What Lives in Your Water?
Monitoring Bacteria in the Chesapeake Bay Watershed
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OBJECTIVES

- Understanding sources and impacts of bacterial contamination in the Chesapeake Bay watershed
- Learning to selectively grow bacteria from an environmental sample
- Analyze bacterial growth data to determine the prevalence of bacteria in environmental samples
- Contribute to ongoing research tracking bacterial contamination in the Chesapeake Bay watershed
- Civically engage to improve water quality in the Chesapeake Bay watershed through improvements at the community level

MARYLAND SCIENCE CORE LEARNING GOALS

Goal 1.0 Skills and Processes
Primary focus: 1.4.2, Addresses: 1.2.7, 1.3.1, 1.3.4, 1.4.1, 1.4.2, 1.4.4, 1.4.6, 1.5.1, 1.5.2, 1.5.3, 1.5.5, 1.6.1, 1.6.2, 1.6.3, 1.6.5, 1.7.1, 1.7.4, 1.7.5, 1.7.6

Goal 3.0 Concepts of Biology
Primary focus: 3.5.3, Addresses: 3.5.1, 3.5.2, 3.5.3, 3.6.1

Goal 6.0 Environmental Science
Primary focus: 6.3.2 Addresses: 6.2.2, 6.2.3, 6.3.2, 6.4.1

Visiting the MdBioLab

Bacterial Counts is a laboratory activity developed for use on the mobile bioscience laboratory, MdBioLab. Students work as microbiologists to monitor bacterial contamination in water samples that they have collected in their local area.

The MdBioLab is a state-of-the-art mobile laboratory for high school teachers and students, designed to bring new opportunities in science education and training to schools interested in expanding their current science curricula. Using this educational tractor trailer, instructors are capable of hosting 32 students in a variety of innovative hands-on laboratory investigations. Visit www.mdbiolab.org for more info.

Teachers and students must first complete a pre-laboratory classroom activity introducing the conceptual aspects of the curriculum before visiting the MdBioLab. There is a pre-laboratory classroom activity included in this document, although teachers may use additional materials they feel are appropriate for their own classroom to introduce the laboratory activity and concepts. Suggestions for extension activities are also included to expand and solidify the students' understanding of the concepts and/or to apply new concepts to real world situations or problems.

MdBioLab will supply all reagents, equipment, appropriate personal protective equipment and instruction for the laboratory activity. Teachers and students will be required to supply their own pencils and copies of the student laboratory handout.
Section I - Pre-Laboratory Classroom Activity
- Discussion of basic principles of watersheds and specifically the Chesapeake Bay watershed
- Basic principles of bacterial growth
- Basic information about bacterial culture in laboratory conditions, including selective growth
- Instructions on finding and collecting water samples from various streams near students' homes

Section II - Laboratory Activities (on the MdBioLab)
- Session 1: Students will filter collected water samples and prepare filters for culture on selective media
- Session 2: Students will count bacterial colonies for each collection site

Section III - Post-Laboratory Extension Activities
- Students will prepare a written summary that reports the results from all samples.
  1. Students will chart bacterial counts against parameters such as water temperature, salinity, pH, impervious surface data and rainfall data.
  2. Students will plot their data on a Google Map of Maryland posted on the MdBioLab website. This will allow them to compare their results with other student results. In the future, students will be able to view their results compared to previous years at the same collection sites.
- Students can engage civically by:
  1. Presenting their data and/or report to the appropriate local water offices
  2. Presenting their local water quality data to their community association leaders, with the hope of engaging those leaders to implement policies to improve water quality in their own community
Introduction to Water Quality Issues

The Clean Water Act of 1972 declares that natural waters of this country should be “fishable and swimmable,” yet many streams and rivers within Maryland and even some beaches of the Chesapeake Bay do not meet these expectations. While there are multiple pollutants that impair our waterways, bacterial contaminants in particular prevent many of our waterways from being available for harvesting shellfish or even swimming after rain storms. Through this MdBioLab project, students throughout Maryland will have an opportunity to determine levels of bacterial contamination of streams in their communities and develop plans to reduce contamination of these natural waters. They will measure bacterial contamination using EPA Method 1600, which is recognized by the Environmental Protection Agency (EPA) as the standard method for enumerating fecal enterococci in natural waters.

Enterococci

Enterococcus species are part of the normal intestinal flora of all birds and animals, including humans. It is estimated that there are about 10 million Enterococci per gram of human feces [1]. They are hardy, surviving in a wide variety of environments. They are facultative anaerobes, tolerating both the presence and absence of oxygen. They tolerate a broad range of growth conditions including temperatures and salt concentrations. Morphologically, they are Gram-positive cocci that tend to grow in chains (Figure 1).

Figure 1- Enterococcus infecting lung tissue. *Enterococcus faecalis* can cause a variety of diseases if it gets into the wrong place in the human body. [2]

Certain species of Enterococci are considered probiotic species, and have been traditionally included in fermented dairy products such as yogurt. They produce a variety of chemicals, similar to antibiotics, that fight off other species of bacteria, including gut pathogens. [3]

There are a variety of sources of fecal bacterial pollution in the Chesapeake Bay. Human feces are released directly into the Bay by boaters, despite laws to restrict this. Many homes in Maryland are not serviced by public sewers, so seepage from failing septic systems also enters the water. Droppings from domestic pets as well as wildlife can also be washed into the Bay by rainfall. Additionally, manure from farm animals often washes into tributaries, and in some cases, cows walk right into unfenced streams.

Enterococci were selected by the EPA as an indicator organism because they are hardy enough to persist in the environment. Enterococci enter the watershed in a number of different ways, including dog droppings that are washed into the watershed, leaking septic systems, bird droppings and even boats that refuse to close their toilet latches and drop raw sewage directly into the water.

However under normal circumstances, the environment is too cold to support much growth of this species. This means that the bacterial counts are truly representative of bacteria from fecal material in the water. The only time when these bacteria grow in the environment is during the hottest days of summer, when there is also a high algae count. Algae excrete sugars into the water, and the sugar and the warm water combine to make an excellent growth medium for Enterococci. This can impact the safety of the water for swimming and fishing.

Enterococci are “opportunistic pathogens”, which means that they only cause disease if they infect areas in which they normally do not belong (such as the bladder, or the blood through a surgical wound) or infect someone who is immunocompromised (such as an AIDS patient). Under these circumstances, Enterococci can cause a number of diseases, including urinary tract infections, endocarditis (infection of the heart lining), bacteremia (bacteria growing in the blood), wound infections, and peritonitis (infection of the abdominal cavity). In rare cases it can also cause meningitis, septic joint infections and respiratory tract infections [4].

Enterococci are naturally resistant to penicillin and many related antibiotics, so when they do cause disease, they are very difficult to treat. Recently, there has been an increase in acquired antibiotic resistance in Enterococci, particularly to vancomycin. This is why they are considered so dangerous in hospital settings.

Culturing Bacteria in a laboratory

Culturing bacteria in a laboratory causes them to grow relatively quickly compared to the way they grow in environmental settings. Generally, bacteria are cultured either in a liquid broth, or on a gelatinous substance called agar, a derivative of seaweed. In both cases, the nutrients needed to optimize growth for the organism that is being cultured are included in the growth medium. In addition, there are often substances added to the medium that inhibit the growth of undesired organisms. The special media used to select only one particular species of microbe is referred to as “selective media.” In this lab, we will be using a combination of chemicals in the media and growth conditions (particularly temperature) to inhibit the growth of everything other than Enterococci.
The laboratory activity

The goal of this lab is to measure levels of fecal pollution from water samples collected throughout the Chesapeake watershed.

The first step is to collect the water samples. Ideally, this would be done the morning of the lab, but collecting samples the day before the lab is acceptable as long as the sample is transported on ice and kept refrigerated.

In the first lab session, students will filter their water sample (2 different volumes) and place the filters on selective media. The plates will then be incubated overnight to allow for growth of Enterococcus colonies (Figure 2).

In the second lab session, students will count bacterial colonies and compute bacteria per 100 mL water.

After the laboratory session, the results will be tallied and the data for each collection site will be documented on a Google Map on the MdBioLab website.

Figure 2- Example of bacterial growth on selective media. Photo courtesy of Hornor Lab, Anne Arundel Community College, Arnold, MD.
SECTION 1 - Pre-Laboratory Classroom Activity

This classroom activity can be used to explore:

1. The basic structure of watersheds (primary/secondary streams, etc.), particularly the Chesapeake Bay watershed
2. The role of primary and secondary order streams in urban, suburban and rural communities as receiving waters for overland flow of storm water
3. Sources of fecal contamination, including the role of land use within communities that influences water quality including:
   a. The prevalence of septic systems and public sewers,
   b. Farm land,
   c. Urban and suburban use (particularly impervious surfaces).
4. Problems associated with bacterial contamination of surface waters
   a. Prevention of swimming at beaches,
   b. Prevention of fishing and oyster harvesting.
5. Culturing bacteria in a laboratory, including concepts of enriched media, selective media, and bacterial growth curves.

Students will also learn how to collect water samples from streams in their communities and will learn how to enumerate bacteria in those samples. Students should utilize GIS-based public websites, such as Google Maps, to locate public-access sites for water collection.

Suggested Lecture Outline

1. How watersheds are organized

   Water in our environment is constantly moving through the water cycle, alternating between evaporation and precipitation.
Rainfall returns water to the oceans by collecting and running through streams of increasing size, until finally it reaches larger rivers and, ultimately, the ocean. These streams and rivers of varying sizes are classified into “orders” of streams, with small numbers being associated with smaller streams, and larger numbers being associated with major rivers. As an example, we only have one tenth order river in the United States— the Mississippi. The highest order river on Earth is the Amazon, a fourteenth order river. [6]

The overwhelming majority of all streams are first order streams. Almost 90% of stream length in the Chesapeake watershed is in small first- through third-order streams. (See Figure 5) Large rivers (fifth- through eighth -order) only make up about 5% of the total length of flowing waters.[8] In fact, in Maryland, most people live just a few minutes away from a first-order stream. Often we are not aware of how close we are to these streams— they often “hide” in public parks and in wooded areas at the back of neighborhoods.

A watershed consists of all of the land that drains into a body of water. Often these watersheds cover large geographical areas. Over time, watersheds and the stream systems in them reach a balance in terms of the dissolved materials, suspended particles and larger materials that are pushed along the bottom of streams. This balance can be affected by changes in land use, such as addition of impervious surfaces like parking lots, disturbing soil and vegetation to support building houses and septic systems, and adding the waste generated by domestic pets. [7]
2. General information about the Chesapeake Bay Watershed

The Chesapeake Bay watershed extends into the states of New York, Pennsylvania, Delaware, Virginia, West Virginia and the District of Columbia. It covers over 64,000 square miles. There are over 100,000 streams and rivers that feed the Chesapeake Bay. [9] The Chesapeake's land-to-water ratio (14:1) is the largest of any coastal water body in the world. This is why our actions on the land have such a significant influence on the health of the Bay.

Figure 6- The Chesapeake Bay Watershed. [10]

The Chesapeake Bay Watershed is divided into eight sub-watersheds as shown in Figure 7.
3. Bacterial Pollution in the Chesapeake Bay Watershed

According to the Report “Maryland at a Glance” from the Maryland Governor’s office, fishing activities in the Chesapeake Bay add over $50M to the Maryland economy. [12] According to the Governor’s Economic Update address, tourism to the state attracts over $14.5B to the state—with one of the largest attractions being the Chesapeake Bay [13]. Fecal contamination of the Chesapeake Bay can make the waters unsafe or undesirable for fishing and swimming, leading to an economic loss in these important areas of the Maryland economy. In fact, there were 44 no-swimming advisories or closures at Maryland beaches in 2008 due to fecal bacteria counts. [14]
Further, some of the bacteria that enters the watershed can cause diseases ranging from diarrheal diseases (for example, *Vibrio cholerae* causes explosive diarrhea, which can result in dehydration and death), skin infections (especially if the swimmer already has a cut), eye infections, and even bacteremia (a potentially fatal infection of the blood).

There are a variety of sources of fecal bacterial pollution in the Chesapeake Bay. Human feces are released directly into the Bay by boaters, despite laws to restrict this. Many homes in Maryland are not serviced by public sewers, so seepage from failing septic systems also enters the water. Droppings from domestic pets as well as wildlife can also be washed into the Bay by rainfall. Additionally, manure from farm animals often washes into tributaries, and in some cases, cows walk right into unfenced streams.

![Figure 8: Cows with direct access to streams (USDA, 2005: http://www.ars.usda.gov/Main/docs.htm?docid=11769)](image)

4. **Enterococci**

![Figure 9: Scanning Electron Micrograph of Enterococcus faecalis. Sources: CDC Public Health Image Library (PHIL), Photo by Janice Haney Carr [15]](image)
Enterococcus species are part of the normal intestinal flora of a wide variety of animals, including humans. They are hardy, surviving in a wide variety of environments. They are facultative anaerobes, growing both in the presence and absence of oxygen. They tolerate a broad range of growth conditions including temperatures, salt concentrations and food sources. This makes them excellent organisms to use as indicators of fecal pollution. Morphologically, they are Gram-positive cocci that tend to grow in chains.

When they gain access to the body, which normally occurs as a result of surgery, Enterococci can cause a number of diseases, including urinary tract infections, endocarditis (infection of heart lining), bacteremia (bacteria growing in the blood), wound infections, peritonitis (infection of the abdominal cavity). In rare cases it can also cause meningitis, septic joint infections and respiratory tract infections. [4]

Enterococci are naturally resistant to penicillin and many related antibiotics. Recently, there has been an increase in acquired antibiotic resistance in Enterococci, particularly to vancomycin, one of the most advanced antibiotics in our clinical arsenal. This means that enterococcal infections can be very difficult to treat.

5. Culturing Bacteria in a Laboratory

Culturing bacteria in a laboratory causes them to grow relatively quickly compared to the way they grow in environmental settings. Generally, bacteria are cultured either in a liquid broth, or on a gelatinous substance called agar, a derivative of seaweed. In both cases, the nutrients needed to optimize growth for the organism being grown are included in the growth medium. In the incubator, temperature and moisture are controlled as well to maximize growth. In addition, there are often substances added to the medium that inhibit the growth of unwanted organisms. When you are trying to select only one particular species of microbe, the media used is referred to as “selective media”. In this lab, we will be using a combination of chemicals in the media and growth conditions (particularly temperature) to inhibit the growth of everything other than Enterococci.

6. Water Collection

Prior to the laboratory activity, students should identify appropriate sites for water sample collection. Google Maps can be used to locate local streams. Places where roads cross these streams normally have bridges or overpasses, and these are often excellent potential sites for sample collection. However, the safety of these sites must be considered. Bridges on busy roads and sites that have a steep slope leading to the water may not be suitable collection sites. Public parks with streams can also be excellent sites for collection. Students are often surprised to learn that there are headwater streams in close proximity to their schools and homes.

The students should then be paired up so that two samples are collected from each collection site. This allows for duplicate data measurements, improving the quality of the data for each site. The students may choose to go to the collection sites together, which would provide additional safety support. For safety reasons, students should be encouraged to bring a parent or companion when they collect water as field work is always safer and easier with two people.

Each student pair will receive a collection kit for collecting water samples. This kit will include:
   a. One pair of Nitrile gloves
   b. 2 Ziplock bags- one to hold ice, the other to hold the collection tubes
   c. 2 125-150 mL water collection bottles
   d. Thermometer (preferably digital)
   e. Collection form to note collection parameters such as time of day, air and water temperature, water color and movement, and other observations.

Immediately before leaving to perform collection, the students should place crushed ice in one of the ziplock bags. When the students arrive at the water collection site, they should record the water temperature, along with the other information requested on the collection form. Each of the two students should then take a collection tube, and collect a water sample. The samples should be taken at the same time, and close together. The water bottles should then be placed into the empty ziplock bags, the air removed from the bag by pressing the bags flat (or wrapping the bag around the tube) and then sealing the bag. The tubes in the sealed ziplock should then be nestled into ice in the other ziplock bag, remove as much air as possible, then seal the ziplock.
Samples should be kept upright (cap facing up) on ice and returned to the MDBioLab within 4 hours of collection if possible. If this is not possible, samples could be held in a refrigerator until the next day. Students should be told to remove the collection vials from the ice (leave them in the sealed inner ziplock bag) and place the collection vials in a coffee mug, cap facing up, in the refrigerator to help keep it upright if overnight storage is required. In the morning, the water samples should be placed in fresh ice in the second ziplock bag, and transported to school. The bag with the sample should immediately be dropped off at the MdBioLab Mobile Laboratory to be refrigerated until the water can be processed.

The students should be instructed to measure water temperature with the thermometer provided in the collection kit, and note that temperature on the collection form, along with an indication of the color, cloudiness and level of water movement in the stream based on the criteria listed on their data collection sheet. They should record these measurements immediately and on-site instead of trying to “remember” the information later.

7. Lab Safety

Enterococci are opportunistic pathogens, and consequently the students will need to follow a few safety rules when working with these organisms in the laboratory. Enterococci are classified as Biosafety Level 2 (BSL2) organisms by the Centers for Disease Control. This classification is used for organisms that cause only mild disease in humans, and are difficult to contract via aerosol in laboratory settings. These safety rules are common practice in microbiology laboratories handling BSL2 organisms.

The students will be required to:

a. Wear personal safety equipment including:
   i. Nitrile gloves on their hands to prevent contamination of hands and fingernails
   ii. Protective glasses over their eyes to protect against splashes (students who regularly wear glasses are considered sufficiently protected)
   iii. Long hair must be tied back.
   iv. Optionally, the student may choose to wear a large T-shirt or men’s shirt over their clothes. Their outer layer of clothing should be washable, in the event of a spill or splash.

b. No food or drinks will be allowed in the laboratory while the experiment is in process. This prevents accidental ingestion of the bacteria, which would potentially cause diarrhea.

Possible questions for discussion: (See resources provided below to help answer these questions)
1. What river is closest to your school?
2. How big is the watershed (acres or square miles) that feeds that river?
3. Are there known pollution problems that affect that river watershed?
4. What kinds of land uses affect your river’s watershed?
   a. Which ones are sources of pollution?
   b. Which ones cause the greatest damage?
5. What kinds of forests and other open spaces with vegetation are present in your watershed area?
6. What kinds of agricultural activities occur in your area?
   a. Are they large or small farms?
   b. Are they primarily livestock- or crop-based operations?
7. What steps do people in your area take to control/pick up waste from dogs to prevent runoff? Are there rules about picking up and disposing of pet waste in your community?
Optional Additional Lecture Materials:

8. General Information About Bacteria

Different bacteria have a number of distinguishing features that allow microbiologists to tell them apart. The following list is not meant to be an exhaustive list of these kinds of distinguishing features, just ones that are of particular interest for Enterococci, which is the group of bacteria this activity focuses on.

Two of these distinguishing features are cell shape and colony morphology. Cell shape generally consists of one of three shapes:

- cocci—round cells
- bacilli—rod or oval shaped cells
- spirochetes—spiral or corkscrew shaped cells

Colony morphology is a description of how groups of bacteria of a particular species grow. Some species grow as individual cells. Others grow in small groups of two or four cells. Enterococci tend to grow in a chain formation as seen in the streptococci above.
Most bacteria are enveloped by a cell wall. There are two basic configurations of bacterial cell wall, which can be distinguished experimentally using a technique called the Gram stain. In Gram staining, a type of stain called crystal violet is applied to the cells. Cells that take up the crystal violet stain appear purple under the microscope, and are referred to as Gram-positive. Gram-negative bacteria do not retain the crystal violet stain, and therefore appear pink under the microscope.

All bacteria have a cell wall made up of peptidoglycan, which has a net-like structure that functions to protect the bacteria. In Gram-positive bacteria, the peptidoglycan coat is so thick that it traps the crystals of the crystal violet stain, making the cells appear purple. This does not happen with the much thinner peptidoglycan coat of Gram-negative bacteria.

Another feature that distinguishes different species of bacteria is the kind of atmosphere they grow in—particularly the presence of oxygen. Bacteria that utilize oxygen for growth are called “aerobes.” Bacteria that cannot grow in the presence of oxygen are called “anaerobes.” There is also a group of bacteria that can grow either with or without oxygen, such as Enterococci, which are called “facultative anaerobes.” These adaptable species often are capable of growing in many environments.

Salt-tolerance (known as “halotolerance”) is another distinguishing feature of bacterial growth. Enterococci are tolerant of a broad range of salt conditions, allowing them to grow in the brackish waters of the Chesapeake as well as the fresh waters of streams.

The final distinguishing growth feature is carbon source metabolism. Different bacteria use different kinds of carbon-containing molecules as food. Most bacteria use carbohydrates (e.g. glucose and other sugars) as a food source. In addition to a wide variety of sugars, Enterococci also consume amino acids, glycerol, and a variety of organic acids produced by the metabolic activities of other microorganisms. This ability allows Enterococci to live in community with other microorganisms, particularly in the digestive tract. Its flexibility also allows it to grow out in the environment.
Additional Resources:

1. Watershed and stream organization
   a. www.dnr.state.md.us/streams/volunteer/k12.html
   b. geography.about.com: http://geography.about.com/od/physicalgeography/a/streamorder.htm
   c. Ohio State University Extension Fact Sheet on Stream Classification: http://ohioline.osu.edu/aex-fact/pdf/AEX44501StreamClassification.pdf
   d. geography.about.com: http://geography.about.com/od/physicalgeography/a/watersheds.htm
   e. Missouri River Basins and Watersheds Middle School Activity Guide: http://www.moststreamteam.org/activity_guide/watershed/missouri_riverBasins_watersheds.htm

2. Chesapeake Bay Watershed
   a. Chesapeake Bay Program Website: http://www.chesapeakebay.net/wshed.htm
   b. Chesapeake Bay & Mid-Atlantic from Space Website “What are Watersheds?”: http://chesapeake.towson.edu/landscape/impervious/all_watersheds.asp
   c. EPA Surf Your Watershed Website Resource: http://www.epa.gov/surf/
   g. Maryland Streams by County: [17]
   h. Chesapeake Bay Subwatersheds: http://svs.gsfc.nasa.gov/goto?3477

3. Sources and Impact of Fecal Pollution
      i. Article 17: Microbes in Urban Watersheds [18],
      ii. Article 126: Understanding Watershed Behavior [19]
   b. The Encyclopedia of Earth Website: http://www.eoearth.org/article/Fecal_pollution_of_water#Human_vs._animal_fecal_pollution_and_source_tracking
   c. Pollution Prevention Fact Sheet: Animal Waste Collection: http://www.stormwatercenter.net/Pollution_Prevention_Factsheets/AnimalWasteCollection.htm

4. General Microbiology

5. Enterococcus organisms
   b. eMedicine Website: http://emedicine.medscape.com/article/216993-overview
   c. Anne Arundel Community College/Hornor Lab Enterococcus tracking data: http://ola4.aacc.edu/sghornor (then click on data for the Severn or Magothy Rivers)
SECTION II – Laboratory Activity

The laboratory portion of this activity is based on EPA Method 1600.[20] It is broken into two sessions. In the first session, students will filter their water samples (Two different volumes will be used to increase the likelihood of getting a plate with between 20 and 60 colonies.). The plates will then be incubated for 24 hours at 41˚C. During the second laboratory session, students will select one of their two plates for counting bacterial colonies. The ideal is to use a plate that has between 20-60 colonies growing on it.

If time permits, students may be able to go back to streams with high Enterococcus counts and sample upstream from where they originally collected so that they could begin to pinpoint the sources of the bacteria in their samples. Running a set of duplicate samples would also be a good exercise for students so that they can see the type of variability that exists from day to day in natural waters. If a rain event occurred within 48 hours of any sample collection, then this would be an excellent opportunity to demonstrate how an influx of storm water causes dramatically increased bacterial counts in natural waters. Comparison of results from before and after a rain event is often an impressive demonstration of urban, suburban or rural runoff.

Equipment List:

**Session 1: Students will filter collected water samples and prepare filters for culture on selective media**

**MdBioLab:**

- Bunsen Burner
- 20x Sterile membrane filtration unit
- Vacuum aspirator with 1 L discard flask
- Forceps and beaker for holding ethanol for flame sterilizing forceps
- Incubator, set to 41˚C
- Lab marking pen
- Dilute Detergent Bucket for used collection bottles and filters
- Digital Vernier meter along with probes to measure salinity and pH.

**Consumables (x20):**

- Disposable 10 mL Syringe
- 2x50 mm petri dishes preloaded with mEI agar
- 150 mL Collection bottles/Tubes
- 2x47 mm membrane filters
- 95% Ethanol
- Sterile buffered water in sterile squeeze bottle

Positive control culture: *Enterococcus faecalis* (*E. faecalis*) ATCC #19433 pre-diluted to approximately 50-100 bacteria per sample.

Negative control culture: *Escherichia coli* (*E. coli*) ATCC #11775 pre-diluted to approximately 50-100 bacteria per sample.

**Session 2: Students will count bacterial colonies for each collection site**

**MdBioLab:**

- 5x Lamp with cool white fluorescent tube
- 5x Magnifying glass
- 5x Hand tally
- Digital Camera
- Bucket of 10% bleach solution for plate disposal
Micr\scope
Blank slides

Nigrosin (India Ink) stain
Toothpicks

**mEI agar:**

In 1 L reagent grade water:
- 10.0 g Peptone- partially digested proteins from milk or meat. Contains amino acids and short peptides, fats, minerals, salts and vitamins
- 15.0 g Sodium chloride (NaCl)
- 30.0 g Yeast extract- yeast waste from brewing- rich in amino acids, vitamins and minerals, esp B vitamins
- 1.0 g Esculin- a nutrient specific to Enterococci, which splits a glucose off the esculin. The remaining molecule turns black in the presence of iron, so functions as an indicator
- 0.05 g Actidione (Cycloheximide)- inhibits the growth of fungi and other eukaryotes by binding to eukaryotic ribosomes
- 0.15 g Sodium azide- inhibits gram- bacteria
- 0.75 g Indoxyl β-D-glucoside- turns blue when modified- only modified by certain Enterococci
- 15.0 g Agar
- 0.24 g nalidixic acid (sodium salt)- antibiotic tolerated by Enterococci
- 0.02 g triphenyltetrazolium chloride (TTC)- indicator (differential) tolerated by Enterococci

The objectives of the laboratory are as follows:
- Filter two quantities of each collected water sample plus positive and negative control
- Culture the filters on media selective for Enterococci
- Calculate the number of colonies per 100 mL (the standard measure) for each collected sample

**Prelab Preparation**

Three hours prior to the first session, one vial of stock positive control culture and one vial of negative control culture should be moved from the freezer to the refrigerator to allow them to thaw. Just prior to the lab, a small beaker at each station should be filled with about \( \frac{1}{2} \) of ethanol for flame sterilization of forceps. Place forceps in beaker with ethanol.

**Session 1: Students will filter collected water samples and prepare filters for culture on selective media (70 min)**

**Prior to Entry to MdBioLab Mobile Lab – Safety Review.** Students should be told to leave any food or beverage outside the MdBioLab laboratory. They should be provided with Nitrile gloves and Safety Glasses and asked to put these items on prior to entering the lab.

**Step 1 – Students label mEI agar plates in preparation for culture.** Each student will be given two mEI plates for their experiment. The students will label each plate with their initials and group number, date and volume of the filtered water sample (~one should be labeled 10 mL and the other 100 mL). This should be demonstrated by the MdBioLab instructor, who should mark one plate for the positive control and one for the negative control.

**Step 2 – Students filter collected water sample, then transfer the mEI filter to mEI agar plates.** Students should work in pairs and follow the following protocol. This procedure should be demonstrated by the MdBioLab Instructor, who should filter and transfer the positive and negative controls. The MdBioLab Instructor should also monitor the vacuum discard flask after each student and empty as needed to avoid overflow

1. Place a new funnel on the vacuum apparatus.
2. Shake the water sample to evenly distribute the bacteria. The “standard shake” technique should be demonstrated to the students by the MdBioLab instructor for the positive and negative control cultures:
   i. Hold the tightly capped bottle in the right hand.
   ii. Strike the bottle firmly against the palm of the left hand 25 times to break up any microcolonies or biofilms that have formed in the collection tube. The right arm should rotate approximately 90˚ (right shoulder down to left hand held at waist level) to give a sufficient force to break up any colonies in the water sample.
3. Place a sterile membrane filter on the filter base, grid side up.
4. Rinse the syringe with sterile water to remove static electricity. This will also function as practice with the syringe. Draw up 10 mL of sterile water, then apply it to the paper filter. This may be repeated until the student is comfortable with the pipetting procedure. The student must be careful not to touch the syringe tip to any surface to avoid contaminating the samples.

5. Remove a 10 mL quantity out of the sample bottle with the syringe, then apply the liquid to the membrane filter. The student must be careful not to touch the syringe tip to the filter. When the sample has been transferred, the syringe should be thrown away.

6. Once the liquid has been completely suctioned through the filter, wash the sides of the filter with approximately 5 mL sterile water from the squeeze bottle. Allow the water to suction through the filter.

7. Transfer the filter to the mEI agar using sterilized forceps.
   a. Dip the tip of the forceps in the beaker of ethanol,
   b. Pass the tips of the forceps through the flame of a Bunsen burner.
   c. When the flame goes out on the forcep tips, remove the lid on the agar plate, transfer the membrane filter from the filter apparatus to the mEI agar plate, rolling the membrane filter onto the agar to avoid bubbles, then replace the lid on the plate.

8. Repeat steps 3 through 7 for a 100 mL sample. Instead of using the syringe, 100 mL of sample will be poured into the filter by filling it up to the 100 mL line.

9. Place the funnel and collection bottle in the detergent bucket for washing.

Step 3 – Place plates in the incubator. The plates should be turned upside-down and placed in a container. Place the container with the plates in the incubator at 41°C for 24 hours. After 24 hours, if the students will not be reading their plates at that time, place the container with the plates in the refrigerator to stop further growth until the students are ready to count bacterial colonies.

Step 4 – measure salinity with refractometer and pH with meter or kit and record on data sheet

Post-Lab Cleanup.

1. Safety Glasses should be sprayed with 95% ethanol.
2. Funnels should be removed from the detergent, rinsed and drip-dried.
3. Any extra collected water samples can be poured down the drain.

Session 2: Students will count bacterial colonies for each collection site (30 min) and optionally view bacteria through a microscope (30 min)

Step 1 – Students count bacterial colonies. Each student will be given the two mEI plates from the samples they collected. The plates were made using two different volumes of water, so one will have many more colonies than the other (although the precise number of colonies will depend on the levels of bacterial contamination in the water. The student should select whichever of the plates has between 20 and 60 colonies. If there are no plates with this range of colonies, count the plate with the number of colonies closest to 20-60. Each colony that is at least 0.5 mm in diameter should be counted. Ignore colonies smaller than 0.5 mm. The MdBioLab Instructor should demonstrate the counting method on the positive control plate on the overhead projector. Each student should then calculate the number of Enterococci in 100 mL of water using the following formula:

\[
\text{Enterococci} / 100 \text{ mL} = (\text{Number of enterococci colonies/Volume of sample filtered (mL)}) \times 100
\]

If possible, students should take a digital picture of the plates that they count to include in post-lab activities.

Step 2 – After counting, as an optional activity, each pair of students can view a colony from their plate under the microscope. Each pair of students should select one colony from one of their growth plates. The lid should be removed from the plate by one student, and the other should scrape the colony off the agar using the flat side of a toothpick. The student with the toothpick should then smear the colony in the middle section of a microscope slide while the student holding the plate replaces the lid. One drop of Nigrosin stain should be applied to the area of smeared bacteria, and a coverslip should be placed on the slide. The slide can then be viewed under a microscope. The procedure should be demonstrated by the MdBioLab Instructor using the positive control plate. The shape (round or coccus shape) of the cells as well as the colony morphology (chains of bacteria) should be recorded by each student based on the microscope view.
Post-Lab Cleanup.
Students should dispose of plates and microscope slides in 10% bleach after counts are complete.

Alternative to Session 2: Digital Photos

Another option is to have the MdBioLab Instructors take digital pictures of each plate, and provide the pictures to the teacher for in-class plate counting. This procedure is not as accurate, nor is it as satisfying to the students as actually holding and counting their cultured plates, however it is a good option in cases where the students are unable to pay a second visit to the MdBioLab, as the plates cannot be safely removed from the MdBioLab lab facility.

Discussion Questions

▪ What is a negative control? What is a positive control? Why are they important?
  The negative control is a sample that contains a pure culture of E. coli that should not grow on the selective media, and that is likely present in the samples collected by the students. It is used to test that the media is truly selective.
  The positive control is a sample that contains a pure culture of E. faecalis, an organism that is expected to grow in the selective media. It is used to verify that the media allows growth of the target organism.
  Both types of controls are important to validate the results of the culture. They let you know that your experimental procedure worked as you intended.

▪ If no colonies were present in the positive control, what would the next step be?
  A positive control is used to verify that the media supports growth of our organism of interest. If no colonies were present then the media is not working correctly and needs to be replaced.

▪ Why are two different volumes of sample cultured?
  The ideal number of colonies on the plate for accurate reading is 20-60 colonies. The small volume should give this number of colonies if there is a lot of fecal pollution at the collection site. The large volume is for samples with a small amount of fecal pollution.
SECTION III – Post-Lab/Extension Activity Suggestions

Summarizing and reporting data – Have students prepare an Excel spreadsheet showing all collected data. An example spreadsheet, which is provided as an Excel Spreadsheet with the curriculum materials, is shown as follows:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Lat</th>
<th>Long</th>
<th>Count</th>
<th>Volume</th>
<th>Bact/100mL</th>
<th>Location</th>
<th>Temp °C</th>
<th>pH</th>
<th>Color</th>
<th>% impervious</th>
<th>Salinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>38.939015</td>
<td>-76.556029</td>
<td>33</td>
<td>100</td>
<td>33</td>
<td>Warehouse Creek</td>
<td>11</td>
<td>7.6</td>
<td>murky brown</td>
<td>15</td>
<td>.2 ppt</td>
</tr>
</tbody>
</table>

The collection points should then be plotted on the MdBioLab Google Map.

![Google Map screenshot](http://maps.google.com)

Figure 12- MdBioLab Google Map.

The points can be added to the Google Map by going to [http://maps.google.com](http://maps.google.com) and logging in to the map using the MdBioLab.ChromeMaps userid. This username and password should only be use by the instructor and not given to the students.

**Userid:** MDBio.Map@gmail.com  
**Password:** teachers

Once logged in, select My places (top of lefthand panel), then MAPS, then MdBio Enterococci. You should see a map with blue markers in the righthand panel and a listing of water collection sites in the lefthand panel (Figure 12). Click on the red EDIT button to add new entries of edit existing ones. If the collection points used for the students had been previously entered, then only the date and count need to be added. If the collection point is being entered for the first time, select the blue data point icon (at the top of the map), and drop it at the actual collection point. The map can be zoomed to make the placement of new markers more accurate. If the icon is dropped at the wrong point, it can be dragged using the mouse. Give the point a name, such as “Main Street Overpass over Blue Creek” so that other classes can easily recognize the location for future collections. To edit an existing entry (for example to add
additional collection dates to a previously visited site), click on the name of the location in the lefthand panel. An editable text box should appear on the map (Figure 13).

![Google Map with markers and a text box](image.png)

**Figure 13. Editing view, showing blue marker icon (top) to create new entries and pop-up text box for editing existing entries (middle).**

**Calculation of Impervious Surface:** The amount of impervious surface surrounding each collection site should then be computed by the students. The pair of students for each collection site should work together to complete this activity. A good online educational training resource that discusses issues related to impervious surface is MathBench, a series of modules developed at the University of Maryland to teach underlying math skills for the chemical and biological sciences.

To access MathBench:
1. Go to: mathbench.umd.edu
2. Select “Environmental Science”
3. Select “What’s in your Watershed?”
4. Work through the impervious surface exercise

You then should compute the percentage of impervious surface surrounding each water sample collection sitepoint.

1. On the MdBioLab Google Map, Move to the largest zoom level with the collection point centered, then zoom out twice. This gives you the immediate area surrounding the collection site. The scale at the lower left corner of the map should be 100 ft/50 meters.
2. Print out the displayed Google map, preferably in color
3. Use the overhead with gridlines every centimeter (approximately every 50 feet of scale)
4. For each cell in the grid, identify whether the cell is primarily impervious (building, parking lot, road, gravel) surface. For cells where it is approximately half, count it as half impervious. Otherwise, treat it as entirely pervious or impervious.
5. Count up the total number of impervious cells on the map, divided by the total number of cells to compute percent impervious surface
6. If possible, compare data to that computed from Landsat Imagery from the Mid-Atlantic RESAC (http://www.geog.umd.edu/resac/impervious.htm)
Preparation for Civic Engagement: The students should then be divided into four groups. They should attempt to correlate the bacterial counts with other parameters they measured, such as temperature, pH, salinity, proximity to septic systems (if your county tracks this information), proximity to water treatment systems (http://www.cbf.org/Page.aspx?pid=939), dog-walking routes, impervious surfaces, rainfall in the days preceding sample collection and so on.

These can be charted by hand or using the charting capabilities in Excel. Students should also include a brief explanation of the results from the positive and negative controls (i.e. were there any signs of contamination or growth of the negative control (“selection failure”) that would bring the validity of the data into question?).

Group 1
Identify the organization in your county that is responsible for monitoring and enforcing water quality standards. Identify the correct point of contact and their mailing address. Identify the same information at the state level. Ask the county agency if there is a volunteer environmental group such as the Severn River Assn that is involved with stream health.

Group 2
Discuss how human population growth affects the Chesapeake Bay Watershed. Focus on your county, but address the overall issues with these factors in the health of the Chesapeake Bay. http://pubs.usgs.gov/circ/circ1316/html/circ1316chap2.html

Group 3
Discuss how land-use changes, especially regarding farming, affect the Chesapeake Bay Watershed. Focus on your county, but address the overall issues with these factors in the health of the Chesapeake Bay. Also, this group should gather information about rainfall for their area. One reliable source is found at www.cocorahs.org, a nationwide weather data collection cooperative.

Group 4
Discuss the amount and location of impervious surface in your local area. Comparing land use to bacterial burden in our surface waters is an excellent way to see the impact of development and impervious surfaces on water quality. Students can discuss ways to prevent such contamination by considering better site design for developments and better ways to keep manure and farm animals out of our streams. http://pubs.usgs.gov/circ/circ1316/html/circ1316chap2.html

Each group will record the main points made during their discussions and share them with the entire class. As a class, synthesize the ideas from all groups into the report that could be submitted to county and state water quality offices. Students should understand that conservationists consider a broad range of problems and use many tools to develop solutions.

A proposed report format:

Introduction:
- Background information on water quality issues, focusing on the local subwatershed
- Significance of tracking fecal pollution for local as well as regional health

Methods:
- Describe the experimental procedures, including the equipment, experimental steps and controls

Results:
- The data, including the charts, maps and data tables.
- Calculation of impervious surface surrounding the collection point (see MathBench activity)

Discussion:
- Identify the relationship between bacterial count and the other parameters measured or considered.
- Draw conclusions about the problem.
- Propose solutions

MdBioLab can also create a database of all the information collected throughout the state. This will allow students to compare their results to those found by other students in the program throughout the State.

Students should then consider how they can become civically engaged with the information they have collected. Some possible activities:

- Send a copy of the class report to their local county water quality officials and/or the Maryland Department of the Environment
• Contact local community organizations near any areas of high fecal contamination. Present the results to a community meeting, with some suggestions for what the community might do to curb the pollution, such as pick up after their dogs or identify failing septic systems.

Contact local farmers to suggest measures such as installing fencing to prevent cows from entering streams. The class may decide to do a community service project of installing a simple wire fence for a farmer who is willing to participate, to keep cows out of a local stream. Check with local ordinances or the Chesapeake Bay Foundation to see if cows are permitted in streams in your local area. If they are, maybe giving a report or meeting with local lawmakers would have a better effect in keeping cows out of streams.

Additional Resources:

1. Mapping Resources
   a. Latitude/Longitude finder: http://www.batchgeocode.com/lookup/
TERMINOLOGY

Primary Stream -
Selective Media -
Incubator -
Probiotic -

Negative control: A substance on which your experiment has no effect. In this lab, your negative control is water. The purpose of a negative control in PCR assays is to detect contamination (the presence of non-target DNA amplification).

Positive control: A sample that demonstrates whether your manipulation of the variable produced the desired affect. In this lab, your positive control is the amplification of a region of a shark genome that occurs in all species of sharks. Thus, if there is any shark template DNA present in your PCR, you should see a band on your agarose gel.

Reference List


   Ref Type: Electronic Citation


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15. Janice Haney Carr. Scanning Electron Micrograph of Enterococcus faecalis. CDC- Public Health Image Library. 2010. 2-8-2010. Ref Type: Electronic Citation


