PROTOZOAN PATHOGENS 7.3

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

Illustrations

Tables

Notes:

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

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

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

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

PROTOZOAN PATHOGENS 7.3

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

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

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

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

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

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

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

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

7.3.1 STERILIZATION PROCEDURES FOR SAMPLING EQUIPMENT

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

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

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

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

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

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

7.3.1.A SODIUM HYPOCHLORITE STERILIZATION METHOD

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

To sterilize sampling equipment using the bleach sterilization method:

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

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

ALTERNATIVE STERILIZATION METHOD 7.3.1.B

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

Equipment Washing

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

To sterilize sampling equipment using the alternative sterilization method:

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

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

Autoclaving

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

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

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

Quality-control tests for autoclave operation are mandatory.

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

7.3.2 SAMPLE COLLECTION, PRESERVATION, TRANSPORT, AND HOLDING TIMES

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

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

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

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

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

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

7.3.2.A SAMPLE COLLECTION

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

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

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

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

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

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

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

Quality Control

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

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

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

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

Isokinetic Sampling Methods

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

1. Select the appropriate isokinetic method (NFM 4.1). The EDI method is preferred at sites where the velocity distribution across a stream section is well established or at a section where the depth varies (for example, at a gaging station) (Edwards and Glysson, 1999).

2. Select the appropriate sampler and equipment and prepare the equipment for use (section 7.3.1). **Sampling equipment must be sterile,** including the collection bottle (or bags for the bag sampler), nozzle, and cap (table 7.3-1).

   - For streams with depths of 5 meters (m) (approximately 16.4 feet) or less, use a US D-95, US DH-95, or US DH-81 sampler (NFM 2.1.1).
   - For stream sections where depths exceed 5 m (16.4 feet), use the US D-96, with either autoclavable Teflon® bags or autoclavable cooking bags. Thermotolerant polymers are described in section 7.3.1.B under "Autoclaving."
   - Use the proper nozzle size and transit rate for the velocity conditions in the section to ensure isokinetic collection of the sample (NFM 4.1.1.A).

Hand-Dip Sampling Method

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

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

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

SAMPLE PRESERVATION, TRANSPORT, AND HOLDING TIMES 7.3.2.B

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

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

To prepare for collecting a halogenated sample:

1. Prepare a 10-percent solution of $Na_2S_2O_3$ as follows:

   a. In a volumetric flask, dissolve 100 grams (g) $Na_2S_2O_3$ into 500 milliliters (mL) of DIW. Stir until dissolved. Fill flask to 1,000 mL with DIW (Bordner and Winter, 1978, p. 6; American Public Health Association and others, 1998, p. 9-19).

   b. Autoclave at 121°C for 30 minutes (U.S. Environmental Protection Agency, 1996, p. VIII–11).

   c. Store the $Na_2S_2O_3$ solution at room temperature or under refrigeration. **After 6 months, prepare a fresh solution** and label the bottle with the contents and date of preparation. **Discard unused solution that has expired, either through a certified laboratory or according to existing regulations for your locale.**

2. Before collecting the sample in the sample bottle, pipet into the sample bottle 0.5 mL of 10-percent $Na_2S_2O_3$ solution for every 1 L of sample. If the sterile $Na_2S_2O_3$ is used, then be sure to use only sterile pipets and sterile sample bottles. If the $Na_2S_2O_3$ is not sterile, then dispense the required volume of $Na_2S_2O_3$ into a sample bottle and autoclave at 121°C for 15 minutes.

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

1. Prepare the EDTA stock solution as follows:

   a. Dissolve 372 milligrams (mg) of EDTA in 1,000 mL of DIW (American Public Health Association and others, 1998, p. 9-19).

   b. Store the EDTA stock solution at room temperature.

   c. Keep the bottles tightly capped between uses. **After 6 months, prepare a fresh solution** and label the bottle with the contents and date of preparation. **Discard unused solution that has expired, either through a certified laboratory or according to existing regulations for your locale.**

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

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

To prepare the samples for transport:

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

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

7.3.3 LABORATORY METHOD: USEPA METHOD 1623

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

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

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

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

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

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

7.3.4 CALCULATION AND REPORTING OF PROTOZOAN PATHOGENS

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

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

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