Method 1601 must fulfill the following general quality-control requirements as described in the method.

- **Initial Demonstration of Capability (IDC)**—The laboratory and analyst must demonstrate the ability to generate acceptable results by performing an IDC test before analyzing any environmental samples.
- **Method Blanks**—The laboratory must analyze reagent-water samples containing no coliphage to demonstrate freedom from contamination. The laboratory must analyze one method blank per spot plate.
- **Positive Controls**—The laboratory must analyze positive control samples (reagent water spiked with a known amount of coliphage) to demonstrate that method reagents are performing properly. The laboratory must analyze one positive control per spot plate.
- **Ongoing Demonstration of Capability (ODC)**—The laboratory must demonstrate that the method is in control on an ongoing basis through analysis of ODC samples. The laboratory must analyze one set of ODC samples after every 20 field and matrix spike samples. For each coliphage type, at a minimum, one out of three reagent-water samples spiked with a known amount of coliphage must be positive.

CALCULATION AND REPORTING OF FECAL INDICATOR VIRUSES 7.2.4

The calculation and reporting protocols differ, depending on the laboratory method used. A list of parameter codes for reporting coliphages in the USGS National Water Information System (NWIS) are given in Appendix A7-A, table 3.

- **SAL method**—Count the total number of plaques from all plates for a sample. If the plaques are not discrete, results should be recorded as "too numerous to count" (TNTC), and the remaining sample should be diluted and reanalyzed if possible within 48 hours of collection. Record the result as the total number of plaques per 100 milliliters (plaques/100 mL).
- **Two-step enrichment method**—Record results as presence (1) or absence (2) of coliphage.

For each sample analyzed, document:

- **the type of coliphage analyzed,**
- **the bacterial host strain used,**
- **the sample volume analyzed, and**
- **the corresponding QC results from the laboratory.**

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PROTOZOAN PATHOGENS 7.3

By R.N. Bushon and D.S. Francy

Illustrations

Tables

Notes:

References for section 7.3, Protozoan pathogens, are located at the end of Chapter A7 in the "Selected References and Documents" section, which begins on page REF-1.

See Appendix A7-A, Table 4, for parameter codes for protozoan pathogens that are used in the National Water Information System (NWIS) of the U.S. Geological Survey.

The citation for this section (7.3) of NFM 7 is as follows:

Bushon, R.N., and Francy, D.S., November 2003, Protozoan pathogens: U.S. Geological Survey Techniques of Water-Resources Investigations, book 9, chap. A7 (3d ed.), section 7.3, accessed ___*date*___, from http://pubs.water.usgs.gov/twri9A/.

PROTOZOAN PATHOGENS 7.3

Protozoan pathogens are widely distributed in the aquatic environment and have been implicated in several outbreaks of waterborne diseases (Lee and others, 2002; Rose and others, 1997). *Cryptosporidium* and *Giardia* are the principal protozoan pathogens that are known to affect the acceptability of water supplies for public use within the United States (U.S.). *Cryptosporidium* and *Giardia* produce environmentally resistant forms (oocysts for *Cryptosporidium* and cysts for *Giardia*) that allow for the extended survival of the organisms in natural and treated waters.

PROTOZOAN PATHOGENS, such as *Cryptosporidium* and *Giardia,* are unicellular microorganisms that cause disease in humans and other animals.

In comparison with fecal indicator bacteria, oocysts and cysts are more resistant to disinfection, survive longer in the environment, and are much larger and more complex. Fecal indicator bacteria are, therefore, inadequate as indicators for *Cryptosporidium* and *Giardia* in source waters. The presence of protozoan pathogens in water must be verified by identification of the pathogens themselves.

Fecal indicator bacteria cannot be used to indicate the presence of *Cryptosporidium* or *Giardia* in source water.

A sampling program for *Cryptosporidium* oocysts and *Giardia* cysts should be conducted over an extended period of time because of cyclical and seasonal variations in their environmental concentrations (LeChevallier and Norton, 1995). For example, seasonal differences in the volume and intensity of precipitation or in the shedding of parasites by animals can account for elevated occurrences of oocysts and cysts in water (Atherholt and others, 1998). The average percentages of *Cryptosporidium* and *Giardia* occurrence in U.S. waters vary considerably among published studies, ranging from 10 to 60 percent for *Cryptosporidium* and 16 to 90 percent for *Giardia* (Atherholt and others, 1998; LeChevallier and Norton, 1995; LeChevallier and others, 2003; Rose and others, 1988; Rose and others, 1991). In these studies, concentrations of protozoan pathogens in environmental waters were considerably lower than concentrations of fecal indicator bacteria; average concentrations of *Cryptosporidium* ranged from 0.7 to 10 oocysts per 10 liters (L) of water and of *Giardia* from 0.8 to 7 cysts per 10 L. Higher concentrations of *Cryptosporidium* and *Giardia* were found in waters receiving industrial and sewage effluents than were found in waters not receiving these wastes and (or) having more extensive watershed-protection practices (LeChevallier and others, 1991).

The U.S. Environmental Protection Agency (USEPA) Method 1623 (Method 1623—filtration/immunomagnetic separation (IMS)/fluorescent antibody (FA)) currently is the method of choice for detecting *Cryptosporidium* oocysts and *Giardia* cysts in water. This method does not identify the species of *Cryptosporidium* and *Giardia*, nor does it determine the viability or infectivity of the detected organisms. Method 1623 is a performance-based method, which means that alternative components not listed in the method may be used, provided that the results meet or exceed the acceptance criteria described in the method. Aspects of the method that may be modified can include, but are not limited to, the type of filter used, the manufacturer of the magnetic beads, and the protocol used to separate the oocysts and cysts from the magnetic beads. Because the method is complex, only experienced analysts should use it (U.S. Environmental Protection Agency, 2001a).

Recoveries of *Cryptosporidium* and *Giardia* are determined in the same manner as are recoveries of chemical constituents, such as pesticides. A suspension is prepared of *Cryptosporidium* oocysts and *Giardia* cysts and quantified by use of an accurate method, such as flow-cytometry, which uses a particle-sorting instrument capable of counting protozoa. The suspension with known concentrations of organisms then is used to spike an environmental water sample in the laboratory. Recoveries of oocysts and cysts from environmental water samples using Method 1623 can vary greatly, which is an important consideration for data interpretation.

TECHNICAL NOTE: Recoveries of *Cryptosporidium* ranged from 2 to 63 percent in 11 stream-water samples (Simmons and others, 2001), 20 to 60 percent in 430 samples from 87 source waters (U.S. Environmental Protection Agency, 2001b), and 9 to 88 percent in samples from 19 surface-water sites (Kuhn and Oshima, 2002). In one large study, average recoveries of *Giardia* were 47 percent, with a relative standard deviation of 32 percent (U.S. Environmental Protection Agency, 2001b).

7.3.1 STERILIZATION PROCEDURES FOR SAMPLING EQUIPMENT

Sterile technique must be implemented and documented when collecting and processing samples for protozoan pathogens. **In addition, the specific equipment and supplies that are needed to collect and analyze samples for protozoan pathogens must be kept clean and sterile** before sampling at each site and for each sample collected at the same site at different times (table 7.3-1, and table 7.3-2 in section 7.3.2).

- All equipment should be cleaned with nonphosphate, laboratory-grade detergent and rinsed thoroughly with deionized/distilled water (DIW) before being sterilized.
- Procedures to sterilize equipment involve either: (1) cleaning selected equipment with a 12-percent sodium hyplochlorite (bleach) solution (section 7.3.1.A), or (2) rigorous washing followed by autoclaving ("Alternative Sterilization Method," section 7.3.1.B).
- Equipment must be wrapped.
  - Wrap equipment that has been sterilized using the sodium hypochlorite method in sterile aluminum foil, sterile autoclavable bags, or sterile kraft paper. The equipment is then ready for storage or for transport.
  - If the sodium hypochlorite method is not used, then equipment first must be wrapped in aluminum foil, autoclavable bags, or kraft paper, and then autoclaved. After autoclaving, equipment must remain wrapped for storage or transport.
- Resterilize equipment if foil, bag, or kraft paper is torn.

Autoclaving kills oocysts and cysts and eliminates infectivity; however, epitopes (proteins on the surface of cells) are not inactivated by autoclaving. Epitopes attach to the fluorescent stain used in Method 1623 and are detected microscopically. To avoid false positives that are caused by residual epitopes from a previous sample, use a strong (12-percent) sodium hypochlorite solution (full-strength swimming-pool bleach) to sterilize the equipment (section 7.3.1.A).

Table 7.3-1. Summary of equipment cleaning and sterilization procedures

[L, liter; DIW, distilled or deionized water; g/L, grams per liter; °C, degrees Celsius]

Equipment and supplies
• Autoclavable 1-L bottle or 3-L bag, nozzle, and cap. • Collapsible low-density polyethylene cubitainer for collection of a 10-L bulk sample. • Regular and sterile DIW. • Nonphosphate, laboratory-grade detergent. • 12-percent sodium hypochlorite solution. • Aluminum foil, autoclavable bag, or kraft paper.
Cleaning and sterilization procedures
Sodium hypochlorite sterilization method: • Scrub equipment with a dilute (1-percent) nonphosphate, laboratory-grade detergent solution. • Rinse three to five times with tap water. • Submerge equipment in a 12-percent (120 g/L) sodium hypochlorite solution for 30 minutes. • Using sterile DIW, rinse thoroughly, inside and out, at least three times. • Wrap equipment in sterile aluminum foil or sterile kraft paper, or place into a sterile bag. **Do not use this method to disinfect equipment used to collect samples for subsequent determination of trace elements and organic substances – metallic and plastic equipment components can be damaged and subject to early deterioration after repeated sterilization with a strong sodium hypochlorite solution.**
Alternative sterilization method: (Use if equipment contact with sodium hypochlorite should be avoided). • Soak equipment in a dilute (1-percent) nonphosphate, laboratory-grade detergent solution for 30 minutes. • Scrub well and rinse three to five times with tap water. • Rinse again three to five times with DIW. • Wrap equipment in aluminum foil or kraft paper, or place into autoclavable bags. • Autoclave at 121°C for 20 minutes.

7.3.1.A SODIUM HYPOCHLORITE STERILIZATION METHOD

As noted previously, to avoid false positives that are caused by residual epitopes from a previous sample, it is necessary to immerse the equipment in a strong sodium hypochorite solution.

To sterilize sampling equipment using the bleach sterilization method:

1. Set up a clean area and assemble the needed equipment and supplies.
2. Scrub equipment with a dilute (1-percent) nonphosphate, laboratory-grade detergent (if equipment is being cleaned in the field, use a 0.1-percent detergent solution and rinse thoroughly with DIW).
3. Rinse three to five times with tap water.
4. Soak equipment for 30 minutes in a 12-percent (120 grams per liter) sodium hypochlorite (full-strength pool bleach) solution.
5. Rinse the equipment a minimum of three times with sterile DIW. Use only sterile DIW to rinse the equipment—**do not use a sodium thiosulfate solution to neutralize the sodium hypochlorite when rinsing the equipment.**
6. Wrap equipment in sterile aluminum foil or sterile kraft paper, or place into a sterile autoclavable bag.

The 12-percent sodium hypochlorite solution is very caustic and, over time, can damage sampling equipment.

ALTERNATIVE STERILIZATION METHOD 7.3.1.B

To avoid deterioration of equipment that also is used to collect samples for trace-element or organic-compound analyses, an alternative sterilization method should be used. As a result of repeated exposure to a strong sodium hypochlorite solution, metallic surfaces can corrode and plastic equipment components can become brittle, shortening the life of the equipment. The alternative sterilization method described below consists of two major steps: (1) rigorous equipment washing, and (2) autoclaving.

Equipment Washing

Rigorous washing of sample-collection and sample-processing equipment is essential before equipment is autoclaved.

To sterilize sampling equipment using the alternative sterilization method:

1. Soak equipment in 1-percent nonphosphate, laboratory-grade detergent for 30 minutes. Scrub the equipment well, using a soft brush.
2. Rinse all parts of the equipment thoroughly three to five times with tap water, followed by three to five rinses with DIW.
3. Wrap equipment in aluminum foil or kraft paper, or place into autoclavable bags.
4. Autoclave equipment, following the guidelines described below.
5. **Collect additional quality-control samples** (for example, equipment and field blanks) to determine whether the alternative sterilization method was effective. **Use sterile DIW as the blank solution.**

The alternative sterilization method avoids use of the strong sodium hypochlorite solution, but requires collection of an equipment blank for quality control of the method's efficacy.

Autoclaving

Sampling equipment must be autoclaved for 20 minutes at 121°C before use. (If the sodium hypochlorite sterilization method is used, autoclaving is not necessary.)

- Use only autoclaves that have temperature, pressure, and liquid- and dry-utensil-cycle controls. **Do not use** steam sterilizers, vertical autoclaves, and pressure cookers without temperature controls.
- Ensure that the materials to be autoclaved are thermally stable. Autoclavable materials include plastics (such as polycarbonate, polypropylene, polyallomer, and polymethylpentene) and Teflon® and Tefzel® (such as perfluoroalkyoxy-polymers (PFA), ethylenetetrafluoroethylene (ETFE), fluorinated ethylene propylene (FEP), and polytetrafluoroethylene polymers (PTFE)). **Note that each of these materials has different thermal characteristics and tolerances to repeated autoclaving.**
- Consult the 20th edition of "Standard Methods for the Examination of Water and Wastewater" (American Public Health Association and others, 1998, Section 9020 B, table 9020:III) for specifications regarding the length of time, temperature, and pressure for autoclave sterilization of various materials.

- When using the autoclave, it is necessary to:
  - Use sterilization indicator tape with each load.
  - Test the autoclave performance at least quarterly, using commercially available biological indicators. Biological indicators are composed of endospores—living cells that are resistant to heat but that can be destroyed by autoclaving.
  - Drain the autoclave at the end of each period of use.
  - Clean the autoclave with mild soap and water once a week during periods of daily use.
  - Avoid overloading the autoclave with equipment or materials; overloading will result in incomplete sterilization.
- Keep a logbook of the autoclave operation.
  - Record the temperature, pressure, date, and time of each autoclave run.
  - Record the date of each cleaning and the procedures used.
  - Enter into the logbook the results from the regularly scheduled quality-control (biological-indicator) checks, noting the date, the test results, and the name of the autoclave operator and (or) analyst.

Quality-control tests for autoclave operation are mandatory.

If the autoclave does not reach the specified temperature and pressure or fails the quality-control test, then service the autoclave, retest the autoclave, and resterilize all materials (American Public Health Association and others, 1998, p. 9-2 to 9-13).

7.3.2 SAMPLE COLLECTION, PRESERVATION, TRANSPORT, AND HOLDING TIMES

The specific procedures that have been developed for the collection, preservation, transport, and holding times of water samples before the samples are analyzed for protozoan pathogens must be followed strictly. These procedures can vary with types of sampling equipment and source of sample (surface water, ground water, treated water, or wastewater).

Maintain sterile conditions throughout sample collection, preservation, transport, and analysis.

Currently, samples for analysis of protozoan pathogens are collected primarily from surface water. Protozoan pathogens are not commonly found in ground water, although they have been known to occur in ground water that is in direct hydraulic connection with ("under the influence of") surface water. A summary of requirements for sample-collection equipment, procedures for sample preservation, and holding-time requirements is given in table 7.3-2.

Table 7.3-2. Summary of equipment and sample-preservation procedures used for surface-water sample collection for protozoan pathogen analysis

[EWI, equal-width increment; EDI, equal-discharge increment; L, liter; *NFM, National Field Manual for the Collection of Water-Quality Data*; mL, milliliter; $Na_2S_2O_3$, sodium thiosulfate; EDTA, ethylenediaminetetraacetic acid; °C, degrees Celsius]

Equipment for sample collection
For EWI or EDI surface-water samples: use US D-95, US DH-95, or US DH-81 samplers with a sterile 1-L wide-mouth bottle, and sterile caps and nozzles, or the US D-96 with a sterile 3-L autoclavable bag (NFM 2.1.1).
For surface-water samples using point samplers or the hand-dip method: use a sterile, narrow-mouth container, 1-L or 3-L capacity.
For preparing sample composites: use a collapsible, low-density polyethylene cubitainer for collection of the 10-L bulk sample (fig. 7.3-1)
Procedures for sample preservation
Before sample collection: if halogen neutralization is needed to preserve the sample, add 0.5 mL of a 10-percent $Na_2S_2O_3$ solution per 1 L of sample (NFM 7.3.2.B). -If sterile $Na_2S_2O_3$ is used, then dispense with sterile pipet into sterile bottle. -If $Na_2S_2O_3$ is not sterile, then dispense with pipet into sample bottle and autoclave.
Before sample collection: if chelation of trace elements is needed to preserve the sample, then add 0.3 mL of a 15-percent EDTA solution per 100 mL of sample (NFM 7.3.2.B).
After sample collection: Chill all samples at 0 to 8°C to preserve the sample until analysis.
Maximum holding time
Do not hold the sample for longer than 96 hours after sample collection and before sample analysis for protozoan pathogens.

7.3.2.A SAMPLE COLLECTION

The spatial and temporal distribution of microorganisms can be as variable as the distribution of suspended sediment in water because microorganisms generally associate with solid particles. **Collection of quality-control (QC) samples is an essential component of the sampling process.**

CAUTION: Always wear gloves when handling sampling equipment and samples. Take care to prevent contaminated water from contacting skin, mouth, nose, or eyes (NFM 9.7).

- **Ground Water:** Follow the guidelines described in NFM 7.1.1.B for the collection of fecal indicator bacteria in ground water, but collect a 10-L bulk sample. The use of the alternative sterilization method is recommended when using a pump with metallic components (see section 7.3.1).
- **Surface Water:** To obtain data that accurately represent the site at the time of sampling, use the same methods for collecting surface-water samples for protozoans as for suspended sediment (Edwards and Glysson, 1999; NFM 4.1).
  - For the isokinetic or hand-dip sample-collection methods described below, collect the water using 1-L bottles or 3-L bags and prepare a 10-L bulk composite sample by pouring the bottle or bag contents into a collapsible, low-density polyethylene cubitainer (fig. 7.3-1).
    - **Flowing water:** use isokinetic depth-and-width-integrating sampling methods[7] (NFM 4.1.1.A).

[7]Sample-collection methods may be modified to ensure consistency with study objectives and as appropriate for site conditions. It is necessary to describe any modifications to methods in a report of the results of the study.

- **Still water** (lakes or reservoirs, or other surface-water conditions for which depth-and-width-integrating methods are not applicable): use the hand-dip method or a sterile point sampler (NFM 4.1.1.B).

— Be sure to fill the cubitainer completely, to ensure collection of a full 10-L sample.

Figure 7.3-1. Samples for protozoan pathogens are collected in a sterile 1-liter or 3-liter bottle and composited into a 10-liter sterile cubitainer. (Photograph by Richard P. Frehs.)

Quality Control

Plan to collect quality-control samples. Although subject to the specific data-quality requirements and site conditions of the study, quality-control samples typically constitute at least 5 to 20 percent of the total number of samples collected over a given period of time at a given location. General requirements and recommendations for the common types of quality-control samples are described below ("Selected Terms" in the Conversion Factors section at the end of NFM 7 contains definitions of these quality-control terms as they apply to protozoan pathogens).

- **Equipment blanks**—An equipment blank is required when equipment is sterilized using the alternative sterilization method, or when study objectives require additional quality-control samples. Equipment blanks are optional for the sodium hypochlorite sterilization method.
- **Field blanks—**Field blanks generally are not required because of the low potential for contamination. Their use depends on study objectives and site conditions.
- **Field replicates—**Field replicates generally are optional because of the low numbers of protozoans in most waters. The use of replicate samples depends largely on site conditions and study objectives.
- **Matrix spikes—**Samples for matrix spikes are collected routinely for studies involving protozoan analyses. **A second 10-L sample must be collected for the matrix spike.** Matrix-spike samples are fortified (spiked) with known amounts of oocysts and cysts by the analyzing laboratory. As previously noted, the recovery of oocysts and cysts from environmental samples using Method 1623 has been found to be highly variable and affected by water chemistry, as well as by streamflow and other characteristics of the water body.

— Collection of a 10-L matrix-spike sample along with the first 10-L sample that is collected from a water source is required (U.S. Environmental Protection Agency, 2001a, Section 9.5).

— Although USEPA guidelines stipulate the collection of additional matrix-spike samples from the same source water after at least every 20th sample, **the USGS recommends collecting matrix-spike samples more frequently, as is appropriate for specific study objectives, streamflow conditions, and chemical characteristics.**

Isokinetic Sampling Methods

Isokinetic sampling methods, including the equal-discharge-increment (EDI) method, equal-width-increment (EWI) method, and single vertical at centroid-of-flow (VCF) method, are the standard USGS methods used for sampling flowing waters and are recommended unless study objectives or site characteristics dictate otherwise (NFM 4.1.1.A).

1. Select the appropriate isokinetic method (NFM 4.1). The EDI method is preferred at sites where the velocity distribution across a stream section is well established or at a section where the depth varies (for example, at a gaging station) (Edwards and Glysson, 1999).
2. Select the appropriate sampler and equipment and prepare the equipment for use (section 7.3.1). **Sampling equipment must be sterile,** including the collection bottle (or bags for the bag sampler), nozzle, and cap (table 7.3-1).
    - For streams with depths of 5 meters (m) (approximately 16.4 feet) or less, use a US D-95, US DH-95, or US DH-81 sampler (NFM 2.1.1).
    - For stream sections where depths exceed 5 m (16.4 feet), use the US D-96, with either autoclavable Teflon® bags or autoclavable cooking bags. Thermotolerant polymers are described in section 7.3.1.B under “Autoclaving.”
    - Use the proper nozzle size and transit rate for the velocity conditions in the section to ensure isokinetic collection of the sample (NFM 4.1.1.A).

Hand-Dip Sampling Method

If the stream depth and (or) velocity are not sufficient to use an isokinetic sampling method, collect a sample using a hand-dip method. Sampling still water or sampling at depth in lakes, reservoirs, estuaries, and oceans requires a sterile point sampler. For example, Niskin, ZoBell, and Wheaton point samplers hold a sterilizable bottle or bag. Wearing nonpowdered nitrile or latex gloves (NFM 2), collect a hand-dip sample as follows:

1. Open a sterile, plastic bottle; grasp the bottle near the base, keeping hand and arm on the downstream side of the bottle.
2. Without rinsing it, plunge the bottle opening downward, below the water surface, with the opening pointed slightly upward into the current. Allow the bottle to fill.
3. Remove the bottle with the opening pointed upward from the water and tightly cap it. Composite several bottles into a 10-L cubitainer until it is full.

CAUTION:
Do not sample in or near an open water body without wearing a correctly fitted personal flotation device (PFD).

SAMPLE PRESERVATION, TRANSPORT, AND HOLDING TIMES 7.3.2.B

Halogens and trace metals that are present in the source water can compromise an accurate analysis of the sample for protozoan pathogens. Residual chlorine commonly is found in treated (disinfected) potable water (for example, public water systems), and in sources such as wastewater effluents or mixing zones directly downstream from wastewater-treatment plants. Most taps or wells (for example, small private water systems) do not contain residual chlorine. Trace metals such as copper, nickel, and zinc that are present at concentrations from 10 to greater than 1,000 micrograms per liter (μg/L) can be toxic to microorganisms; the concentration at which toxicity occurs varies in the literature (Britton and Greeson, 1989, p. 56; Bordner and Winter, 1978, p. 6; American Public Health Association and others, 1998, p. 9-19). The sample must be treated at the time of collection to prevent halogen and trace-metal interferences.

- **Add sodium thiosulfate ($Na_2S_2O_3$)** to sample bottles, either before sterilization or immediately after sample collection, if the water to be collected is suspected to contain residual chlorine or other halogens.
- **Add ethylenediaminetetraacetic acid (EDTA)** to sample bottles before filling the bottles with sample, if trace-metal concentration is suspected at levels that could be toxic to protozoan pathogens.

To prepare for collecting a halogenated sample:

1. Prepare a 10-percent solution of $Na_2S_2O_3$ as follows:
   a. In a volumetric flask, dissolve 100 grams (g) $Na_2S_2O_3$ into 500 milliliters (mL) of DIW. Stir until dissolved. Fill flask to 1,000 mL with DIW (Bordner and Winter, 1978, p. 6; American Public Health Association and others, 1998, p. 9-19).
   b. Autoclave at 121°C for 30 minutes (U.S. Environmental Protection Agency, 1996, p. VIII–11).
   c. Store the $Na_2S_2O_3$ solution at room temperature or under refrigeration. **After 6 months, prepare a fresh solution** and label the bottle with the contents and date of preparation. **Discard unused solution that has expired, either through a certified laboratory or according to existing regulations for your locale.**
2. Before collecting the sample in the sample bottle, pipet into the sample bottle 0.5 mL of 10-percent $Na_2S_2O_3$ solution for every 1 L of sample. If the sterile $Na_2S_2O_3$ is used, then be sure to use only sterile pipets and sterile sample bottles. If the $Na_2S_2O_3$ is not sterile, then dispense the required volume of $Na_2S_2O_3$ into a sample bottle and autoclave at 121°C for 15 minutes.

To prepare for collecting samples with potential trace-metal toxicity:

1. Prepare the EDTA stock solution as follows:
   a. Dissolve 372 milligrams (mg) of EDTA in 1,000 mL of DIW (American Public Health Association and others, 1998, p. 9-19).
   b. Store the EDTA stock solution at room temperature.
   c. Keep the bottles tightly capped between uses. **After 6 months, prepare a fresh solution** and label the bottle with the contents and date of preparation. **Discard unused solution that has expired, either through a certified laboratory or according to existing regulations for your locale.**

2. Before sterilization, add 0.3 mL of the EDTA stock solution per 100 mL of sample to sample bottles. EDTA can be combined with the $Na_2S_2O_3$ solution in the sample bottle.
3. Autoclave the sample bottle containing EDTA stock solution at 121°C for 15 minutes.

$Na_2S_2O_3$ and EDTA solutions have a 6-month shelf life.

To prepare the samples for transport:

1. **Chill—do not freeze—**the 10-L sample cubitainer in an ice chest or refrigerator at 0 to 8°C **immediately after the samples have been collected and treated.**
2. Check that the sample identification and relevant information for use by the laboratory have been recorded correctly on the sample container and on the analytical services request (ASR) form and, if being used, on a chain-of-custody form.
   - Seal the ASR form and chain-of-custody form in plastic bags and tape the bags to the inside lid of the ice chest to be shipped to the laboratory.
   - Upon receipt, the laboratory should record the temperature of the samples and store them at 0 to 8°C until processed.
   - It is best for the laboratory to process the samples as soon as possible, but **sample analysis must be within 96 hours of sample collection.**

The holding time for samples to be analyzed using USEPA Method 1623 is 96 hours from sample collection.

7.3.3 LABORATORY METHOD: USEPA METHOD 1623

Project personnel should be aware of the analytical method to be used by a laboratory on samples to be analyzed for protozoan pathogens, and the requirements for quality control for the method. The field and laboratory procedures for protozoan samples that are described in this chapter are specific to analysis by USEPA Method 1623: *Cryptosporidium* and *Giardia* in water by filtration/IMS/FA (U.S. Environmental Protection Agency, 2001a). Method 1623 must be performed in a certified laboratory by a qualified analyst, and involves the following steps:

1. **Filtration**—*Cryptosporidium* oocysts and *Giardia* cysts from the water sample are concentrated on a filter, eluted from the filter with an elution buffer, and reconcentrated by centrifugation.
2. **Immunomagnetic separation (IMS)**—The oocysts and cysts are magnetized by attachment of magnetic beads conjugated to antibodies and then separated from extraneous materials in the sample with a magnet.
3. **Immunofluorescence assay (FA)**—Fluorescently labeled antibodies and vital dye are used to make the final microscopic identification of the oocysts and cysts. The organisms are identified when the size, shape, color, and morphology agree with specified criteria.

Quality Control. Laboratory performance is compared to established performance criteria to determine whether the results of the analyses meet the performance characteristics of the method, as described in U.S. Environmental Protection Agency, 2001a, Section 9.0. Any laboratory that uses USEPA Method 1623 must fulfill the following minimum quality-control requirements: Initial Precision and Recovery (IPR) tests, Ongoing Precision and Recovery (OPR) tests, and the use of method blanks.

- **Initial Precision and Recovery (IPR)**—Each analyst in the laboratory must establish the ability to demonstrate control over the analytical system and to generate acceptable precision and recovery. IPR tests consist of reagent-water samples that are spiked with known amounts of *Cryptosporidium* and *Giardia* and that are analyzed exactly like environmental samples. The IPR test must be completed before the analysis of any environmental samples (U.S. Environmental Protection Agency, 2001a, Section 9.4).

- **Ongoing Precision and Recovery (OPR)**—The laboratory must demonstrate that the method is under control by analyzing OPR samples. OPR samples consist of reagent-water samples that are spiked with known amounts of *Cryptosporidium* and *Giardia* and that are analyzed exactly like environmental samples. The laboratory must analyze one OPR sample for each week in which environmental samples are analyzed, or after every 20th environmental sample, whichever comes first (U.S. Environmental Protection Agency, 2001a, Section 9.7).

- **Method Blank**—The laboratory must analyze reagent-water samples containing no protozoans to demonstrate freedom from contamination. Method blanks should be analyzed immediately before conducting the IPR and OPR tests. The laboratory should analyze one method blank for each week in which environmental samples are analyzed, or after every 20th environmental sample, whichever comes first (U.S. Environmental Protection Agency, 2001a, Section 9.6).

7.3.4 CALCULATION AND REPORTING OF PROTOZOAN PATHOGENS

As prescribed by USEPA Method 1623, report the total number of *Cryptosporidium* oocysts and (or) *Giardia* cysts counted.

- Record the result as the total number of oocysts or cysts per 10 L.
- Record the percent recovery for matrix spikes analyzed.

Use the list of parameter codes shown in Appendix A7-A, table 4, when reporting protozoans in the USGS National Water Information System (NWIS).

CONVERSION FACTORS, SELECTED TERMS, SYMBOLS, CHEMICAL FORMULAS, AND ABBREVIATIONS

CONVERSION FACTORS

Multiply	By	To obtain
micrometer (µm)	3.937×10^{-5}	inch (in.)
	3.3×10^{-6}	foot (ft)
millimeter (mm)	0.03937	inch
centimeter (cm)	0.3937	inch
square centimeter (cm^2)	0.155	square inch (in^2)
meter (m)	3.281	foot
nanometer (nm)	3.93×10^{-8}	inch
liter (L)	0.264	gallon (gal)
milliliter (mL)	0.0338	ounce, fluid (oz)
gram (g)	0.03527	ounce, avoirdupois
microgram (µg)	3.527×10^{-8}	ounce, avoirdupois
milligram (mg)	3.527×10^{-5}	ounce, avoirdupois
kilopascal (kPa)	0.1450	pound per square inch (lb/in^2)

Temperature: Water and air temperature are given in degrees Celsius (°C), which can be converted to degrees Fahrenheit (°F) by use of the following equation:

$$°F = 1.8(°C) + 32$$
$$°C = (°F/1.8) – 32$$

SELECTED TERMS

Distilled or deionized water: ASTM type 1 water or better.

Equipment blank: A quality control sample that consists of a blank solution processed sequentially through each component of the equipment system to be used in sample collection, processing, preservation, and handling in a controlled environment. The sample is prepared by passing sterile water or buffer through the sampling equipment (if applicable) and into a sterile sample container. Positive results for the equipment blank indicate sampling and analytical bias caused by contamination from equipment and supplies.

Field blank: A quality-control sample that consists of a laboratory-certified blank solution processed through all the equipment used in the various stages of sample collection, processing, preservation, and handling under field conditions. **For quality control of water samples for microbial analyses,** the blank sample is prepared by passing either sterile deionized or distilled water (DIW) or sterile buffered water through the sampling equipment (if applicable) into a sterile sample container. Positive growth on the field blank indicates sampling and analytical bias caused by contamination from equipment, supplies, and (or) ambient environmental conditions.

Field-generated sequential replicate and split replicate: Quality-control samples that measure the variability in all or part of the sampling and analysis system. Replicates—environmental samples collected in duplicate, triplicate, or higher multiples and collected close in time and space—are considered identical in composition and are analyzed for the same properties. **For quality control of water samples for microbial analyses,** two samples are collected sequentially in the field (sequential replicates) and each sample is analyzed twice (split replicate). The relative percent difference between the results is calculated as a measure of variability.

Filter blank (membrane-filtration): As applied to the quality control of water samples for microbial analyses, the filter blank measures the sterility of the equipment and supplies used during the membrane-filtration procedure for bacterial indicators. A 50- to 100-mL sample of sterile buffered water is passed through the filtration apparatus onto a sterile membrane filter before processing the sample. Positive growth on the filter after incubation on selective media indicates poor technique in analysis and positive bias (contamination) of results.

Matrix spike (laboratory matrix spike): A quality-control sample that determines the effect of the sample composition (matrix) on the recovery efficiency of the analytical method. For quality control of water samples for microbiological analyses, a sample is prepared in the laboratory by adding a known quantity of organisms to a specified amount of sample matrix for which an independent estimate of target analyte concentration is available.

Micrometer (µm): The millionth part of the meter. The pore diameter of filter media is expressed in micrometer units.

Negative control: A quality-control sample that measures the selectivity of the membrane-filtration medium for the test organism. A pure culture of nontarget organisms is passed through the filtration apparatus onto a membrane filter and cultured on a selective medium. The absence of growth on the filter after incubation on a selective medium indicates the selective medium is meeting its specifications for culture of the target organism.

Normality, *N* (equivalents per liter): The number of equivalents of acid, base, or redox-active species per liter of solution. Examples: a solution that is 0.01 *F* (formal) in HCl is 0.01 *N* in the hydronium ion (H^+). A solution that is 0.01 *F* in H_2SO_4 is 0.02 *N* in acidity.

Positive control: A quality-control sample that ensures the analytical method is correctly performed and that target organisms are correctly identified and detected. A pure culture of the target organism is passed through the filtration apparatus onto a membrane filter and cultured on a selective medium. Positive growth within a recommended range is considered indicative of the quality of the test medium and procedures to support growth under typical working conditions.

Procedure blank: A quality-control sample that measures the effectiveness of the analyst's rinsing technique during the membrane-filtration procedure for bacterial indicators. A 50- to 100-mL sample of sterile buffered water is passed through the filtration apparatus onto a sterile membrane filter after processing the sample. Positive growth on the filter after incubation on a selective medium indicates poor rinsing technique.

SELECTED SYMBOLS AND CHEMICAL FORMULAS

>	greater than
≥	equal to or greater than
<	less than
≤	equal to or less than
±	plus or minus
g/L	gram per liter
µm	micrometer
µg/L	microgram per liter (equivalent to parts per billion)
$CaCl_2$	calcium chloride
Cu	copper
$FeCl_3$	ferric chloride
H_2SO_4	sulfuric acid
K_2HPO_4	potassium hydrogen phosphate
KH_2PO_4	potassium dihydrogen phosphate
$MgSO_4$	magnesium sulfate
Na_2SO_3	sodium sulfite
$Na_2S_2O_3$	sodium thiosulfate
$NaHPO_4$	sodium phosphate
NaOH	sodium hydroxide
Ni	nickel
NH_4Cl	ammonium chloride
Zn	zinc

ABBREVIATIONS

BOD	biochemical oxygen demand
BOD_5	biochemical oxygen demand (5 day)
CBOD	carbonaceous biochemical oxygen demand
$CBOD_u$	ultimate carbonaceous biochemical oxygen demand
col/100mL	colonies per 100 milliliters
DIW	deionized or distilled water
DO	dissolved oxygen
E. coli	*Escherichia coli*
EDI	equal-discharge increment

EDTA	ethylenediaminetetraacetic acid
EIA	enterococcus confirmation medium (esculin substrate)
+ ETFE	ethylenetetraflouroethylene
EWI	equal-width increment
FEP	fluorinated ethylene propylene
GWUDISW	ground water under the direct influence of surface water
IPR	initial precision recovery
KF	streptococcus medium
mCP	*Clostridium perfringens* medium
mE	membrane filter—Enterococci medium
mEI	enterococcus medium
mENDO	membrane filter—total coliform medium
mF	membrance filter technique
mFC	membrane filter—Fecal Coliform medium
MI	total coliform and *Escherichia coli* medium
MPN	most probable number
mTEC	membrane filter—Thermotolerant *Escherichia coli* media
N	normal
NA–MUG	nutrient agar-4-methylumbelliferyl-b-D-glucuronide
+ NFM	National Field Manual (*National Field Manual for the Collection of Water-Quality Data*)
NWIS	National Water Information System of the U.S. Geological Survey
OPR	ongoing precision recovery
OWQRL	USGS Ocala Water Quality & Research Laboratory, Ocala, Florida
PDF	personal flotation device
PFA	perflouroalkyoxy polymers
PTFE	polytetraflouroethylene polymers ("Teflon")
QC	quality control
TCMP	2-chloro-6-(trichloro methyl) pyridine
TD	to deliver
TNTC	Too Numerous To Count
TTC	triphenyltetrazolium chloride
USEPA	U.S. Environmental Protection Agency
USGS	U.S. Geological Survey

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7.0—SELECTED REFERENCES FOR FIVE-DAY BIOCHEMICAL OXYGEN DEMAND

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APPENDIX A7-A.

Parameter Codes Used in the National Water Information System (NWIS) of the U.S. Geological Survey

Table 1. Parameter code for 5-day biochemical oxygen demand

Determination	Parameter code	Comments
Five-day biochemical oxygen demand (BOD)	00310	Parameter codes for methods other than the 5-day BOD can be found by accessing "Support Files" in the QWDATA database and searching for "Parameter Code Dictionary."

Table 2. Parameter codes for fecal indicator bacteria

[mENDO, total coliform medium; MI, total coliform and *Escherichia coli* medium; mFC, fecal coliform medium; µm, micrometer; mTEC, *Escherichia coli* medium; NA-MUG, *Escherichia coli* medium; mEI, enterococcus medium; EIA, enterococcus confirmation medium; mE, enterococcus medium; KF, fecal streptococcus medium; mCP, *Clostridium perfringens* medium]

Fecal indicator bacteria type[1]	Medium	Parameter code[2]
Total coliform bacteria	mENDO	31501
	MI	90900
Fecal coliform bacteria	mFC, 0.65-µm pore-size filter	31625
	mFC, 0.45-µm pore-size filter	31616
Escherichia coli	mTEC followed by urea phenol	31633
	Modified mTEC	90902
	MI	90901
	NA-MUG	50278
Enterococci bacteria	mEI followed by EIA	90909
	mE	31649
Fecal streptococci bacteria	KF	31673
Clostridium perfringens	mCP	90915

[1]Membrane-filtration method: all units are in colonies per 100 milliliters.
[2]The parameter codes listed are those that are in common (2003) use in the National Water Information System (NWIS) of the U.S. Geological Survey.

Table 3. Parameter codes for somatic and F-specific coliphages

[SAL, single-agar layer; *E. coli*, *Escherichia coli*; mL, milliliter; L, liter]

Type of coliphage	*E. coli* host strain[1]	Parameter code[2]	Unit of measurement[3]
SAL method			
Somatic	*E. coli* CN-13	90903	plaques/100 mL
F-specific	*E. coli* F-amp	90904	plaques/100 mL
Somatic	*E. coli* C	90905	plaques/100 mL
Two-step enrichment method			
Somatic	*E. coli* C	99328	Presence or absence/100 mL
Somatic	*E. coli* C	99329	Presence or absence/1 L
Somatic	*E. coli* C	99330	Presence or absence/4 L
Somatic	*E. coli* CN-13	99331	Presence or absence/100 mL
Somatic	*E. coli* CN-13	99332	Presence or absence/1 L
Somatic	*E. coli* CN-13	99333	Presence or absence/4 L
F-specific	*E. coli* F-amp	99334	Presence or absence/100 mL
F-specific	*E. coli* F-amp	99335	Presence or absence/1 L
F-specific	*E. coli* F-amp	99336	Presence or absence/4 L

[1]Bacterial host strain used to detect the specified coliphage type.
[2]The parameter codes listed are those that are in common use (2003) in the National Water Information System of the U.S. Geological Survey.
[3]Parameter codes vary by the sample volume associated with the unit of reporting.

Table 4. Parameter codes for *Cryptosporidium* and *Giardia*

[**Parameter code:** Analysis by U.S. Environmental Protection Agency Method 1623]

Parameter name	Parameter code	Unit of measurement
Cryptosporidium	99599	oocysts per 10 liters
Cryptosporidium—spike efficiency	99600	percent recovery
Giardia	99597	cysts per 10 liters
Giardia—spike efficiency	99598	percent recovery