Voluntary Estuary Monitoring Manual

Chapter 17: Bacteria
Indicators of Potential Pathogens

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Unit Three

Biological Measures

Bacteria: Indicators of Potential Pathogens

Submerged Aquatic Vegetation • Other Living Organisms

Chapter 17

Bacteria: Indicators of Potential Pathogens

Direct testing for pathogens is very expensive and impractical, because pathogens are rarely found in waterbodies. Instead, monitoring for pathogens uses "indicator" species—so called because their presence indicates that fecal contamination may have occurred. The four indicators most commonly used today by both volunteer and professional monitors—total coliforms, fecal coliforms, E. coli, and enterococci—are bacteria that are normally prevalent in the intestines and feces of warm-blooded animals.

Overview

"Is the water safe?" This is one of the major water quality questions every user of an estuary wants to know when preparing for a day of swimming, boating, fishing, shellfishing, or other pursuit. Whether the water is safe depends in part on the presence or absence of pathogens—viruses, bacteria, and protozoans that can cause disease. Increasingly, monitoring and regulatory emphasis are focused on the potential for pathogens that may lead to waterborne diseases. Pathogens can enter a waterbody via fecal contamination as a result of inadequately treated sewage, faulty or leaky septic systems, runoff from urban areas, boat and marina waste, combined sewer overflows, and waste from pets, farm animals, and wildlife. Human illness can result from drinking or swimming in water that contains pathogens or from eating shellfish harvested from such waters.

Direct testing for pathogens is very expensive and impractical, because pathogens are rarely found in waterbodies. Instead, monitoring for pathogens uses "indicator" species—so called because their presence indicates that fecal contamination may have occurred. The four indicators most commonly used today by both volunteer and professional monitors—total coliforms, fecal coliforms, *E. coli*, and enterococci—are bacteria that are normally prevalent in the intestines and feces of warm-blooded animals, including wildlife, farm animals, pets, and humans. The indicator bacteria themselves are not usually pathogenic.

This chapter discusses factors that should be considered when establishing a volunteer monitoring program for bacteria and reviews the major bacterial indicators and the analytical methods most commonly used to test for them. Case studies provide further examples and illustrations.

Why Monitor Bacteria?

Waterfowl are among the many non-human sources of bacteria in estuaries (photo by S. Schultz).

Shellfish beds are closed when bacteria concentrations exceed established criteria (photo by R. Ohrel).

Pathogenic microorganisms (including bacteria, viruses, and protozoans) are associated with fecal waste and can cause a variety of diseases including typhoid fever, cholera, giardiasis (a parasitic infection of the small intestine), and hepatitis, either through the consumption of contaminated shellfish or ingestion of tainted water. Since these pathogens tend to be found in very low concentrations in the water, and there are many different pathogens, it is difficult to monitor them directly. Also, pathogens are shed into the waste stream inconsistently. For these reasons, direct testing for pathogens is expensive and nearly impossible.

Instead, monitoring for pathogens uses "indicator" species whose presence in the water suggests that fecal contamination may have occurred. The four indicators most commonly used today by volunteer and professional monitors—total coliforms, fecal coliforms, *E. coli*, and enterococci—are bacteria that are normally prevalent in the intestines and feces of warm-blooded animals, including:

- wildlife (e.g., deer, geese, raccoons);
- farm animals (e.g., swine, cattle, poultry);
- pets; and
- humans.

States routinely monitor shellfish harvesting areas for fecal coliform bacteria and close them to harvesting when the bacterial count exceeds an established criterion. States may also close bathing beaches if officials find sufficiently high levels of fecal coliform bacteria. In addition to bacteria, shellfish are also monitored for hazards such as viruses, parasites, natural toxins, and chemical contaminants (e.g., pesticides, mercury, PCBs). (See the U.S. Food and Drug Administration's Web site, provided at the end of this chapter, for more information.)

States monitor heavily used beach and recreation areas as well as the water overlying shellfish beds for total and fecal coliforms, but there are limits to the coverage they can provide. Volunteers can supply valuable data to assist established programs by monitoring areas where officials are not sampling, thereby augmenting a state's network of stations. State officials can use this information to screen for areas of possible contamination. Such expanded coverage helps states make beach- and shellfish-closing decisions on a more localized basis.

Fecal coliform contamination can frequently occur in conjunction with other inorganic pollutants. Runoff from a livestock area washing into an estuary, for instance, may contain not only fecal coliforms, but high levels of nutrients as well (see Chapter 10 for more information on nutrients). By including bacterial counts as one of a suite of monitoring parameters, a program manager can design a program that provides a good characterization of the chosen sites. This sort of data collection may reveal problem areas that were not previously recognized.

Volunteers can also perform fecal coliform monitoring with an eye toward regulatory compliance. For example, the program may establish monitoring sites near known or suspected bacterial discharges. Monitoring sites can be set up adjacent to the discharge, but the effluent itself can also be sampled. Program managers should be aware of the legal issues affecting this type of sampling, such as trespass laws and the violation of privacy and property rights.

Why do volunteer groups decide to do bacteria testing themselves? The first and foremost reason is that volunteers are concerned about their watershed and want the opportunity for more community involvement and ownership of the data. Cost is also a factor; unless you find a lab that will donate the analysis, charges run $10-$35 per sample, whereas some volunteer monitoring groups spend approximately $2 for each sample they process themselves.

The Role of Bacteria in the Estuarine Ecosystem

Bacteria are microscopic single-celled organisms that function as decomposers in an estuary, breaking down plant and animal remains. This activity releases nutrients previously locked up in the organic matter into the estuarine food web.

Bacteria live in water, on the surface of water, in the bottom (benthic) sediments, on detritus (dead organic material), and in and on the bodies of plants and animals. They exhibit round, spiral, rod-like, or filamentous shapes (Figure 17-1). Some bacterial organisms are mobile and many congregate into colonies. In the estuary, bacteria are often found densely packed on suspended particulate matter.

Bacteria serve as food for other organisms; they are also involved in many chemical reactions within the water. For example, certain bacteria convert ammonia to nitrite. Another species converts nitrite to nitrate. These nutrients are used by plants. Some bacteria exist only under **aerobic** (oxygenated) conditions; others live in **anaerobic** (no oxygen) environments. Some versatile bacteria can function under either condition.

Bacterial Contamination

While bacteria normally inhabit estuaries as an integral part of the food web, human activities may introduce pathogenic (disease-causing) bacteria to the system. Of greatest concern to public health is the introduction of fecal waste from humans or warm-blooded animals. Sources of fecal bacterial contamination include faulty wastewater treatment plants, livestock congregation areas, sanitary landfills, inefficient septic systems, fecal waste from pets, stormwater runoff, boat and marina waste, sewage sludge, and untreated sewage discharge. Wildlife also add bacteria to waterways, and can be the dominant source of fecal coliform bacteria in some areas (Figure 17-2). ■
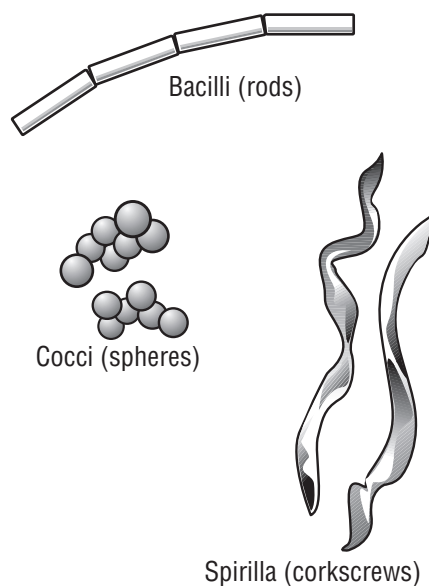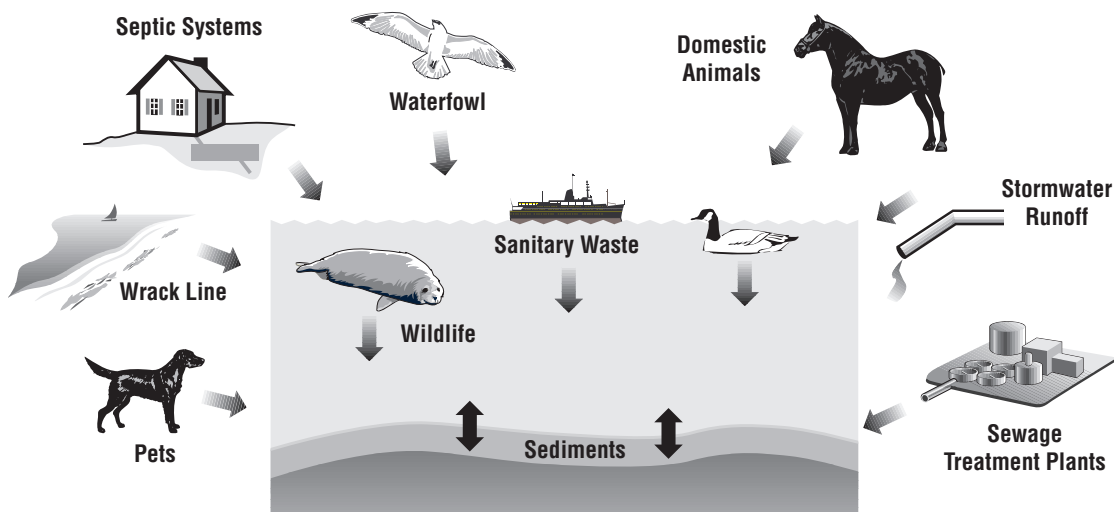

Bacilli (rods)

Cocci (spheres)

Spirilla (corkscrews)

Figure 17-1. Three shapes assumed by bacteria.

Figure 17-2. Potential sources of bacteria in an estuary *(redrawn from Ely, 1997).*

Septic Systems

Waterfowl

Domestic Animals

Sanitary Waste

Stormwater Runoff

Wrack Line

Wildlife

Pets

Sediments

Sewage Treatment Plants

BACTERIA SOURCE TRACKING

Part of interpreting fecal coliform data involves trying to understand the sources of bacteria in the estuary. If your monitoring indicates high counts of bacteria, the next step is to examine the possible sources. To begin "bacteria source tracking," volunteers should note the number of wildfowl in the area and observe the scat (excrement) of animals along the beach or shore. To establish if wild animals are large contributors of bacteria, compare bacteria counts in an area with few signs of wildlife with an area heavily populated with birds and other animals.

Also investigate whether parts of the watershed have residential areas where dog droppings can be readily found. It is recommended that monitoring programs work with local agencies to research possible sources. It is important to look at all the possible sources of bacteria (see Figure 17-2 for examples), rather than immediately assume that faulty sewage treatment or failing septic systems are the only culprits.

In addition to careful observation of possible sources and comparing bacteria counts in different apparent situations, there are other more complex methods used by laboratories to track bacteria sources. One method uses the fact that some bacteria in humans and domesticated animals have developed resistance to antibiotics. Colonies of bacteria are exposed to various antibiotics to help determine if the source of the bacteria is human, domesticated animals, or wildlife. Other methods, carried out in a few universities and laboratories, involve the analysis of bacterial DNA.

The Bacterial Indicators

In this section, the four main indicator bacteria are discussed. But before we can understand these indicators, we need to understand the criteria that were used to select them as indicators. To be an ideal assessor of fecal contamination, an indicator organism should meet as many of the following criteria as possible:

- The organism should be present whenever enteric (intestinal) pathogens are present.

- The organism should be useful for all types of water.

- The organism should have a longer survival time than the hardiest enteric pathogen.

- The organism should not grow in water.

- The organism should be found in warm-blooded animals' intestines.

- The testing method should be easy to perform.

- The density of the indicator organism should have some direct relationship to the degree of fecal pollution (Gerba, 2000).

Total Coliforms and Fecal Coliforms

Coliform bacteria live in the lower intestines of warm-blooded animals and may constitute as much as 50 percent of fecal waste. Although coliform bacteria are not usually pathogenic themselves, their presence indicates sewage contamination, perhaps accompanied by disease-causing pathogens.

Public health agencies have used total coliforms and fecal coliforms as indicators since the 1920s. **Total coliforms** are a group of closely related bacterial genera that all share a useful diagnostic feature: the ability to

metabolize (ferment) the sugar lactose, producing both acid and gas as byproducts. There are many selective growth media available that take advantage of these metabolic characteristics in traditional testing protocols.

Total coliforms are not very useful for testing recreational or shellfishing waters. Some species in this group are naturally found in plant material or soil, so their presence doesn't necessarily indicate fecal contamination. Total coliforms are useful, however, for testing treated drinking water where contamination by soil or plant material would be a concern.

A more fecal-specific indicator is the fecal coliform group, which is a subgroup of the total coliform bacteria. **Fecal coliforms** are widely used to test recreational waters and are approved as an indicator by the U.S. Food and Drug Administration's National Shellfish Sanitation Program (NSSP) for classifying shellfishing waters. However, even this group includes some species that can have a nonfecal origin (e.g., *Klebsiella pneumoniae*, which grows well in paper pulp and is sometimes found in high concentration near paper mills). Studies have found that all members of the coliform group can regrow in natural surface water depending on the water temperature and the amount of organic matter in it (Gleeson and Gray, 1997). Some warm tropical waters have sufficient organic matter for the bacteria to increase in numbers. The effluents from pulp mills, paper mills, and wastewater treatment plants may, in some cases, also provide conditions under which coliform bacteria can grow.

Even though fecal coliform bacteria have some deficiencies when it comes to being a "perfect" indicator, they are generally considered the best available indicators of contamination at the present time. Many citizen programs and state agencies use fecal coliform testing to assess potential bacterial contamination in an estuary.

One major question often asked about fecal coliforms and estuaries is: "How long do fecal coliform bacteria persist in an estuary?" The answer may vary, depending on where the bacteria are located in the estuary. For example, bacteria may survive for weeks in the sediment

or in fecal pellets from wildfowl that have sunk to the bottom. During a storm or other event that disturbs the sediment, fecal coliform bacteria can become reintroduced to the water column. Fecal matter also collects in the line of seaweed and organic material (called **wrack**) that can be seen when the high tide goes out. Birds and other animals forage for food and defecate in this wrack line. When the wrack line enters the water during high tide or a storm, the fecal material and associated bacteria also enter the water.

After incubation, any fecal coliform bacteria in the water sample will have grown into a colony (when using mFC medium or broth). These are called colony forming units (cfu) (photo by University of Maine Cooperative Extension).

Escherichia Coli and Enterococci

Other commonly used indicator bacteria are *Escherichia coli*, a single species within the fecal coliforms group, and **enterococci**, another group of bacteria found primarily in the intestinal tract of warm-blooded animals. Enterococci are unrelated to the coliforms; instead, they are a subgroup of the fecal streptococci group.

The method approved by the U.S. Environmental Protection Agency (EPA) for enterococci testing requires the use of an expensive growth medium that contains a toxic ingredient. Volunteer programs interested in monitoring for enterococci bacteria could partner with a university or lab to conduct these tests.

Other Bacteria as Indicators

In addition to the four main indicators discussed above, there are other bacteria that can also serve useful indicators of contamination. These include *Aeromonas hydrophila* (a noncoliform), which can be tested using the membrane filtration method described later in this chapter. One medium, ECA Check (made by Micrology Laboratories), identifies and quantifies *Aeromonas* as well as *E. coli* and total coliforms. Consult with suppliers for availability of medium (see Appendix C).

How Effective Are the Indicators?

Total coliforms, fecal coliforms, *E. coli*, and enterococci are easy to grow in a lab, and all will be present in large numbers if recent fecal contamination has occurred. Unfortunately, one problem with the indicators is the question of source. All the indicators can come from animals and some can also come from plants or soil. Another problem is that none of the indicators accurately reflect the potential for human health effects, though some do a better job than others. Because of these and other complications, microbiologists are still looking for better indicators. In the meantime, volunteer monitors and public health agencies alike must do their best with the presently available indicators.

In 1986, EPA issued a revision to its bacteriological ambient water quality criteria recommendations to include *E. coli* and enterococci, as they provide better correlations with swimming-associated gastrointestinal illness than fecal coliforms. As an indicator, *E. coli* has a major advantage over the fecal coliforms: it is more fecal-specific (*E. coli* occurs only in the feces of warm-blooded mammals).

Why Fecal Coliforms Are the Indicator of Choice

Even though EPA recommends enterococci or *E. coli* for testing recreational waters, many states still use fecal coliforms. This is partly for the sake of continuity, so that new data can be directly compared with historical data. Another reason fecal coliforms are the indicator of choice for many states and volunteer monitoring programs is due to economics: the EPA-approved method for testing enterococci can be more expensive than the fecal coliform test. ∎

Bacterial Sampling and Equipment Considerations

Chapter 6 summarized several factors that should be considered when determining monitoring sites, where to monitor, and when to monitor. In addition to the considerations in Chapter 6, a few additional ones specific to monitoring bacteria are presented here.

Due to the costs and training associated with analyzing water samples for bacterial contamination, programs just starting up or those without adequate lab facilities should strongly consider allowing a professional, university, or other lab facility to run the bacterial analyses. Often these labs will run samples free of charge or at a reduced rate for volunteer monitoring programs.

Where to Sample

The selection of bacterial monitoring sites depends on the ultimate purpose of the data. If the data are to supplement state efforts, for example, the program should choose sites based on gaps in the state's array of monitoring stations. Areas suspected of contamination that are not routinely monitored by state officials should receive the highest priority.

If data will serve as regulatory compliance documentation, sites should cluster near dischargers believed to be in noncompliance. State health or water quality agencies can provide information on where additional data

are needed. Government managers are more likely to use the data if volunteers monitor more than one site near discharge sources.

To better understand bacterial contamination in a particular estuary, it is necessary to establish the relationship between flow into the estuary and the extent of bacterial contamination. Choose sample sites above and below the area of suspected contamination, at the effluent's entry into the estuary, and even the discharge itself to obtain a scientifically valid set of data (Figure 17-3). Bacterial data collected by volunteers can help assess the relationship between bacterial density and estuarine conditions, and help identify bacterial sources.

As previously mentioned, bacteria may survive for weeks in the sediment, or in fecal pellets which have sunk to the bottom. Bacteria in sediment can be tested by stirring up the sediment before collecting a water sample. To facilitate data analysis, volunteers should be careful to identify samples that contain sediment.

When to Sample

Volunteers should monitor bacteria on a weekly, biweekly, or monthly basis. In addition, it may be extremely helpful to monitor during or immediately after storm events. It is important to create a monitoring schedule that is sustainable. Set reasonable goals for the frequency of monitoring given your program's number of volunteers and financial resources. In areas where volunteers sample primarily to assess the health risks in seasonal areas, such as bathing beaches, monitoring can cease or be conducted much less frequently during cold-weather months. Sampling to determine possible contamination of shellfish beds, however, should continue on a regular basis throughout the harvesting season. ■
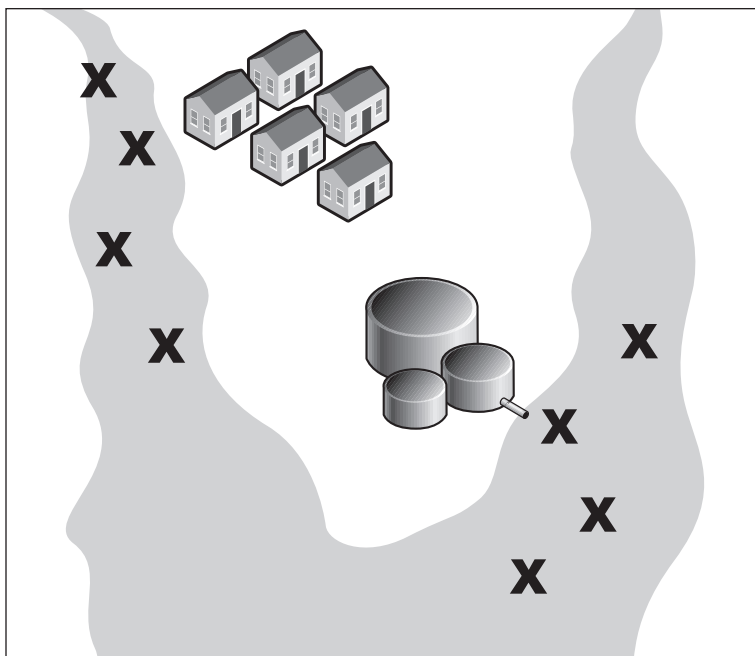

Figure 17-3. Sites to monitor for bacterial contamination.

Reminder!

To ensure consistently high quality data, appropriate quality control measures are necessary. As discussed in Chapter 5, it is very important for volunteers to carefully follow established protocols so that the resulting data are of the highest quality. With bacteria testing, two quality assurance/quality control procedures are especially critical. First, the bacteria monitoring program should require periodic split samples, in which one sample is divided equally into two or more sample containers and then analyzed by different analysts or labs. Careful handling of the water sample is also critical. Some programs have chain-of-custody forms to identify the responsible person at every step of the process. While most volunteer programs don't require these forms, the chain-of-custody can become important if the data will be used in cases where legal or corrective actions need to be taken.

In the Field:
Collecting Water Samples for Bacterial Analysis

A volunteer with the Friends of the Estuary/Morro Bay NEP Volunteer Monitoring Program collects a sample in a plastic bottle for bacteria testing (photo by E. Ely).

Some citizen monitoring programs use volunteers to conduct the lab analysis of fecal coliform bacteria, and others use volunteers to collect the water samples, leaving the responsibility of sample analysis to a professional lab. In either case, the procedure for collecting the water samples requires strict adherence to quality assurance and quality control guidelines. Analysis of the sample should be done within six hours of the time when the sample was collected.

Before proceeding to the monitoring site and collecting samples, volunteers should review the topics addressed in Chapter 7. It is critical to confirm the monitoring site, date, and time; have the necessary monitoring equipment and personal gear; and understand all safety considerations. Once at the monitoring site, volunteers should record general site observations, as discussed in Chapter 7. In particular, they should keep alert for signs of bacteria sources (e.g., wildfowl or other wildlife, pets, nearby residences, foul smells, etc.).

STEP 1: *Check equipment.*

In addition to the standard sampling equipment and apparel listed in Chapter 7, the volunteer should bring an ice cooler (with ice packs to keep samples cool) and sterilized wide-mouth sample bottles (over 150 ml) or Whirl-pak bags.

Sampling Hint:

If using a boat to reach the sampling location, make sure that it is securely anchored. It is critical not to bring up the anchor until the sampling is completed, since mud (with associated bacteria) may become stirred into the water.

STEP 2: *Collect the sample.*

Strict adherence to protocol guidelines is critical in sampling for bacteria. Contamination from any outside source will skew the results and invalidate the data.

Volunteers must take several precautions to ensure good samples: stay clear of algal blooms, surface debris, oil slicks, and congregations of waterfowl; avoid agitating the bottom sediments; and do not allow the boat propeller to stir up the water. Wear gloves when collecting water samples.

Plastic Bottles or Whirl-pak Bags?

For collecting water samples, both plastic bottles and Whirl-pak bags meet the basic criteria of being both sterile and nontoxic. The pre-sterilized, disposable Whirl-pak bags are convenient, but plastic bottles can be washed and reused practically indefinitely, making them cheaper in the long run. In addition, the bottles are easier to work with because they stand up on a benchtop. However, they need to be sterilized in an **autoclave**, and this procedure may require the assistance of a certified lab. Volunteers should ensure that the bottles they purchase are autoclavable—some plastics are not.

(Excerpted and adapted from Miceli, 1998.)

Check to see if a current or tide is running by examining the movement of water or surface debris. If it is running, sample on the upstream side of the boat or pier.

Sampling Hint: Protect Yourself!

Volunteers should take particular care in collecting samples, especially near wastewater discharge pipes, as the effluent may contain highly pathogenic organisms. Avoid splashing water, wash hands thoroughly after water contact, and minimize the breathing of water vapor. Most importantly, all volunteers should wear gloves and protective eyeglasses or goggles.

If using a bottle

- Using a waterproof pen, label the bottle with site name, date, time, data collector, and analysis to be performed.

- Making sure to wear gloves, plunge the bottle into the water upside-down.

- Open the sample bottle below the water surface, keeping hands off the bottle mouth and the inside of the cap. Hold the lid; do not set it down as it may become contaminated.

- Reach down into the water as far as possible (at least 12-18 inches), still holding the bottle with its mouth down. Make sure you keep the bottle above the bottom so as not to disturb the sediment. In a single motion, rotate the bottle mouth so that is it facing up, and sweep the bottle up and out of the water. Make sure that the sweeping motion continues until the bottle is fully out of the water.

- Pour out enough water to leave about 1 inch of air space in the bottle so that the lab technician can shake the sample prior to analysis.

- Replace the lid, again making sure not to touch the inside of the cap or bottle rim.

- Place the bottle in the cooler. Transport samples back to the lab in a cooler regulated to between 1°- 4°C. Do not allow water that may have accumulated in the cooler from melting ice to

submerge the bottles. To prevent this problem, use ice cubes packed in plastic bags, water frozen in plastic jars, or sealed ice packs.

If using a Whirl-pak bag

- Using a waterproof pen, write the following on the outside of the Whirl-pak bag: site name, date, time, data collector, and analysis to be performed.

- Tear off the perforated top of the bag.

- Making sure to wear gloves, pinch the white tabs on the top of the Whirl-pak between your fingers, and place the bag into the water.

- Open the bag below the water surface, keeping hands away from the inside of the bag.

- Fill the bag about two-thirds full, and remove from the water.

- Leave an inch or so of air space in the bag. Hold the plastic-coated wire tabs at the top of the bag with both hands, and "whirl" the bag quickly around and around in circles. This will cause the top of the bag to fold over on itself several times.

- Seal the bag by pinching the plastic-coated wire tabs together and twisting them. The bag should not leak.

- Place the bag in the cooler. Transport samples back to the lab in a cooler regulated to between 1°- 4°C. Do not allow water that may have accumulated in the cooler from melting ice to submerge the bags. To prevent this problem, use ice cubes packed in plastic bags, water frozen in plastic jars, or sealed ice packs.

A measured amount of a water sample is being removed from a Whirl-pak bag prior to testing for the presence of bacteria. The sample has been kept cold since it was collected 3 hours before. Note that the volunteer is wearing gloves (photo by K. Register).

STEP 3: *Check data sheets, and send off the sample for analysis.*

Volunteers should make sure the samples remain at the optimal temperature, adding additional ice if necessary. Recheck the data sheets for accuracy and account for all samples. Transport the samples to the designated lab. Processing of the samples should start within six hours of sample collection. Ensure that the data survey forms are complete and legible. Send the forms to the appropriate person or agency. As with all data sheets, the volunteer should make a copy in case the original becomes lost. ■

In the Lab: Analytical Methods

Membrane filtration equipment. Clockwise from left: membrane filtration apparatus; hand vacuum pump (syringe and tubing); petri plate with absorbent pad; membrane filter (photo by M. Redpath).

When testing for the presence of bacteria, laboratories generally use one of two analysis procedures: **membrane filtration (MF)** or **most probable number (MPN)**. Volunteer monitoring groups generally use the MF procedure, but may also use **presence-absence tests** or one of the simplified test methods described below. Any procedure can be used for any of the indicator bacteria, simply by varying such factors as growth media and incubation temperature. Read the summaries of each analysis procedure before deciding which is appropriate for your bacterial monitoring program.

Membrane Filtration (MF): The Classic Method for Bacteria Testing

Membrane filtration for fecal coliforms is the method most widely used by volunteer groups, who select this method because it is EPA-approved, it conforms to what many state labs use, and it is a long-established, well-recognized method. For programs that monitor shell-fishing waters, MF for fecal coliforms represents a practical way to approximate the methods used by their state shellfishing lab. State shellfish labs, in accordance with NSSP mandate, use the MPN method for fecal coliforms; volunteer groups tend to use the same indicator (fecal coliforms) but not the MPN method.

Since bacteria are too tiny to count individually, MF relies on an incubation step, followed by a count of the resultant bacteria colonies. A known volume of sample water is pulled through a filter with suction from a vacuum pump. Bacteria are collected on the top of the

What Levels Are Significant?

Interpreting bacterial data can be tricky. There is a great deal of variability in the test procedure as well as in the environment, so a firm conclusion cannot be drawn based on just one sample.

Waterbodies almost always contain some level of fecal coliform bacteria; therefore, it is strongly recommended that volunteer groups do routine monitoring in dry weather so that they can know the baseline conditions for their specific sampling sites. Take samples during different weather conditions and, if possible, collect data during rain events. Consult your appropriate state agency to learn your state's standards for bacteria in surface waters.

filter, which is then placed in a petri dish on top of either solid mFC medium or an absorbent pad soaked with mFC broth.

The petri dishes are inverted and incubated for 24 hours (plus or minus 2 hours) at 44.5°C (Hach, 1997). The incubation temperature is the crux of the membrane filtration with mFC method, since the ability to grow and ferment lactose at 44.5°C is the key distinguishing feature of the fecal coliforms group. To obtain accurate counts, the temperature must be held absolutely steady (within 0.2°C): a bit too warm, and the fecal coliforms can't grow; a bit too cool, and nonfecal bacteria start growing. A good-quality waterbath incubator, while not a cheap piece of equipment, is the least expensive incubator that can provide sufficient results. Air incubators capable of maintaining the required temperature are even more expensive. Some volunteer programs have tried building their own waterbath incubators, with mixed success. Another option is to purchase a reconditioned waterbath incubator. Check the Yellow Pages or ask local laboratories to recommend companies that specialize in used and reconditioned equipment.

After incubation, it is necessary to count the number of blue-colored fecal coliform colonies. A 10- to 15-power microscope or illuminated magnifier is needed to count the colonies. Each colony has grown from a single bacterial cell, so by counting the colonies you can obtain a count of the bacteria present in the water sample. Results are reported as colony forming units (cfu)/100 ml, using the following formula:

cfu/100 ml = (coliform colonies counted x 100)/ (ml sample filtered)

In addition to using mFC medium to investigate the possible presence of fecal coliforms, the membrane filtration method can be used with other media to analyze other indicator bacteria. The medium used depends on which indicator you are looking for. Some media contain ingredients that give the target organisms a distinctive appearance, such as a color. Other media require incubation at very specific temperatures. The amount of time of incubation also varies according to the medium used.

Some volunteer monitoring groups use membrane filtration with mTEC agar, a method that provides counts for both fecal coliforms and *E. coli*. However, this procedure is extra-challenging. In addition to all the steps described above for fecal coliforms, this procedure requires the plates to be incubated at two temperatures (first 35°C and then 44.5°C), and then a special reagent is used to distinguish the *E. coli* colonies from the other fecal coliforms.

Equipment Requirements for Membrane Filtration

Unquestionably, equipment requirements present the biggest hurdle to volunteer groups who want to use an EPA-approved method. The two approved methods volunteers use—membrane filtration with mFC or with mTEC—both require an incubator, an autoclave (for sterilizing equipment), and a membrane filtration apparatus. On the other hand, once the initial investment is made, routine testing by these methods is inexpensive. Many volunteer programs arrange to use high school or university laboratories to sterilize equipment, prepare media, incubate plates, and dispose of wastes. Others set up the equipment at a central program lab.

Most Probable Number (MPN)

The traditional "most probable number" (MPN) technique (using test tubes) may not be practical for volunteer groups because it is labor-intensive, takes up significant incubator space, and requires up to four days for a final result. However, it is important for volunteer estuary monitoring groups to be aware of this method because MPN for fecal coliforms is the only method that is NSSP-approved for classifying shellfish-growing waters.

Unlike membrane filtration, which gives you a plate of colonies to count, MPN does not yield a direct count of bacteria. Instead, the water sample is added to a series of tubes that contain a liquid medium. After incubation, each tube shows either a positive or negative reaction for the target organism. In the case of fecal coliforms, for example, a positive tube is one

that shows growth and gas in lactose broth medium. A second step is required to "confirm" the positive tubes. The number of confirmed positives corresponds to a statistical probability that the sample contained a certain number—the "most probable number"—of bacteria. The accuracy of the MPN method can be increased by inoculating more tubes and by using several dilutions of the water sample.

Comparing Membrane Filtration and MPN

Professional labs mainly use the MF method of analyses, although some use the MPN method. The MF technique is good for large numbers of samples and produces results more rapidly. It should be noted that highly turbid water or water with high counts of noncoliform bacteria can limit the utility of the MF procedure. If a water sample is very turbid, the filter in the MF procedure can become clogged by sediment, algae, etc.

Presence-Absence Tests

Volunteers using membrane filtration equipment. The person on the right is using a hand-operated vacuum pump to pull rinse water through the membrane filter (photo by E. Ely).

Presence-absence (P-A) tests are the easiest method for answering the simple question of whether the target bacteria are present in the water sample. Many volunteer monitoring programs use P-A tests to determine if more extensive testing is needed. The P-A test procedure requires that a bacterial growth medium (selected based on the bacteria indicator you are interested in monitoring) be added to a water sample in a sterile, transparent test tube. The test tube is capped, and the contents are shaken until the medium is dissolved or totally mixed. The sample is then incubated for the prescribed length of time at the required temperature. After

incubation, reading the results usually requires comparing the color of the sample to a standard.

For example, if using the Colilert reagent (see below) in your P-A test because you are interested in monitoring total fecal coliforms and *E. coli*, you will check the color of the sample after incubation. A yellow color confirms the presence of total coliforms. If yellow is observed, the next step is to check the sample for fluorescence by placing an ultraviolet (UV) light within five inches of the test tube. If the sample's fluorescence is greater or equal to the fluorescence of the standard, the presence of *E. coli* is confirmed. Several companies sell P-A test kits; be sure to carefully read and follow all directions before using them.

Special Note About Disposing of Bacteria Cultures:

After counting the colonies that have grown in petri dishes, you will need to safely destroy the bacteria cultures. Here are two methods:

Autoclave

Place all petri dishes in a container in an autoclave. Heat for 15 to 18 minutes at 121°C and at a pressure of 15 pounds per square inch. Throw away the petri dishes.

Bleach

Disinfection with bleach should be done in a well-ventilated area, since it can react with organic matter to produce toxic and irritating fumes. Pour a 10-25 percent bleach solution into each petri dish. Let the petri dishes stand overnight. Place all petri dishes in a sealed plastic bag and throw away.

Simplified Testing Methods

Because traditional laboratory methods are complex and can be expensive, several volunteer monitoring groups have started using simplified methods to test for total coliforms, *E. coli*, and enterococci. The

products and procedures outlined below are alternatives to the approved methods and, in some cases, can have simpler equipment requirements. New bacteria monitoring products are introduced often, so check with scientific supply houses for new options (see Appendix C).

With these simplified methods, there are a couple of important caveats to keep in mind:

- These methods are not EPA-approved for recreational waters (although Colilert is approved for drinking water) and thus are appropriate for screening only.

- None of the quick methods provides a fecal coliforms count. They only assess total coliforms, *E. coli*, or enterococci. This may be problematic for volunteer groups whose data users utilize or require fecal coliform indicators.

The big advantage of these simplified methods is that they make it possible for individual volunteer monitors to perform the tests in their own homes. Incubation is at 35°C or even at room temperature. Some of the popular simplified methods use the products listed below. See Appendix C for addresses of suppliers.

Coliscan Easygel and Coliscan-MF Membrane Filtration

Coliscan (from Micrology Labs—see Appendix C) is a product used by many volunteer monitoring programs to monitor for total coliform and *E. coli*. Coliscan comes in two pre-packaged kits: Coliscan Easygel (which is used in a plate-count method) and Coliscan-MF (which uses membrane filtration).

Both Coliscan products make use of a patented medium on which total coliform colonies other than *E. coli* appear pink and *E. coli* colonies appear purplish blue. With the Coliscan-MF Membrane Filtration Kit, water samples are processed by the membrane filtration technique and the filter is placed on the special Coliscan medium.

Coliscan Easygel is a very easy pour-plate method. It is self-contained and relatively inexpensive. You simply add the water sample (unfiltered) directly to a bottle of liquid Coliscan medium, mix it, and pour it into a special petri plate which is coated with a substance that causes the medium to gel. Easygel is appropriate only for counts higher than about 20 colony forming units per 100 milliliters (20 cfu/100 ml), since there is no filtration step to concentrate the bacteria and the maximum sample water volume is 5 ml.

For both Coliscan-MF and Coliscan Easygel, the manufacturer recommends an incubation temperature of 35°C, but says that plates can also be incubated at room temperature (though growth will be slower). However, room temperature can vary with season or even day to day, making it difficult to compare results obtained at different times. Using an incubator ensures a consistent temperature.

After incubation, colonies that have formed in the petri dish are counted. Some users have found colony counting somewhat tricky with the Easygel plate because many colonies are embedded in the agar (since it is a pour plate). Nevertheless, Easygel can be an effective screening tool.

Colilert, Colilert-18, and Enterolert

Some health care agencies, pollution dischargers, and volunteer monitoring groups have adopted the use of Colilert and Enterolert test kits (all made by Idexx Laboratory—see Appendix C) as alternative methods for detecting and enumerating total coliforms, *E. coli*, and enterococci. Colilert and Colilert-18 are the media used in MPN tests to determine if total coliforms and *E. coli* are present in the water sample. **Colilert is not intended for marine waters**, but

After the water sample is pulled through the membrance filter (using a vacuum), the glassware above the filter is rinsed so all bacteria present in the sample will accumulate on the filter. In this laboratory, the membrane filtration process occurs under a hood for added quality control (photo by K. Register).

Colilert-18 is. These kits use either multiple tubes or multiple wells, with an MPN approach, to detect the presence or absence of total coliforms and *E. coli*. As with the classic MPN method, the more tubes inoculated, the more sensitive the count. Five tubes are enough for a rough screen.

Results are read after 18 hours for Colilert-18 and after 24 hours for Colilert. Incubation is required at 35°C (plus or minus 0.5°C). With Colilert, the detection of total coliforms is based upon a color change and *E. coli* is detected when the sample fluoresces under UV light. This modified MPN test provides more information about the amount of bacteria in the water than a presence-absence test, but not as much information as an MF or MPN test.

Enterolert is used to detect enterococci in a water sample using MF, MPN, P-A, or the modified MPN procedure discussed above. Incubation is 24 hours at 41°C (plus or minus 0.5°C). ■

Case Study: Bacteria Monitoring in California

In California, several chapters of Surfrider Foundation (a nonprofit environmental organization dedicated to the protection of the world's waves, oceans, and beaches) use Colilert to monitor the surf zone. Surfrider volunteers carry out the tests in their homes or local school laboratories, using relatively inexpensive incubators. Supplies for each sample cost about $5.

Surfrider volunteers publish their results in local newspapers and present them at public meetings. Their efforts are helping to raise awareness about bacteria and nonpoint source pollution.

(Excerpted from Ely, 1998.)

Which Method and Which Medium Should You Use?

In deciding what method to use, a number of questions must be considered. Some of them are:

- How do you hope to use your data?
- Will you be testing the freshwater or saltwater portion of the estuary?
- Will you be testing water where shellfish are harvested?
- What methods does your state lab currently use?
- Do you have access to laboratory facilities?
- What kind of equipment can you afford?
- Which bacteria are used as indicators by your state?

If your budget allows, select your bacterial indicator and analysis method based on the intended use of your data. If the primary objective of the volunteer monitoring program is to evaluate water for compliance with state water quality standards, the program should use the same or similar method used by state labs. The program should keep apprised of any changes in state requirements.

On the other hand, groups that are primarily interested in raising community awareness and/or screening for high counts may find that a simpler, non-approved method is adequate for their needs. ■

Case Study: Bacteria Monitoring in Maine

The Clean Water Program of the University of Maine Cooperative Extension was established in 1988. It provides organizational and technical support to 18 citizen water quality monitoring groups (approximately 600 volunteers). The Clean Water Program works in collaboration with the Maine State Planning Office Partners in Monitoring Program, the Maine Department of Marine Resources, and the Maine Department of Environmental Protection to form the umbrella program known as the Maine Shore Stewards Program. Water quality groups study the health of estuarine water by monitoring for dissolved oxygen, temperature, pH, salinity, and fecal coliform bacteria.

Students participating in Maine's Shore Stewards Program run estuary samples for fecal coliform bacteria using the membrane filtration method (photo by University of Maine Cooperative Extension).

The primary objective of the program is to assist in determining bacterial pollution sources and to work with local and state officials to remediate those sources (Figure 17-4). The program focuses at the local community level. Labs for fecal coliform bacteria analysis are set up in local high schools or community group locations.

Water Quality Samples 1999
Fecal Coliform Bacteria
Boothbay Harbor Lab

Shore Stewards Volunteers
55%
(5027 samples)

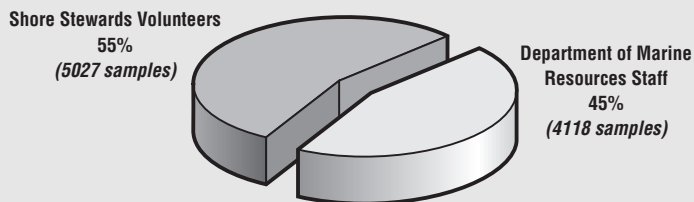Department of Marine Resources Staff
45%
(4118 samples)

Figure 17-4. Volunteers participating in Maine's Shore Stewards Program are instrumental in supplementing state agency-collected fecal coliform data. Their efforts have helped identify the causes of many shellfish bed closures *(reprinted from Maine Department of Marine Resources).*

Through their monitoring efforts, citizen groups have discovered many bacterial sources causing shellfish bed closures, including unregulated septic storage and failing septic systems. Working with local officials and state agencies, the groups helped remedy the problems and reopen the beds. Due in large part to these monitoring efforts, 100,000 acres of clam flats in Maine have been reopened in the past five years.

Other objectives of the program are to monitor coastal swimming areas and provide baseline data. Recently, a coastal community with a failing septic system used volunteer data to determine when bacteria levels were safe for swimming. In addition, volunteer data has identified recreational boats as major bacterial sources in many communities during the summer.

The Maine Shore Stewards Program has built on the strengths of communities by providing them with water quality and marine resources education, and by assisting them with their work on environmental issues. Partly from their program participation, many high school students have been inspired to go on to study environmental science in universities and to become involved in community conservation efforts. Watershed communities have begun working together to resolve water quality problems, and hundreds of citizens have become active in environmental education and conservation efforts.

For More Information:

Maine Shore Stewards
University of Maine Cooperative Extension
235 Jefferson Street
P.O. Box 309
Waldoboro, ME 04572
Phone: 207-832-0343
Fax: 207-832-0377
http://www.ume.maine.edu/ssteward

Bacteria Testing Q & A

Bacteria testing is a very important—and demanding—part of many monitoring programs. Here are some helpful answers to common questions that may arise (excerpted from Miceli, 1998).

What does it mean when I get a high bacteria count?

The first action to take is to return to the same location and get more samples. If some or all of these sample results are high, too, then you should follow your organization's procedures—for example, calling your state agency to notify them.

A little detective work plays a big role in determining where contamination is coming from and whether it is of human origin. Always make observations—the presence of animals and birds, abundant leaf matter, any strange debris, any unusual smells, etc. Also note weather conditions since results can vary tremendously if it is raining.

Remember, too, that variability and unusual test results will occur and that a high level of fecal coliforms is not abnormal, especially since wildlife frequent estuaries. A long-term monitoring effort will provide baseline information about a sampling site and will enable you to quickly recognize any unusual results.

What exactly am I looking at and counting anyway?

A single bacterium in the water sample that is caught on the filter, if able to grow on the medium, can reproduce at a fast rate. Some bacteria multiply every 20 minutes, so after 24 hours, when you retrieve your plates, you are looking at a clump of about a million bacteria—visible to the naked eye!

I am using the membrane filtration method. Why do I see . . .

(a) a big blob of growth on only one spot on the filter?
This may occur when the sample aliquot being analyzed is small (1-10 ml) and is not distributed evenly on the filter. To ensure even distribution, be sure to add enough buffer or rinse water (5-10 ml) to the funnel prior to adding the sample—and prior to applying the vacuum. The sample will disperse in the buffer (picture the way a small dollop of cream spreads out in a cup of coffee), and the colonies should be evenly distributed on the filter.

(b) all the growth on only one side of the filter?
The funnel base may be clogged so that the vacuum is only pulling through one part of the base. Remove the base and thoroughly clean it of any buildup. It is recommended that funnels and bases be cleaned periodically.

(c) colonies that look runny and oblong?
First, you may be incubating the plates in the wrong position. Plates should be incubated in an inverted position—that is, medium side up—so that condensation will fall down on the cover, not on the growing colonies. Second, excessive moisture may remain on the filter if it is

(continued)

(Bacteria Testing Q & A, continued)

removed before all the sample is filtered. This may cause the bacterial growth to spread out. These "spreaders" should be counted as one colony.

There's a lot of background growth. Can I still count all my target colored colonies?

There is a maximum number of total colonies allowable on a plate. For the small-size membrane filtration plates, 80 (or even 60, depending on the method) is the maximum. The larger plates used with Coliscan Easygel can accommodate up to 300 colonies.

All those organisms compete for the limited nutrients in the medium. The ones that grow are those that were able to outcompete the others. This competition may mask what the actual numbers are. If the total number of colonies exceeds the allowable number, the count is invalid and the result should be reported as an estimate based on the quantity of sample analyzed and the plate size.

I have a hard time assessing if a colony is the "right" color.

Including positive and negative control organisms when you analyze your samples will give you a reference to compare to. It takes practice to learn which questionable colonies are positive for your method. When starting out, it's a good idea to pick a representative colony you are unsure about and verify what it is, perhaps with assistance from a professional lab. This is especially helpful if an entire plateful of a strange-looking colony appears. Identifying what it is may uncover an unknown problem in the area or point to a problem with your quality control.

On mTEC medium (before you add the urease reagent) some yellow colonies are bigger, some are smaller, and some are pinpoint, but they should all be considered fecal coliform colonies. Some may even start to turn a brown-yellow.

Plates of mFC media are usually easy to count; the one potential problem is crowding, because the colonies are big and flat.

Pour plates (such as the Coliscan Easygel plate) can be difficult to read since colonies grow both on top of and within the medium. The colonies may be smaller and more difficult to assess when there is a lot of growth. Total coliforms appear pink-red, *E. coli* appears purple, and non-coliforms, which are also able to grow, are usually green or white. Lots of background growth may interfere with "reading" the plates.

How do I store a plate that I want to send to a laboratory?

If you want to send a plate to a lab for help with identification, place it in a ziplock bag labeled "biohazard" and store it in the refrigerator, media-side up. Transport the plate to a laboratory as soon as possible, but the plates can be stored for a week or longer in the refrigerator because the cold temperature slows bacterial growth.

(continued)

(Bacteria Testing Q & A, continued)

I gave another laboratory a duplicate sample bottle and their results are very different! Why?

First, be clear about what you are duplicating. If you collect two separate samples from the same site, you are replicating collection. Since organisms are not homogenous in the environment, it is very possible that two separate grabs from the same area may yield different results.

Most often, what volunteer groups really want to replicate is the analysis. Never use two separate grab samples to test for comparability of analysis with another laboratory; rather, collect a single sample in a large container (you may need to buy a few larger sample bottles for this purpose), mix it well, then immediately pour half into another sterile container which you will provide to the other laboratory for analysis.

Both laboratories should use the same test method, and preferably both should analyze the sample at approximately the same time. If the results are not within acceptable limits of variability, determine where the discrepancy lies. (NOTE: Defining acceptable limits of variability is a complex problem; consult with a professional lab for guidance.) Common problems include not mixing the sample well enough prior to analysis, not measuring accurately, and incorrect incubation temperature.

What minimum quality control should I be doing?

Briefly, you should maintain records of positive and negative controls, incubator temperatures, and split sample results. Maintaining proof that your results were generated in a consistent, reproducible manner that adheres to the requirements of the method will allow others to accept your results. Quality control testing should not take too much extra time, but it will instill confidence that you are producing valid data.

Can I combine my results with others in my program who are using a different method?

No. When reporting results, it is necessary to specify the method used, the media used, and the lower limit of detection (the smallest number of test bacteria that could be found considering the method and the quantity of sample). Different methods have different precision and recovery ability. It is important to separate results that were generated by different test methods and under different conditions. ■

References and Further Reading

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Web sites:

U.S. Food and Drug Administration, National Shellfish Sanitation Program (NSSP): http://vm.cfsan.fda.gov/~mow/sea-ill.html and http://vm.cfsan.fda.gov/seafood1.html.

Many manufacturers of bacteria-testing equipment have Web sites that are informative and up-to-date on the bacteria-growing media they offer. See Appendix C for Web addresses.

Other resources:

Educational Video on Processing Fecal Coliform Samples

To assist volunteer organizations, a short educational video is available that describes and demonstrates the analysis of fecal coliform sampling. It includes information for the layperson on everything from sterilization techniques to QA/QC (quality assessment/quality control) procedures. The video costs $14 and can be ordered through the New Hampshire Sea Grant Communications Office, Kingman Farm House, University of New Hampshire, Durham, NH 03824; phone: 603-749-1565; fax: 603-743-3997.