Introduction

The purpose of this laboratory exercise is to familiarize you with the most common assays for the detection of bacteria in water. We will be using two types of assays: membrane filtration, which, and the Most Probable Number (MPN) assay. We will simultaneously detect three groups of organisms: total coliforms, fecal coliforms, and E. coli.

The Most Probable Number Assay

Purpose

In this lab, you will use the MPN assay to measure the numbers of total coliforms, fecal coliforms and E. coli in water from Morgan Creek.

Principle

Indicator bacteria

Traditionally, indicator bacteria have been used to determine the possible presence and to estimate the amount of fecal contamination in water, foods and other samples. The detection of indicator bacteria is preferred over direct pathogen detection because the former are considered to be normal, non-pathogenic intestinal inhabitants that are present in feces, wastewater and other fecal wastes in much higher numbers than pathogenic microorganisms and because they are technically easier to detect and quantitate than pathogens. Present criteria and standards for the sanitary quality of water, foods and other materials with respect to fecal contamination are expressed in terms of concentrations of indicator bacteria.

The most widely-used indicator bacteria in the U.S. and worldwide are the so-called total and fecal coliforms and specifically Escherichia coli, the bacterium in these groups that is most feces-specific. The word "coliform" has been used historically to represent various genera of the family Enterobacteriaceae that ferment lactose. The definition of a coliform has now been updated to include those Gram-negative bacteria that have the ability to metabolize Beta-galatosides as substrates (because they possess the enzyme Beta-galactosidase). The ability to ferment lactose depends upon the possession of the enzyme beta-galactosidase as well as a galactoside permease which facilitates lactose entry into the cell.

It should be emphasized that "coliforms" are actually defined in operational or functional terms based upon the media, biochemical reactions and incubation conditions used for their
isolation and quantitation. None of these operational definitions detects all lactose-fermenting (or Beta-galactosidase-producing) members of the family *Enterobacteriaceae* and some include members of other families. The ability to ferment lactose (and produce Beta-galactosidase) is thus a characteristic of considerable diagnostic importance in distinguishing among the various groups of enteric bacteria.

**Fecal Coliforms**

Some of the bacteria detected by coliform procedures are sometimes also found in soil (*Citrobacter*, *Enterobacter* and *Klebsiella*), on various plants, including grains and trees (*Serratia*, *Klebsiella* and *Enterobacter*) and in certain industrial wastes. For these reasons, another group, the "fecal coliforms" or “thermotolerant coliforms” has been established in an attempt to exclude those total coliforms that are non-fecal and detect only those of fecal origin. The basis for this exclusion and selection is a higher incubation temperature of 44.5°C, at which presumably coliforms of only fecal origin will grow because they are adapted to the higher temperature of the mammalian intestinal tract of about 36-37°C.

Coliforms from non-fecal, environmental sources are generally incapable of growing at this elevated temperature. Thus, fecal or thermotolerant coliforms can be defined as gram-negative, non-sporeforming, rod-shaped bacteria which ferment lactose with the production of gas at 44.5°C within 24 hr. The fecal coliform test is now widely applied to surface and ground water, sewage treatment systems, biosolids (treated sludges) and general monitoring of natural waters for sanitary quality, including recreational and shellfish waters, and water quality standards have been developed for it. However, the coliform test is still used in the examination of potable waters in the USA and some other countries, because coliform bacteria of any kind are considered undesirable and are not tolerated in a finished drinking water.

**Escherichia coli**

Because both the total and fecal (thermotolerant) coliform tests often still detect bacteria of non-fecal origin, test have been devised to detect *E. coli* exclusively as the coliform bacterium most specific for fecal contamination. Today, both the total and fecal coliform tests are used in the examination of water, even though *E. coli* has been identified as the most feces-specific member of the coliform group.
Methods

The MPN method uses liquid media in a multi-step assay that detects total coliforms, fecal coliforms, and *E. coli* all in one method. The assay for total and fecal coliforms actually consists of three successive steps or tests: Presumptive, Confirmed and Completed. For the Presumptive Test, dilutions of the sample are inoculated into fermentation tubes of lactose or lauryl tryptose broth and incubated at 35°C for 24 hr. At the end of the first 24 hours, results for total coliforms are read. For the Confirmed Test, organisms from all positive fermentation tubes (those with growth plus gas) of the Presumptive Test are transferred to fermentation tubes of brilliant green lactose bile broth or EC broth with MUG (the broth we will use in this lab) and incubated at 44.5°C for 24 hr. Tubes showing both growth and gas are considered positive Confirmed tubes. At this point, results for fecal coliforms and *E. coli* are read. For the Completed Test, organisms from positive Confirmed tubes are isolated in pure culture on agar plates of differential/selective media (Endo or eosin methylene blue agar) and then tested for: (1) growth and gas production in fermentation tubes of lactose or lauryl tryptose broth incubated at 35°C for 48 hr.; and a negative reaction in the Gram stain. For a positive Completed Test, the organisms must show growth plus gas production in the fermentation tubes and be Gram negative. The Completed test is rarely done in practice due to its complexity and additional time.

We are going to perform a two-step procedure for total coliforms, fecal coliforms, and *E. coli*. For the two-step Multiple Tube Fermentation Technique, positive tubes from the Presumptive total coliform test (using lauryl tryptose broth) are inoculated into fermentation tubes of EC medium with MUG and incubated at 44.5°C for 24 hr. Tubes showing growth with gas production are considered confirmed positives. In this lab exercise, we will stop at the Confirmed test step. Further confirmation will be part of the next laboratory exercise.

The concentration of total coliform bacteria is estimated from the numbers of confirmed positive and negative culture tubes that received specified and often different volumes of a water sample. This estimate is based on computing the Most Probable Number of bacteria using so-called maximum likelihood estimates and the assumption that the bacteria conform to a Poisson distribution.

Materials:

- Three 90 mL dilution bottles of PBS
pipettes
- tubes of lauryl tryptose
- Tubes of EC broth (second day)
- sterile sticks

Protocol

First day: inoculation
1. Mix the sample by shaking well before diluting.
2. Make 3 dilutions of the water sample by adding 10 mL to 90 mL of PBS in a dilution bottle.
3. Mix vigorously 25 times.
4. Place 25 fermentation tubes of lauryl tryptose in a test tube rack as groups of 5 dilutions, and mark the tubes as to their dilutions. You will inoculate 4 sets of tubes—one set with undiluted water sample, and three dilutions.
5. Using a pipet, inoculate 1-ml volumes of undiluted sample into the first set of 5 replicate tubes.
6. Inoculate 1-ml volumes from the first dilution bottle into the first each sample dilution to be tested into the second set of 5 replicate tubes.
7. Incubate the tubes in a 35°C air incubator for 24 hours.

Second day: Reading of presumptive positive tubes and inoculation of tubes for Confirmed Total and Fecal Coliform Tests and for E. coli.
1. Gently shake the rack of tubes back and forth several times to release gas in positive tubes.
2. Examine all tubes for the presence of growth (turbidity or cloudiness) and gas (look in the small inverted tube), and score tubes showing both growth and gas as presumptive positive for total coliforms.
3. Submit all lactose broth fermentation tubes that are Presumptive positive to the confirmed tests.
4. Insert a sterile wood applicator into the broth of a positive tube to a depth of >1 inch to wet the end. Transfer the organisms on the wetted end of the applicator to a fermentation tube of EC with MUG (E. coli confirmatory broth) by briefly immersing the wet end of the applicator into the broth.
5. Discard the applicator.
6. For each positive Presumptive tube, use a fresh applicator stick and repeat these steps above.
7. Incubate the EC-MUG tubes in a 44.5°C water bath.

*Third day: Reading Confirmed Tubes*

1. Examine the EC tubes growth plus gas. Tubes showing both are scored as confirmed positive for fecal coliforms.
2. Examine the positive EC tubes under a long wavelength UV light. Tubes showing bluish fluorescence under long wavelength UV light (in addition to growth plus gas) are scored as positives for *E. coli*.

*Calculation of Most Probable Number*

Calculate the total and fecal coliform and *E. coli* densities of your sample from the number of positive and negative tubes using the MPN calculator provided.
The membrane filtration assay

Purpose
Membrane filtration is a method to count the numbers of bacteria in a water sample. It is one of the USEPA-approved gold standard methods for determining whether drinking water is of acceptable microbial quality. It is also applicable to a range of other water types and water sampling needs. You will measure the levels of fecal coliforms and E. coli in water from Morgan Creek, which receives effluent discharges from the OWASA wastewater treatment plant.

Principle
Membrane filtration uses filter membranes with pore sizes small enough to catch bacteria in a water sample as the water passes through the membrane. The standard membrane filter has a pore size of 0.45µm, small enough to retain most bacteria in water. In the procedure, the membrane is placed at the bottom of a filter funnel. Water samples are pulled through the funnel using a vacuum, and the bacteria are retained on the surface of the membrane. The membrane is then placed on the surface of an agar plate. Nutrients can diffuse up through the membrane to the bacteria, and they will grow and form colonies on the surface of the membrane which can be counted. The MF method is thus a direct count method (as opposed to the MPN, which is a quantal method). One of the advantages of the membrane filter is that you can pass a large volume of sample through the filter, making it possible to detect low numbers of bacteria in large volumes of water. The MPN, on the other hand, is limited to the amount that can be inoculated into a relatively small tube of media.

The use of solid media instead of liquid media is an alternative approach to the simultaneous detection and quantitation of coliforms and E. coli in water and other samples. There are many types of media available for simultaneously detecting fecal coliforms and E. coli in water. Many are based on 1) detection of lactose breakdown for identifying coliforms (caused by the activity of the β-galactosidase enzyme) and 2) detection of enzymatic activity that is specific to E. coli.

One such activity is the presence of the enzyme beta-glucuronidase, which is found only in E. coli and some Salmonella and Shigella. Tests for this enzyme have been developed in which a beta-glucuronidase substrate, a hydrolysable beta-glucuronide compound, such as MUG (4-methylumbelliferyl-beta-D-glucuronide) is incorporated into the agar medium. These substrates work because when they are broken down by beta-glucuronidase, they change color. For example, if E. coli is present, the MUG is hydrolyzed to yield a fluorogenic product.
(4-methylumbelliferone) whose blue fluorescence can be readily seen by shining a long wavelength UV light onto the culture. The MUG substrate can be added to coliform and fecal coliform media so that when E. coli is present and grows in the culture medium broth, the medium fluoresces blue under long wavelength UV light.

Other tests for coliforms and E. coli have been devised in which the substrates can be detected because the hydrolysis products have a unique and distinguishable color. That color appears in the broth culture medium or in the bacterial colonies if the bacteria are cultured on a solid medium.

Some commercially available media and test systems for E. coli and coliforms use defined substrates for beta-galactosidase and beta-glucuronidase to simultaneously detect and quantify coliforms and E. coli, respectively, by characteristic color changes resulting from hydrolysis of the defined substrates. These substrates are present as the only food supply for the bacteria, resulting in tests with high specificity in detecting the target bacteria, coliforms or specifically E. coli. We will use such a test, based on defined chromogenic substrates that detect the target bacteria as colonies on a membrane filter on a solid medium. For this test, we will use BioRad Rapid E. coli 2 agar.

**Materials**

- pipettes
- vacuum flask
- membrane filters
- BioRad plates
- forceps
- filter funnels
- Bunsen burner
- beaker of 70% ethanol
Protocol

First day: Filtration

1. You will filter 200 mL, 20 mL, and 2 mL samples in duplicate. Label six plates.
2. Set up a vacuum assembly and connect the lower section (base) of a filter unit to it. Be sure to handle the base using aseptic technique (avoid touching the top surface of the base on which the membranes are placed).
3. Dip forcep tips in ethanol and pass through flame to sterilize.
4. Use a sterile forceps to put (aseptically) a membrane filter, grid side up, on the surface of the filter base. Align and attach the upper, funnel section of the filter unit to the base.
5. Starting with the 2 mL sample, shake the sample bottle well.
6. Pipet exactly 2 ml into the funnel.
7. Turn on the vacuum to filter the water through the membrane.
8. As soon as the sample has filtered, squirt sterile diluent water onto the inside of the filter funnel and allow it to filter through the membrane.
9. Turn off the vacuum.
10. Aseptically remove the filter funnel from the filter base and set aside (turn upside down on a sterile surface).
11. Using a pair of flat-blade forceps, lift the membrane filter from the base by its outermost edge.
12. Place it grid side up onto the surface of a plate. The filter is applied to the medium by "rolling" it onto the surface. Avoid trapping air bubbles under the filter.
13. Repeat with the 20 mL and 200 mL samples.
14. Incubate all plates inverted (lid down) in a 35°C air incubator for 2 hours.
15. After 2 hours of incubation remove plates and place in a 44.5°C incubator for an additional 18 hours.

Second day Reading Results (after 22 +/- 2 hours of incubation, total).

Remove the plates from the incubator. Count the colonies of both colors. You may have to carefully remove the lids from the plates to clearly see the colonies on the filter surface. If possible, count plates having 20-80 TC colonies and 20-60 FC colonies. However, count all plates, even if they have fewer than 20 colonies per plates. Plates with >100 colonies are
considered "too numerous to count"; do not count colonies in these plates. Bio-Rad TC/E. coli chromogenic medium: TC colonies are blue to blue-green (Gal+/Gluc-) and E. coli colonies are violet to pink colonies (Gal+/Gluc+), Figure 1 below.

![Figure 1](image)

**Recording Data and Calculations**

Record the numbers of FC and E. coli colonies for each of the media tested above. Calculate the fecal coliform and E. coli numbers. Bring your data to the next laboratory class.

**Computing Bacteria Concentrations**

Using the individual colony counts of each replicate filter at the most countable dilution(s), calculate the total and fecal coliform and E. coli concentrations of each replicate sample per 100 ml. To do these calculations, you must first determine which sample dilution(s) is (are) most countable. For total coliforms and E. coli, the desired counting range is 20 to 80 colonies per filter; for fecal coliforms it is 20 to 60 colonies per filter. If the number of colonies per filter are above the countable range at all dilutions at all dilutions tested, estimate the number of colonies by counts from plates having >80 (or >60) but <200 colonies per filter. Compute the estimated concentration per 100 ml and report as a "greater than (>)" value. If the membrane filter counts of all dilutions are below 20 per filter, sum the colony counts for all filters and the sample volumes filtered for all filters and then compute the number of colonies per 100 ml; report as an approximate count.

Additionally, estimate the 95% confidence intervals. Using the RAW data for all countable filters (all filter counts used in the calculation of concentration per 100 ml), compute the 95%
confidence interval from the relationship between the variance and the mean of the Poisson distribution:

\[ S^2 = X, \]

where \( S^2 \) = the variance and \( X \) = the mean count (or total count in this lab)

Then compute \( S \), the square root of the mean:

\[ S = \pm (X^{1/2}) \]

We will take the upper and lower 95% confidence limits as \( \pm 2S \).

After computing \( \pm 2S \) values, apply the appropriate dilution factors to obtain the upper and lower 95% confidence limits per 100 ml, as done for the mean value of counts. For the membrane filter results, answer the same questions posed for the results of the Multiple Fermentation Tube (MPN) analysis.
Instructions for lab Write-up

Each group will calculate the results from their own experiments, and we will then pool the class data.

1. Using your data from laboratory #1, calculate the titer of the *E. coli* culture you did by spread plating in CFU per mL.
2. From the MPN assay, calculate the MPN of total coliforms, fecal coliforms, and *E. coli* in Morgan Creek water from your experiment.
3. From the membrane filtration assay, calculate the number of fecal coliforms, and *E. coli* in Morgan Creek water from your experiment in CFU per mL.
4. Put your data on the whiteboard in the lab. Record the class data in your notebook.

Questions

**Lab 1: titer assay**

1. Graph the results from all lab groups for the titer assay from lab 1.
2. For the titer assay: What is the average result for the class? How do the numbers compare among groups? How is your technique?

**Lab 2: Morgan Creek water**

1. Graph the results for Morgan Creek water from all lab groups for both methods.
2. For the two methods (multiple fermentation tube and solid media) how well do the results for the replicate analyses of each sample agree? Specifically, are they identical? If not, does the MPN value for one replicate fall within the 95% confidence limit of the other replicate?
3. For each sample, compare the traditional multiple fermentation tube MPN concentrations of total coliforms, fecal coliforms and *E. coli*. Which indicator concentration is highest? Which is lowest? Are these results consistent with what you would expect, based upon the definitions of each indicator and the expected relationships among them?
4. How do the numbers compare among groups? How is your technique?
5. Considering that the raw sewage was treated and then discharged to Morgan Creek, what is the potential impact of the sewage effluent discharge on the concentrations of indicator bacteria in Morgan Creek?