
FECAL INDICATOR BACTERIA 7.1

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	Page
7.1 Fecal indicator bacteria	FIB–5
7.1.1 Sampling equipment and equipment sterilization procedures	10
7.1.1.A Autoclaving	16
7.1.1.B Flame sterilization of the Hydrosol® field filtration unit	18
7.1.1.C Sterilization of equipment by ultraviolet irradiation	20
7.1.1.D Sterilization of equipment with sodium hypochlorite.....	20
7.1.2 Sample collection, preservation, storage, and holding times.....	22
7.1.2.A Surface-water sample collection	24
Depth- and-width-integrating methods	25
Point-sampling methods	26
7.1.2.B Ground-water sample collection.....	28
Supply wells	29
Monitoring wells	30
7.1.2.C Bed-sediment sample collection	34
7.1.2.D Sample preservation, storage, and holding times	37

7.1.3 Identification and enumeration methods.....	38
7.1.3.A Culture media and reagents.....	40
7.1.3.B Processing bed sediments	43
7.1.3.C Membrane filtration	45
7.1.3.D Enzyme substrate tests in the presence- absence format	58
7.1.3.E Enzyme substrate tests in the most-probable- number format	59
7.1.4 Calculating and reporting fecal indicator bacteria densities	62
7.1.5 Selected references	71
7.1.6 Acknowledgments	73

Illustrations

7.1–1. Photograph showing procedure to flame sterilize the Millipore Hydrosol® field filtration unit	19
7.1–2. Diagram showing preparation of sample volumes by dilution	46
7.1–3. Photograph showing steps in membrane-filtration procedure	49
7.1–4. Diagram showing a method for counting colonies on gridded membrane filters	54
7.1–5. Photographs of typical colonies of fecal indicator bacteria on culture media	56

Tables

7.1–1. Recreational water criteria under the Beaches Environmental Assessment and Coastal Health Act of 2000	8
7.1–2. Equipment and supplies used for membrane-filtration and liquid broth analyses (presence-absence or most- probable-number format) for fecal indicator bacteria in water or sediment samples.....	11
7.1–3. Equipment cleaning and sterilization procedures	14
7.1–4. Recommended times to autoclave media and materials....	17
7.1–5. Summaries of equipment for sample collection, procedures for sample preservation, and holding times for indicator bacteria	23
7.1–6. Fecal indicator test media, typical applications, incubation times and temperatures, and types of rinse or dilution waters	39
7.1–7. Positive- and negative-control test organisms for specific media types.....	42
7.1–8. Detection ranges achieved by analyzing various sample-water volumes by membrane filtration	46
7.1–9. Test (medium type), ideal colony count, and typical colony color, size, and morphology for indicator bacteria colonies	47

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FECAL INDICATOR BACTERIA 7.1

Fecal indicator bacteria are used to assess the microbiological quality of water. Although these bacteria are not typically disease causing, they are associated with fecal contamination and the possible presence of waterborne pathogens. The density of indicator bacteria¹ is a measure of water safety for body-contact recreation or for consumption.

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

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

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

Criteria for selecting an indicator of fecal contamination in water

The preferred fecal indicator:

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

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

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

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

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

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

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

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

- Enterococci are the preferred indicator bacteria in marine waters because of their salt tolerance.
- Either *E. coli* or enterococci are recommended for monitoring fresh water (U.S. Environmental Protection Agency, 2004).

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

[mL, milliliters]

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

¹Designated beach areas are frequently lifeguard protected, provide parking and other public access, and are heavily used by the public (U.S. Environmental Protection Agency, 1986, p. 7).

²Other recreational uses, which involve various levels of full-body contact, are designated by individual State water-quality standards (U.S. Environmental Protection Agency, 1986, p. 7).

- ▶ **Shellfish-growing area.** Water-quality criteria for shellfish-growing areas have been developed by the U.S. Food and Drug Administration under the National Shellfish Sanitation Program. The 2005 guide for the control of molluscan shellfish (U.S. Food and Drug Administration, 2005) specifies criteria, based on total coliform and fecal coliform densities, to indicate the sanitary quality of water in shellfish-growing areas.
- ▶ **Potable water supplies: treated, untreated, private, and public.** Water-quality criteria for drinking water, based on total coliform density, are specified in the Safe Drinking Water Act, as amended in 1986 (U.S. Environmental Protection Agency, 1986).
 - Under the provisions of the Safe Drinking Water Act, all public water supply systems must disinfect their water unless criteria are met that ensure equivalent protection.
 - Under the Total Coliform Rule (U.S. Environmental Protection Agency, 2001), public water supply systems also must monitor distribution systems for contamination. When total coliforms are detected, follow-up tests for fecal coliforms or *E. coli* are required and a more intensive monitoring schedule may be required.
 - Water-quality criteria for ground water are specified in the Ground Water Rule, which was passed by Congress in October 2006. The Rule covers public water systems, which are defined as those that serve 25 or more people or have 15 or more service connections and operate at least 60 days per year. In addition to total coliforms and *E. coli* as indicators of sanitary quality in ground water, the Rule includes enterococci and coliphage viruses (U.S. Environmental Protection Agency, 2006). Ground water typically contains substantially lower densities of indicator bacteria compared to bodies of surface water.

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

7.1.1 SAMPLING EQUIPMENT AND EQUIPMENT STERILIZATION PROCEDURES

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

- ▶ **Equipment for the collection and analysis of bacterial samples must first be cleaned and then sterilized** (table 7.1–3). Sterilize the filtration unit and sampling equipment before traveling between sites or before each sample collected at the same site at different times. There are several sterilization methods, but autoclaving is preferred.
- ▶ **Quality assurance and quality control of sterilization procedures must be documented.** Keep a logbook of autoclave operation or other sterilization procedure(s) used. In the log, include a brief description of the quality-assurance procedures used and quality-control tests run; note the date, the test results, and the name of the autoclave operator and (or) analyst.
- ▶ **If sample water contains residual chlorine or other halogens:** Add sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) to the sample bottles before the bottles are autoclaved. Residual chlorine commonly is found in samples collected from sources such as treated drinking water (withdrawn from taps), wastewater effluents, and in the mixing zones directly downstream from wastewater-treatment plants, or from the residue of sodium hypochlorite used to sterilize nonautoclavable sampling equipment (section 7.1.1.D).

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

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

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

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

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

- ▶ **Equipment that has been decontaminated using a methanol rinse can affect the viability of the microbial population** for which analyses will be performed. Ensure the removal of methanol residue from sampling equipment before samples are collected for bacteria analysis.
 - Allow the methanol to evaporate completely from the interior and exterior surfaces of equipment. In an office setting, filtered argon or nitrogen gas under pressure can be forced through equipment to help evaporate the methanol from interior spaces that cannot be exposed adequately to the atmosphere.
 - After evaporating the methanol, rinse the equipment with pesticide grade blank water (PBW) or volatile/pesticide grade blank water (VPBW) and autoclave the equipment. For nonautoclavable equipment, rinse thoroughly with PBW or VPBW that has been autoclaved. **Methanol-tainted water must be collected and disposed of as a hazardous substance: follow local and (or) State and Federal regulations.**
 - Collection and analysis of a blank sample for volatile organic compound analysis can help document the absence (or presence) of methanol in the sample.
 - Collect bacteria samples last.
 - As an alternative to the procedures described above, use completely separate sampling equipment that is dedicated for microbial sample collection.

- ▶ **When using a pump system to collect ground-water samples for both chemical and bacteria analysis**, it is recommended that individual lengths of tubing be dedicated to, and prepared for, each well at which samples will be collected.
 - To clean the tubing, (a) follow the office cleaning procedures described in NFM 3, removing the methanol by pushing it out with at least two tubing volumes of PBW or VPBW or (and) by forcing clean gas through the tubing, as described above; and (b) autoclave the tubing. Be certain, first, that the tubing can be autoclaved. Sterilization by autoclave is the preferred method. If the tubing cannot be autoclaved, use autoclaved PBW or VPBW to push methanol from, and subsequently rinse, the tubing.

- Tubing should be dry if it will be stored and transported under warm conditions, to prevent microbial growth. Forced gas can be used to dry the tubing; however, the tubing should be autoclaved afterwards.
- Between sites, clean the pump using the field procedures described in NFM 3, taking extra care to remove methanol residue from the pump interior either by using a forced gas method or rinsing copiously with autoclaved VPBW or PBW.

Store and transport sterile equipment in a sterile container.

Table 7.1–3. Equipment cleaning and sterilization procedures

[DIW, distilled or deionized water; mL, milliliter; Na₂S₂O₃, sodium thiosulfate; L, liter; EDTA, ethylenediaminetetraacetic acid; UV, ultraviolet light; psi, pounds per square inch; °C, degrees Celsius]

Equipment	Procedures
Cleaning	
All equipment (this includes water-level tape measure, all sample-collection and sample-processing equipment used in the field and laboratory)	Wash equipment thoroughly with a dilute, nonphosphate laboratory-grade detergent. Rinse three times with tap water. Rinse again three to five times with DIW Wipe the wetted portion of water-level tapes with disinfectant (0.005 percent bleach solution or 70-percent methyl or ethyl alcohol) and rinse thoroughly with sterile water.
Sterilization (Refer to table 7.1–4 for recommended times for autoclaving glassware, liquids, and other media and materials.)	
Magnetic plastic filtration field units, glass, plastic, and Teflon bottles and containers, volumetric flasks, pipets and pipettors, and other autoclavable materials	If sample may contain residual chlorine or other halogens, add 1 mL of 10-percent Na ₂ S ₂ O ₃ solution per liter volume of sample. This can be added to the sample bottle before autoclaving. If sample may contain toxic trace metals, add 3 mL of a sterile 15-percent EDTA stock solution per 1 L of sample. This can be added to the sample bottle before autoclaving. Wrap equipment in kraft paper, aluminum foil, or place into autoclavable bags. Autoclave at 121°C, 15 psi, for 15 minutes. NOTE: If an autoclave is not available, refer to sections 7.1.1.B, 7.1.1.C, and 7.1.1.D for alternative sterilization techniques.

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

Equipment	Procedures
Stainless steel filtration field units	Autoclave at 121°C, 15 psi, for 15 minutes, flame sterilize with methanol (Millipore Hydrosol® units only, section 7.1.1.B), or use ultraviolet irradiation for 15 minutes (section 7.1.1.C).
Portable submersible pumps and pump tubing	<p>Autoclavable equipment (preferred): Wrap components in kraft paper, aluminum foil, or place into autoclavable bags. Autoclave at 121°C, 15 psi, for 15 minutes.</p> <p>Non-autoclavable equipment: (1) Submerge sampling system in pH-neutral sodium hypochlorite solution prepared from household laundry bleach (section 7.1.1.D). (2) Circulate solution through pump and tubing for 30 minutes. (3) Follow step 2 by thoroughly rinsing, inside and out, with a working solution of 1 mL 10-percent sterile Na₂S₂O₃ per liter of water, and circulate solution for 5 minutes. (4) Pump Na₂S₂O₃ to waste, then circulate sterile DIW through pump, followed by sample water pumped from the well. Dispose of waste solutions according to regulatory requirements.</p> <p>CAUTION: Prolonged or repeated use of a hypochlorite solution on interior or exterior metallic surfaces of a pump can cause corrosion or other damage to the pump and compromise the quality of samples collected for a trace-element or organic-compound analysis.</p>

To prepare a 10-percent stock solution of sodium thiosulfate (Na₂S₂O₃) for treatment of samples:

1. Dissolve 100 grams (g) of Na₂S₂O₃ into 500 milliliters (mL) of deionized or distilled water; stir until dissolved, and fill a flask to 1,000 mL (Bordner and Winter, 1978, p. 6; American Public Health Association and others, 1998, p. 9-19). Sterilize by autoclaving (table 7.1-3).
2. Dispense 1 milliliter (mL) of 10-percent Na₂S₂O₃ stock solution for every liter of sample (final concentration is 0.01 percent).
3. Store the Na₂S₂O₃ stock solution at room temperature in a tightly capped bottle that is labeled with its contents and expiration date. Discard after 6 months and prepare a fresh solution.

- ▶ **If sample water contains toxic trace metals:** Add ethylenediaminetetraacetic acid (EDTA) to sample bottles when water to be collected contains toxic concentrations of trace metals. EDTA can be combined with the $\text{Na}_2\text{S}_2\text{O}_3$ solution in the sample bottle before sterilization.

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

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

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

7.1.1.A AUTOCLAVING

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

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

- ▶ Before autoclaving, wrap clean equipment in Kraft paper, autoclavable plastic bags, or aluminum foil. Wrap loosely to allow steam to penetrate the wrapping. Cap tubing ends with aluminum foil.
 - Sterilize and store the equipment in a clean area.
 - Resterilize equipment if the foil, bag, or Kraft paper is torn.
- ▶ Consult table 7.1–4 for recommended times for autoclave sterilization of various media and materials.
 - Liquids must be exposed to 121°C at 15 psi (pounds per square inch (lbs/in²)) for the specified time for effective sterilization—larger volumes of liquid take longer to reach 121°C.
 - If the autoclave does not reach the specified temperature and pressure or fails a quality-control test, service the autoclave and then resterilize all materials (American Public Health Association and others, 1998, p. 9-2 to 9-14).
- ▶ In addition to the guidance listed above, it is necessary to:
 - Use sterilization indicator tape with each load.
 - Use commercially available biological indicators at least quarterly to test autoclave performance. Biological indicators are composed of endospores—living cells that are resistant to heat but are killed by effective autoclaving.
 - Drain the autoclave daily. Clean with mild soap and water once per week during periods of daily use. Record cleaning procedures in the logbook.
 - Avoid overloading the autoclave with equipment or materials; overloading will result in incomplete sterilization.

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

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

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

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

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

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

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

CAUTION:

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

To flame sterilize the Hydrosol unit, carefully:

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

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



7.1.1.C STERILIZATION OF EQUIPMENT BY ULTRAVIOLET IRRADIATION

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

7.1.1.D STERILIZATION OF EQUIPMENT WITH SODIUM HYPOCHLORITE

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

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

3. Clean the equipment and submerge it in the sodium hypochlorite solution, or completely fill the equipment with the sodium hypochlorite solution. Maintain contact for 30 minutes.
4. Remove or drain the equipment.
5. Thoroughly rinse the equipment, inside and out, with sterile $\text{Na}_2\text{S}_2\text{O}_3$ solution (prepared as 1 mL of 10-percent stock per liter of water) to remove residual chlorine. Maintain contact for 5 minutes.
6. Remove or drain the equipment.
7. Rinse the equipment thoroughly with sterile deionized or distilled water.
8. If adding EDTA to the sample bottle, use a sterile pipet and sterile EDTA.

CAUTION:

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

7.1.2 SAMPLE COLLECTION, PRESERVATION, STORAGE, AND HOLDING TIMES

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

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

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

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

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

7.1.2.A SURFACE-WATER SAMPLE COLLECTION

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

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

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

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

Depth- and width-integrating methods

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

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

Point-sampling methods

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

To collect a hand-dipped sample:

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

CAUTION:

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

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

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

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

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

- ▶ **Analytical blanks**—Process **filter blanks** and **procedure blanks** (for membrane filtration) or **method blanks** (for liquid broth tests) during sample processing to document that equipment and rinse or dilution water were sterile.
 - A filter blank is processed for each sample before the sample is filtered.
 - A procedure blank is processed through the filtration unit after the sample has been filtered, at a frequency of one blank for every 10 to 20 samples.
 - Method blanks are processed at a frequency of one blank for every 10 to 20 samples.
 - ▶ **Field concurrent replicates**—Collect and analyze one field replicate for every 10 to 20 samples. A split concurrent replicate is recommended. Two samples are collected and each sample is analyzed in duplicate by membrane filtration. Replicate data are used to quantify the uncertainty in density estimates (see Francy and Darner, 1998, for an example).
 - ▶ **Positive and negative controls**—These types of quality-control samples are required if media are prepared from basic ingredients by field or laboratory personnel, and are recommended if media are purchased from a commercial supplier. The frequency of analysis depends on project objectives and the type of medium. Positive controls test the medium's ability to recover target bacteria; negative controls are used to ensure that nontarget bacteria are inhibited or recognized. Refer to section 7.1.3.A for details.
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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. Collecting a 100-mL ground-water sample for bacteria analysis is standard procedure because ambient ground water flowing through aquifers typically contains much fewer particulates, and bacteria density is expected to be low. Applying the protocols for purging wells before collecting water-quality samples (NFM 4.2) is necessary to ensure that the particulate content and bacteria density sampled represent ambient aquifer conditions.

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

Supply wells

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

- ▶ For aquifer-monitoring studies, locate the point of ground-water withdrawal upgradient of (before the water reaches) a chlorination or other treatment system (unless study objectives dictate otherwise).
- ▶ For drinking-water studies, sample the ambient water in the well regardless of the history of treatment. Dechlorination with $\text{Na}_2\text{S}_2\text{O}_3$ is required if the sample is collected after the water has passed through a chlorination unit (section 7.1.1).

To sample a supply well for indicator bacteria:

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

Monitoring wells

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

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

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

To disinfect a pump with a sodium hypochlorite solution:

1. Follow the instructions for cleaning equipment with a hypochlorite solution (bleach) (section 7.1.1.D and table 7.1–3)
2. Lower the pump carefully into the well. Purge the residual chlorine and $\text{Na}_2\text{S}_2\text{O}_3$ from the system by pumping three tubing volumes of well water through the system; contain or appropriately discard this waste water. Take care not to contaminate samples for chemical analysis with residual chlorine or $\text{Na}_2\text{S}_2\text{O}_3$. **The pump must have a backflow check valve (an antibacksiphon device) to prevent residual chlorine from flowing back into the well.**

To use a pump that cannot be disinfected:

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

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

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

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

Exercise the following precautions when collecting samples from monitoring wells:

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

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

- ▶ **Pump blanks**—This type of blank should be collected ahead of sampling so that results can be evaluated before field sampling. Thereafter, collect pump blanks with ground-water samples at a frequency of one blank for every 10 to 20 samples, or as required by the data-quality objectives of the study. Collect pump blanks by passing sterile buffered water (for membrane filtration) or sterile distilled or deionized water (for liquid broth tests) through the sampling equipment and into a sterile sampling container. A standpipe may be used to collect a pump blank, but it first must be cleaned and disinfected. Analyze pump blanks for fecal indicator bacteria and record results. If no growth is observed, the use of sufficiently sterile procedures is confirmed and documented.
- ▶ **Analytical blanks**—Process **filter blanks** and **procedure blanks** (for membrane filtration) or **method blanks** (for liquid broth tests) during sample processing to document that the equipment and the rinse or dilution water were sterile. A filter blank is processed for each sample before the sample is filtered. A procedure blank is processed through the filtration unit after the sample has been filtered, at a frequency of one blank for every 10 to 20 samples. Method blanks also are processed at a frequency of one blank for every 10 to 20 samples.
- ▶ **Field sequential replicates**—Because few ground-water samples test positive for indicator bacteria, it may be necessary to collect field sequential replicates for every sample. A lower frequency may be used if a large percentage of wells are positive or study objectives do not require quantification of variability.
- ▶ **Positive and negative controls**—These types of quality-control samples are required if media are prepared from basic ingredients, and is recommended if the medium is purchased from a commercial supplier. The frequency of analysis depends on project objectives and the type of medium. Positive controls test the medium's ability to recover target bacteria; negative controls are used to ensure that nontarget bacteria are inhibited or recognized. Refer to section 7.1.3.A and table 7.1–6 for details.

7.1.2.C BED-SEDIMENT SAMPLE COLLECTION

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

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

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

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

1. The sampler needs to be sterilized before collecting samples for bacterial analysis. Because of the dimensions of these samplers, autoclaving generally is not practical. **If more than one site is to be sampled, the sampler needs to be resterilized in the field at each of the sites.** To field-sterilize the sampler:
 - a. Put on laboratory gloves.
 - b. Wash and scrub the sampler in dilute nonphosphate, laboratory-grade detergent and rinse with tap water and then deionized or distilled water.
 - c. Soak the sampler in a 0.005-percent sodium hypochlorite solution for 15 minutes (section 7.1.1.D)
 - d. Soak in a sterile 0.01-percent $\text{Na}_2\text{S}_2\text{O}_3$ solution for 5 minutes (section 7.1.1).

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

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

- ▶ **Field blanks**—Collect and analyze field blanks when using a sampler to collect bed-sediment samples at a frequency of one blank for every 10 to 20 samples, or as required by study objectives, to document that sampling and analysis equipment have not been contaminated. Process field blanks before sample collection if the sampler does not need to be sterilized in the field. If the sampler does need to be sterilized in the field, process field blanks after collection of a sample and resterilization of the sampler. This will demonstrate that the field-sterilization procedure is working appropriately.
 1. Pass sterile buffered water (for membrane filtration) or sterile distilled or deionized water (for liquid broth tests) over the sterile sampler and into a sterile washtub. Collect the field blank into a sterile bottle or jar.
 2. Analyze field blanks for fecal indicator bacteria. If no growth is observed, then the sample was collected by use of sufficiently sterile procedures.
- ▶ **Analytical blanks**—Process **filter blanks** and **procedure blanks** (for membrane filtration) or **method blanks** (for liquid broth tests) during sample processing to document that the equipment and the rinse or dilution water were sterile. A filter blank is processed for each sample before the sample is filtered. A procedure blank is processed through the filtration unit after the sample has been filtered, at a frequency of one blank for every 10 to 20 samples, or as otherwise required. Method blanks also are processed at a frequency of one blank for every 10 to 20 samples.
- ▶ **Field replicates**—Collect and analyze one field replicate for every 10 to 20 samples, or as otherwise required by study objectives. A split sequential replicate is recommended. For samples collected from wading sites, an additional three jars of sediment are collected and treated as a separate sample. For samples collected using a sampler, the sampler is resterilized before collection of the replicate; the sediment is deposited into a new, sterile washtub, composited in the field, and treated as a separate sample. In the laboratory, each sequential replicate is analyzed twice to produce a total of four split sequential replicate samples.
- ▶ **Positive and negative control samples**—These types of quality-control samples are required if media are prepared from basic ingredients by field or laboratory personnel and recommended if media are purchased from a commercial supplier. The frequency of analysis depends on project objectives and the type of medium. Positive control samples test the medium's ability to recover target bacteria; negative control samples are used to ensure that nontarget bacteria are inhibited or recognized. Refer to section 7.1.3.A for details.

SAMPLE PRESERVATION, STORAGE, AND HOLDING TIMES 7.1.2.D

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

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

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

7.1.3 IDENTIFICATION AND ENUMERATION METHODS

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

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

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

[mENDO, total coliform medium; ±, plus or minus; °C, degrees Celsius; MI, total coliform and *Escherichia coli* medium; MgCl₂, magnesium chloride; NA-MUG, *E. coli* medium; mTEC, *E. coli* medium; mFC, fecal coliform medium; KF, fecal streptococci medium; mEI, enterococci medium; mCP, *Clostridium perfringens* medium.]

Test (medium)	Typical application	Incubation time and temperature	Type of rinse and (or) dilution water ¹
Total coliform bacteria (mENDO)	Drinking water and ground water	24 ± 2 hours at 35.0 ± 0.5°C	Phosphate-buffered water with MgCl ₂
Total coliform bacteria (MI)	Drinking water and ground water	24 ± 2 hours at 35.0 ± 0.5°C	Phosphate-buffered water with MgCl ₂
Total coliform bacteria (Colilert or Colilert-18)	Drinking water and ground water	24–28 hours at 35.0 ± 0.5°C (Colilert) 18–22 hours at 35.0 ± 0.5°C (Colilert-18)	Distilled or deionized water
<i>Escherichia coli</i> (NA-MUG)	Drinking water and ground water	4 hours at 35 ± 0.5°C after primary culture on mENDO medium	(See mENDO)
<i>Escherichia coli</i> (MI)	Drinking water and ground water	24 ± 2 hours at 35.0 ± 0.5°C	Phosphate-buffered water with MgCl ₂
<i>Escherichia coli</i> (modified mTEC)	Fresh waters—recreational and other surface water	Resuscitate, 2 hours, 35.0 ± 0.5°C Incubate, 22 to 24 hours, 44.5 ± 0.2°C	Phosphate-buffered water with MgCl ₂
<i>Escherichia coli</i> (on urea substrate broth after primary culture on mTEC)	Fresh waters—recreational and other surface water	Resuscitate, 2 hours, 35.0 ± 0.5°C Incubate, 22 to 24 hours, 44.5 ± 0.2°C Transfer filter to urea substrate broth, 15 to 20 minutes, before counting	Phosphate-buffered water with MgCl ₂
<i>Escherichia coli</i> (Colilert or Colilert-18)	Fresh waters—recreational and other surface water, drinking water and ground water	24–28 hours at 35.0 ± 0.5°C (Colilert) 18–22 hours at 35.0 ± 0.5°C (Colilert-18)	Distilled or deionized water
Fecal coliform bacteria (mFC)	Recreational water, shellfish-harvesting water	24 ± 2 hours at 44.5 ± 0.2°C	Phosphate-buffered water with MgCl ₂
Fecal streptococci (KF)	Recreational water	48 ± 2 hours at 35.0 ± 0.5°C	Phosphate-buffered water with MgCl ₂
Enterococci (mEI)	Fresh and saline recreational waters, proposed for ground water	24 hours at 41.0°C ± 0.5°C	Phosphate-buffered water with MgCl ₂
Enterococci (Enterolert)	Fresh and saline recreational waters, proposed for ground water	24–28 hours at 41.0°C ± 0.5°C	Distilled or deionized water
<i>Clostridium perfringens</i> (mCP)	All waters	24 ± 2 hours at 44.5 ± 0.2°C	Phosphate-buffered water with MgCl ₂

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

7.1.3.A CULTURE MEDIA AND REAGENTS

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

- ▶ Sterile phosphate-buffered water amended with magnesium chloride (U.S. Environmental Protection Agency, 2000) is used to dilute samples and to rinse the filtration unit and utensils.
 - Sterile buffered water can be obtained in 99-mL dilution bottles and in 500-mL volumes.
 - **Do not use sterile buffered water that exceeds the expiration date indicated on the label.**
 - Obtain buffered water from a commercial vendor that provides quality-control documentation. Buffered water also can be prepared according to the instructions found at <http://oh.water.usgs.gov/micro/qcmanual/appendm.pdf> (accessed January 17, 2007). Buffered water prepared according to these instructions must be autoclaved and checked for sterility before use.
- ▶ Culture media (including dehydrated media) for enumeration of fecal indicator bacteria for USGS studies are obtained commercially. Instructions for preparation are printed on the labels of dehydrated media bottles and should be followed carefully. For studies that require small amounts of media, or that require media with complex preparation steps (such as mEI and MI agars), the use of pre-poured plates is recommended. Sources of dehydrated and pre-poured media are listed in Office of Water Quality Technical Memorandum 2005.02 (U.S. Geological Survey, 2005). Updated, detailed information about media and reagent preparation also can be found at http://oh.water.usgs.gov/micro_qaqc.htm <http://oh.water.usgs.gov/micro/qcmanual/manual.html> (accessed January 16, 2007 July 16, 2013).

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

To store media and reagents:

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

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

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

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

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

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

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

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

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

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

PROCESSING BED SEDIMENTS 7.1.3.B

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

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

To process bed sediments:

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

4. Elute bacteria from the sediment as soon as possible after compositing the sample.
 - a. Place 20 g of the sediment composite into a bottle containing 200 mL of phosphate buffered water with magnesium chloride (U.S. Environmental Protection Agency, 2000). NOTE: If preparing a sample for split replicate analysis, increase the amount of sediment and buffered water appropriately. For example, place 30 g of the sediment composite into a bottle containing 300 mL of buffered water.
 - b. Label the lid of this bottle with the time the bottle should be removed from the shaker (the bottle will be shaken for 45 minutes).
 - c. Place the bottle on a wrist-action shaker.
 - d. After 45 minutes, remove the bottle from the shaker and let it stand for 30 seconds undisturbed. Pour off the supernatant into a new, labeled sterile bottle.
5. Analyze the supernatant using the membrane-filtration method (section 7.1.3.C) or by the enzyme substrate MPN method (section 7.1.3.E). Autoclave the sediment and supernatant and discard.

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

6. Remove the dish for proportional dry weight of sediment after 24 hours or until a constant weight is obtained.
 - a. Record the constant weight obtained as "weight after drying."
 - b. Use the following equation to calculate the proportional dry weight:

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

where,

W_{tare} = Tare weight of empty dish,

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

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

MEMBRANE FILTRATION 7.1.3.C

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

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

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

To prepare to filter samples:

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

To filter samples:

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

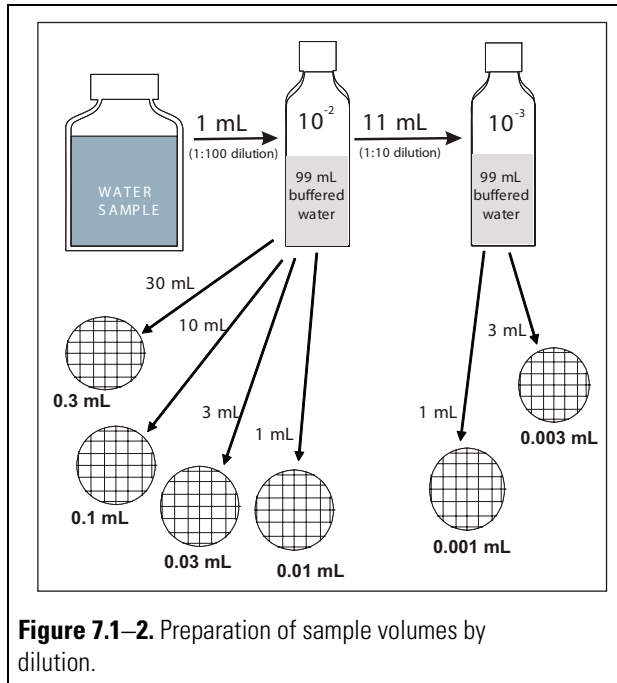


Figure 7.1–2. Preparation of sample volumes by dilution.

Table 7.1–8. Detection ranges achieved by analyzing various sample-water volumes by membrane filtration
[mL, milliliter; CFU, colony-forming units]

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

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

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

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

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

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

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

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

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

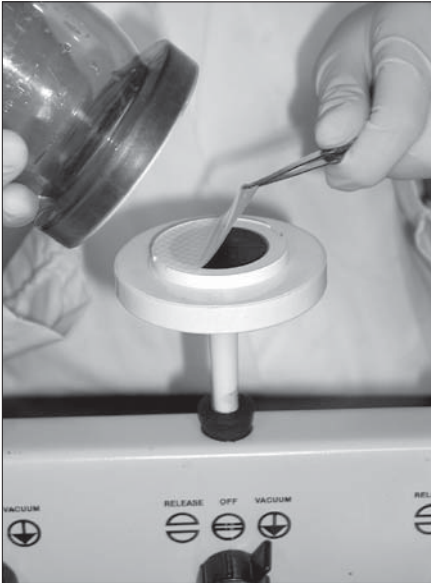
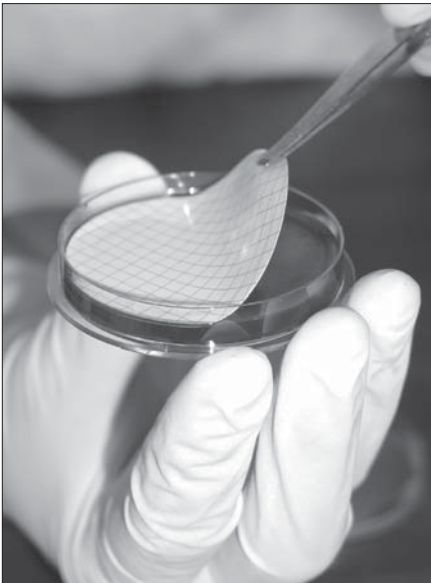
3. If the sample volume to be plated is less than 1 mL, prepare dilutions with sterile buffered water in 99-mL dilution bottles (fig. 7.1–2 and table 7.1–8).
 - Transferring 11 mL of sample to a 99-mL dilution bottle creates a 1 to 10 dilution. Transferring 1 mL of sample to a 99- mL dilution bottle creates a 1 to 100 dilution.
 - These can be diluted in series, as needed. For example, transferring 1 mL of the 1 to 100 dilution to another 99-mL dilution bottle creates a 1 to 10,000 dilution.
 - When preparing a dilution series, use a sterile pipet to measure each sample volume. After each sample-volume transfer, close and shake the dilution bottle vigorously at least 25 times.
 - **Filter the diluted samples within 20 minutes after preparation.** Keep dilution bottles out of sunlight and do not transfer less-concentrated sample volumes with pipets that were used to transfer more-concentrated sample volumes.
4. Assemble the filtration unit by inserting the base of the sterile filter-holder assembly into a side-arm flask or manifold (fig. 7.1–3). Connect the filtration unit to a hand-held pump, vacuum pump, or peristaltic pump.
5. If flame sterilization is used (Hydrosol units), rinse the inside of the filtration unit with sterile buffered water to remove any residue of formaldehyde.
6. Sterilize stainless steel forceps:
 - a. Immerse tips in a small bottle or flask containing 70- or 90-percent ethanol.
 - b. Pass forceps through the open flame of an alcohol burner. Allow the alcohol to burn out and allow the forceps to cool to avoid scorching the membrane filter.
7. Remove the filter from its sleeve. Remove the sterilized funnel from the base. Always hold the funnel in one hand while placing or removing the membrane filter. Placing the funnel on anything other than the filter unit base might result in contamination of the funnel.
8. Using the sterile forceps, place a sterile, gridded membrane filter (47 mm) on top of the filter base, gridded side up (fig. 7.1–3). Carefully replace and secure the filter funnel on the filter base. Avoid tearing or creasing the membrane filter.

**A****B****C**

PROCEDURE

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

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

**D****E**

PROCEDURE

8. Sterilize forceps and remove filter (D). Replace funnel on filtration apparatus.
9. Roll filter onto media in plate (E). Place inverted plate in incubator.
10. Repeat steps 4–9 for each sample volume on order of the smallest to the largest volume. A filter blank is processed before each sample. Filter a procedure blank after every 10 to 20 samples or once per day at each site, according to study objectives.
11. Filter a replicate sample after every 10 to 20 samples or at each site, according to study objectives.

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

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

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

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

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

12. Remove the required volume by pipet or by pouring into a graduated cylinder (>10 mL) within 5 seconds of shaking the sample. If pipetting, place the pipet tip in the center of the sample volume and use a pipettor or pipet bulb with a valve for volume control. It is acceptable to use the upper and lower graduations to measure the volume (line-to-line) or simply draw up the selected volume.
13. Pour or pipet the measured volume of sample into the filter funnel (fig. 7.1-3B or C).
 - a. **If the volume of sample to be filtered is from 1 to 10 mL,** pour about 20 mL of sterile buffered water into the funnel before pipetting the sample to allow even distribution of bacteria on the membrane filter.
 - b. **If the volume of sample to be filtered is more than 10 mL,** transfer the sample with a sterile pipet or graduated cylinder directly into the funnel.
14. Allow the pipet to drain, touching the pipet to the inside of the funnel to remove any remaining sample (fig. 7.1-3C). 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.

15. Apply a vacuum. **To avoid damage to bacteria, do not exceed a pressure of about 5 lb/in² (25 cm of mercury).**
16. Rinse the inside of the funnel twice with 20 mL to 30 mL of sterile buffered water while applying a vacuum. If a graduated cylinder is used, rinse the cylinder with sterile buffered water and deliver rinse water to the filtration unit.
17. Remove the funnel and hold it in one hand—do not set the funnel on the counter top.
18. Remove the membrane filter using sterile forceps (fig. 7.1–3D).

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

19. Replace the funnel on the filter base and release the vacuum. (Releasing the vacuum after removing the filter prevents backflow of sample water onto the filter. Unnecessarily wet filters promote confluent growth of colonies and poor results.)
20. Open a labeled plate and place the membrane filter on the medium, grid side up, and starting at one edge by use of a rolling action (fig. 7.1–3E). 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 plates to direct sunlight.**
21. Close the plate by pressing the top firmly onto the bottom. Invert the plate. Incubate within 20 minutes to avoid growth of interfering microorganisms.
22. Continue to filter the other sample volumes in order, from smallest to largest volume. Record on field forms the volumes filtered and the time of processing. For USGS personnel, the microbiology section of the Personal Computer Field Form (PCFF) version 5.2.1 and above is a tool to help record and maintain analytical data and perform key calculations.

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

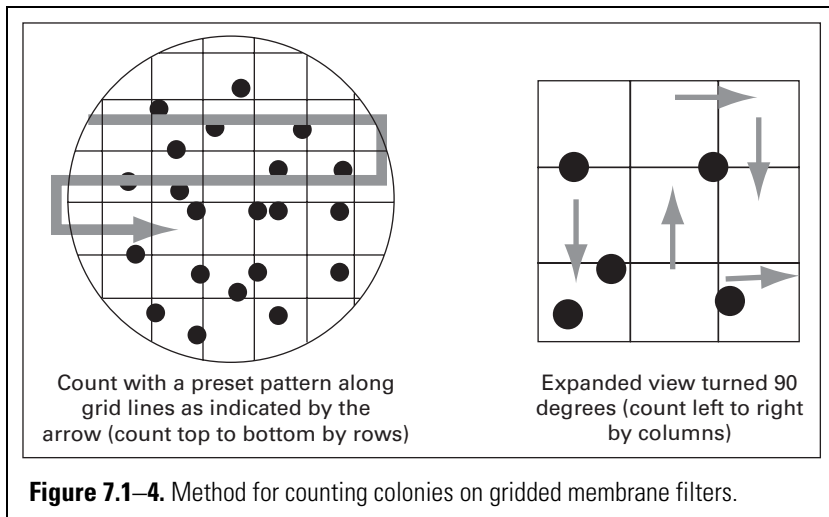
23. Place the inverted plates into a preheated aluminum heater-block or into water-tight plastic bags and then into a water-bath incubator. Incubate at the prescribed times and temperatures (table 7.1–6).
24. Wash the counter top between each sample with an antibacterial cleaning solution (see “To prepare to filter samples” at the beginning of section 7.1.3.C). Wash and sterilize the filter apparatus before the next use.

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

To count colonies and calculate results:

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

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



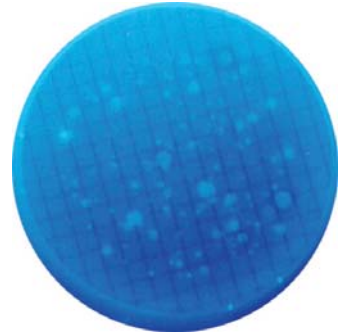
Media-specific guidance for making colony counts:

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

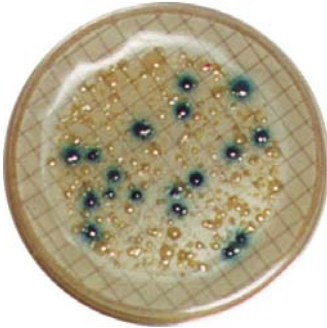
- For *E. coli* on mTEC medium, transfer the filter to a filter pad saturated with urea-phenol reagent; count only colonies that are yellow, yellow-green, or yellow-brown after 15 to 20 minutes at room temperature (U.S. Environmental Protection Agency, 2002a; fig. 7.1–5E).
 - For *E. coli* on modified mTEC medium, count colonies that are red to magenta under natural light (U.S. Environmental Protection Agency, 2002c; fig. 7.1–5F).
 - For fecal coliforms on mFC medium, count colonies that are light to dark blue, in whole or in part, under natural light (fig 7.1–5G).
 - For fecal streptococci on KF medium, count colonies, using magnification, that are glossy pink or red under natural light (fig. 7.1–5H).
 - For enterococci on mEI medium, count colonies of any color that have a blue halo under magnification with a small fluorescent lamp. (U.S. Environmental Protection Agency, 2002b; fig. 7.1–5J). Always use 2 to 5 times magnification when counting colonies on mEI agar.
 - For *C. perfringens* on mCP medium, count colonies that are straw yellow, turning dark pink to magenta under natural light when exposed to ammonium hydroxide in a laboratory fume hood (U.S. Environmental Protection Agency, 1996; fig. 7.1–5K).
2. Check quality-control blanks for colony growth, and report results on the field forms. The presence of colonies on blanks indicates that results of the bacterial analyses are suspect and should not be reported or the results should be clearly qualified. It is not valid to subtract colony counts on blanks from results calculated for samples.
 - One or more colonies on the field or filter blank indicates inadequate sterilization of either the equipment or the buffered water, or contamination during the sampling and analysis process.
 - One or more colonies on the procedure blank indicates either inadequate rinsing or contamination of the equipment or the buffered water during sample processing.
 3. Calculate the number of colonies per 100 mL of sample as described in section 7.1.4.
 4. Put all plates to be discarded into an autoclavable bag and **autoclave at 121°C for 45 minutes before discarding in the trash**. If plates cannot be autoclaved immediately, they may be held in a freezer or refrigerator for up to a week before being autoclaved. Other contaminated, disposable supplies should also be placed in autoclavable bags for autoclaving. Reusable equipment that contains contaminated sample water, including sample bottles and dilution bottles, should be autoclaved before disposing of the water.



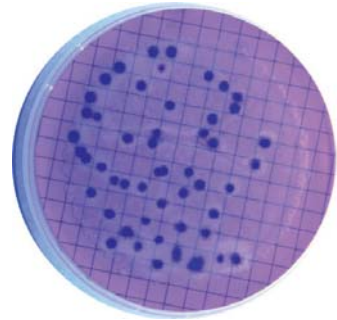
(a) Total coliform bacteria
on mENDO medium



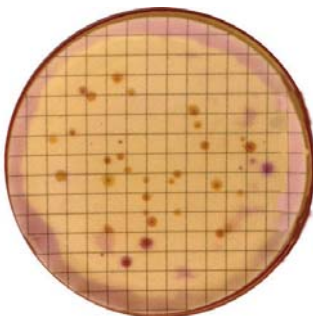
(b) Total coliform bacteria
on MI medium



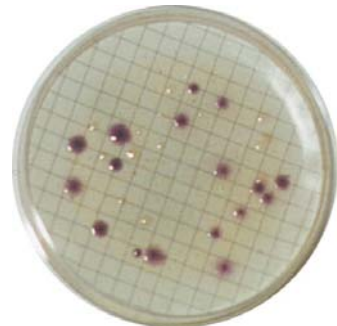
(c) *Escherichia coli* on
MI medium



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



(e) *Escherichia coli* on
mTEC medium

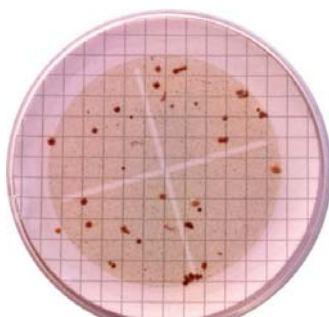


(f) *Escherichia coli* on
modified mTEC medium

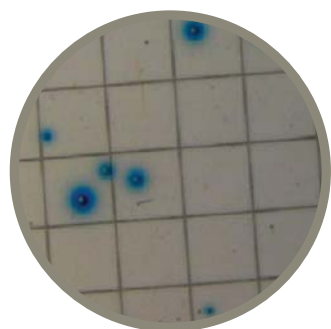
Figure 7.1–5. Photographs of typical colonies of fecal indicator bacteria on culture media.



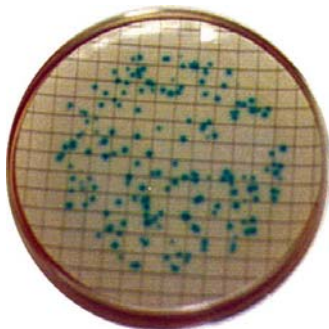
(g) Fecal coliform bacteria
on mFC medium



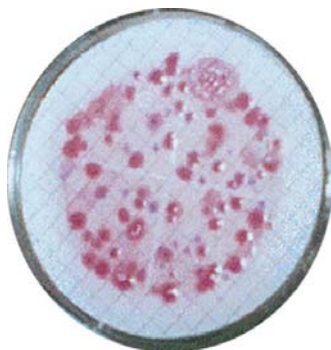
(h) Fecal streptococci
bacteria on KF medium



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



(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*

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

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

To prepare to process samples:

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

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

To process samples and read results:

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

Quality control. Verify the incubator temperature before beginning the analysis.

2. Record the site name, date, time of sample collection, and time of sample processing on the culture bottle and on the record sheet or field form.
3. Shake the sample at least 25 times.

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

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

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

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

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

To prepare to process samples:

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

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

To process samples and read results:

1. Step one for reading results updated on 5/12/2014.

Quality control. Verify the incubator temperature before beginning the analysis.

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

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

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

7.1.4 CALCULATING AND REPORTING FECAL INDICATOR BACTERIA DENSITIES

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

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

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

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

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

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

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

Case 1. Colony counts in the ideal range.

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

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

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

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

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

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

Case 1: Colony counts in the ideal range.

Example 1: Ideal colony count on one filter

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

$$\text{Density} = (21 \times 100)/10 = 210 \text{ CFU}/100 \text{ mL}$$

Example 2: Ideal colony counts on two or more filters

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

$$\text{Density} = (79 \times 100)/40 = 200 \text{ CFU}/100 \text{ mL}$$

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

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

$$\text{Density} = (50 \times 100)/0.1 = 50,000 \text{ CFU}/100 \text{ mL}$$

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

CFU, colony-forming unit

TNTC, too numerous to count

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

Example 1: Less than ideal range on all filters

<u>Sample volume</u>	<u>Colony count</u>
3	2
10	6
30	18
<u>Sum 43</u>	<u>26</u>

$$\text{Density} = (26 \times 100)/43 = 60 \text{ CFU}/100 \text{ mL}$$

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

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

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

$$\text{Density} = (100 \times 100)/13 = 770 \text{ CFU}/100 \text{ mL}$$

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

CFU, colony-forming unit

TNTC, too numerous to count

Case 3: No typical colonies on any of the filters

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

$$\text{Density} = (1 \times 100)/100 < 1 \text{ CFU}/100 \text{ mL}$$

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

CFU, colony-forming unit

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

Example 1: Only one filter has colonies

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

$$\text{Density} = (5 \times 100)/30 = 17 \text{ CFU}/100 \text{ mL}$$

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

Example 2: More than one filter has colonies

<u>Sample volume</u>	<u>Colony count</u>
3	1
10*	0
30	5
<u>Sum 43</u>	<u>6</u>

$$\text{Density} = (6 \times 100)/43 = 14 \text{ CFU}/100 \text{ mL}$$

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

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

CFU, colony-forming unit

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

<u>Sample volume</u>	<u>Colony count</u>
10	112
30	TNTC (do not use)
100	TNTC (do not use)
<u>Sum 10</u>	<u>112</u>

$$\text{Density} = (112 \times 100)/10 = 1,100 \text{ CFU}/100 \text{ mL}$$

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

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

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

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

$$\text{Density} = (80 \times 100)/10 > 800 \text{ CFU}/100 \text{ mL}$$

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

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

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

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

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

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

- ▶ To calculate CFU/g_{DW} for membrane filtration results:

$$\text{CFU/g}_{\text{DW}} = (\text{density} \times \text{dilution factor}) / (\text{proportional DW}),$$

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

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

$$\text{MPN/g}_{\text{DW}} = (\text{density} \times \text{dilution factor}) / (\text{proportional DW}),$$

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

Two scenarios are provided to illustrate these calculations:

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

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

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

Calculate proportional dry weight

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

Proportional dry weight 0.46
 $(13.6 \text{ g} - 1.86 \text{ g}) / (27.4 \text{ g} - 1.86 \text{ g})$

Calculate density in supernatant

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

Density in supernatant = $(21 \text{ CFU} / 10 \text{ mL}) \times 100 = 210 \text{ CFU}/100 \text{ mL}$

Calculate density in sediment

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

Density in sediment 48 CFU/g_{DW}
 $(210 \text{ CFU}/100 \text{ mL} \times 10.5 \text{ mL/g}) / 0.46$

CFU, colony-forming unit

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

Calculate proportional dry weight

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

$$\text{Proportional dry weight} = 0.46 \\ (13.6 \text{ g} - 1.86 \text{ g}) / (27.4 \text{ g} - 1.86 \text{ g})$$

Calculate density in supernatant

Positive large wells	46
Positive small wells	10

Density in supernatant 150 MPN/100 mL
from MPN table

Calculate density in sediment

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

$$\text{Density in sediment} = 34 \text{ MPN/g}_{\text{DW}} \\ (150 \text{ MPN/100 mL} \times 10.5 \text{ mL/g}) / (0.46)$$

MPN, most probable number

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