Coliform

VT WSMD Wastewater Program Lab Manual Section #13

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Escherichia coli Membrane Filtration Method

Background

Some facts about *E. coli* that help us to identify and enumerate include:

1) It is thermotolerant at 44℃. Some bacteria cannot survive at this temperature, but *E. coli* can function well at 44℃.
2) It is a Gram-Negative Bacteria. The inclusion of sodium laurel sulfate and sodium deoxycholate in the media offer selectivity against gram-positive bacteria. It is easily differentiated from gram-positive bacteria.
3) It ferments lactose. This is an important point as fermentation of lactose results in the production of acid and gas at both 35℃ and 44.5℃. Many bacteria cannot ferment lactose.
4) It can hydrolyze tryptophan to indole. The indole test is one method of identifying *E. coli*.
5) It hydrolyzes methyl umbellyferyl beta D glucuronide to glucuronic acid umbellyferyl. This causes fluorescence which is easily detected in the MUG test.

Just as important for identification purposes is what *E. coli* does not do:

1) It does not hydrolyze urea. This makes it easily identified when transferred to the urea substrate.
2) It does not utilize sodium citrate. This makes confirmation of colonies easy. By transferring questionable colonies to Simmon's citrate agar and incubating for 24 hours at 35℃ we can easily confirm *E. coli* as other coliform will grow colonies but *E. coli* will not.

Equipment

- Autoclave, dry oven or other type of sterilizer
- Kraft paper
- Aluminum foil
- Autoclave indicator tape
- Masking tape
- Funnel assembly - 47 mm, autoclavable
- Membrane filters - made specifically for microbiology
    - cellulose nitrate or cellulose nitrate/cellulose acetate
    - 47 mm, sterile, white, gridded on one side
    - 0.45 um ± 0.02 um pore size
- Absorbent pads - 47 mm, sterile
- Petri dishes - 50 mm x 12 mm, plastic, sterile, tight-fitting halves
- Forceps, straight or curved, with smooth tips to handle filters without damage
    - Beakers - 50 mL, 250 mL
- Vacuum pump
- Vacuum flask 500 mL
    - Fitted with rubber stopper and glass tubing or a filter manifold for holding a number of filter bases
- Vacuum flask 1000 mL

  - For safety trap-placed between the filter flask and the vacuum source.
- Tubing - thick walled tygon or rubber
- Plastic bags - 18 oz. Whirl-pak or similar waterproof bag, e.g. Ziploc
- Water bath - must be able to maintain a temperature of 44.5℃ ±0.2℃.
- Incubator - air, must be able to maintain a temperature of 35.0℃ ±0.5℃.
- Thermometer† - range of 20℃ to 50℃ with no larger than 0.2℃ graduations.
- Sample bottles - glass or plastic (polypropylene), autoclavable
- Fluorescent lamp - top lighting only
- Microscope or magnifying glass to give at least 5x magnification
- Colony counter - hand held
- Graduated cylinders - 100 mL, 1000 mL
- Volumetric flasks - 250 mL
- Erlenmeyer flasks - 250 mL, 250 mL screw top, 2000 mL screw top
- Graduated pipet - 5 mL, 25 mL
- Bacteriological pipets - 10 mL, 1 mL
- Refrigerator - capable of maintaining 4℃
- Stirrer magnet - bar
- Magnetic stirrer
- Burner - alcohol or Bunsen
- Parafilm or volumetric flask covers
- 0.45 um filters (Millipore HA, Gelman GN 6 Metric, MFS (Micro Filtration Systems), or similar)

† Thermometers should be checked at least once a year against a certifiable NIST (National Institute of Standards Technology) thermometer or one traceable to an NIST thermometer. Any deviation from the NIST thermometer should be noted and taped to the thermometer, e.g. reads 0.1℃ high at 44.5℃. Readings taken from this thermometer should always be adjusted according to the deviation.

Cleaning and Sterilization of Equipment

As discussed earlier in this manual, glassware used for bacteria testing should not be acid washed. This also applies to any equipment such as spatulas, stirrer bars, etc. with which reagents are made for coliform tests. Any acid residue on the glassware will kill bacteria. Wash bacteria glassware in hot, soapy water with a detergent that doesn't inhibit growth, e.g. Micro. Rinse glassware thoroughly in hot tap water and then with distilled or deionized water at least 3 times.

Sterilization of bacterial glassware such as sample bottles, dilution bottles, funnel setups and pipets can be accomplished using an autoclave set at 15 psi and 121℃ or a dry oven set at 170℃. Keep in mind that reusable plasticware or liquids should never be sterilized in a dry oven.

Items such as funnels and pipets should be wrapped in Kraft paper and secured with autoclave indicator tape before being placed in the autoclave to allow the steam to penetrate. (Glassware sterilized in a dry oven may be wrapped in aluminum foil.) Also, always loosen the caps of sample and dilution bottles before autoclaving them.

The 10% sodium thiosulfate solution should be added to the sample bottles BEFORE they are sterilized. 0.1 mL (2 drops) will neutralize the chlorine in 100 mL of effluent. For effluents disinfected by ultra-violet light, the addition of sodium thiosulfate isn't necessary.

It is advisable to place a piece of autoclave indicator tape on equipment to be sterilized so that it can easily be identified later as having been processed. Autoclave indicator tape has invisible writing which appears after 15 minutes in the autoclave.

The following chart lists several items commonly used in Fecal Coliform analysis along with the required sterilization time for each. If your autoclave includes instructions indicating longer sterilization periods, follow those instructions except in the case of media, which should never be autoclaved for more than 15 minutes.

Item	Autoclave	Dry Oven
Sample Bottles	30 min	2 hours
Pipets	30 min	2 hours
Funnels	30 min	2 hours
1 L Dilution H_2O	35 min	
Used Petri Dishes	30 min	2 hours

Sample Collection, Preservation and Holding Time

E. coli samples should be taken at the end of the outfall pipe or at the weir in the chlorine contact chamber, or at the outfall from the de-chlorination tank if your facility is required to dechlorinate. These samples must be taken as grabs into clean, sterile, sodium thiosulfate treated bottles. It is important to take the sample directly into the sterile bottle. Do not transfer an *E. coli* sample from a dipper to the sterile sample bottle because of contamination problems. Allow about 1 inch of headspace for efficient mixing and shake the sample to mix it with sodium thiosulfate. It is usually required that you take the chlorine residual at the same time to establish a correlation between the two. Process the sample as soon as possible, but always within 8 hours. If immediate processing isn't possible, refrigerate the sample at 4℃. If a sample is sent to another lab for analysis, it **must** be kept in a cooler with ice and arrive at the other lab within 6 hours.

Reagents

Order only reagent grade chemicals. Store chemicals and media as indicated on the container label. **IT IS HIGHLY ADVISABLE TO USE ONLY COMMERCIALLY PREPARED MEDIA.** Refrigerate media. Discard media if the expiration date has passed. Also use only high-grade distilled water for reagent preparation.

- 70% Ethanol, methanol, or isopropanol in a small wide mouth container for flame-sterilizing forceps
- Phosphate Buffered Dilution Water:

NaH2PO4	-	Sodium Dihydrogen Phosphate	0.58 g
NaHPO4	-	Sodium Monohydrogen Phosphate	2.50 g
NaCl	-	Sodium Chloride	8.50 g

Preparation: Dissolve the ingredients in 1 L of high-grade distilled water in a flask and dispense in appropriate amounts in screw-cap bottles or culture tubes, and/or into containers for use as rinse water. Autoclave after preparation at 121℃ (15 lbs pressure) for 15 minutes. Final pH should be 7.4 ± 0.2.

- mTEC Agar (Difco 0334-15-0)

Composition:

Proteose Peptone	5.0 g
Yeast Extract	3.0 g
Lactose	10.0 g
NaCl	7.5 g
Dipotassium Phosphate	3.3 g
Monopotassium Phosphate	1.0 g
Sodium Laurel Sulfate	0.2 g
Sodium Deoxycholate	0.1 g
Brom Cresol Purple	0.08 g
Brom Phenol Red	0.08 g
Agar	15.0 g

mTEC prepared Agar plates and urea substrate can be purchased from multiple sources, including:

Northeast Laboratory
PO Box 788
Waterville, Maine 04901
Telephone: 207-873-7711.

Preparation: Use commercially prepared media if possible. If you have no other option prepare the media by adding 45.26 g of dehydrated mTEC medium to 1 L of reagent grade water in flask and heat to boiling, until ingredients dissolve. Autoclave at 121℃ (15 lb pressure) for 15 minutes and cool in a 44-46 ℃ water bath. Pour the medium into each 30 x 10 mm culture dish to a 4-5 mm depth (approximately 4-6 mL) and allow to solidify. Final pH should be 7.3 ± 0.2. Store in refrigerator. Hold time 1 month.

- Urea Substrate Medium

Composition:

Urea	2.0 g
Phenol Red	0.01 g

Preparation: Use commercially prepared urea, substrate medium if possible. If commercially prepared reagent is not available. Add dry ingredients to 100 mL reagent grade water in a flask. Stir to dissolve to pH 5.0 with a few drops of 1N HCl. The substrate solution should be a straw yellow color at pH 5.0 ± 0.2. Store at 6℃ - 8℃ for 1 week.

Calibration and Standardization

Check temperatures in incubators daily to insure operation within stated limits.

Check thermometers at least annually against an NIST certified thermometer or one traceable to NIST.

Procedure

Prepare the mTEC agar and urea substrate. Mark the petri dishes and report forms with sample identification and sample volumes. It is best to label the bottom and top portions of the petri dishes. Pour about 1" of ethanol into a 50 mL beaker. Keep the forceps in it all the time except when handling a filter. This ethanol keeps the forceps sterile. Unwrap a funnel and place it aseptically on the vacuum flask. Open the filter by the edge with the forceps. Place the filter on the funnel base grid side up and the funnel head on the filter. Pour 100 mL sterile dilution water onto the filter. This is the blank. Turn on the vacuum pump and draw the blank through the filter. Rinse the sides of the funnel twice with 20-30 mL of dilution water. Turn off the vacuum pump and loosen the funnel head. Open the petri dish labeled "Blank 100 mL," and place the cover inside up on the work surface. Loosen the funnel head. Flame the forceps. Take the funnel head off with one hand and pick up the filter with the other. Always grasp the filter by the edge. Place the funnel head back on the base. Place the filter mTEC on the media. Pull back the leading edge of the filter and roll it forward to exclude air as described below.

Try to place the filter into the plate without dragging it across the edge of the plate. Repeat this procedure at the other 3 "corners" of the filter. Bubbles prevent media from soaking into the filter, and any bacteria over a bubble won't grow. Although it may be tempting to poke at bubbles to get rid of them, the rolling back procedure works better. Cover the plate, invert, and incubate at 35℃ for 2 hours. Shake the sample bottle vigorously to distribute the bacteria uniformly and measure the desired volume of sample into the funnel. Filter the sample just as you did the "blank" beginning with the smallest volume. For 10 mL or less, pour about 20 mL of dilution water onto the filter before adding the sample. This allows the bacteria to disperse and not grow in one clump. Always filter 3 different volumes of sample, e.g. 0.1 mL, 1 mL and 10 mL, to hopefully produce a readable filter. Shake the sample vigorously 25 times each time before pipetting off a volume. After the sample has been filtered, run a "positive". A positive is generally a drop of influent, or effluent collected before disinfection, filtered the same as the blank and the sample. Growth on the positive filter assures that the media will grow *E. coli* colonies.

As described above for the "blank" use sterile forceps to aseptically remove the membrane filter from the filter base and roll it onto the mTEC agar to avoid the formation of bubbles between the membrane and the agar surface. Reseat the membrane if bubbles occur. Close the dish, invert, and incubate at 35℃ for 2 hours.

After 2 hours incubation at 35℃, transfer the plates to Whirl-Pak bags, seal and place inverted in a 44.5℃ water bath for 22-24 hours.

After 22-24 hours, remove the dishes from the water bath. Place absorbent pads in new petri dishes or the lids of the same petri dishes and saturate with urea broth. Aseptically transfer the membranes to absorbent pads saturated with urea substrate and hold at room temperature.

After 15-20 minutes incubation on the urea substrate at room temperature, count and record the number of yellow or yellow-brown colonies on those membrane filters ideally containing 20-80 colonies.

Verification Procedure

Questionable colonies from the urease test can be verified by many means. One fairly simple and effective method of verification includes the transfer of questionable colonies to a slant or plate containing Simmon's citrate agar.

Simmon's Citrate Agar (BBL 11619, Difco 0091-02)

Composition:

Magnesium Sulfate	0.2 g
Monoammonium Phosphate	1.0 g
Dipotassium Phosphate	3.3 g
Sodium Citrate	2.0 g
Sodium Chloride	5.0 g
Brom Thymol Blue	0.08 g
Agar	15.0 g

Preparation: Add 24.28 g of Simmon's citrate agar to 1 L of reagent water. Heat in boiling water bath with mixing for complete solution. Dispense in screw-cap tubes and sterilize at 121℃ (15 lb pressure) for 15 minutes. Cool tubes and slant. The final pH should be 6.9 ± 0.2.

Transfer questionable colonies onto Simmon's Citrate Agar slant (or plate). Incubate at 35℃ for 24 hours. It there is any growth the colonies are NOT *E. coli*.

Other verification procedures are described in "Test Methods for Escherichia Coli and Enterococci in Water by the Membrane Filter Procedure". Copies of this publication can be obtained from:

National Technical Information Services
5285 Port Royal Road
Springfield, Virginia 22161
Order #PB 86-158-052

The following section describing counting colonies and calculating results is edited from EPA Microbiological Methods for Monitoring the Environment.

Calculations of Results

Escherichia Coli results are reported as colony forming units (CFUs) per 100 mL.

The equation for calculating results is:

$$\frac{E.coli\ CFUs}{100\ mL} = \frac{\#\ of\ E.coli\ Colonies}{Sample\ Volume\ (mL)} * 100$$

The desired number of colonies to be counted on a plate is 20-80. Plates containing more than 80 colonies should be avoided as there is a great likelihood of false positive.

Therefore, if all plates produce more than 80 *E. coli* colonies the plate with the least # of countable colonies is used in calculating the results. But be sure to note that the result is an estimate based on a plate containing >80 colonies. For example:

> If sample volumes of 10, 30, and 50 mL produced results of 86, 120, 150 colonies respectively the result would be calculated as:
>
> $$\frac{86\ CFUs}{10\ mL} * 100 = Estimated\ \frac{860\ CFUs}{100\ mL}$$
>
> with a note in the "comments" section of the report stating that the result was based on a 10 mL sample which produced >80 colonies.

If only one plate produces 20-80 colonies, then only that plate would be used in calculating the result. For example:

> If sample volumes of 10, 30, and 50 mL produce results of 5, 15 and 25 colonies respectively, the result would be calculated as:
>
> $$\frac{25\ CFUs}{50\ mL} * 100 = \frac{50\ CFUs}{100\ mL}$$

If more than one plate produces colonies in the 20-80 range,an average must be calculated. That is: All values within the acceptable range of 20-80, are added together and their sum is divided by the total of their volumes. For example:

> If sample volumes of 10, 30, and 50 mL produce results of 20, 50 and 95 colonies respectively, the result would be calculated as:
>
> $$\frac{(20 + 50)\ CFUs}{(10 + 30)\ mL} * 100 = \frac{175\ CFUs}{100\ mL}$$

If none of the plates produce at least 20 colonies, the result is calculated using all the colonies counted divided by the total volume used. For example:

> If sample volumes of 10, 30, and 50 mL produce results of 1, 3 and 5 mL respectively, the result is calculated:
>
> $$\frac{(1 + 3 + 5)\ CFUs}{(10 + 30 + 50)\ mL} * 100 = \frac{10\ CFUs}{100\ mL}$$

If no colonies are produced on any of the plates (except of course the positive control) the results would be calculated using the largest volume and assuming 1 colony was produced on that plate. A < sign would be included with that result. For example:

> If sample volumes of 10, 30, and 50 mL all produce results of zero then the result would be calculated as:

$$\frac{1\ CFU}{50\ mL} * 100 = < \frac{2\ CFUs}{100\ mL}$$

Escherichia coli Troubleshooting Guide

PROBLEM	MOST LIKELY CAUSE	SOLUTION
Colonies are observed on the blank.	Autoclave/sterilizer not performing properly/non-sterile technique.	Verify that all equipment, glassware is sterile, forceps are dipped in alcohol and flamed before each use and good sterile technique is used throughout the procedure.
	Dilution water not sterile.	Watch dilution water carefully for growth or discoloration. Discard when either is observed.
No colonies observed in positive control.	Bacterial inhibitors in sample, sample container, or equipment.	Use sodium thiosulfate in sample collection bottle. Addition of EDTA to the collection bottle (before sterilization) can eliminate inhibition by several heavy metals.
All colonies are bundled together in center of filter.	Poor dilution technique.	Add dilution water to funnel first then add sample to allow dispersion of bacteria.
None of the dilutions used produced the desired 20-80 colonies (<20 on all plates).	Insufficient sample volume.	Increase sample volume - up to 100 mL (undiluted).
All dilutions used produce more than 60 colonies.	Too much sample used.	Dilute sample.
White areas with no colony growth appear on filter.	Air bubbles under filter inhibit media transfer.	Be sure there are no bubbles under filter before placing plates in incubator.
	Condensation from top of petri dish has precipitated onto filter.	Place plates into water bath in inverted position.

Quality Control for *Escherichia coli* Membrane Filter Procedure

Document the Following (Including but not limited to):

Supply Water Quality

- Chlorine and Ammonia Free
- No Chlorine Demand
- Conductivity
- Copper, Lead, Zinc, Nickel, Cadmium, Chromium, Iron, Silver levels (annual) <.01 mg/l*.

Sample Collection

- GRAB - Exact time collected
    - Exact time analyzed
    - Exact location
- Must be collected directly into sterilized sample container.
- DO NOT overfill container - Lose sodium thiosulfate
- **HOLD TIME: 8 hours at 10℃ (max)**
- *EDTA addition eliminates metal toxicity problem.

Sampling Container & Analytical Glassware

- Sterility! Sterility! Sterility!
- Container must be sterilized. Sodium Thiosulfate solution must be placed in container before sterilization.
- All glassware must be sterilized and remain wrapped until analysis is performed.

***NOTE**: Supply water quality verification is recommended at this time but may soon be required.

Equipment

- Autoclave
    - Use indicator tape each use
    - Sterility indicator/monthly
    - Annual calibration/professional
    - Record date, time, and temp of each use
    - Water bath incubator
    - Constant temp 44.5℃ ± 0.2℃
    - Thermometer:
        - must have 0.1℃ graduations
        - must be calibrated (NIST)
    - Second incubator
        - constant temp of 35℃ ± 1℃

Reagents

- Media
- NIST Traceability
- Preparation (received) date and expiration date

- Verify pH of prepared media 1 per lot
- Dilution water - sterility
- Blank - dilution water only - 0 colonies
- Positive control - 1 mL primary effluent (etc.) - should produce a large number of colonies or colonies Too Numerous To Count TNTC

Duplication Schedule

- 100% replication of single dilution
- 5% minimum duplication

Validity

- Count plates with 20-80 CFU's
- Use weighted average of plates which were in 20-80 range

Reporting Escherichia Coliform Results- Membrane Filtration Method

Escherichia Coliform Bench Sheet- MF Method
Analyst:
Exact time and date sampled:
Exact time and date analyzed:
Exact time into and out of 35℃ incubator:
Exact time into and out of water bath:
Incubation Temperature:
Blank - colonies on plate:
Sample Dilution #1 - # of CFU's on plate: Sample Dilution #2 - # of CFU's on plate: Sample Dilution #3 - # of CFU's on plate:
Replicate Dilution - # of CFU's on plate:
Show calculations to determine CFU's/100 mL: NOTE: "TNTC" is <u>not</u> a valid result. An actual number must be calculated.

Escherichia Coli Analysis Enzyme Substrate Coliform Test

This method, just as in the modified membrane filtration method, depends upon the fact that the 4-methyl-umberlifery-B-D-glucuronide (MUG), included in the fluorogenic substrate, will be hydrolyzed by B-D-glucuronidase enzyme produced in most *E. coli*. This reaction produces a bluish fluorescence that can be seen under an ultraviolet light at 365-366nm. In this method, other chromogenic (develops a color reaction) substrates are included. These chromogenic substrates are hydrolyzed by the enzyme B-D-galactosidase (produced by total coliform) to cause a color change which indicates the presence of total coliform. A member of the Total Coliform group (yellow in color) that fluoresces can be identified as an *E. coli*.

Use of these methods is perfectly acceptable. However, keep in mind that Quality Control requirements must be met. These requirements might exceed those described in the method. Check with your assigned Wastewater Program analyst to determine what additional quality control procedures might be required. These QC requirements may include a blank and positive control to be run with each analysis performed **and** the establishment of a minimum 10% duplication schedule.

All NPDES reporting requires methods be sufficiently sensitive to assure compliance with permit limits. Some facility permits specifically require that the membrane filtration method be used - Be sure to check your permit.

Here is a link to the IDEXX Colilert® method for simultaneous detection of Total and Escherichia Coliforms.

https://www.idexx.com/files/colilert-procedure-en.pdf

Colilert 18 - IDEXX US This link includes an excellent description of the chemistry involved as well as an instructional demonstration of the method.

Thermotolerant (Fecal) Coliform Membrane Filtration Method

Background

Coliform bacteria occur in a variety of environments. Soil, decaying vegetation and natural waters all contain coliform bacteria, but fecal coliforms originate in the intestinal tracts of warm-blooded animals.

The coliform bacteria tests were developed to give a measure of the presence of pathogenic (disease causing) organisms. Although most coliforms are not pathogenic, their numbers are related to the numbers of pathogenic organisms. A good kill of coliforms will indicate a good kill of pathogens, and therefore coliforms are called indicator organisms.

In past years, wastewater treatment operators were permitted to test their effluent for total or fecal coliforms. Coliform results are always expressed as colonies or MPN (**m**ost **p**robable **n**umber) per 100 mL of sample water. The maximum allowable number of coliforms was 500/100 mL for total coliforms and 200/100 mL for fecal coliforms. Next only the fecal coliform test (Now referred to as "Thermotolerant Coliform Bacteria test) was acceptable because these bacteria are more closely associated with sewage. Now most Vermont National Pollutant Discharge Elimination Systems (NPDES)

permits require analysis of *E. coli* bacteria specifically, while a few still require fecal coliform analysis, so we will describe it here.

There are two approved methods for the analysis of fecal coliform. These methods are the **m**embrane **f**ilter (MF) procedure and the multiple-tube fermentation procedure. (Also referred to as the most probable number (MPN) method.) The MPN test may be used for primary or secondary effluents, but the membrane filter test may be used only for secondary or tertiary effluents. Since primary effluent is often turbid, it may be difficult to filter a sufficient amount to adequately test this effluent. Also, colonies growing on a filter with many solids present, tend to run together, making it quite difficult to count these colonies.

The membrane filter method involves filtration of three different volumes of sample. The filters are placed in petri dishes, containing agar or liquid broth absorbed onto a pad, and incubated for 24 hours.

Equipment

- Autoclave, dry oven or other type of sterilizer
- Kraft paper
- Aluminum foil
- Autoclave indicator tape
- Masking tape
- Funnel assembly - 47 mm, autoclavable
- Membrane filters - made specifically for microbiology
- cellulose nitrate or cellulose nitrate/cellulose acetate
    - 47 mm, sterile, white, gridded on one side
    - 0.45 um or 0.7 um pore size
- Absorbent pads - 47 mm, sterile
- Petri dishes - 50 mm x 9 mm, plastic, sterile, tight-fitting halves
- Forceps - flat tipped
- Beakers - 50 mL, 250 mL
- Vacuum pump
- Vacuum flask - 500 mL, fitted with rubber stopper and glass tubing
- Vacuum flask - 1000 mL
- Tubing - thick walled tygon or rubber
- Plastic bags - 18 oz. Whirl-pak or similar waterproof bag, e.g., Ziploc
- Water bath - must be able to maintain a temperature of 44.5℃ ± 0.2℃.
- Incubator - air, must be able to maintain a temperature of 35.0℃ ± 0.5℃
- Thermometer† - range of 20℃ to 50℃ with no larger than 0.2℃ graduations.
- Sample bottles - glass or plastic (polypropylene), autoclaveable
- Fluorescent lamp
- Microscope or magnifying glass to give 10x - 15x magnification
- Colony counter - hand held
- Graduated cylinders - 100 mL, 1000 mL
- Volumetric flasks - 250 mL
- Erlenmeyer flasks - 250 mL, 250 mL screw top, 2000 mL screw top
- Graduated pipet - 5 mL, 25 mL
- Bacteriological pipets - 10 mL, 1 mL
- Refrigerator
- Glass stirring rod
- Stirrer magnet - bar
- Magnetic stirrer
- Burner - alcohol or Bunsen
- Parafilm or volumetric flask covers
- 0.45 um filters (Millipore HA, Gelman GN 6 Metric, MFS (Micro Filtration Systems), or similar)

† Thermometers should be checked at least once a year against a certifiable NIST (National Institute of Science and Technology) thermometer or one traceable to an NIST thermometer. Any deviation from the NIST thermometer should be noted and taped to the thermometer, e.g. reads 0.1℃ high at 44.5℃. Readings taken from this thermometer should always be adjusted according to the deviation.

Cleaning and Sterilization of Equipment

Glassware used for bacteria testing should not be acid washed. This also applies to any equipment such as spatulas, stirrer bars, etc. with which reagents are made for coliform tests. Any acid residue on the glassware will kill bacteria. Wash bacteria glassware in hot, soapy water with a detergent that doesn't inhibit growth, e.g., Micro. Rinse glassware thoroughly in hot tap water and then with distilled or deionized water at least 3 times.

Sterilization of bacterial glassware such as sample bottles, dilution bottles, funnel setups and pipets can be accomplished using an autoclave set at 15 psi and 121℃ or a dry oven set at 170℃. Keep in mind that reusable plasticware or liquids should never be sterilized in a dry oven.

Items such as funnels and pipets should be wrapped in kraft paper and secured with autoclave indicator tape before being placed in the autoclave, so as to allow the steam to penetrate. (Glassware sterilized in a dry oven may be wrapped in aluminum foil.) Also, always loosen the caps of sample and dilution bottles before autoclaving them.

The 10% sodium thiosulfate solution should be added to the sample bottles BEFORE they are sterilized. 0.1 mL (2 drops) will neutralize the chlorine in 100 mL of effluent. For effluents disinfected by ultra-violet light, the addition of sodium thiosulfate isn't necessary.

It is advisable to place a piece of autoclave indicator tape on equipment to be sterilized so that it can easily be identified later as having been processed. Autoclave indicator tape has invisible writing which appears after 15 minutes in the autoclave.

The following chart lists several items commonly used in Fecal Coliform analysis along with the required sterilization time for each. If your autoclave includes instructions indicating longer sterilization periods, follow those instructions except in the case of media such as lauryl tryptose broth, which should never be autoclaved for more than 15 minutes.

Item	Autoclave	Dry Oven
Sample Bottles	30 min	2 hours
Dilution Bottles	30 min	2 hours
Pipets	30 min	2 hours
Funnels	30 min	2 hours
1 L Dilution H_2O	35 min	
100 mL Lauryl Tryptose Broth	15 min	
Used Petri Dishes	30 min	2 hours

Reagents

NOTE: It is highly recommended that dilution water and prepared media (in petri dishes) be purchased rather than prepared on site. Therefore, we have eliminated the reagent list for preparation of the agar plates and those needed to prepare dilution water.

Sample Collection, Preservation and Holding Time

Coliform samples should be taken at the end of the outfall pipe after all treatment including de-chlorination if applicable, or at the weir at the end of the chlorine contact chamber. These samples must be taken as grabs into clean, sterile, sodium thiosulfate treated bottles. It is important to take the sample directly into the sterile bottle. Do not transfer a coliform sample from a dipper to the sterile sample bottle because of contamination problems. Allow about 1 inch of headspace for efficient mixing, and shake the sample to mix it with the sodium thiosulfate. It is usually required that you take the chlorine residual at the same time to establish a correlation between the two. Process the sample as soon as possible, refrigerate the sample at 10°C or less. If a sample is sent to another lab for analysis, it **must** be kept in a cooler with ice and arrive at the other lab for analysis to be analyzed within 8 hours. Never mail a bacteria sample.

Filtration

1) Fill out the paperwork, e.g. dilutions you will use.
2) Set up the filtration equipment on a clean surface. Always use a trap flask to protect the vacuum pump from any water that may overflow from the vacuum flask.
3) Label the bottom half of petri dishes with the volume (e.g.,0.1 mL, 1 mL, 10 mL, etc.) of sample or blank they will contain.
4) If you are using agar plates, they are ready to be used with a filter. If you are using liquid media and absorbent pads, aseptically transfer an absorbent pad to the bottom half of each petri dish. Flame the top of the ampule before breaking it off and pour the entire contents onto the pad.
5) Pour 70% ethanol to the 40 mL line of a 50 mL beaker. Keep the forceps in it all the time except when handling a filter. This ethanol keeps the forceps sterile.
6) Unwrap a funnel and place it aseptically on the vacuum flask.
7) Open the filter package, pass the forceps through a flame to burn off the ethanol, and grasp the filter by the edge with the forceps.
8) Place the filter on the funnel base and the funnel head on the filter.
9) Pour 100 mL sterile dilution water onto the filter. This is the blank.
10) Turn on the vacuum pump and draw the blank through the filter. Rinse the sides of the funnel twice with 20-30 mL of dilution water.
11) Turn off the vacuum pump and loosen the funnel head.
12) Open the petri dish labeled "Blank 100 mL," and place the cover inside up on the work surface. Pour off any excess media. Loosen the funnel head.
13) Flame the forceps. Take the funnel head off with one hand and using the forceps pick up the filter with the other. Always grasp the filter by the edge. Place the funnel head back on the base.

14) Place the filter on the agar or absorbent pad. Pull back the leading edge of the filter and roll it forward to exclude air as described below.
    - Try to place the filter into the plate without dragging it across the edge of the plate. Repeat this procedure at the other 3 "corners" of the filter. If you are using agar plates, any air bubbles will show up on the underside of the plate. Bubbles prevent media from soaking into the filter, and any bacteria over a bubble won't grow. Although it may be tempting to poke at bubbles at get rid of them, the "rolling" procedure works better.
15) Cover the plate.
16) Filter the sample similarly beginning with the smallest volume. For 10 mL or less, pour about 20 mL of dilution water onto the filter before adding the sample. This allows the bacteria to disperse and not grow in one clump. Shake the sample about 25 times before pipetting off a volume. Always filter 3 different volumes of sample, e.g. 1 mL,10 mL and 50 mL to hopefully produce a readable filter.
17) After the sample has been filtered, run a "positive" control. A positive control is generally a drop of influent, or primary effluent collected before disinfection, filtered the same as the blank and sample. Growth on the positive filter assures that the media will grow fecal coliforms.

Incubation

The prepared Petri dishes should be placed into waterproof “whirl-pack” bags. Remove as much air as possible from the bags before sealing. Then invert the bags so that the dishes are upside down in the water bath. Make sure the dishes are completely submerged with anchors or by putting weights on them. This will help ensure that the important constant temperature of 44.5 ℃ +/- 0.2℃ is maintained for the entire 24 hour +/- 2 hours incubation time.

NOTE: Be sure that the water bath is at the required temperature before putting the samples in for incubation.

Incubation within a very strict temperature range of only 0.2 ℃ is very important for accurate measurement of Fecal Coliform. Although there are dry incubators that might be capable of maintaining the required temperature range if left unopened for the entire incubation period, water baths are preferred.

Calculations

Fecal Coliforms (Thermotolerant Coliform) will appear blue. Only blue colonies should be counted. Disregard any gray or cream-colored colonies. These are NOT Fecal Coliform.

Fecal Coliform (Thermotolerant Coliforms) results are reported as #Colony Forming Units (CFU) per 100 mL. The equation for calculating the results is:

$$\frac{\#\ of\ CFUs}{Volume\ of\ Sample\ (mL)} * 100$$

The desired number of colonies to be counted on a plate is 20-60.

If only one plate produces 20-60 colonies, then only that plate would be used in calculating the result. For example:

If sample volumes of 10, 30, and 50 mL produce results of 5, 15 and 25 colonies respectfully, the result would be calculated as:

$$\frac{25\ CFUs}{50\ mL} * 100 = \frac{50\ CFUs}{100\ mL}$$

If more than one plate produces colonies in the 20-60 range. A "weighted" average must be calculated. That is: All volumes within the acceptable range of 20-60, are added together and their sum is divided by the total of their volumes. For example, if 10 and 30 mL samples produce 20 and 50 CFUs:

$$\frac{(20\ CFUs + 50\ CFUs)}{(10\ mL + 30\ mL)} * 100 = \frac{175\ CFUs}{100\ mL}$$

If none of the plates produce at least 20 colonies the result is calculated using all the colonies counted divided by the total volume used. For example, if 10, 30, and 50 mL samples produce 1, 3, and 5 CFUs:

$$\frac{(1\ CFU + 3\ CFUs + 5\ CFUs)}{(10\ mL + 30\ mL + 50\ mL)} * 100 = \frac{10\ CFUs}{100\ mL}$$

If all plates produce more than 60 colonies the plate with the least number of countable colonies is used in calculating the result. For example:

If sample volume of 10, 30, and 50 mL produce results of 90, 150, and 200 colonies respectively, the result would be calculated as:

$$\frac{90\ CFUs}{10\ mL} * 100 = \frac{900\ CFUs}{100\ mL}$$

If no colonies are produced on any of the plates (except of course the positive control) the result would be calculated using the largest volume and assuming 1 colony was produced on that plate. A < sign would be included with that result. For example:

If sample volumes of 10, 30 and 50 mL all produce results of zero then the result would be calculated as:

$$\frac{1\ CFU}{50\ mL} * 100 = < \frac{2\ CFUs}{100\ mL}$$

When calculating monthly averages for Fecal Coliforms you must use the geometric mean. (*Note: At the time this manual was printed monthly averages for Fecal Coliform are not required in Vermont NPDES permits but are in some other states.)

To calculate geometric mean:

1) Find the log of the results
2) Find the sum of the logs
3) Divide the sum of the logs by the number of results
4) Find the antilog of the number

For example, if the results of 3 weekly Fecal Coliform analyses are:

50 CFUs/100 mL

30 CFUs/100 mL

20 CFUs/100 mL

The monthly average would be calculated like this:

1) Find the log of the results:

$$\log(50) = 1.69897$$

$$\log(30) = 1.47712$$

$$\log(20) = 1.30103$$

2) Find the sum of the logs:

$$1.69897 + 1.47712 + 1.30103 = 4.47712$$

3) Divide the sum of the logs by the number of the result:

$$\frac{4.47712}{3} = 1.49237$$

4) Find the antilog of the number:

$$10^{1.49237} = 31.07229$$

5) **Answer:** 31 CFUs/100 mL

Fecal Coliform Troubleshooting Guide

PROBLEM	MOST LIKELY CAUSE	SOLUTION
Colonies are observed on the blank.	Autoclave/sterilizer not performing properly/non-sterile technique. Dilution water not sterile.	Verify that all equipment, glassware is sterile, forceps are dipped in alcohol and flamed before each use and good sterile technique is used throughout the procedure. Watch dilution water carefully for growth or discoloration. Discard when either is observed.
No colonies observed in positive control.	Bacterial inhibitors in sample, sample container, or equipment.	Use sodium thiosulfate in sample collection bottle. Addition of EDTA to the collection bottle (before sterilization) can eliminate inhibition by several heavy metals.
All colonies are bundled together in center of filter.	Poor dilution technique.	Add dilution water to funnel first then add sample to allow dispersion of bacteria.
None of the dilutions used produced the desired 20-60 CFUs (<20 on all plates).	Insufficient sample volume.	Increase sample volume - up to 100 mL (undiluted).
All dilutions used produce more than 60 CFUs.	Too much sample used.	Dilute sample.
White areas with no colony growth appear on filter.	Air bubbles under filter inhibit media transfer. Condensation from top of petri dish has precipitated onto filter.	Be sure there are no bubbles under filter before placing plates in incubator. Place plates into water bath in inverted position.

Quality Control for Fecal Coliform Membrane Filter Procedure Standard Method 9222-D

Document the Following (Including but not limited to):

Supply Water Quality

- Chlorine and Ammonia Free
- No Chlorine Demand
- Conductivity
- Copper, Lead, Zinc, Nickel, Cadmium, Chromium, Iron, Silver levels (annual) <0.01 mg/l*.

Sample Collection

- GRAB - Exact time collected
    - Exact time analyzed
    - Exact location
- Must be collected directly into sterilized sample container.
- DO NOT overfill container - Lose sodium thiosulfate
- **HOLD TIME: 8 hours at 10℃(max)**
- *EDTA addition eliminates metal toxicity problem.

Sampling Container & Analytical Glassware

- Sterility! Sterility! Sterility!
- Container must be sterilized. Sodium Thiosulfate solution must be placed in container before sterilization.
- All glassware must be sterilized and remain wrapped until analysis is performed.

NOTE: Supply water quality verification is recommended at this time but may soon be required.

Equipment

- Autoclave
    - Use indicator tape each use
    - Sterility indicator (use monthly)
    - Annual calibration/professional
    - Record date, time and temp of each use
    - Water bath incubator
        - Constant temp 44.5℃ ± 0.2℃
        - Thermometer:
            - must have 0.1℃ graduations
            - must be calibrated (NIST)

Reagents

- Media
- NIST Traceability
- Preparation, date received and expiration date
- Verify pH of prepared media 1/lot

- Dilution water - sterility
- Blank - dilution water only - 0 CFU's
- Positive control -1 mL primary effluent (etc.) – should produce very high number of colonies or colonies Too Numerous To Count (TNTC)

Duplication Schedule

- 100% replication of single dilution
- 5% minimum duplication

Validity

- Count plates with 20-60 CFU's
- Use weighted average of plates which were in 20-60 range

Reporting Fecal Coliform Results

Fecal Coliform Bench Sheet
Analyst:
Exact time and date sampled:
Exact time and date analyzed:
Exact time into and out of incubator:
Incubation Temperature:
Blank – CFU's on plate:
Sample Dilution #1 - # of CFU's on plate: Sample Dilution #2 - # of CFU's on plate: Sample Dilution #3 - # of CFU's on plate:
Replicate Dilution - # of CFU's on plate:
Show calculations to determine CFU's/100 mL: NOTE: "TNTC" is <u>not</u> a valid result. An actual number must be calculated.

Thermotolerant (Fecal) Coliform Enzyme Substrate Method (MPN)

Enzyme substrate methods use chromogenic (cause a color reaction) and fluorogenic (cause a light reaction).

The 23rd edition of Standard Methods has 28 pages of quality control procedures related to Coliform analysis. Section 9223B2 "Quality Control" refers to required quality control methods for use of enzyme substrate tests such as Colilert® and Colisure®. This Section specifically states that "Method users must adhere to the quality assurance (QA)/QC guidelines in Section 9020, including, but not limited to, analytical QC (Section 9020B.9), instrumentation/equipment (Sections 9020 B.4 and 9030B) and supplies (Section9020B.5) Refer to Table 9020: I for key QC procedures." Whereas the State has the right to require more stringent requirements than EPA, less stringent requirements are prohibited. Therefore, for analyses performed at or for NPDES regulated wastewater facilities quality control procedures beyond those described in IDEXX methods may be required. At a minimum reagent blanks and positive controls may be required for each analysis.

Here is a link to the IDEXX Colilert 18 method for Fecal Coliform:

Colilert 18 - IDEXX US

This link gives an excellent description of the method and the chemistry involved. There is also a very handy instructional video. The video demonstrates the method for Total Coliform and Escherichia Coliform method first. To view the Fecal Coliform method demonstration simply fast forward to that section of the video.

References

Fecal Coliform: Standard Methods for the Examination of Water and Wastewater, 23rd Edition, Method 9222D, Pages 9-60 through 9-61.

Escherichia coliform: EPA Method #1103.1, 2010, Test Methods for Escherichia Coli and Enterococci in Water by the Membrane Filter Procedure

Standard Methods for the Examination of Water and Wastewater, 23rd Edition, Method #9213 D 3.