

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*



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**U.S. DEPARTMENT OF THE INTERIOR**  
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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

# Techniques of Water-Resources Investigations

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## Book 9 Handbooks for Water-Resources Investigations

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# National Field Manual for the Collection of Water-Quality Data



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**Notes:**

The citation for this third edition of chapter A7 of the *National Field Manual* is as follows:

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# Chapter A7. BIOLOGICAL INDICATORS

*Third Edition*

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

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## 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).

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## 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.

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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.

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## 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

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+ 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.

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## 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<sup>1</sup> 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.

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<sup>1</sup>"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.

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### 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.

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Comments on the *National Field Manual*, and suggestions for updates or revisions, should be sent to [nfm-owq@usgs.gov](mailto: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.

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## 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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# FIVE-DAY BIOCHEMICAL OXYGEN DEMAND 7.0

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

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**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/>.

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# FIVE-DAY BIOCHEMICAL OXYGEN DEMAND 7.0

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).

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.

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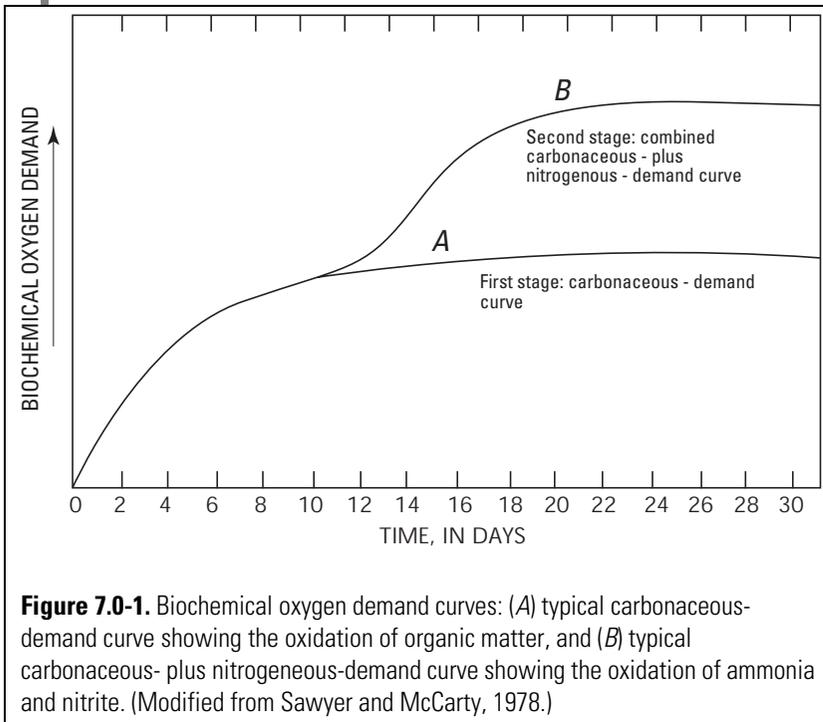
**Biochemical oxygen demand represents the amount of oxygen consumed by bacteria and other microorganisms while they decompose organic matter under aerobic conditions at a specified temperature.**

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**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 nitrogenous 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<sub>5</sub> 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 nitrogenous-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 ( $^{\circ}\text{C}$ ) ( $\text{BOD}_5$ ). The  $\text{BOD}_5$  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  $\text{BOD}_5$  and  $\text{CBOD}_5$  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  $\text{BOD}_5$ .
- ▶ An ultimate carbonaceous BOD ( $\text{CBOD}_u$ ) 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  $\text{CBOD}_u$  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,  $18.3^{\circ}\text{C}$ , 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  $18.3^{\circ}\text{C}$ . The temperature has been rounded upward to  $20^{\circ}\text{C}$ , but the 5-day time period has become the universal scientific and legal reference."

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## 7.0.1 EQUIPMENT AND SUPPLIES

Table 7.0-1 lists equipment and supplies commonly used in the BOD<sub>5</sub> 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;  $\text{KH}_2\text{PO}_4$ , potassium dihydrogen phosphate;  $\text{KHPO}_4$ , potassium monohydrogen phosphate;  $\text{Na}_2\text{HPO}_4$ , sodium monohydrogen phosphate;  $\text{NH}_4\text{Cl}$ , ammonium chloride; *N*, normality; DO, dissolved oxygen; KCl, potassium chloride;  $\text{CoCl}_3$ , cobalt chloride]

Item	Description
<b>Equipment and supplies</b>	
Constant-temperature chamber or water bath	Thermostatically controlled to maintain $20 \pm 1^\circ\text{C}$ . 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 $40^\circ\text{C}$ with $0.5^\circ\text{C}$ 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.
<b>Chemical reagents<sup>1</sup> and preparation of dilution water</b>	
Calcium chloride ( $\text{CaCl}_2$ ) solution <sup>2</sup>	Dissolve 27.5 g of $\text{CaCl}_2$ 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 ( $\text{FeCl}_3$ ) solution <sup>2</sup>	Dissolve 0.25 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in deionized water and dilute to 1 L.
Magnesium sulfate ( $\text{MgSO}_4$ ) solution <sup>2</sup>	Dissolve 22.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in deionized water and dilute to 1 L.
Phosphate buffer solution <sup>2</sup>	Dissolve 8.5 g of $\text{KH}_2\text{PO}_4$ , 21.8 g of $\text{KHPO}_4$ , 33.4 g of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , and 1.7 g of $\text{NH}_4\text{Cl}$ 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
<b>Chemical reagents for sample pretreatment and preparation of chemical solutions</b>	
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 <sub>2</sub> SO <sub>3</sub> ) or sodium thiosulfate (Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> ) for residual chlorine pretreatment solution	Dissolve 1.575 g of Na <sub>2</sub> SO <sub>3</sub> or NaS <sub>2</sub> O <sub>3</sub> 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 <sub>2</sub> SO <sub>4</sub> ) for caustic alkalinity pretreatment	Slowly and while stirring add 28 mL of concentrated H <sub>2</sub> SO <sub>4</sub> to about 900 mL of deionized water. Mix and dilute acid solution to 1 L (1 N H <sub>2</sub> SO <sub>4</sub> ).
<b>DO equipment and supplies (refer to NFM 6.2)</b>	
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 <sup>TM</sup> 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 <sub>2</sub> SO <sub>3</sub> and a few crystals of CoCl <sub>3</sub> in 1 L water. Prepare fresh zero DO solution before each use.

<sup>1</sup> Properly discard chemical reagents if there is any sign of biological growth or if past the expiration date.

<sup>2</sup> Can be purchased from the HACH<sup>TM</sup> Instrument Company in the form of nutrient buffer pillows ready for immediate use.

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## 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<sub>5</sub>, 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<sub>5</sub> 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. +

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## FIVE-DAY TEST FOR BIOCHEMICAL OXYGEN DEMAND 7.0.3

The BOD<sub>5</sub> 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<sub>5</sub> 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.

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### SAMPLE PREPARATION 7.0.3.A

Most relatively unpolluted streams have a BOD<sub>5</sub> that ranges from 1 to 8 mg/L (milligrams per liter) (Nemerow, 1974). If the BOD<sub>5</sub> value of a sample is less than 7 mg/L, sample dilution is not needed. A BOD<sub>5</sub> 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<sub>5</sub> sample (American Public Health Association and others, 1995). The BOD<sub>5</sub> 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<sub>5</sub> (Sawyer and McCarty, 1978).

**BOD<sub>5</sub> 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<sub>5</sub> 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<sub>5</sub> of the stream water, use a minimum of four volumes to accommodate a range of BOD<sub>5</sub> 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<sub>5</sub> 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<sub>5</sub>, 5-day biochemical oxygen demand]

Anticipated range of BOD <sub>5</sub> (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<sub>5</sub> test procedure:*

1. Prepare dilution water 3 to 5 days before initiating BOD<sub>5</sub> 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<sub>2</sub>SO<sub>4</sub>) 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<sub>2</sub>SO<sub>3</sub>) 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<sub>5</sub> TEST PROCEDURE**

*Use the following procedure for the BOD<sub>5</sub> 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<sub>5</sub> 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<sub>5</sub> 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  $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$  for 5 days.
  17. At the end of 5 days  $\pm$  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 ( $\text{DO}_{0 \text{ days}} - \text{DO}_{5 \text{ days}}$ ) 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  $\text{BOD}_5$  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<sub>5</sub>) Worksheet

Site/station: \_\_\_\_\_ Collection date and time: \_\_\_\_\_  
 Project: \_\_\_\_\_ Personnel: \_\_\_\_\_

#### Dilution-water blanks

Bottle number	Initial DO reading ( <i>D</i> <sub>1</sub> )	Date/time of reading	Final DO reading ( <i>D</i> <sub>2</sub> )	Date/time of reading	BOD ( <i>D</i> <sub>1</sub> - <i>D</i> <sub>2</sub> )	BOD average (<0.2 mg/L)

#### Environmental sample

Bottle number	Sample size (mL)	Initial DO reading ( <i>D</i> <sub>1</sub> )	Date/time of reading	Final DO reading ( <i>D</i> <sub>2</sub> )	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

$$\text{BOD}_5 \text{ (mg/L)} = \frac{D_1 - D_2}{P}$$

where

*D*<sub>1</sub> = initial sample dissolved-oxygen (DO) concentration (in mg/L)

*D*<sub>2</sub> = 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.

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## CALCULATIONS 7.0.4

The general equation for the determination of a BOD<sub>5</sub> value is:

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

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<sub>5</sub> 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<sub>5</sub>, 5-day biochemical oxygen demand; mg/L, milligrams per liter; HCl, hydrochloric acid]

Symptom	Possible cause and corrective action
DO readings drift downward	<ul style="list-style-type: none"> <li>Weak batteries for stirring unit result in inadequate flow across membrane—replace batteries.</li> </ul>
BOD <sub>5</sub> demand in dilution water is greater than the acceptable 0.2 mg/L	<ul style="list-style-type: none"> <li>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.</li> <li>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.</li> <li>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.</li> </ul>
Sample BOD values are unusually low in the diluted sample (BOD <sub>5</sub> dilution water is within the acceptable range)	<ul style="list-style-type: none"> <li>Dilution water contains interferences inhibiting the biochemical oxidation process—increase purity of dilution water or obtain from another source.</li> <li>Use deionized water that has been passed through mixed-bed resin columns. <b>Never use copper-lined stills.</b> Distilled water may be contaminated by using copper-lined stills or copper fittings—obtain from another source.</li> </ul>

---

## REPORTING 7.0.6

When reporting results of a BOD<sub>5</sub> test, be sure to use the correct parameter code (Appendix A7-A, table 1).

- ▶ Report BOD<sub>5</sub> values less than 2 mg/L as <2 mg/L rather than as 2.0 mg/L.
- ▶ Report BOD<sub>5</sub> values less than 10 mg/L to the nearest 0.1 mg/L.
- ▶ Report BOD<sub>5</sub> 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<sub>5</sub> results.

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# FECAL INDICATOR BACTERIA 7.1

*By D.N. Myers*

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**Notes:**

References for section 7.1, Fecal indicator bacteria, 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 2, for parameter codes for fecal indicator bacteria that are used in the National Water Information System (NWIS) of the U.S. Geological Survey.

The citation for this section (7.1) of NFM 7 is as follows:

Myers, D.N., November 2003, Fecal indicator bacteria: U.S. Geological Survey Techniques of Water-Resources Investigations, book 9, chap. A7 (3d ed.), section 7.1, accessed     date    , from <http://pubs.water.usgs.gov/twri9A/>.

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# FECAL INDICATOR BACTERIA 7.1

## BACTERIA

Fecal indicator bacteria are used to assess the microbiological quality of water because although not typically disease causing, they are correlated with the presence of several waterborne disease-causing organisms. The concentration of indicator bacteria (the term indicator bacteria is used synonymously with fecal indicator bacteria in this section) is a measure of water safety for body-contact recreation or for consumption.

---

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

---

- ▶ Wastes from warm-blooded animals contribute a variety of intestinal bacteria that are disease causing, or pathogenic, to humans.
- ▶ Body contact with and consumption of water that contains pathogens of the genera *Salmonella*, *Shigella*, and *Vibrio*, for example, can result in several types of disease in humans, including gastroenteritis and bacillary dysentery, typhoid fever, and cholera.

- ▶ The presence of *Escherichia coli* (*E. coli*) in water is direct evidence of fecal contamination from warm-blooded animals and indicates the possible presence of pathogens (Dufour, 1977). A few strains of *E. coli* are pathogenic, such as *E. coli* O157:H7, but most strains are not.
- ▶ The presence of *Clostridium perfringens* (*C. perfringens*) in water, as spores and (or) vegetative cells, indicates the contamination of water with treated or untreated sewage or similar wastes (Bisson and Cabelli, 1980; Fujioka and Shizumura, 1985).

Bacteriological tests are used to assess the sanitary quality of water and the potential public health risk from waterborne diseases. This section describes tests that can be completed in the field for identifying and enumerating five types of fecal indicator bacteria: total and fecal coliform bacteria, *E. coli*, fecal streptococci, and enterococci (Britton and Greeson, 1989; U.S. Environmental Protection Agency, 1985, 1991a, 1996, 1997, 2000a, 2000b). These are the most widely used indicator bacteria. Information for collecting samples for analysis of *C. perfringens* is included in this section, although membrane-filtration analysis of samples for *C. perfringens* is to be performed in a microbiological laboratory and not in the field.

**CAUTION:**

**When sampling for fecal indicator bacteria, wear protective apparel to prevent nose, mouth, eye, and direct skin contact with polluted water.**

Water-quality criteria have been developed by the U.S. Environmental Protection Agency (USEPA) for concentrations of indicator bacteria in recreational waters, shellfish-growing waters, and in ambient waters (U.S. Environmental Protection Agency, 1986). In 1986, *E. coli* and enterococci bacteria became the recommended indicator bacteria for recreational waters, replacing fecal coliform and fecal streptococci bacteria (U.S. Environmental Protection Agency, 1986, 2000b). Public Law 106-284 (Beaches Environmental Assessment and Coastal Health Act of 2000) requires the use of *E. coli* and (or) enterococci to assess beach-water quality in all bathing beach monitoring programs.

*E. coli* and enterococci are the preferred indicators to be used for primary-contact recreational waters because both are superior to total coliform, fecal coliform, and fecal streptococci bacteria as predictors of swimming-associated gastroenteritis in marine and fresh waters (Cabelli, 1977; Dufour and Cabelli, 1984). “Primary-contact recreational waters are waters where people engage in or are likely to engage in activities that could lead to ingestion or immersion in the water and are designated for use as such in state and tribal water-quality standards” (U.S. Environmental Protection Agency, 2002). Examples of primary-contact recreational activities are swimming, canoeing, and water skiing.

- ▶ Typical concentrations of indicator bacteria in contaminated and uncontaminated surface waters are shown in table 7.1-1.
- ▶ Ground water typically contains substantially lower concentrations of indicator bacteria than surface water.
- ▶ Although membrane-filtration procedures for total coliform and fecal coliform bacteria and *E. coli* are accepted for analysis of potable water, the Most Probable Number tests may provide superior results because they usually result in comparatively greater recoveries of microorganisms.
- ▶ USEPA regulations stipulate the methods to be used for monitoring and assessment of drinking water<sup>2</sup> and recreational water (U.S. Environmental Protection Agency, 1991b, 2002).

<sup>2</sup>The term “drinking water” is used when the information presented is within the context of U.S. Environmental Agency regulations; otherwise, the more general term “potable water” is used.

**Table 7.1-1.** Ranges of fecal indicator bacteria typically found in uncontaminated surface water and contaminated surface water

[Modified from Bordner and Winter, 1978, p. 127; American Public Health Association and others, 1998; U.S. Environmental Protection Agency, 1986, 1996, 1997, 2000a, and 2000b; Francy and others, 2000. <, less than; >, greater than]

Bacterial group	Uncontaminated surface water, colonies per 100 milliliters	Fecal-contaminated surface water, colonies per 100 milliliters
Total coliform	<1 to 80,000	1,200 to > 4,000,000
Fecal coliform	<1 to 5,000	200 to > 2,000,000
<i>Escherichia coli</i>	<1 to 576	126 to > 2,000,000
Fecal streptococcus	<1 to 1,000	400 to > 1,000,000
Enterococcus	<1 to 100	100 to > 1,000,000
<i>Clostridium perfringens</i>	<1 to 100	100 to > 10,000

## SAMPLING EQUIPMENT AND EQUIPMENT STERILIZATION PROCEDURES 7.1.1

Sterile technique must be followed and documented when collecting and processing samples for fecal indicator bacteria. Specific equipment and supplies are needed for collection and membrane-filtration analysis of samples for indicator bacteria. The equipment and supplies listed in tables 7.1-2 and 7.1-3 should be sufficient to begin (a) membrane-filtration analysis of fecal indicator bacteria and (b) equipment cleaning and sterilization procedures, respectively. The equipment and procedures described in the following paragraphs may be used for fecal indicator bacteria and fecal indicator viruses.

- ▶ **Equipment for collection and analysis of bacterial samples must first be cleaned and then sterilized** (table 7.1-3). There are several sterilization methods, but autoclaving is preferred. Before autoclaving, wrap clean equipment in kraft paper, autoclavable bags, or aluminum foil. Sterilize and store the equipment in a clean area. Resterilize equipment if the foil, bag, or kraft paper is torn.
- ▶ **Quality assurance and quality control of sterilization procedures are mandatory.** 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. 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, service the autoclave and then reesterilize all materials (American Public Health Association and others, 1998, p. 9-2 to 9-14).
- ▶ **Add sodium thiosulfate ( $\text{Na}_2\text{S}_2\text{O}_3$ ) to sample bottles before sterilization if the water to be collected contains residual chlorine or other halogens.** 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.

***To prepare a 10-percent solution of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>:***

1. In a volumetric flask, dissolve 100 g (grams) of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> into 500 mL of deionized or distilled water; stir until dissolved, and fill flask to 1,000 mL (Bordner and Winter, 1978, p. 6; American Public Health Association and others, 1998, p. 9-19). +
2. Using a pipet, dispense 0.5 mL of 10-percent Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> solution for every 1 L of sample.
3. Store the Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> solution in a dark bottle and keep it refrigerated; discard after 6 months and prepare a fresh solution. The Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> solution may be sterilized before storing, but will need to be autoclaved again if the bottle is left open and in contact with air.

**Add ethylenediaminetetraacetic acid (EDTA)** to sample bottles when water to be collected contains trace elements. Although references to toxic concentrations vary somewhat, concentrations of trace metals such as copper, nickel, or zinc that are present at concentrations greater than 10–1,000 µg/L (micrograms per liter) can be toxic to bacteria (Britton and Greeson, 1989, p. 56; Bordner and Winter, 1978, p. 6; American Public Health Association and others, 1998, p. 9-19). When in doubt, add EDTA to sample bottles prior to collection. EDTA is prepared by dissolving 372 mg (milligrams) in 1,000 mL of deionized or distilled water (American Public Health Association and others, 1998, p. 9-19). Before sterilization, add 0.3 mL of the EDTA solution per 100 mL of sample to sample bottles. EDTA can be combined with the Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> solution in the sample bottle before sterilization. Keep bottles tightly capped between uses, and prepare a fresh stock solution every 6 months; discard any expired stock solution. +

Sterilize the filtration apparatus and sampling equipment between sites or for each sample collected at the same site at different times. Table 7.1-3 shows specifications for sterilizing equipment and supplies using a variety of techniques, including autoclaving. Autoclaving is the preferred method. +

**Table 7.1-2.** Equipment and supplies used for membrane-filtration analysis of samples for fecal indicator bacteria testing

[mENDO, total coliform medium; mTEC, *Escherichia coli* medium; mL, milliliters; °C, degrees Celsius; μm, micrometer; mm, millimeters; TD, to deliver; TTC, triphenyltetrazolium chloride; NIST, National Institute of Standards and Technology; NA-MUG, *Escherichia coli* medium; MI, total coliform and *Escherichia coli* medium; nm, nanometers; lb/in<sup>2</sup>, pounds per square inch; cm, centimeters]

✓	Item	Description
	Absorbent pads	For use with total coliform test on mENDO medium and with <i>Escherichia coli</i> on mTEC medium for urease test
	Alcohol burner	Glass or metal, containing ethanol for flame sterilizing forceps
	Alcohol bottle	Wide mouth, 100 mL, containing ethanol for forceps sterilization
	Autoclave	For sterilization, capable of maintaining 121°C
	Bottles	Dilution bottles, 99 mL, with autoclavable screwcaps for sample dilution
	Buffered water	Specific to the test method and prepared in advance of field work
	Counter	Handheld, for counting bacterial colonies
	Filter disk	Sterile, disposable, 0.2-μm pore size for sterilizing reagents, to fit on 5-mL barrel syringe
	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
	Flasks, stir bars	Narrow mouth, Erlenmeyer type flasks with stir bars for media preparation, 250 or 500 mL
	Forceps	Stainless steel, smooth tips
	Hot plate	With magnetic stirrer or boiling water bath for media preparation
	Graduated cylinders	Borosilicate glass or plastic, 25 and 100 mL, wrapped in sterile aluminum foil, autoclavable bag, or kraft paper
	Incubator	Aluminum heat sink (heater block) or water bath, capable of maintaining specified temperature ranges during incubation
	Membrane filters	47-mm, sterile, white, gridded, mixed cellulose ester, cellulose acetate, or cellulose nitrate composition, 0.45-μm (or 0.65-μm) pore size depending on the test

(Table 7.1-2 is continued on the next page.)

**Table 7.1-2.** Equipment and supplies used for membrane-filtration analysis of samples for fecal indicator bacteria testing—*Continued*

✓	Item	Description
	Media and reagents	Liquid or solid media and reagents specific to the test method, prepared in advance of field work
	Microscope	Wide field type with 5 to 15 magnifications, dissecting type with fluorescent lamp
	Pipets	Sterile, TD, bacteriological or Mohr, glass or plastic with cotton plugs; 1, 10, and 25 mL
	Pipettor or pipet bulb	For use with pipets ( <b>do not pipet by mouth</b> )
	Petri dishes	Sterile, plastic, disposable top and bottom plates, 50 x 12 mm
	Syringe	5 mL, disposable, for delivering TTC
	Thermometer	Range of 40-110°C, glass mercury or dial, calibrated in 0.2°C increments, checked against a NIST-certified thermometer
	Ultraviolet lamp, long wave	For use with NA-MUG and MI tests, 366-nm, 6-watt bulb
	Vacuum source	Either a hand pump with gage or electric vacuum or peristaltic pump; vacuum pressure not to exceed 5 lb/in <sup>2</sup> or 25 cm of mercury
	Wrapping for equipment	Kraft paper, aluminum foil, autoclavable plastic bags

**Table 7.1-3.** Equipment cleaning and sterilization procedures

[DIW, distilled/deionized water; mL, milliliter; Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, sodium thiosulfate; µg/L, micrograms per liter; EDTA, ethylenediaminetetraacetic acid; °C, degrees Celsius; mg/L, milligrams per liter; >, greater than value shown]

Equipment	Procedures
<b>Cleaning</b>	
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 hot tap water. Rinse again three to five times with deionized or distilled water. 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.
<b>Sterilization</b>	
Autoclavable glass, stainless steel, plastic, and Teflon bottles and containers, volumetric flasks, and pipets and pipettors	If sample will contain residual chlorine or other halogens, add 0.5 mL of a 10-percent Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> solution per liter of sample to sample bottle before autoclaving. If sample will contain greater than 10 µg/L trace elements, add EDTA to sample bottle before autoclaving. Wrap equipment in kraft paper, aluminum foil, or place into autoclavable bags. <sup>1</sup> Autoclave at 121°C for 15 minutes.
Stainless steel field units	Flame sterilize with methanol (Millipore Hydrosol <sup>®</sup> units only), or autoclave at 121°C for 15 minutes.
Portable submersible pumps and pump tubing	<p><b>Autoclavable equipment</b> (preferred):</p> Wrap components in kraft paper, aluminum foil, or place into autoclavable bags. Autoclave at 121°C for 15 minutes. <p><b>Non-autoclavable equipment:</b> (1) Submerge sampling system in 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<sub>2</sub>S<sub>2</sub>O<sub>3</sub> solution per liter of water and circulate solution for 5 minutes. (4) Pump Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> to waste, then circulate sterile deionized or distilled water through pump, followed by sample water pumped from the well.</p> <p><b>CAUTION:</b> 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.</p>

<sup>1</sup>Equipment to be wrapped in kraft paper, aluminum foil, or placed into autoclavable bags includes, for example, bottles, tubing, flasks, bailers, pump components. The Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> solution is also autoclaved.

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## 7.1.1.A AUTOCLAVING

Use only autoclaves that have temperature, pressure, and liquid- and dry-utensil-cycle controls (table 7.1-3). 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® (such as perfluoroalkoxy-polymers (PFA), ethylene tetrafluoroethylene (ETFE), fluorinated ethylene propylene (FEP), and polytetrafluoroethylene polymers (PTFE)). Note that each material type 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, p. 9-2 to 9-14) for specifications for the length of time, temperature, and pressure for autoclave sterilization of various media and materials.
- ▶ In addition to the guidance listed above, it is necessary to:
  - Use a 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.
  - Wrap silicone tubing, in addition to other autoclavable equipment, in kraft paper, aluminum foil, or place in an autoclavable plastic bag.
  - 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 procedures in the logbook.
  - Avoid overloading the autoclave with equipment or materials; overloading will result in incomplete sterilization.

**Autoclaving is the preferred method for sterilizing equipment for fecal indicator bacteria.**

## **FLAME STERILIZATION OF THE 7.1.1.B HYDROSOL<sup>®</sup> FIELD FILTRATION UNIT**

The older style Millipore Hydrosol<sup>®</sup> field filtration units are designed to be flame sterilized with methanol. Formaldehyde gas, a byproduct of methanol combustion, kills all bacteria in the unit. The following sterilization procedure is acceptable for the Hydrosol<sup>®</sup> 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.

*To flame sterilize the Hydrosol<sup>®</sup> unit, carefully:*

1. Remove the 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 Teflon squeeze bottle or with an eye dropper from a screw-top glass container.
3. Ignite the methanol on the asbestos wick and allow 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 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 sterilize the Millipore Hydrosol<sup>®</sup> field filtration unit.

**CAUTION:**

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

## SAMPLE COLLECTION, 7.1.2 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 7.1-4).

**Table 7.1-4.** Summary 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, narrow-mouth 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 0.5 mL of a 10-percent sodium thiosulfate solution per 1 L of sample for halogen neutralization.

If necessary, add 0.3 mL of a 15-percent EDTA solution per 100 mL of sample for chelation of trace elements.

Chill all samples at 1- 4°C before analysis.

### Maximum holding times for samples obtained from surface water, ground water, or treated drinking water

Do not exceed the 6-hour maximum holding time period after sample collection and initiation of analysis for *E. coli*, fecal coliform bacteria, total coliform bacteria, enterococci, and fecal streptococci in **surface water and ground water** (Bordner and Winter, 1978, p. 30; U.S. Environmental Protection Agency, 2000b, 2002).

A 24-hour holding time for *C. perfringens* may be used. A 6-hour maximum holding time period after sample collection for *C. perfringens* (U.S. Environmental Protection Agency, 1996, p. XI-8) 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).

Do not exceed the 30-hour maximum holding time period after sample collection for total coliform bacteria, fecal coliform bacteria, and *E. coli* collected from treated **drinking-water sources** (Bordner and Winter, 1978 p. 30).

## 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 (Edwards and Glysson, 1999; NFM 4.1 and table 7.1-4).

- ▶ **Flowing water**—use depth-and-width-integrating sampling methods (NFM 4.1.1.A).<sup>3</sup>
- ▶ **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 4.1.1.B).
- ▶ **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 6 to 12 inches (15 to 30 centimeters) below the water surface.
  - Sample near known or suspected pollution sources, in areas of concentrated activity (for example, near lifeguard chairs), or for every quarter mile (approximately every 500 meters) of beach length (U.S. Environmental Protection Agency, 2002) and record sampling location.
  - Position the sampler downstream from any water currents to collect the sample from the incoming flow (U.S. Environmental Protection Agency, 2002). 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).

<sup>3</sup>Sample-collection methods may be modified to ensure consistency with study objectives and as appropriate for site conditions.

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

## 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 methods to use when sampling flowing waters and generally are required unless study objectives or site characteristics dictate otherwise (NFM 4.1.1.A and table 7.1-4).

1. The EDI method is preferred to the EWI method for sites where the velocity distribution across a stream section is well established; for example, at a gaging station or at a section where the depth varies (Edwards and Glysson, 1999).
2. Select the appropriate sampler and equipment. **Sampling equipment 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 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<sup>®</sup> bags or autoclavable cooking bags. Thermotolerant polymers are described in more detail in 7.1.1 under “Sampling Equipment and Equipment Sterilization Procedures.”
  - To composite 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.

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## Hand-dip method

If the stream depth and (or) velocity is not sufficient to use a depth-and-width-integrating method to collect a sample, then use the 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 toward 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 avoids contact with the streambed.

### **CAUTION:**

**Do not sample in or near a 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 samples (field blanks, procedure and filter blanks, positive and negative controls, and field replicates) generally constitute from 5 to 20 percent or more of the total number of samples collected over a given period of time. Quality-control terms (shown below in bold type) are defined at the end of this chapter, beginning on page CF-2.

- ▶ **Field blank**—Collect and analyze field blanks at a frequency of 1 in every 10 to 20 samples to document that sampling and analysis equipment have not been contaminated. If sampling in a beach environment, collect a field blank at least once per sampling trip (U.S. Environmental Protection Agency, 2002, p. J-2). Process field blanks before collecting the water sample.
  1. Pass sterile buffered water through sterile sampling equipment and into a sterile sampling container.
  2. Analyze sterile buffered water for fecal indicator bacteria. If no growth is observed, then the sample was collected by use of sterile procedures.
- ▶ **Filter blank, procedure blank**—Collect and analyze filter blanks and procedure blanks during sample processing to document that filtration equipment and buffered water were sterile and not contaminated by the analyst. A filter blank is processed for each sample before the sample is filtered. A procedure blank is processed through the filtration apparatus after the sample has been filtered at a frequency of 1 in every 10 to 20 samples.
- ▶ **Positive and negative controls**—These types of quality-control samples are required if media are prepared from basic ingredients (not dehydrated media) by field or laboratory personnel. The analyses of positive and negative controls are performed in the laboratory, and not in the field.
- ▶ **Field replicate**—Collect and analyze 1 field replicate for every 10 to 20 samples. A split sequential replicate is recommended. Two samples are collected and each sample is analyzed in duplicate by membrane filtration.

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## 7.1.2.B GROUND-WATER SAMPLE COLLECTION

As with surface water, most bacteria 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 collection of a representative ground-water sample for indicator bacteria 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 sample quality has not been compromised.

- ▶ Collect samples for analysis of bacteria after all other water-quality sampling has been completed.
- ▶ If using the same equipment for chemical-analysis and bacteria-analysis samples, then clean the equipment by first using standard procedures (NFM 3), followed by the disinfecting and rinsing procedures described in section 7.1.1. Purge the well as described in NFM 4.2 before collecting samples.
- ▶ If different equipment will be deployed in a well for bacteria sampling, then first check for stable turbidity and dissolved-oxygen readings to ensure collection of a representative sample.

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### 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 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). Avoid sampling after downhole chlorination. Dechlorination with  $\text{Na}_2\text{S}_2\text{O}_3$  is required if you cannot avoid collecting the sample before the water has passed through the treatment unit (refer to Section 7.1.1).

**Do not sample from leaking taps.**

*To sample a supply well for indicator bacteria:*

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 deionized or distilled water.
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 7.1-4).
  - If the well is used infrequently, then
    - (a) 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); and
    - (b) collect the sample directly from the tap into a sterile container (table 7.1-4).

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## 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). The sampling devices, as well as the sample line, must be sterilized if possible or else disinfected (table 7.1-3). If disinfected, 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-collection objectives before using a disinfectant. Disinfectants are corrosive; they can damage the metal parts of a pump, and can render the pump inadequate for trace-element sampling and other constituent sampling.
- ▶ 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.

*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 that has been opened for more than 60 days before use may not be full strength. Prepare solutions fresh with each use, because concentrations will diminish 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 Protection Agency, 1982, p. 253 and 1996, p. VIII-41). This concentration is sufficient for waters with a range of pH between 6 and 8 and for temperatures greater than 20°C. Outside of 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. Afterwards, rinse the pump thoroughly with a sterile Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> solution. The Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> solution is prepared by adding 0.5 mL of a 10-percent sterile solution to every 1 L of sterile deionized or distilled water. Recirculate this solution for 5 minutes.
4. Rinse the exterior of the pump and tubing thoroughly with sterile deionized or distilled water.
5. Lower the pump carefully into the well. Purge the residual chlorine and Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> 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 disinfectant or Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>. **The pump must have a backflow check valve (an antibacksiphon device) to prevent residual disinfectant from flowing back into the well.**

**The type of well, its use, construction, composition, and condition could alter samples for fecal indicator bacteria analysis.**

*To use a pump that cannot be disinfected:*

1. Handle the pump and tubing carefully to avoid contamination. If the pump is a downhole dedicated pump, skip 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 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 during pump removal and bailing that would not otherwise be included in the sample.

Sampling-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.

Be aware that the type of well, its use, construction, composition, and condition can 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.

*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 bacteriocidal metals. If samples must be collected from these types of wells, add 0.3 mL of EDTA solution per 100 mL of sample to the sample bottle prior to autoclaving. Collect the sample directly into the bottle.
2. Purge the well (see NFM 4.2 and 6.0.3.A) 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 measurements and proceed with sampling.

**Quality control for ground-water sample collection.** Depending on the data-quality requirements of the study, quality-control samples (field blanks and field replicates) will constitute from a small to a large percentage of the total number of samples collected over a given time period. Quality-control terms (shown below in bold type) are defined at the end of this chapter, beginning on page CF-2.

- ▶ **Field blanks**—Collect field blanks with ground-water samples 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 by passing sterile buffered water through sterile sampling equipment and into a sterile sampling container. Analyze sterile buffered water for fecal indicator bacteria and record results. If no growth is 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 sampling so that results can be evaluated before field sampling.**

- ▶ **Filter blank, procedure blank**—Collect and analyze a filter blank and procedure blank to document that filtration equipment and buffered water were sterile and not subsequently contaminated by the analyst. A filter blank is processed with each sample through the filtration apparatus before the sample is filtered. A procedure blank is processed through the filtration apparatus after the sample has been filtered at a frequency of 1 in every 10 to 20 samples.
- ▶ **Field sequential replicates**—Collect and analyze one set of sequential field replicates for every 10 to 20 samples. (Usually, a single 100-mL sample is collected from ground water.) Because few ground-water samples test positive for indicator bacteria, it may be necessary to collect field replicates at a higher frequency to determine variability.

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## SAMPLE PRESERVATION, STORAGE, 7.1.2.C AND HOLDING TIMES

After collection, immediately chill samples in an ice chest or refrigerator at 1 to 4°C. **Do not freeze samples.** Except for samples collected from drinking-water sources, **process samples as quickly as possible, preferably within 1 hour but not more than 6 hours after sample collection for initiation of analysis for the coliform and enterococci groups** (U.S. Environmental Protection Agency, 1985, 2000a, 2000b, and 2002). Adhering to holding times minimizes changes in the concentration of indicator bacteria. For treated drinking water, do not exceed 30 hours prior to initiation of analysis. *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). Holding times are summarized in table 7.1-4.

*C. perfringens* is analyzed at the laboratory, and not in the field. Ship samples for *C. perfringens* analysis 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. Information on analysis of *C. perfringens* can be accessed at <http://oh.water.usgs.gov/micro/clos.html> (accessed November 25, 2003).

**Strictly adhere to the maximum  
holding-time requirements for samples.  
Chill at 1°C to 4°C and store samples  
in the dark until analysis.**

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## 7.1.3 IDENTIFICATION AND ENUMERATION METHODS

Membrane-filtration (MF) and most probable number (MPN) methods are used for the presumptive identification, confirmation, and enumeration of indicator bacteria. Procedures to analyze water samples using the MF method are described in this section. For general use, the MF method is preferable to the MPN method, with a few exceptions. The MPN method is preferred if toxic substances are present in the sample or if, after filtration, a residue heavy enough to block the micropores of the membrane filter is visible. The MPN method also is preferable for the analysis of treated drinking water. The MPN method is not typically used in the field. Instructions for the MPN method are described in “Standard Methods for the Examination of Water and Wastewater” (“Standard Methods”), 20th edition (American Public Health Association and others, 1998, p. 9-47 to 9-56) and in Britton and Greeson (1989).

Fecal indicator bacteria are operationally defined by the method employed for identification and enumeration, as shown in table 7.1-5. Presumptive identification and enumeration are made from colonies that develop on selective media after filtration of one or more sample volumes onto gridded membrane filters. Detailed confirmation, identification, and enumeration of these bacteria require additional culturing and biochemical testing, the details of which are beyond the scope of this manual. Additional confirmation methods are needed under certain circumstances, such as use of the data in support of environmental regulation and enforcement. Although all of the methods described herein are suitable for analysis of water, methods should be selected that are appropriate for the source of water. 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 7.1-5).

**Table 7.1-5.** Fecal indicator test definitions, incubation times, and incubation temperatures

[mENDO, total coliform medium;  $\pm$ , plus or minus;  $^{\circ}\text{C}$ , degrees Celsius; MI, total coliform and *Escherichia coli* medium;  $\text{MgCl}_2$ , magnesium chloride; NA-MUG, *E. coli* medium; mTEC, *E. coli* medium; mFC, fecal coliform medium; KF, fecal streptococci medium; mE, enterococci medium; EIA, enterococci medium; mEI, enterococci medium; mCP, *Clostridium perfringens* medium]

Test (medium)	Typical application	Incubation time and temperature	Buffered water type
Total coliform bacteria (mENDO)	Drinking water and ground water	$24 \pm 2$ hours at $35.0 \pm 0.5^{\circ}\text{C}$	Phosphate-buffered water with peptone
Total coliform bacteria (MI)	Drinking water and ground water	$24 \pm 2$ hours at $35.0 \pm 0.5^{\circ}\text{C}$	Phosphate-buffered water with $\text{MgCl}_2$
<i>Escherichia coli</i> (NA-MUG)	Drinking water and ground water	4 hours at $35 \pm 0.5^{\circ}\text{C}$ after primary culture on mENDO medium	Phosphate-buffered water with peptone
<i>Escherichia coli</i> (MI)	Drinking water and ground water	$24 \pm 2$ hours at $35.0 \pm 0.5^{\circ}\text{C}$	Phosphate-buffered water with $\text{MgCl}_2$
<i>Escherichia coli</i> (modified mTEC)	Recreational and surface water	First resuscitate for 2 hours at $35.0 \pm 0.5^{\circ}\text{C}$ , then incubate for 22 to 24 hours at $44.5 \pm 0.2^{\circ}\text{C}$	Phosphate-buffered saline water or phosphate-buffered water with $\text{MgCl}_2$
<i>Escherichia coli</i> (on urea substrate broth after primary culture on mTEC)	Recreational and surface water	First resuscitate for 2 hours at $35.0 \pm 0.5^{\circ}\text{C}$ , and then incubate for 22 to 24 hours at $44.5 \pm 0.2^{\circ}\text{C}$ After 22 to 24 hours, transfer filter to urea substrate broth for 15 to 20 minutes before counting	Phosphate-buffered saline water or phosphate-buffered water with $\text{MgCl}_2$
Fecal coliform bacteria (mFC)	All waters	$24 \pm 2$ hours at $44.5 \pm 0.2^{\circ}\text{C}$	Phosphate-buffered water with peptone
Fecal streptococci (KF)	All waters	$48 \pm 2$ hours at $35.0 \pm 0.5^{\circ}\text{C}$	Phosphate-buffered water with peptone
Enterococci (mE and EIA)	All waters, but primarily recreational waters	48 to 50 hours at $41.0 \pm 0.5^{\circ}\text{C}$ on mE medium. Transfer filter to EIA medium for 20 minutes at $41.0^{\circ}\text{C}$ before counting	Phosphate-buffered saline water or phosphate-buffered water with $\text{MgCl}_2$
Enterococci (mEI)	All waters, but primarily recreational waters	24 hours at $41.0^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$	Phosphate-buffered saline water or phosphate-buffered water with $\text{MgCl}_2$
<i>Clostridium perfringens</i> (mCP)	All waters	$24 \pm 2$ hours at $44.5 \pm 0.2^{\circ}\text{C}$	Phosphate-buffered water with $\text{MgCl}_2$

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## 7.1.3.A CULTURE MEDIA AND REAGENTS

MF analysis requires the use of several types of culture media and reagents, the types being dependent on the indicator bacteria and method. The necessary media and reagents include sterile buffered water, agar- or broth-based selective and differential growth media, and media and reagents for additional biochemical identification. **The preparation of selective and differential culture media for indicator bacteria is an important part of analysis. Adhering to proper preparation, storage, and holding-time requirements will help ensure the quality of the analysis.** Instructions for the preparation of 100 mL of primary culture media for MF tests and additional confirmation media or broth for three MF confirmation tests are described in section 7.1.5, “Instructions for Media Preparation.”

Sterile buffered water (distilled or deionized water that has been buffered and autoclaved)<sup>4</sup> is used to dilute samples and to rinse the membrane-filter apparatus and utensils. USGS personnel can purchase sterile buffered water from the Ocala Water Quality and Research Laboratory in Ocala, Fla. (OWQRL or Ocala Laboratory). Sterile buffered water is provided in 250-mL bottles and in 99-mL dilution bottles. Three types of buffered water are available: (1) phosphate-buffered water with peptone, (2) phosphate-buffered saline water, and (3) phosphate-buffered dilution water with magnesium chloride (MgCl<sub>2</sub>). Table 7.1-5 provides information on the types of buffered water to use with various types of media. **Buffered water that exceeds the expiration date indicated on the label should not be used.** Buffered water not obtained from the Ocala Laboratory should be prepared and autoclaved ahead of time. Instructions for preparing sterile buffered water are described in Britton and Greeson (1989, p. 18).

Culture media for enumeration of fecal indicator bacteria for USGS studies are provided in kits from the Ocala Laboratory. These kits include instructions for media preparation. Dehydrated media also can be purchased from scientific suppliers.

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<sup>4</sup>Sterile buffered water is described in Britton and Greeson (1989, p. 18), “Standard Methods” (American Public Health Association and others, 1998, p. 9-18), and USEPA (1996, 1997, 2000a, 2000b),

***To store media and reagents:***

1. Store media kit (supplied by the Ocala Laboratory) or dehydrated, commercially prepared media in a desiccator. Store other reagents in a dust-free laboratory cabinet (not in a field vehicle).
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 necessary. When diluting a sample, use the sterile buffered water immediately after opening the bottle and discard any remainder.
4. Label all prepared petri dishes to identify the media type, the preparation date, and the analyst.
5. Store prepared petri dishes upside down in a plastic bag and refrigerate before use.

**Quality control for culture media and reagents.** Supplies of dehydrated media purchased either from the Ocala Laboratory or through established vendors of scientific supplies have been quality-control tested. Each batch of media that is prepared from basic ingredients by the analyst also must be quality-control tested. If sterile buffered water is prepared by the user, it should be prepared under laboratory conditions. In addition, quality-control procedures must be used to ensure that the sterile buffered water will produce a suitable medium for transfer of bacteria from samples to filters. To test the sterility of the buffered water, collect 100-mL blank samples and process them along with each set of environmental samples. 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).

If field work is conducted on a daily or weekly schedule, then analyze a **positive control** and **negative control** at least quarterly, when media are prepared from basic ingredients by field personnel. Analyze positive- and negative-control samples annually if sampling is performed intermittently. The positive-control sample tests the ability of the medium and reagents to support growth of the target microorganism. Negative-control samples are used to ensure that the test does not support the growth of nontarget organisms. Additional detailed information on positive and negative controls can be found in the 20th edition of "Standard Methods" (American Public Health Association and others, 1998, p. 9-10).

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## 7.1.3.B MEMBRANE-FILTRATION METHOD

After collecting the sample and selecting the appropriate sample volumes, label the petri dish with the station number (or other site identifiers), the volume of sample filtered, and the date and time of sample collection. Select those sample volumes that are anticipated to yield one or two filters in the ideal colony count range.

**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 different sample volumes may suffice. However, where past analyses have shown the variation to be large or where the variation is not known, filtering five or more different sample volumes is recommended.

**Always wear laboratory gloves when processing samples for fecal-indicator analysis.**

### *To prepare to filter samples and make colony counts:*

1. When collecting and processing samples, work inside the field vehicle, if possible, and out of direct sunlight and wind.
2. Prevent contamination of the work area. Before and after processing the samples, clean counter tops in field vehicles with an antibacterial cleaning solution, such as a 7-percent phenolic solution; 50- to 70-percent isopropyl or ethyl alcohol; 5-percent bleach; or a 7-percent ammonia solution.

- +
3. Before beginning analysis, preheat incubators for at least 2 hours, according to specifications for each test (table 7.1-5). 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).
  4. Using sterile equipment, collect field, filter, and procedure blank samples.
    - One or more colonies on the field or filter blank indicates inadequate sterilization of either the equipment or the sterile 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.

***To filter samples and make colony counts:***

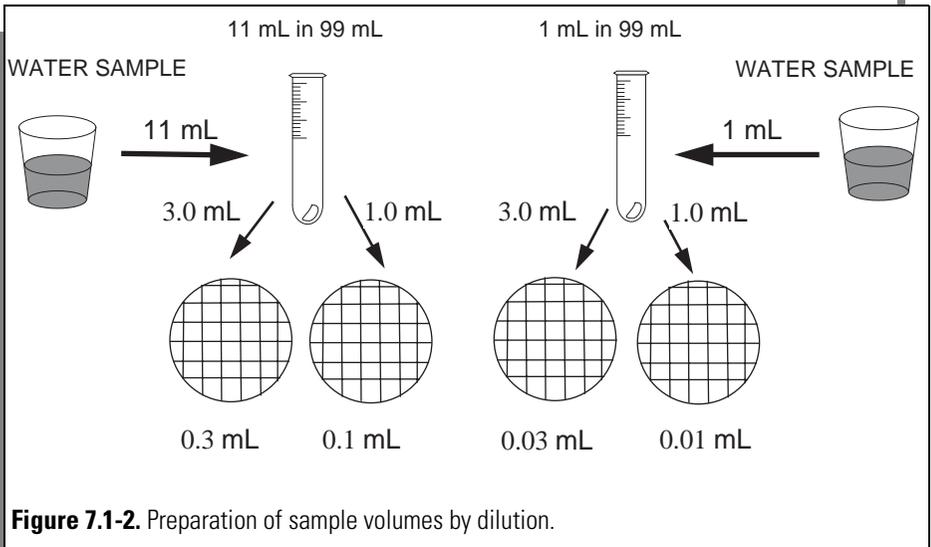
- +
1. Select sample volumes (table 7.1-6, fig. 7.1-2) to provide at least one filter having colonies in the ideal counting range. The ideal range and number of sample volumes to filter depend on the test and the expected bacterial concentrations.
    - Record the site name, date, time of sample collection, and sample volume on the petri dish and on the record sheet or field form. Record the time of sample processing on the record sheet or field form.
    - Label replicates, blanks, filter blanks, and procedure blanks and other quality-control samples.
  2. Assemble filtration equipment by inserting the base of the filter-holder assembly into a flask or manifold (fig. 7.1-3). Draw a vacuum by use of a hand-held pump, vacuum pump, or battery-operated peristaltic pump. If flame sterilization is used, rinse the inside of the filtration apparatus with sterile buffered water to remove any residue of formaldehyde.
- +

**Table 7.1-6.** Recommended sample volumes for membrane-filter analyses based on ideal colony count and concentration range

[col/100 mL, colonies per 100 milliliters; mL, milliliters; &lt;, less than value shown; &gt;, greater than value shown]

Ideal colony-counting ranges for number of colonies per membrane filter within a range of concentrations from <1 to 2,000,000 col/100 mL					
20–60 colonies			20–80 colonies		
Sample volume (mL) <sup>1</sup>	Added as (mL) <sup>2</sup>	Concentration range	Sample volume (mL) <sup>1</sup>	Added as (mL) <sup>2</sup>	Concentration range
100	100	<1–60	100	100	<1– 80
30	30	60–200	25	25	80–320
10	10	200–600	6.0	6.0	330–1,300
3.0	3.0	600–2,000	1.5	1.5	1,300–5,300
1.0	1.0	2,000–6,000	.4	4.0 of a 1:10 dilution	5,000–20,000
.3	3.0 of a 1:10 dilution	6,000–20,000	.1	1.0 of a 1:10 dilution	20,000–80,000
.1	1.0 of a 1:10 dilution	20,000–60,000	.025	2.5 of a 1:100 dilution	80,000–320,000
.03	3.0 of a 1:100 dilution	60,000–200,000	.006	6.0 of a 1:1,000 dilution prepared by diluting 11 mL of a 1:100 in 99 mL <sup>1</sup>	330,000–1,300,000
.01	1.0 of a 1:100 dilution	200,000–600,000			
.003	3.0 of a 1:1,000 dilution prepared by diluting 11 mL of a 1:100 in 99 mL <sup>1</sup>	600,000–2,000,000			
20–100 colonies					
Sample volume (mL) <sup>1</sup>	Added as (mL) <sup>2</sup>	Concentration range			
100	100	<1–100			
20	20	100–500			
5.0	5.0	400–2,500			
1.0	1.0	2,000–10,000			
.2	2.0 of a 1:10 dilution	10,000–50,000			
.05	5.0 of a 1:100 dilution	50,000–200,000			
.01	1.0 of a 1:100 dilution	200,000–1,000,000			

<sup>1</sup>All sample volumes less than 1.0 mL require dilution in a 99-mL dilution bottle containing sterile buffered water.<sup>2</sup>Sample volumes smaller than those indicated may be needed when bacterial concentrations are greater than those listed.



3. 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 for several seconds to prevent heat damage to the membrane filter.
  - c. Resterilize forceps before each use. Return cooled forceps to alcohol container between transfers. **Do not set forceps on the countertop.**
4. 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 apparatus might result in contamination of the funnel.) Using sterile forceps, place a sterile, gridded membrane filter (47 mm (millimeters)) on top of the filter base, gridded side up (fig. 7.1-3). Be sure to use the correct pore-size membrane filter for the test procedure (table 7.1-7).
5. Carefully replace and secure the filter funnel on the filter base. Avoid tearing or creasing the membrane filter.
 

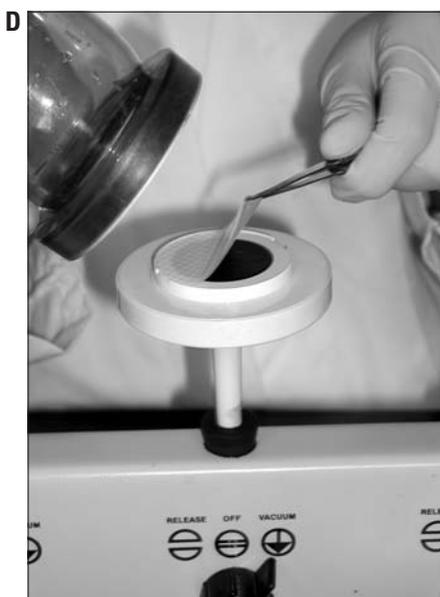
**Quality control. Rinse the funnel with 100 mL of sterile buffered water before filtering sample volumes to obtain a filter blank.**



### PROCEDURE

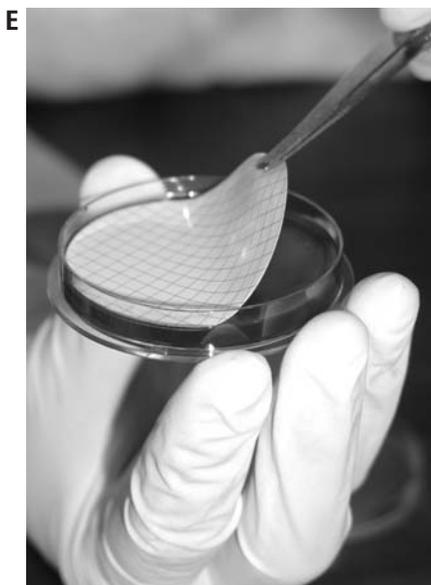
1. Preheat the incubator; prepare work areas.
2. Select sample volumes. If needed, prepare dilutions for filtration of sample volumes less than 1.0 mL (tables 7.1-6 and 7.1-8; and figure 7.1-2).
3. Label petri dishes.
4. Assemble sterile filtration apparatus.
5. Place sterile filter on filtration apparatus 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-filtration procedure.



#### PROCEDURE

8. Sterilize forceps and remove filter (D). Replace funnel on filtration apparatus.
9. Roll filter onto media in petri dish (E). Place inverted petri dish in incubator.
10. Repeat steps 4-9 for each sample volume in 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-filtration procedure—*Continued.*

**Table 7.1-7.** Membrane-filter pore sizes for fecal indicator bacteria tests

Test	Pore size of membrane filter (in micrometers)
Total coliform	0.45
Fecal coliform	0.45 or 0.65
<i>Escherichia coli</i>	0.45
Fecal streptococci	0.45
Enterococci	0.45
<i>Clostridium perfringens</i>	0.45

6. Filter the sample in order of smallest to largest sample volume. If the sample volume is less than 1.0 mL, prepare dilutions with sterile buffered water in a 99-mL dilution bottle and transfer the appropriate volume of dilution to the membrane filter (fig. 7.1-2 and table 7.1-8).
  - When preparing dilutions, 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 concentrated sample volumes.
7. 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.
  - a. Pour or pipet the shaken sample into the filter funnel within 5 seconds (fig. 7.1-3B or C). Pipet the sample from the center of the sample volume. You can use the upper and lower graduations (line-to-line) to measure the volume. Proceeding from smallest to largest sample volume, deliver the sample volume to the membrane filter by use of a pipettor or pipet bulb with a valve for volume control.

- **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 facilitate distribution of bacteria on the membrane filter.
  - **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.
- b. Allow the pipet to drain, touching the pipet to the inside of the funnel to remove remaining sample (fig. 7.1-3C). 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.**

**Table 7.1-8.** Preparation guidelines for dilution of samples to volumes less than 1.0 milliliter for fecal indicator bacteria analysis

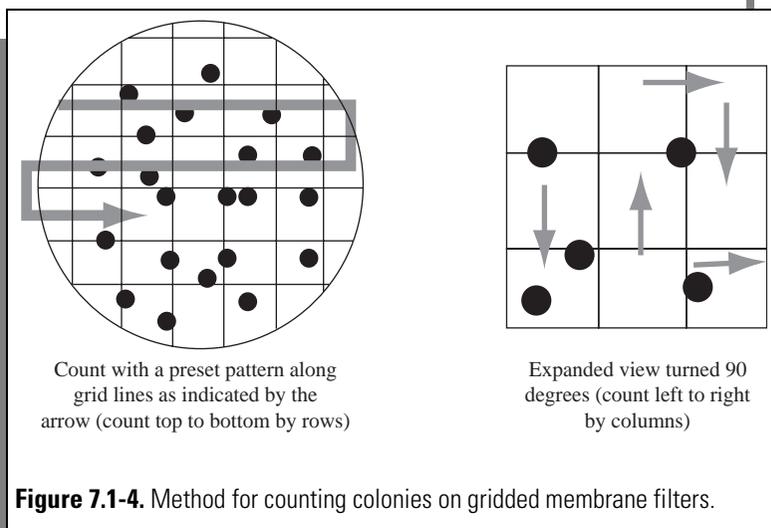
[mL, milliliter]

Dilution factor	Volume (mL) of sample added to 99 mL sterile dilution water	To obtain this dilution, filter this volume
1:10	11.0 mL of original sample	for 0.3, use 3.0 mL of 1:10 for 0.1, use 1.0 mL of 1:10
1:100	1.0 mL of original sample	for 0.3, use 30 mL of 1:100 for 0.1, use 10 mL of 1:100 for 0.03, use 3.0 mL of 1:100 for 0.01, use 1.0 mL of 1:100
1:1,000	1.0 mL of 1:10 dilution	for 0.003, use 3.0 mL of 1:1,000 for 0.001, use 1.0 mL of 1:1,000

8. Apply a vacuum with a hand, peristaltic, or vacuum pump. **To avoid damage to bacteria, do not exceed a pressure of about 5 lb/in<sup>2</sup> (pounds per square inch) (25 cm of mercury).** +
9. 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 apparatus.
10. Remove the funnel and hold it in one hand—do not set the funnel on the counter top. Remove the membrane filter using sterile forceps (fig. 7.1-3D). 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.) Replace funnel on filter base.
11. Open a petri dish and place the membrane filter on the medium, grid side up, and starting at one edge by use of a rolling action (fig. 7.1-3E). 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 petri dishes to direct sunlight.** +
12. Close the petri dish by pressing the top firmly onto the bottom. Invert the petri dish. Incubate within 20 minutes to avoid growth of interfering microorganisms.
13. Continue to filter the other sample volumes in order, from smallest to largest volume. Record on the field forms the volumes filtered and the time of processing.  
**Quality control.** After filtrations are complete, place a sterile, gridded-membrane filter onto the funnel base and rinse the funnel with 100 mL of sterile buffered water to obtain a procedure blank. Procedure blanks are analyzed at a frequency of 1 in every 10 to 20 samples.
14. Place the inverted petri dishes 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 7.1-5). Wash, then flame sterilize or autoclave filter apparatus before the next use. Wash counter top between each sample with an antibacterial cleaning solution (see “To prepare to filter samples and make colony counts” in this section). Wash hands with bacteriocidal soap. +

**Quality control.** Verify the incubator temperature on a regular schedule against a National Institute of Standards and Technology (NIST) thermometer or 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.

15. After the prescribed length of incubation, remove the petri dishes from the incubator. For each sample volume filtered, count and record on the field forms the number of typical 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 slightly different angle. 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.



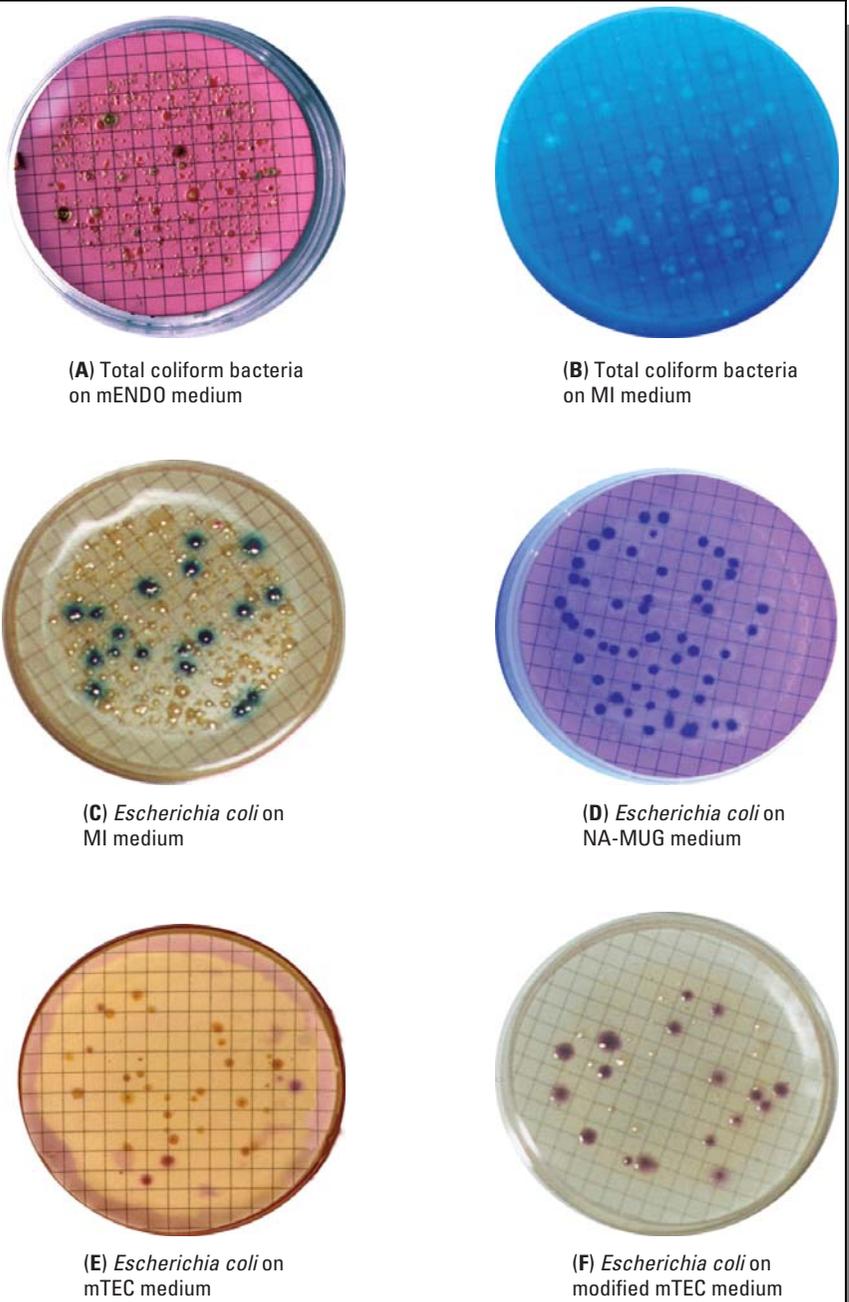
- For total coliform colonies on mENDO medium, enhance sheen production by removing filters from media and placing them on absorbent pads to dry for at least 1 minute before counting (fig. 7.1-5A).
- For total coliform on MI medium, count under a long-wave ultraviolet light in a completely darkened room (U.S. Environmental Protection Agency, 2000a) or in a viewing box (fig. 7.1-5B).
- For *E. coli* on MI medium, count under natural light (with magnification) (fig. 7.1-5C).
- If the NA-MUG test is done for *E. coli*, transfer the total coliform filter onto NA-MUG petri dishes and incubate for 4 hours at 35°C. Afterwards, count under a long-wave ultraviolet light in a completely darkened room (U.S. Environmental Protection Agency, 1991b) or in a viewing box (fig. 7.1-5D).
- For *E. coli* on mTEC medium, transfer the filter to a filter pad saturated with urea-phenol reagent; count only yellow colonies after 15 to 20 minutes at room temperature (U.S. Environmental Protection Agency, 1985; fig. 7.1-5E).
- For *E. coli* on modified mTEC medium, count colonies directly from the petri dish under natural light (fig. 7.1-5F).
- For fecal coliform on mFC medium and fecal streptococci on KF medium, count colonies, using magnification, under fluorescent or incandescent light (fig. 7.1-5G and H, respectively).
- For enterococci on mE medium, transfer the filter to EIA medium after incubation for 20 minutes at 41°C; count colonies from the underside of the petri dish placed over a fluorescent illuminator (fig. 7.1-5I).
- For enterococci on mEI medium, count colonies directly from the petri dish under fluorescent or incandescent light (fig. 7.1-5J).
- For *C. perfringens* on mCP medium, count colonies directly from the petri dish under magnification, and under fluorescent or incandescent light (7.1-5K).

+ 16. 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. Discard sample results bracketed by contaminated blanks.

**Quality control.** A second analyst should recount the colonies on the petri dishes and record the results for 1 in every 20 samples. Table 7.1-9 and figure 7.1-5 contain information on colony identification.

17. Calculate the number of colonies per 100 mL of sample as described in section 7.1.4, “Calculation and Reporting of Fecal Indicator Bacteria.”

18. Put all petri dishes to be discarded into an autoclavable bag. Freeze or chill any petri dishes to be discarded until they can be autoclaved in the laboratory. **Autoclave all cultures at 121°C for a minimum of 30 minutes before discarding.**



(A) Total coliform bacteria on mENDO medium

(B) Total coliform bacteria on MI medium

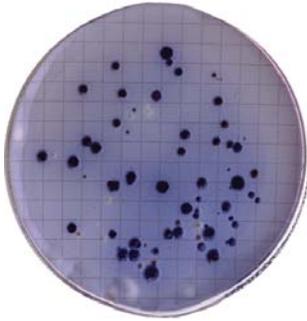
(C) *Escherichia coli* on MI medium

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

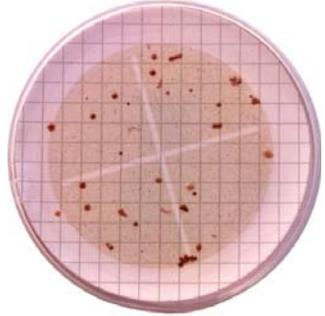
(E) *Escherichia coli* on mTEC medium

(F) *Escherichia coli* on modified mTEC medium

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



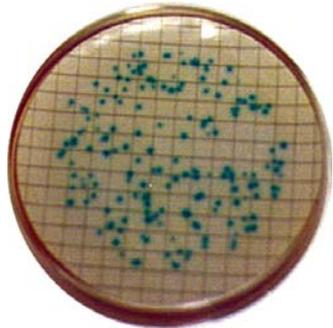
(G) Fecal coliform bacteria on mFC medium



(H) Fecal streptococci bacteria on KF medium



(I) Enterococci bacteria on EIA medium



(J) Enterococci 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.*

**Table 7.1-9.** Test (medium type), ideal colony count, 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 count range (colonies per filter)	Typical colony color, size, and morphology
Total coliform bacteria (mENDO)	20 to 80	Colonies are round, raised, and smooth; 1 to 4 mm in diameter, and red with a golden-green metallic sheen.
Total coliform bacteria (MI)	20 to 80	Colonies are blue white, blue green, or blue green with fluorescent halos under long-wave ultraviolet light (366 nm); 1 to 3 mm in diameter. Count under a long-wave ultraviolet lamp in a completely darkened room.
<i>Escherichia coli</i> After primary culture as total coliform colonies on mENDO (NA-MUG)	Much fewer in number than total coliforms on the same filter	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.
<i>Escherichia coli</i> (mTEC)	20 to 80	Colonies are round, raised, and smooth; 1 to 4 mm in diameter, remain yellow to yellow brown after urease test; may have darker raised centers.
<i>Escherichia coli</i> (modified mTEC)	20 to 80	Colonies are round, raised, and smooth; 1 to 4 mm in diameter, deep pink to magenta.
<i>Escherichia coli</i> (MI)	Much fewer in number than total coliforms on the same filter	Colonies are blue under ambient light, and blue green with or without fluorescent edges under long-wave ultraviolet light (366 nm); 1 to 3 mm in diameter.
Fecal coliform bacteria (mFC)	20 to 60	Colonies are round, raised, and smooth with even to lobate margins; 1 to 6 mm in diameter, and 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; about 0.5 to 3 mm in diameter; glossy pink or red.
Enterococci (mE and EIA)	20 to 60	Colonies are round, smooth, and raised; 1 to 6 mm in diameter; pink to red with a black or red dish-brown precipitate on underside.
Enterococci (mEI)	20 to 60	Colonies have blue halos regardless of colony color; 1 to 6 mm in diameter. Count under a fluorescent lamp.
<i>Clostridium perfringens</i> (mCP)	20 to 80	Colonies are round and straw yellow before exposure to ammonium hydroxide, dark pink to magenta afterward; 1 to 4 mm in diameter.

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## CALCULATION AND REPORTING OF FECAL INDICATOR BACTERIA 7.1.4

The range of ideal colony counts depends on the fecal indicator group to be enumerated (table 7.1-9). Crowding and insufficient media to support full development of colonies can result if the bacterial concentration on the filter exceeds the upper limit of the ideal range. The lower limit of the ideal range is set as a number below which statistical validity becomes questionable (Britton and Greeson, 1989, p. 14). For potable waters, results are routinely reported from counts of 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. The computation for concentration is the sum of the colony counts for each sample volume, multiplied by 100, and divided by the sum of the sample volumes.

- ▶ Enumeration results for the membrane-filtration method are expressed as a concentration in units of col/100 mL.
- ▶ Whole numbers are reported for results less than 10, and two significant figures are reported for results greater than or equal to 10.

Parameter codes for use in reporting fecal indicator bacteria in the USGS National Water Information System are listed in Appendix A7-A, table 2.

Examples of scenarios that are commonly experienced when counting colonies are presented in the following five cases:

Case 1. Ideal colony counts.

Case 2. Colony counts less than or greater than 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 are too numerous to count.

**Case 1: Ideal colony counts.**

Example 1: Ideal colony count on one filter

Sample volume	Colony count
6.0	7 (do not use)
25	21
100	101 (do not use)
<u>Sum 25</u>	<u>21</u>

$$\text{col}/100 \text{ mL} = (21 \times 100) / 25 = 84$$

Example 2: Ideal colony counts on two or more filters

Sample volume	Colony count
6.0	7 (do not use)
25	21
100	58
<u>Sum 125</u>	<u>79</u>

$$\text{col}/100 \text{ mL} = (21 + 58) \times 100 / (100 + 25) = 63$$

Example 3: If dilutions of the original sample were made before filtration (sample volumes less than 1 mL), calculate the volume (in mL) of original sample as follows:  
(TNTC, Too Numerous To Count)

Sample volume	Colony count
0.1 (1:10)	50
0.3 (3:10)	TNTC (do not use)
1.0	TNTC (do not use)
<u>Sum 0.1</u>	<u>50</u>

$$\text{Colony count} = 50$$

$$\text{Volume of original sample} = 0.1 \text{ mL}$$

$$\text{Volume of diluted sample} = 1.0 \text{ mL}$$

$$\text{col}/100 \text{ mL} = 50 \times 100 / 1 \times 0.1 = 50,000$$

**Case 2: Colony counts less than or greater than the ideal range but not zero or TNTC (Too Numerous To Count).**

Results are reported with the statement,  
 "Estimated count based on non-ideal colony count."

Example 1: Less than the ideal range on all filters

Sample volume	Colony count
3.0	2
10	6
30	18
Sum 43	26

$$\text{col}/100 \text{ mL} = (2 + 6 + 18) \times 100 / (3 + 10 + 30) = 60$$

Example 2: Either greater than or less than the ideal range

Sample volume	Colony count
4	18
20	101
100	TNTC (do not use)
Sum 24	119

$$\text{col}/100 \text{ mL} = (18 + 101) \times 100 / (4 + 20) = 500$$

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

Assume a colony count of one on the filter with the largest sample volume.

Result is reported as less than the calculated number per 100 mL. It is a maximum estimated number.

Sample volume	Colony count
3.0	0 (do not use)
10	0 (do not use)
30	assume 1
Sum 30	1

$$\text{col}/100 \text{ mL} = (1 \times 100) / 30 = < 3$$

**Case 4: Less than the ideal range—including some zero counts—and no filters with colonies that are TNTC (Too Numerous To Count).**

The result is reported as "Estimated count based on non-ideal colony count."

Sample volume	Colony count
3.0	0 (do not use)
10	0 (do not use)
30	5
<b>Sum</b> 30	5

$$\text{col}/100 \text{ mL} = 5 \times 100 / 30 = 17$$

**Case 5: Colony counts on all filters are TNTC.**

Assume maximum ideal count on filter with the smallest volume filtered.

Result is reported as greater than the calculated number per 100 mL. It is a minimum estimated number.

Sample volume	Colony count
3.0	TNTC (assume 60)
10	TNTC (do not use)
30	TNTC (do not use)
<b>Sum</b> 3	60

$$\text{col}/100 \text{ mL} = 60 \times 100 / 3 = >2,000$$

---

## INSTRUCTIONS FOR MEDIA PREPARATION 7.1.5

1. mENDO medium for total coliform analysis.
  - a. Empty the vial containing 4.8 g of dehydrated mENDO medium into a 250-mL flask and add 100 mL of a 2-percent ethanol solution.
  - b. Stir the mixture well for several minutes to break up clumps and prevent agar from adhering to the flask.
  - c. Place foil-covered flask in a heated water bath or on a hot plate and heat slowly to boiling. If using a hot plate, stir the mixture constantly or use a stir bar and magnetic stirring hot plate to prevent scorching. **Do not autoclave.**
  - d. When medium reaches the boiling point, promptly remove from the heat. **Do not boil.**
  - e. Cool the medium to a temperature of about 50°C and pour 6 to 7 mL into 50-mm petri-dish bottoms. Quickly place petri-dish tops loosely onto the bottoms to allow condensation to escape.
  - f. When the medium has solidified (about 10 minutes), close the petri dishes by pressing firmly on the tops. The plates are suitable for use after the medium has solidified. About 15 to 20 plates can be prepared from 100 mL of medium. **Label and date the petri dishes.**
  - g. Petri dishes that are not used immediately after preparation should be placed into small plastic bags to prevent drying and stored in darkness in a refrigerator for a **maximum of 5 days.**

2. NA-MUG medium for confirmation of *E. coli* after primary culturing of total coliform bacteria with mENDO medium.
  - a. Add 2.3 g of NA-MUG medium to 100 mL of deionized or distilled reagent-grade water in a 250-mL flask.
  - b. Stir the mixture well for several minutes to break up clumps and prevent medium from adhering to the flask.
  - c. Place the flask into a heated water bath or on a hot plate and heat slowly to boiling. If using a hot plate, stir the mixture constantly or use a stir bar and magnetic stirring hot plate to prevent scorching.
  - d. Autoclave at 121°C and 15 lb/in<sup>2</sup> for 15 minutes. Allow to cool to 44-46°C, or when the flask is cool enough to pick up barehanded.
  - e. Pour 6 to 7 mL of medium into the bottom of a 50-mm petri dish. About 15 to 20 plates can be prepared from 100 mL NA-MUG medium. Quickly place petri-dish tops loosely onto the bottoms to allow condensation to escape.
  - f. When the medium has solidified (about 10 minutes), close petri dishes by pressing firmly on the tops. The plates are suitable for use after the medium has solidified. Label and date the plates. Prepared petri dishes that are sealed in small plastic bags to prevent drying can be **stored in a refrigerator for up to 2 weeks.**

### 3. MI medium for total coliform bacteria and *E. coli* analysis.

Commercial medium may be available; if so, prepare according to instructions. If not, prepare fresh medium in the laboratory prior to departing for the field site. Information on reagents and media preparation can be obtained from the U.S. Environmental Protection Agency (2000a).

- a. Prepare fresh agar according to U.S. Environmental Protection Agency (2000a) by weighing and adding 36.52 g of the combined reagents to 900 mL of reagent-grade deionized or distilled water. Mix to break up clumps. Adjust the volume to 1,000 mL. If dehydrated medium is used, add 36.52 g of dehydrated MI medium to 1 L of reagent-grade deionized or distilled water in a flask and heat to boiling until the ingredients dissolve. This volume will produce approximately 200 petri dishes. To prepare fewer, proportionately reduce the quantities of reagents and volume of deionized or distilled water.
- b. Prepare Cefsulodin (which is added to the MI medium after the medium is autoclaved and cooled). To prepare Cefsulodin solution, add 0.02 g of Cefsulodin to 20 mL of reagent-grade deionized or distilled water, sterilize the Cefsulodin solution by aseptically filtering through a disposable, sterile 0.22- $\mu\text{m}$ -pore-size syringe filter. Store in a sterile tube in the refrigerator at about 5°C until needed. Prepare fresh solutions each time the test is used. Do not save any unused portion. Write the preparation date on the tube.
- c. Dispense MI medium into 100-mL dilution bottles. Autoclave the medium for 15 minutes at 121°C, then cool in a 50°C water bath.
- d. **If the plates will be poured right away:** allow the medium to cool to 45–50°C. Add 0.5 mL of freshly prepared Cefsulodin solution to each dilution bottle of tempered agar medium. The final pH should be  $6.95 \pm 0.2$ . Proceed to step (e).  
**If the plates will NOT be poured right away:** store the medium in the dilution bottles, under refrigeration (at approximately 5°C) for up to 6 months. When ready to prepare the petri dishes, melt the medium by autoclaving the MI-medium-containing dilution bottles (as needed) for 5 minutes at 121°C or by placing them into a boiling water bath. Be sure to loosen the screw tops on the bottles before

they are heated to allow air to escape. Cool the medium to 45–50°C. Add 0.5 mL of freshly prepared Cefsulodin solution to each dilution bottle of tempered agar medium. The final pH should be  $6.95 \pm 0.2$ . Proceed to step (e).

- e. Pour 6 to 7 mL of the medium into each 50-mm petri dish.
  - f. When the medium has solidified (about 10 minutes), close the petri dishes by pressing firmly on the tops. These dishes are suitable for use after the medium has solidified. About 15 to 20 plates can be prepared from 100 mL of medium.
  - g. Label and date the petri dishes. Prepared petri dishes, sealed in small plastic bags to prevent drying, can be **stored in a refrigerator for up to 2 weeks**.
  - h. All freshly prepared medium must be quality-control checked by the preparing laboratory through the use of positive and negative controls. Refer to “Standard Methods” (American Public Health Association and others, 1998, p. 9-1 to 9-78) for instructions.
4. mTEC medium for *E. coli* analysis.
- a. Empty the vial containing 4.53 g of dehydrated mTEC medium into 100 mL of deionized or distilled water in a 250-mL flask.
  - b. Stir this mixture for several minutes to break up clumps. Make sure that none of the medium adheres to the bottom or side of the flask.
  - c. Place the flask containing the medium solution in a heated water bath or on a hot plate and begin heating slowly to boiling. If using a hot plate, stir the mixture constantly or use a stir bar and magnetic stirring hot plate to prevent scorching. After boiling begins, remove the flask from the hot plate or boiling water bath and autoclave at 121°C and 15 lb/in<sup>2</sup> for 15 minutes. Allow to cool to 45–50°C.
  - d. Pour 6 to 7 mL of the medium into 50-mm petri-dish bottoms. Quickly place the petri-dish tops loosely onto the bottoms to allow condensation to escape.

- e. When the medium has solidified (about 10 minutes), close the petri dishes by pressing firmly on the tops. These plates are suitable for use after the medium has solidified. About 15 to 20 plates can be prepared from 100 mL of medium. Label and date.
- f. Prepared petri dishes, sealed in small plastic bags to prevent drying, can be **stored in a refrigerator for up to 2 weeks.**
- g. Begin preparation of urea-phenol broth for mTEC. Identification of *E. coli* using urea-phenol broth is required.
  - i. After sample incubation, prepare the urea-phenol red broth by adding 100 mL of sterile deionized or distilled water to 2.0 g of urea and 0.01 g phenol red crystals in a 250-mL flask and mix thoroughly. Although all the phenol red crystals will not dissolve, the solution is nevertheless acceptable to use.
  - ii. Adjust the pH of the urea-phenol solution to 5.0 with a few drops of 1 N HCl (hydrochloric acid). The substrate solution should be a straw-yellow color at this pH.
  - iii. When preparing the filter pads for counting colonies, use a clean pipet or dropper and add 2.0 mL of the solution to each absorbent pad before placing the filter on the pad. Before transferring the filters from mTEC to urea-phenol broth, carefully drain excess solution from each pad by tilting the petri dish against a clean lab wipe.
  - iv. Urea-phenol broth can be stored in the refrigerator for up to 2 weeks, as long as the solution remains straw-yellow in color.

## 5. Modified mTEC medium for *E. coli* analysis.

Commercial dehydrated medium may be available; if so, prepare according to instructions. If not, prepare fresh medium in the laboratory prior to departing for the field site. Information on reagents and media preparation can be obtained from the U.S. Environmental Protection Agency (2000b).

- a. Prepare fresh agar by weighing and adding 45.6 g of combined reagents to 900 mL of reagent-grade deionized or distilled water. Stir the mixture to break up clumps. After the reagents are dissolved, adjust the volume to 1,000 mL. This volume will produce approximately 200 plates. To prepare fewer petri dishes, proportionately reduce the quantities of reagents and volume of deionized or distilled water.
- b. Autoclave the medium at 121°C for 15 minutes. Cool the medium. Store the medium in dilution bottles for up to 6 months in the refrigerator (at about 5°C). Prepare petri dishes as needed from individual bottles of medium. Write type of medium and date prepared on bottles.
- c. When ready to prepare petri dishes, melt the modified mTEC medium by autoclaving dilution bottles containing MI medium for 5 minutes at 121°C or by placing them in a boiling water bath. Be sure to loosen the screw tops on the bottles prior to heating to allow air to escape.
- d. Cool the medium to 45–50°C. Pour 6 to 7 mL of the medium into each 50-mm petri-dish bottom to a depth of about 1/8 inch (0.32 mm). Allow to cool 4 to 8 minutes and replace petri-dish tops tightly.
- e. When the medium has solidified (about 10 minutes), close the petri dishes by pressing firmly on the tops. The plates are suitable for use after the medium has solidified. About 15 to 20 plates can be prepared from 100 mL of medium. Label and date the plates. Prepared plates, sealed in small plastic bags to prevent drying, can be **stored in a refrigerator for up to 2 weeks.**

- f. All freshly prepared medium must be quality-control checked by the preparing laboratory by means of positive and negative controls. Refer to American Public Health Association and others (1998, p. 9-1 to 9-78) for instructions.
  - g. Sterile agar petri dishes prepared ready for use can be purchased from commercial suppliers and do not need to be refrigerated during shipping. These plates can be stored in the refrigerator at about 5°C for up to 1 year. Write the date received on the plates.
6. mFC medium for fecal coliform bacteria analysis.
- a. Prepare a rosolic acid solution by adding 10 mL of 0.2 *N* NaOH (sodium hydroxide) to 0.1 g of rosolic acid crystals. Shake the mixture to dissolve crystals. Do not heat. The crystals will dissolve in 15 minutes. Prepare a new solution for each analysis.
  - b. Empty the vial containing 5.2 g of dehydrated medium into 100 mL of deionized or distilled water in a 250-mL flask.
  - c. Stir the mixture well for several minutes to break up clumps and prevent medium from adhering to the flask.
  - d. Place the flask in a heated water bath or on a hot plate and heat slowly to 90°C. If using a hot plate, stir the mixture constantly or use a stir bar and magnetic stirring hot plate to prevent scorching. Do not autoclave.
  - e. With a clean pipet, add 1 mL of rosolic acid solution per 100 mL of medium when the medium reaches 90°C. Continue heating until boiling begins; then remove from the heat.
  - f. Cool the medium to a temperature of about 45–50°C and pour 6 to 7 mL in 50-mm petri-dish bottoms. Quickly place petri-dish tops loosely on petri-dish bottoms to allow condensation to escape.

- g. When the medium has solidified (about 10 minutes), close petri dishes by pressing firmly on the tops. The plates are suitable for use after the medium has solidified. About 15 to 20 plates can be prepared from 100 mL of medium. Label and date the petri dishes. Prepared petri dishes, sealed in small plastic bags to prevent drying, can be **stored in a refrigerator for up to 72 hours**.

7. KF medium for fecal streptococci analysis.

- a. Empty the vial containing 7.64 g of dehydrated medium into 100 mL of deionized or distilled water in a 250-mL flask.
- b. Stir the mixture well for several minutes to break up clumps and prevent medium from adhering to the flask.
- c. In a separate small bottle, add 10 mL of deionized or distilled water to the 0.1 g of TTC (triphenyltetrazolium chloride crystals).
- d. Place the flask in a heated water bath or on a hot plate and heat slowly to boiling. If using a hot plate, stir the mixture constantly or use a stir bar and magnetic stirring hot plate to prevent scorching. After boiling begins, reduce to a simmer for 5 minutes.
- e. Remove the medium solution from the heat and cool to 50–60°C. Sterilize the TTC solution by aseptically filtering through a disposable sterile 0.22-mm pore-size membrane filter. Add 1 mL of sterile TTC solution to 100 mL of medium and stir. Prepare a fresh TCC solution each time the medium is prepared.
- f. Cool the medium to a temperature of about 50°C and pour 6 to 7 mL into 50-mm petri-dish bottoms. Quickly place the petri-dish tops loosely on the petri-dish bottoms to allow condensation to escape.

- +
- g. When the medium has solidified (about 10 minutes), close the petri dishes by pressing firmly on the tops. The plates are suitable for use after the medium has solidified. About 15 to 20 plates can be prepared from 100 mL of medium. Label and date the plates. Prepared plates, sealed in small plastic bags to prevent drying, can be stored in a refrigerator for up to 2 weeks if a sterile TTC solution is used. **If the TTC solution is not sterilized, use the medium within 24 hours.**
8. mE medium, followed by EIA medium, for enterococci bacteria analysis.
- a. Pour 5 to 10 mL of deionized or distilled water into a bottle containing 0.015 g of TTC crystals. Cap and shake the bottle to dissolve the crystals. If using a USGS kit, retain the remaining 90 mL of deionized or distilled water. Do not heat.
- b. Empty the vial containing 7.12 g of mE medium into the remainder of the deionized or distilled water (approximately 90 mL) in a 250-mL flask. Stir this mixture for several minutes to break up clumps. It is important that none of the medium adheres to the bottom or the sides of the flask.
- +
- c. Prepare a nalidixic acid solution by adding 10 mL of 0.2 N NaOH solution to a bottle containing 0.25 g of nalidixic acid crystals. Shake the bottle to dissolve the crystals. Do not heat. Make sure all of the crystals dissolve.
- d. Place the flask containing the medium solution in a heated water bath or on a hot plate and stir the solution constantly, or use a stir bar and magnetic stirring hot plate to prevent scorching. After ingredients dissolve, autoclave at 121°C and 15 lb/in<sup>2</sup> for 15 minutes. Allow to cool long enough to pick up barehanded.
- e. Once cooled, the dissolved reagents can be added. Using a sterile pipet, add 1.3 mL of the nalidixic acid solution. If the medium is intended to be used within 24 hours, add all of the TTC solution directly from the bottle. If the prepared medium will be stored for a longer period before use, sterilize the TTC solution by passing it through a 0.22-mm membrane-filter syringe in an aseptic manner.
- +

- f. Pour 6 to 7 mL of the medium into 50-mm petri-dish bottoms. Quickly place the petri-dish tops loosely on the petri-dish bottoms to allow condensation to escape. +
- g. When the medium has solidified (about 10 minutes), close the petri dishes by pressing firmly on the tops. The petri dishes are suitable for use after the medium has solidified. About 15 to 20 petri dishes can be prepared from 100 mL of medium. Label and date the petri dishes. Prepared petri dishes, sealed in small plastic bags to prevent drying, can be stored in a refrigerator for up to 2 weeks, if sterile TTC is used. **If the TTC solution is not sterilized, use the medium within 24 hours.**
- h. Begin preparation of EIA medium for enterococci bacteria. Use of the EIA medium is required.
  - i. If using the USGS kit from the Ocala Laboratory, a second bottle of 100 mL deionized or distilled water is used to prepare the EIA medium.
  - ii. Empty the vial containing 1.65 g of EIA medium into 133 mL of deionized or distilled water in a 250-mL flask.
  - iii. Stir this mixture for several minutes to break up clumps. Make sure that none of the medium adheres to the bottom or the side of the flask. +
  - iv. Place the flask containing the medium solution in a heated water bath or on a hot plate and stir the solution constantly, or use a stir bar and magnetic stirring hot plate to prevent scorching.
  - v. After ingredients dissolve, autoclave at 121°C and 15 lb/in<sup>2</sup> for 15 minutes. Allow the flask to cool long enough to pick up barehanded.
  - vi. Pour 6 to 7 mL of EIA medium into the bottom of a 50-mm petri dish. Quickly place the petri-dish tops loosely on the petri-dish bottoms to allow condensation to escape. +

vii. When the medium has solidified (about 10 minutes), close the petri dishes by pressing firmly on the tops. The plates are suitable for use after the medium has solidified. About 15 to 20 plates can be prepared from 100 mL of medium. Label and date the plates. Prepared plates, sealed in small plastic bags to prevent drying, can be stored in a refrigerator for up to 2 weeks.

9. mEI medium for enterococci bacteria analysis.

- a. Prepare the nalidixic acid solution by adding a few drops of 0.1 *N* NaOH solution to a bottle containing 5 mL deionized or distilled water and 0.24 g of nalidixic acid. Shake the mixture thoroughly to dissolve. Do not heat.
- b. In another bottle, add 0.1 g of TTC to 10 mL of reagent-grade deionized or distilled water. Cap and shake for about 30 seconds to dissolve. Do not heat.
- c. Add 71.2 g of mE agar to 900 mL of reagent-grade deionized or distilled water in a 1,000-mL flask. Add 0.75 g of the indoxyl  $\beta$ -D-glucoside reagent (rinsing thoroughly), and adjust the volume to 1,000 mL.
- d. Stir this mixture for several minutes to break up clumps. It is important to make sure that none of the agar adheres to the bottom or sides of the flask.
- e. Place the flask containing the agar solution on a stove or burner and begin heating slowly. Stir the solution constantly to prevent scorching. After ingredients have dissolved, autoclave at 121°C and 15 lb/in<sup>2</sup> for 15 minutes. Allow the solution to cool to 44–46°C. The pH of the medium should be  $7.1 \pm 0.2$ .

- f. The dissolved reagents may now be added. If the medium is to be used within 24 hours, add the nalidixic acid solution and mix thoroughly. Add 2 mL of the TTC solution per 1 L of medium using a clean pipet. **If, however, the prepared medium will be stored for a longer period prior to use, sterilize the nalidixic acid and TTC solutions by passing them through a 0.22- $\mu$ m pore-size membrane filter aseptically, using a sterile syringe.** Mix thoroughly.
  - g. Pour 6 to 7 mL of mEI medium into 50-mm petri-dish bottoms. Quickly place the petri-dish tops loosely on the petri-dish bottoms to allow condensation to escape.
  - h. When the medium has solidified (about 10 minutes), close the petri dishes by pressing firmly on the tops. The plates are suitable for use after the medium has solidified. About 15 to 20 plates can be prepared from 100 mL of medium. Label and date the plates. Prepared plates, sealed in small plastic bags to prevent drying, can be stored in a refrigerator for up to 2 weeks.
10. mCP media for *C. perfringens* analysis.

The mCP medium for the analysis of *C. perfringens* is prepared by the analyzing laboratory, so preparation instructions are not included in this section. A culture of *C. perfringens* requires anaerobic conditions and a special incubator, as well as a fume hood in which to expose colonies to the ammonium-hydroxide reagent for identification.

# FECAL INDICATOR VIRUSES 7.2

By R.N. Bushon

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**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/>

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# 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.<sup>5</sup> 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:

- ▶ **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).

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

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<sup>5</sup>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 ( $\text{Na}_2\text{S}_2\text{O}_3$ ) to sample bottles before sterilization if the water to be collected is suspected to contain residual chlorine or other halogens.**  $\text{Na}_2\text{S}_2\text{O}_3$  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<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, 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 <sub>2</sub> S <sub>2</sub> O <sub>3</sub> solution per liter of sample to the sample bottles. Wrap all autoclavable equipment in aluminum foil, kraft paper, or place into autoclavable bags. <sup>1</sup> Autoclave at 121°C for 15 minutes.
Portable submersible pumps and pump tubing	<b>Autoclavable equipment</b> (preferred): Wrap components in aluminum foil, kraft paper, or place into autoclavable bags. Autoclave at 121°C for 15 minutes. <b>Non-autoclavable equipment:</b> (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 <sub>2</sub> S <sub>2</sub> O <sub>3</sub> solution per liter of water and circulate solution for 5 minutes; (4) pump Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> , 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. <b>CAUTION:</b> 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.

<sup>1</sup>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<sub>2</sub>S<sub>2</sub>O<sub>3</sub> solution also is autoclaved.

*To prepare for collecting a halogenated sample:*

- +
1. Prepare a 10-percent solution of  $\text{Na}_2\text{S}_2\text{O}_3$  as follows:
    - a. In a volumetric flask, dissolve 100 g of  $\text{Na}_2\text{S}_2\text{O}_3$  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  $\text{Na}_2\text{S}_2\text{O}_3$  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  $\text{Na}_2\text{S}_2\text{O}_3$  solution for every 1 L of sample. If the sterile  $\text{Na}_2\text{S}_2\text{O}_3$  is used, be sure to use only sterile pipets and sterile sample bottles. If the  $\text{Na}_2\text{S}_2\text{O}_3$  is not sterile, dispense the required volume of  $\text{Na}_2\text{S}_2\text{O}_3$  into the sample bottle and autoclave at 121°C for 15 minutes.

+

**$\text{Na}_2\text{S}_2\text{O}_3$  solution has a 6-month shelf life. Discard unused solution that has expired, prepare fresh solution, and label bottle with date of preparation.**

+



- + ▶ 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).

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## **SAMPLE COLLECTION, 7.2.2 PRESERVATION, TRANSPORT, AND HOLDING TIMES**

+ 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<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, 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<sub>2</sub>S<sub>2</sub>O<sub>3</sub> solution per 1 L of sample.

- If sterile Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> is used, dispense with sterile pipet into sterile bottle.
- If Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> 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<sup>6</sup> (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).

<sup>6</sup>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).**

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## 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<sup>®</sup> 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.
- +

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## 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  $\text{Na}_2\text{S}_2\text{O}_3$  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  $\text{Na}_2\text{S}_2\text{O}_3$  solution. +  
The  $\text{Na}_2\text{S}_2\text{O}_3$  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  $\text{Na}_2\text{S}_2\text{O}_3$ . Take care not to contaminate samples for chemical analysis with residual disinfectant or  $\text{Na}_2\text{S}_2\text{O}_3$ . **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.

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## SAMPLE PRESERVATION, 7.2.2.C TRANSPORT, AND HOLDING TIMES

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.**

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## 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).

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### 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.

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## 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

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**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/>.

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## 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.

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

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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).

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## 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 hypochlorite (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]

<b>Equipment and supplies</b>
<ul style="list-style-type: none"> <li>• Autoclavable 1-L bottle or 3-L bag, nozzle, and cap.</li> <li>• Collapsible low-density polyethylene cubitainer for collection of a 10-L bulk sample.</li> <li>• Regular and sterile DIW.</li> <li>• Nonphosphate, laboratory-grade detergent.</li> <li>• 12-percent sodium hypochlorite solution.</li> <li>• Aluminum foil, autoclavable bag, or kraft paper.</li> </ul>
<b>Cleaning and sterilization procedures</b>
<p><b>Sodium hypochlorite sterilization method:</b></p> <ul style="list-style-type: none"> <li>• Scrub equipment with a dilute (1-percent) nonphosphate, laboratory-grade detergent solution.</li> <li>• Rinse three to five times with tap water.</li> <li>• Submerge equipment in a 12-percent (120 g/L) sodium hypochlorite solution for 30 minutes.</li> <li>• Using sterile DIW, rinse thoroughly, inside and out, at least three times.</li> <li>• Wrap equipment in sterile aluminum foil or sterile kraft paper, or place into a sterile bag.</li> </ul> <p><b>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.</b></p>
<p><b>Alternative sterilization method:</b> (Use if equipment contact with sodium hypochlorite should be avoided).</p> <ul style="list-style-type: none"> <li>• Soak equipment in a dilute (1-percent) nonphosphate, laboratory-grade detergent solution for 30 minutes.</li> <li>• Scrub well and rinse three to five times with tap water.</li> <li>• Rinse again three to five times with DIW.</li> <li>• Wrap equipment in aluminum foil or kraft paper, or place into autoclavable bags.</li> <li>• Autoclave at 121°C for 20 minutes.</li> </ul>

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## 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 hypochlorite 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.**

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## ALTERNATIVE STERILIZATION 7.3.1.B METHOD

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.

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### 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.**

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## 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 perfluoroalkoxy-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.

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- + ▶ 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).

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## 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;  $\text{Na}_2\text{S}_2\text{O}_3$ , 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  $\text{Na}_2\text{S}_2\text{O}_3$  solution per 1 L of sample (NFM 7.3.2.B).

-If sterile  $\text{Na}_2\text{S}_2\text{O}_3$  is used, then dispense with sterile pipet into sterile bottle.

-If  $\text{Na}_2\text{S}_2\text{O}_3$  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.

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## 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.**

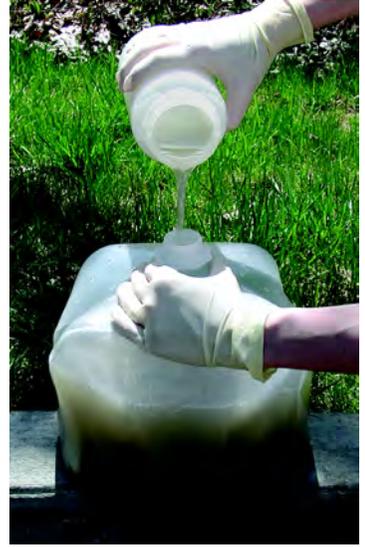
**CAUTION: Always wear gloves when handling sampling equipment and samples. Take care to prevent contaminated water from contacting skin, mouth, nose, or eyes (NFM 9.7).**

- ▶ **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<sup>7</sup> (NFM 4.1.1.A).

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<sup>7</sup>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.)

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## 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.**

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## 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<sup>®</sup> 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).

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## 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).**

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## SAMPLE PRESERVATION, 7.3.2.B TRANSPORT, AND HOLDING TIMES

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 ( $\mu\text{g/L}$ ) 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 ( $\text{Na}_2\text{S}_2\text{O}_3$ )** 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  $\text{Na}_2\text{S}_2\text{O}_3$  as follows:
  - a. In a volumetric flask, dissolve 100 grams (g)  $\text{Na}_2\text{S}_2\text{O}_3$  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  $\text{Na}_2\text{S}_2\text{O}_3$  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  $\text{Na}_2\text{S}_2\text{O}_3$  solution for every 1 L of sample. If the sterile  $\text{Na}_2\text{S}_2\text{O}_3$  is used, then be sure to use only sterile pipets and sterile sample bottles. If the  $\text{Na}_2\text{S}_2\text{O}_3$  is not sterile, then dispense the required volume of  $\text{Na}_2\text{S}_2\text{O}_3$  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  $\text{Na}_2\text{S}_2\text{O}_3$  solution in the sample bottle.
  3. Autoclave the sample bottle containing EDTA stock solution at  $121^\circ\text{C}$  for 15 minutes.

**$\text{Na}_2\text{S}_2\text{O}_3$  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  $8^\circ\text{C}$  **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  $8^\circ\text{C}$  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.**

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### 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).

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

**Temperature:** Water and air temperature are given in degrees Celsius ( $^{\circ}\text{C}$ ), which can be converted to degrees Fahrenheit ( $^{\circ}\text{F}$ ) by use of the following equation:

$$^{\circ}\text{F} = 1.8(^{\circ}\text{C}) + 32$$

$$^{\circ}\text{C} = (^{\circ}\text{F}/1.8) - 32$$

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## 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 ( $\mu\text{m}$ ):** 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  $\text{HCl}$  is 0.01  $N$  in the hydronium ion ( $\text{H}^+$ ). A solution that is 0.01  $F$  in  $\text{H}_2\text{SO}_4$  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.

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**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 <sub>2</sub>	calcium chloride
Cu	copper
FeCl <sub>3</sub>	ferric chloride
H <sub>2</sub> SO <sub>4</sub>	sulfuric acid
K <sub>2</sub> HPO <sub>4</sub>	potassium hydrogen phosphate
KH <sub>2</sub> PO <sub>4</sub>	potassium dihydrogen phosphate
MgSO <sub>4</sub>	magnesium sulfate
Na <sub>2</sub> SO <sub>3</sub>	sodium sulfite
Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub>	sodium thiosulfate
NaHPO <sub>4</sub>	sodium phosphate
NaOH	sodium hydroxide
Ni	nickel
NH <sub>4</sub> Cl	ammonium chloride
Zn	zinc

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**ABBREVIATIONS**

BOD	biochemical oxygen demand
BOD <sub>5</sub>	biochemical oxygen demand (5 day)
CBOD	carbonaceous biochemical oxygen demand
CBOD <sub>u</sub>	ultimate carbonaceous biochemical oxygen demand
col/100mL	colonies per 100 milliliters
DIW	deionized or distilled water
DO	dissolved oxygen
<i>E. coli</i>	<i>Escherichia coli</i>
EDI	equal-discharge increment

	EDTA	ethylenediaminetetraacetic acid
	EIA	enterococcus confirmation medium (esculin substrate)
+	ETFE	ethylenetetrafluoroethylene
	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	<i>Clostridium perfringens</i> medium
	mE	membrane filter—Enterococci medium
	mEI	enterococcus medium
	mENDO	membrane filter—total coliform medium
	mF	membrane filter technique
	mFC	membrane filter—Fecal Coliform medium
	MI	total coliform and <i>Escherichia coli</i> medium
	MPN	most probable number
	mTEC	membrane filter—Thermotolerant <i>Escherichia coli</i> media
	N	normal
	NA–MUG	nutrient agar-4-methylumbelliferyl-b-D-glucuronide
+	NFM	National Field Manual ( <i>National Field Manual for the Collection of Water-Quality Data</i> )
	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	perfluoroalkoxy polymers
	PTFE	polytetrafluoroethylene 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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# **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.**

**Table 2. Parameter codes for fecal indicator bacteria.**

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

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

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**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;  $\mu$ m, 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 <sup>1</sup>	Medium	Parameter code <sup>2</sup>
Total coliform bacteria	mENDO	31501
	MI	90900
Fecal coliform bacteria	mFC, 0.65- $\mu$ m pore-size filter	31625
	mFC, 0.45- $\mu$ m pore-size filter	31616
<i>Escherichia coli</i>	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
<i>Clostridium perfringens</i>	mCP	90915

<sup>1</sup>Membrane-filtration method: all units are in colonies per 100 milliliters.

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

<sup>1</sup>Bacterial host strain used to detect the specified coliphage type.<sup>2</sup>The parameter codes listed are those that are in common use (2003) in the National Water Information System of the U.S. Geological Survey.<sup>3</sup>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
<i>Cryptosporidium</i>	99599	oocysts per 10 liters
<i>Cryptosporidium</i> —spike efficiency	99600	percent recovery
<i>Giardia</i>	99597	cysts per 10 liters
<i>Giardia</i> —spike efficiency	99598	percent recovery