INTRODUCTION AND BACKGROUND

Traditionally, indicator bacteria have been used to determine the possible presence of fecal contamination and to estimate the amount of 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 and wastewater in much higher numbers than are pathogenic microorganisms and because they are technically easier to detect and quantitate than pathogens. Present standards for the sanitary quality of water, foods and other materials, with respect to fecal contamination, are based on concentrations of indicator bacteria.

The most widely used indicator bacteria in the U.S. are the so-called total and fecal coliforms. Although taxonomically meaningless, the word “coliform” has been used to describe various genera of the family Enterobacteriaceae that ferment lactose. It should be emphasized that “coliforms” are actually defined in operational terms that are based upon the media and incubation conditions used for their isolation and quantitation. None of these operational definitions detects all members of the family Enterobacteriaceae and some include members of other families.

Historically, the term “coliform” was meant to include all of the lactose-fermenting species of the family Enterobacteriaceae which are commonly found in the feces of man and the higher animals and to exclude genera of non- or slowly-lactose-fermenting bacteria, some of which are enteric pathogens in man and animals and some of which exist naturally in the environment. Thus, lactose fermentation is a characteristic of considerable diagnostic importance in distinguishing among the various groups of enteric bacteria. 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.
Two major operational definitions of coliforms have been distinguished: “total coliforms” and “fecal coliforms” or “thermotolerant coliforms”. The latter group (fecal or thermotolerant coliforms) are considered a more specific indicator of fecal contamination because the former group may include bacteria of non-fecal origin. Fecal or thermotolerant coliforms are able to grow at higher incubation temperatures and are considered more likely to have come from the intestinal tract of a warm-blooded animal.

**Total Coliforms**

Total coliforms are operationally defined in two different ways in the 17th edition of *Standard Methods for the Examination of Water and Wastewater*

(1). The Multiple-Tube Fermentation Technique defines coliforms as “all aerobic and facultative anaerobic, gram-negative, non-sporeforming, rod-shaped bacteria that ferment lactose with gas formation within 48 hr. at 35°C.” In this method the coliform bacteria are detected and quantitated by their ability to grow and produce gas in lactose-containing liquid medium under specified incubation conditions. This technique 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 48 hr. 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 and incubated at 35°C for 48 hr. Tubes showing both growth and gas are considered positive Confirmed tubes. 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.

(2) The Membrane Filter Technique defines total coliforms as “all organisms that produce a colony with a golden-green metallic sheen within 24 hr. of incubation at 35°C on Endo-type medium containing lactose and are aerobic or facultative anaerobic, gram-negative, non-spore-forming rods.” In this method, measured volumes of sample or sample dilution are vacuum-filtered through 47mm diameter, microporous cellulose membrane filters that retain bacteria. The membranes are transferred to petri dishes containing endo-type lactose medium and incubated under specified conditions for the development of bacteria colonies. Colonies having the characteristic appearance of coliforms are counted and the coliform bacteria density is computed.

Although the total coliform group as presently operationally defined includes bacteria usually found in the feces of humans and other warm-blooded animals, some of the bacteria detected by these procedures are sometimes also found in soil (*Citrobacter, Enterobacter* and *Klebsiella*) on various plants, including grains and trees (*Klebsiella* and *Enterobacter*) and in certain industrial wastes. Furthermore, some coliform bacteria can also be pathogenic for humans and animals when present either in the gut (enteropathogenic *E. coli*) or in other parts of the body (*Klebsiella pneumonia* in the respiratory tract). In addition, organisms that are not members of the family
Enterobacteriaceae and not of strictly intestinal origin, such as the members of the genus Aeromonas, may be detected in some forms of the total coliform test. For these reasons, another group, the “fecal” or “thermo-tolerant coliforms,” has been established in an attempt to separate the total coliforms into those of fecal and non-fecal origin.

Fecal Coliforms

The basis for this distinction is a higher incubation temperature of 44.5ºC, at which presumably coliforms of only fecal origin will grow. Coliforms from non-fecal, environmental sources are generally incapable of growing at this elevated temperature. Thus, fecal coliforms can be defined as gram-negative, non-spore-forming, rod-shaped bacteria which ferment lactose with the production of gas at 44.5ºC within 24 hr. Although the fecal coliform test is applicable to investigations of surface and ground water pollution, sewage treatment systems and general monitoring of natural waters for sanitary quality, including recreational and shellfish waters, it is so far not considered a substitute for the coliform test in the examination of potable waters. Coliform bacteria of any kind are not to be tolerated in a finished (treated) drinking water.

There are actually two approved Multiple Tube Fermentation Techniques for fecal coliforms: a single-step and a two-step procedure. The latter is the traditional test that has been widely used for many years, and the former is a more recent test that appears in the 17th edition of Standard Methods. In this single-step procedure dilutions of the sample are inoculated into fermentation tubes of A-1 medium (see Standard Methods 17th ed.). The tubes are first incubated for 3 hr at 35ºC and then transferred to a 44.5ºC water bath for an additional 21 hr of incubation. Tubes showing growth plus gas are considered positive for fecal coliforms. For the two-step Multiple Tube Fermentation Technique for fecal coliforms, positive tubes from the Presumptive (total) coliform test are inoculated into fermentation tubes of EC medium and incubated at 44.5ºC for 24 hr. Tubes showing growth with gas production are considered confirmed positives.

In the Membrane Filter procedure, samples are filtered onto membranes as in the total coliform test, the membranes are placed onto plates of m-FC medium, sealed in water-tight plastic bags, and submerged in a 44.5ºC water bath incubator for 24 hr. Colonies with a characteristic fecal coliform appearance are then counted and fecal coliform density is computed.

\textit{Escherichia coli} (E.coli)

In recent years, there has been increased interest in detecting \textit{E. coli} exclusively as the lactose-fermenting “coliform” that invariably indicates fecal contamination. An early approach to this effort was the use of four biochemical tests to separate \textit{E. coli} from other lactose-fermenting \textit{Enterobacteriaceae}:

\textbf{Indole test} – detects indole production from tryptophane. \textit{E. coli} is positive (+); many other coliforms are negative.

\textbf{Methyl Red test} – detects acid production in the medium; intended to distinguish between type of fermentation reaction (mixed acid vs. butylenes glycol). \textit{E. coli} is (+) and some other
coliforms are (-).

**Voges-Proskauer test** – detects acetoin, an intermediate in the butylene glycol pathway. Acetoin is oxidized to diacetyl under alkaline conditions in the presence of air, and when reacted with creatine, it forms a pink color. *E. coli* is (-) and some other coliforms are (+).

**Citrate** utilization as sole carbon source. *E. coli* is (-) and many other coliforms are (+).

Therefore, in this series of four tests, called the IMViC tests, *E. coli* is typically ++-- and *Enterobacter* aerogenes is typically --++. Other coliforms give different reaction patterns and are designated “intermediates”. However, the IMViC reactions have been found to be imperfect in speciating *E. coli*, and subsequently other approaches to *E. coli* and coliform speciation have been developed. Two approaches are worth noting here. One is the use of rapid, commercial, biochemical test kits designed to carry out several biochemical tests simultaneously in incubation periods of 4 to 24 hours. The results of each test are scored as (+) or (-) and assigned a number based on the relative reliability of the test. These results constitute a “code” which is compared against a data base of accumulated reaction codes for most of the medically important *Enterobacteriaceae*. The code that is identical or closest to the organism tested is taken as its identity at the genus and species level.

Another approach is the use of a single property or biochemical test in addition to lactose fermentation to definitively identify *E. coli* among other coliforms. One such property is the presence of the enzyme beta-glucuronidase, which is found only in *E. coli* and some *Salmonella* and *Shigella*. A rapid test for this enzyme has been developed in which the beta-glucuronidase substrate MUG (4-methylumbelliferyl-beta-D-glucuronide) is incorporated into a coliform medium. If *E. coli* is present, the MUG is hydrolyzed to yield a fluorogenic product (4-methylumbelliferone) whose fluorescence can be readily seen by shining a long-wavelength UV light onto the culture.

**Membrane Filter Methods for Total and Fecal Coliforms and *E. coli***

As noted above, *E. coli* and other coliform bacteria can be analyzed in water and other environmental samples by membrane filter (MF) as well as other methods. The methods for total and fecal coliform analyses are well-known and are described in standard reference works (APHA, 1985; 1989). A membrane filter method for *E. coli* was developed more recently (Dufour et al., 1981), and it is now standardized for routine use in recreational waters (U.S. EPA,
1985; APHA, 1989). A more recent development in MF methods for *E. coli* is the transfer of TC and FC membrane filters with their colonies to nutrient agar containing MUG. After 4 hours of incubation at 35°C, the colonies are examined under long wavelength UV light, and those colonies fluorescing blue are considered *E. coli* (Mates and Shaffer, 1989; Fed. Reg., 1991).

**TODAY’S EXPERIMENT:**

**PURPOSE:** To estimate the total and fecal coliform and *E. coli* concentrations in samples of surface water and wastewater by Multiple-Tube Fermentation and Membrane Filtration Techniques.

Work in pairs.

**MATERIALS (per group):**
- Samples of water or wastewater for analysis. You will be assigned samples.
- Dilution bottles, each containing 99ml of peptone water (0.1%) diluent.
- Pipets: 1, 5, 10 and 20 ml volume, serological.
- Marking pen or grease pencil.
- Water bath: set at 44.5°C for fecal coliforms and *E. coli* analysis.
- Air incubator: set at 35°C for total coliform analysis.

**Multiple Fermentation Tube Methods**
- Lauryl tryptose or lactose broth tubes containing inverted gas tubes (25 tubes per sample).
- Brilliant green lactose bile broth (BGLB) tubes containing inverted gas tubes (no more than 25 per sample).
- EC+MUG medium tubes containing inverted gas tubes (no more than 25 per sample).
- Long wavelength UV light, to shine on EC+MUG tubes when looking for blue fluorescence indicative of MUG hydrolysis (beto-glucuronidase activity).
- Wooden applicator sticks, sterile.
- Test tube racks, for fermentation tubes.

Use **Aseptic Technique** for all operations; do not mouth pipet.

**PROCEDURES**

**Sample Dilution:**

Dilute the samples(s) serially 10-fold as directed by the instructor.
Mix the sample by shaking 25 times before diluting.

To make 10-fold dilutions, pipet 11 ml of sample into a 99 ml dilution blank. Seal the top of the dilution container and mix vigorously 25 times.
NOTE: Before making dilutions, adjust the diluent volumes in dilution bottles to 99ml as directed by the instructor.

PROCEDURES: MULTIPLE FERMENTATION TUBE METHODS

Two-Step-Tube Fermentation Technique for Total and Fecal Coliforms and E. coli (5 tubes per dilution):

Day 0: Inoculation of tubes for presumptive test:

Place 25 fermentation tubes in a test tube rack as groups of 5 dilutions, and mark the tubes as to their dilutions.
Using a 5 or 10 ml pipet, inoculate 1 ml volumes of each sample dilution to be tested into the 5 replicate tubes.
Incubate the tubes in a 35°C air incubator.

After 24 and 48 hr: Reading of presumptive tubes and inoculation of tubes for Confirmed Total and Fecal Coliform Tests and for E. coli:

Gently shake the rack of tubes back and forth several times to release gas in positive tubes.
Examine all tubes for the presence of growth (turbidity or cloudiness) and gas (look in the small inverted tube), and score tubes showing both as presumptive positive.
Submit all lactose broth fermentation tubes that are Presumptive positive at about 24 hr and all additional Presumptive positive tubes at 48 ±3 hr to the confirmed tests.

Insert a sterile wood applicator into the broth of the positive tubes 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 brilliant green lactose bile (BGLB) broth by briefly immersing the wet end of the applicator into the BGLB broth.
Use the same applicator and same procedure to transfer material from the same Presumptive positive tube to a fermentation tube of EC medium containing MUG. Discard the applicator. For each positive Presumptive tube, use a fresh applicator stick.
Incubate the BGLB broth tubes in a 35°C incubator and the EC tubes in a 44.5°C water bath.

Reading Confirmed Tubes

Examine the EC tubes at 24 ±2 hr for growth plus gas.
Tubes showing both are scored as Confirmed positive for fecal coliforms.
Examine the positive EC tubes under a long wavelength UV light. Tubes showing bluish fluorescence are scored as E. coli.
Examine the BGLB tubes at 48 ±3 hr for growth plus gas.
Tubes showing both are scored as Confirmed positive for total coliforms.
Calculations of Most Probable Number

Calculate the total and fecal coliforms and *E. coli* densities of your sample(s) from the number of positive and negative tubes of three sample dilutions according to the procedures described in *Standard Methods* and below. Bring these results to the next scheduled laboratory period.

MATERIALS: MEMBRANE FILTER METHODS

Water or wastewater sample – assigned by instructor.
Membrane filter apparatus – consists of membrane filter holder, vacuum flask and vacuum manifold assembly.
Membrane filter – mixed cellulose esters, 47 mm diameter, 0.45 μm pore size, gridded (“up” side has a grid pattern).
Flat blade forceps – for holding petri dishes of media.
50-60 mm diameter petri dishes of media:
  - mEndo agar for total coliforms
  - mFC agar for fecal coliforms
Nutrient agar containing 100 μg/ml MUG for *E.coli*.
Washing bottles – containing sterile peptone water; to rinse interior of filter funnel after filtering samples.

PROCEDURES: MEMBRANE FILTER METHOD

Starting with the highest sample dilution to be tested, shake the sample 25 times, remove the bottle cap and pipet exactly 20 ml into the funnel.

Then turn on the vacuum to filter the 20 ml through the membrane.
For each sample dilution, filter a 20 ml volume per medium (2 filtrations).
Rinse the interior walls of the funnel by filtering about 20 ml of dilution water dispensed from a squeeze bottle.

After filtration, turn off the vacuum. Each filter holder section of the vacuum manifold has its own shut-off valve.

Carefully remove the funnel from the base. Use a sterile forceps to remove the filter, and apply it, grid side up, to the surface of a 50 or 60 mm diameter petri dish containing either m-Endo (total coliform) or m-FC (fecal coliform) agar. The filter is applied to the agar by “rolling” it onto the surface. Avoid trapping air bubbles under the filter.

Apply new filters to the filter base and repeat the sample filtration procedure so that individual plates are prepared for total and fecal coliform/*E.coli* tests.
Repeat this membrane filtration procedure for the next three lowest sample dilutions (1:1,000, 1:100, and 1:10; check with instructor for raw sewage dilutions to be filtered).

Incubate all plates inverted in a 35°C air incubator. After 2 hours of incubation remove fecal coliform (mFC) plates from the 35°C incubator, and place them in a 44.5°C incubator/water bath.

**Reading Results** (after 22 ±2 hours of incubation, total).

Remove the plates from the air incubator. Count the total and fecal coliform colonies. 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 colonies. However, count all plates, even if they have fewer than 20 colonies per plate. Plates with >100 colonies are considered “too numerous to count”.

Total coliform colonies are pink to dark red and some (but not all) have a greenish gold metallic sheen.

Fecal coliform colonies are blue.

**Detection of E. coli on Fecal Coliform Membrane Filters (mFC medium)** (Procedure of Mates and Shaffer)

Transfer membranes having countable colonies on mFC to nutrient agar-MUG plates. Incubate 4 hr at 35°C. Then, observe the colonies under long wavelength UV light. Colonies having typical FC appearance and fluorescing blue are *E. coli*.

Calculate the total and fecal coliform and *E. coli* densities as described in Standard Methods and in the next section of the lab handout. Bring your data to the next laboratory class.

**RESULTS AND DATA TABULATION AND ANALYSIS**

**Multiple Fermentation Tube Method**

Compute and record the Most Probable Number of total and fecal coliforms and *E. coli* per 100 ml using the data from the confirmed tubes (BGLB and EC/MUG). Also record the upper and lower 95% confidence limits for these MPN values. The MPNs and confidence limits are found in the table for three dilutions and five tubes per dilution in *Standard Methods for the Examination of Water and Wastewater*, 17th edition.

Note that the MPN table in *Standard Methods* is set up for a dilution series of 10, 1, and .01 ml sample volumes. The sample volumes examined in your samples are smaller (1, 0.1, and 0.01 or even less), and therefore, the MPN values in the table have to be multiplied by factors of 10 (or more for some samples) to correct for the difference in sample volumes analyzed.

**Computing Bacteria Concentrations for Membrane Filter Results**
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 is 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 is above the countable range at all dilutions, 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 11 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 amount.

**Estimating 95% confidence intervals**

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

\[ s^2 = \bar{x}, \quad \text{where} \ s^2 = \text{the variance and} \ x = \text{the mean count (or total count)}. \]

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 counts.

**QUESTIONS**

How well do the results for the multiple fermentation tube (Most Probable Number) and membrane filtration methods compare? Are the estimated bacterial concentrations equivalent?

Specifically, do the MPN values and MF values fall within the calculated 95% confidence limits of the other method? For each sample, compare the MPN and MF concentrations of total and fecal coliforms and *E. coli*. Which indicator concentration is the highest? Which is lowest? Are these results consistent with what you would expect, based upon the definition of each indicator and the expected relationships among them?

Recall that the four samples analyzed were: raw sewage, treated sewage effluent, Morgan Creek (the receiving water) upstream of the sewage effluent discharge, and Morgan Creek downstream of the sewage effluent discharge. Compare the levels of the three indicators in the four samples. Which sample had the highest levels of indicators? Which the lowest? How effective was the sewage treatment plant in reducing the concentrations of indicator bacteria in raw sewage? (Sewage treatment at this plant consists of (i) primary settling, (ii) secondary (biological) treatment and (iii) chlorine disinfection of the secondary-treated effluent.) Considering that the raw sewage was treated and then discharged to Morgan Creek, what is the impact of the sewage effluent discharge on the concentrations of indicator bacteria in Morgan Creek?
REFERENCES

American Public Health Association (1985) and (1989), Sections 908 and 909, Standard Methods for the Examination of Water and Wastewater, 16th and 17th editions, American Public Health Association, Washington, D.C.


Colonies of fecal coliform bacteria filtered from water samples and grown on mFC nutrient agar are indicators of fecal contamination of the water.(www.ars.usda.gov)