COLIFORMS

FECAL COLIFORM

Background

Coliform bacteria occur in a variety of environments. Soil, decaying vegetation and natural waters all contain coliform bacteria, but fecal coliforms are found only 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 (most probable number) 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 was acceptable because these bacteria are more closely associated with sewage. A few Vermont National Pollutant Discharge Elimination Systems (NPDES) permits 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 membrane filter (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 MPN test involves 25 test rubes of liquid broth into which 5 different volumes of sample are placed. The test tubes are incubated for up to 48 hours. Any tubes which show gas production are inoculated into other tubes containing a different kind of liquid broth and are then incubated for an additional 24 hours. Obviously, this test requires the use of a great deal of time and equipment and therefore is not the method of choice for secondary treatment plant operators. Detailed instructions for the MPN procedure are not included in this manual but can be found on pages 9-52 of the 18th Edition of Standard Methods for the Examination of Water and Wastewater.

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. This test is much quicker, uses less equipment than the MPN procedure, and is the method detailed in this manual.

Years of testing by the U.S. Environmental Protection Agency and many state and private laboratories have shown that MF results are close to MPN results although bacteria do grow better in the MPN test and often they cannot grown in the MF test but will grown in the MPN test. For this reason, if there is ever a controversy over the true coliform count in an effluent, the MPN results will be considered accurate and will override MF results.

However, as mentioned above, the MPN procedure is long and not recommended for secondary plant operators. To get an idea of how well the MF results compare with MPN results, operators might occasionally take 2 samples of their effluent at once. They would test one as they usually do by the MF procedure and might take the other sample to a private lab and request that it be run for fecal coliforms by the MPN procedure. More information on both the MPN and MF procedures can be found in the references listed at the end of this section.

Equipment

Autoclave, dry oven or other 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°C ± 0.2°C.
Incubator - air, must be able to maintain a temperature of 35.0°C ± 0.5°C or 58°C ± 2°C.
+Thermometer - range of 20°C to 50°C with no larger than 0.2°C graduations.
Sample bottles - glass or plastic (polypropylene), autocalve
Milk dilution bottles - glass, marked at 99 ml, autocalve
Fluorescent lamp
Microscope or magnifying glass to give 10x - 15x magnification

Equipment (continued.)

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
Heat source - hot plate or bunsen burner to heat media
Balance - four place analytical balance
pH meter - probe with flat surface tip (for determining pH of a single drop of media etc.)
Parafilm or volumetric flask covers
Millipore HC - 0.7 um - this is especially for stressed bacteria
Millipore HA - 0.45 um
Gelman GN 6 Metricel - 0.45 um
MFS (Micro Filtration Systems) - 0.45 um

Thermometers should be checked at least once a year against a certifiable NBS (National Bureau of Standards) thermometer or one traceable to an NBS thermometer. Any deviation from the NBS thermometer should be noted and taped to the thermometer, e.g. reads 0.1 °C high at 44.5 °C. 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 °C or a dry oven set at 170 °C. 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 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 mls 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 laurel tryptose broth, which should never be autoclaved for more than 15 minutes.

ITEM	AUTOCLAVE	DRY OVEN
Sample Bottles	30 minutes	2 hours
Dilution Bottles	30 minutes	2 hours
Pipets	30 minutes	2 hours
Funnels	30 minutes	2 hours
1 L dilution H_2O	35 minutes	-------
100 ml laurel tryptose broth	15 minutes	-------
Used petri dishes	30 minutes	2 hours

Reagents

Order only reagent grade chemicals. Store chemicals and media as indicated on the container label. Store opened jars of dehydrated media in a desiccator for up to 6 months, and discard the media sooner if it becomes caked. Discard any unopened media containers if the expiration date has passed. Refrigerate media ampules.

Ethanol - 95%, may be denatured
Bacto agar
M-FC broth base - dehydrated media or prepared liquid in ampules
Laurel trypotose broth - dehydrated media
Magnesium chloride - $MgCL_2$, anhydrous or $MgCL_2$, $6H_2O$
Potassium phosphate, monobasic - KH_2PO_4
Sodium hydroxide - NaOH
Sodium thiosulfate - $Na_2S_2O_3$
Spore suspensions or strips - BBL kilit ampules or spore strips

MEDIA FOR FECAL COLIFORM ANALYSIS

Always prepare media on a clean surface. Mix 3.7 g M-FC broth base with 1.5 g bacto agar in a 250 ml Erlenmeyer flask. Add 100 ml distilled water and swirl until the broth dissolves. Over the mouth of the flask with aluminum foil.

Heat the flask over a bunsen burner or hot plate, swirling frequently until the agar dissolves and the liquid starts to boil. Allow the media to cool until the flask can be handled comfortably. Dispense 4 ml of sterile media into sterile petri dishes with a sterile 25 ml graduated pipet. Allow the media to harden, replace the petri dish cover and store the plates upside down in a refrigerator at 4°C for up to 2 weeks.

This same media can be made without the agar and used by pouring over sterile absorbent pads in the petri dishes. Use about 2 to 3 ml of media per dish and pour it just before you are going to filter your sample. Pour off any excess media just before placing the filter on the absorbent pad. This liquid media may be stored, covered tightly in a refrigerator at 4°C for up to 96 hours.

LAUREL TRYPTOSE BROTH FOR STERILITY CHECKS

Mix 3.56 g of laurel tryptose broth (LTB) with 100 ml distilled water in a 250 ml screw top Erlenmeyer flask. Swirl the flask until all the LTB dissolves. Screw the cap on loosely and sterilize the LTB in an autoclave at 121°C and 15 psi for 15 minutes. If possible, have the autoclave preheated. The total time the LTB is in the autoclave shouldn't exceed 45 minutes. Tighten the cap when the LTB has cooled, and store it in a refrigerator at 4°C for up to 3 months.

Place 9.5 g anhydrous $MgCL_2$ or 20.3 g $MgCL_2.6H_2O$ in a 250 ml volumetric flask. Add about 50 ml distilled water and swirl until the $MgCL_2$ dissolves. Fill the flask to the mark with distilled water, cover and invert the flask several times to mix the solution.

1N NaOH

Add 10 g NaOH to about 100 ml distilled water in a 250 ml volumetric flask. Swirl it until the chemical dissolves and the flask is cool enough to hold. Fill it up to the mark with distilled water, cover it, and invert it several times for mixing. Store it in a polyethylene or borosilicate glass bottle at room temperature for up to 6 months.

POTASSIUM PHOSPHATE STOCK SOLUTION

Place 8.5 g KH_2PO_4 and about 125 ml distilled water in a 500 ml beaker. Dilute to 250 with distilled water. Swirl this or mix it with a magnetic stirrer until the chemical dissolves. Adjust the pH to 7.2 ± 0.5 with 1N NaOH. Transfer the solution to a sample bottle, cap it loosely, and autoclave it for 30 minutes at 121°C and 15 psi. Store it in the sample bottle in a refrigerator at 4°C for up to 6 months. Discard the solution if it becomes cloudy or shows other signs of bacterial growth.

SODIUM THIOSULFATE, 10% SOLUTION

Place 25 g $Na_2S_2O_3$ in a 250 ml volumetric flask, and add about 125 ml distilled water. Swirl the flask until the chemical dissolves. Fill the flask to the mark with distilled water, cover it, and invert it several times to mix. Store it in a sample bottle refrigerated at 4°C for up to 6 months.

BUFFERED OR DILUTION WATER

Fill a 1000 ml graduated cylinder with distilled water. Add 1.25 ml of the KH_2PO_4 stock solution and 5 ml of the $MgCL_2$ stock solution. Stir it with a glass stirring rod and transfer it to a 2000 ml screw to Erlenmeyer flask. With the cap screwed on loosely, autoclave the dilution water for 35 minutes or however long your autoclave says it necessary for this volume. Store the water at room temperature for up to 2 weeks in the summer or 3 weeks in the winter. If blank show growth, discard the dilution water no matter how long it has been stored.

Sampling

Coliform samples should be taken at the end of the outfall pipe or at the weir in 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 4°C. 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. 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 as shown below. 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., .1 ml, 1 ml, 10 mls, etc.) of sample or blank they will contain.

4) If you are using agar plates, they are all set for a filter. If you are using liquid media and absorbent pads, aspectically 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. If you have made up your own media, flame the mouth of the flask and pour about 2 ml of media onto each pad. Close the plates while filtering the sample. To allow the pad to absorb all the media it can, don't pour off any excess until you are ready to place the filter on the pad.

5) Pour about 1 to 5" of 95% 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.

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 illustrated 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. 0.1 ml, 1 ml and 10 ml, to hopefully produce a readable filter.

17) After the sample has been filtered, run a "positive". A positive is generally a drop of influent, or effluent before disinfection, filtered the same as the blank and sample. Growth on the positive filter assures that the media will grow fecal coliforms.

Calculations

Fecal Coliform results are reported as colonies per 100 mls. The equation for calculating the results is:

$$\frac{\text{\# of colonies} \times 100 \times \text{dilution factor}}{\text{volume of sample in mls}}$$

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 mls produce results of 5, 15 and 25 colonies respectfully, the result would be calculated as:

$$\frac{25 \times 100 \times 1}{50} = 50 \text{ colonies/100 ml}$$

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:

$$\frac{(20 + 50) \times 100 \times 1}{(10 + 30)} = 175 \text{ colonies/100 mls}$$

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:

$$\frac{(1 + 3 + 5) \times 100 \times 1}{(10 + 30 + 50)} = 10 \text{ colonies/100 mls}$$

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 mls produce results of 90, 150, and 200 colonies respectively, the result would be calculated as:

$$\frac{90 \times 100 \times 1}{10} = 900 \text{ colonies/100 mls}$$

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 mls all produce results of zero then the result would be calculated as:

$$\frac{1 \times 100 \times 1}{50} = < 2 \text{ colonies/100 mls}$$

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 colonies/100 mls
30 colonies/100 mls
20 colonies/100 mls

The monthly average would be calculated like this:

STEP 1	log (50) =	1.69897
	log (30) =	1.47712
	log (20) =	1.30103
STEP 2		4.47712
STEP 3		4.47712 ÷ 3 = 1.49237
STEP 4		Antilog of 1.49237 = 31.07229
ANSWER		= 31 colonies/100 mls

FECAL COLIFORM
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 20-60 colonies (<20 on all plates).	Insufficient sample volume.	Increase sample volume - up to 100 mls (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 FECAL COLIFORM
Membrane Filter Procedure
Standard Method 9222-D

Document

*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: 6 hours at 4°C (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
- Waterbath incubator
    - Constant temp 44.5°C ± 0.2°C
    - Thermometer:
        must have 0.1°C graduations
        must be calibrated (NIST)

Reagents

- Media
- NIST Traceability
- Preparation (received) date and expiration date
- Verify pH of prepared media 1/lot
- Dilution water - sterility
- Blank - dilution water only - 0 colonies
- Positive control - ≅ 1 ml primary effluent (etc.) - should be TNTC

Duplication Schedule

100% replication of single dilution
5% minimum duplication

Validity

- Count plates with 20-60 colonies after calculating
    colonies/100 mls
- Use weighted average of plates which were in 20-60 range (20-60 colonies on filter before applying dilution multiplication factor)

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 - colonies on plate:
Sample Dilution #1 - # of colonies on plate: Sample Dilution #2 - # of colonies on plate: Sample Dilution #3 - # of colonies on plate:
Replicate Dilution - # of colonies on plate:
Show calculations to determine colonies/100 mls: NOTE: "TNTC" is not a valid result. An actual number must be calculated.

ESCHERICHIA COLI

Background

The E Coli test, like the fecal coliform tests described earlier in this manual, was developed to measure the presence of pathogenic (disease causing) organisms in wastewater. E Coli, again like the other coliforms, is not necessarily pathogenic. But E Coli is a much better indicator of pathogenic organisms than other coliforms because this bacteria is a natural inhabitant only of the intestinal tract of warm blooded animals. Also, unlike fecal coliform there is much less chance of other coliforms reacting as positive in the analysis. (That is E Coli is more easily differentiated from other coliforms than is Fecal Coliform.)

Extensive studies have shown that there is a direct relationship between the number of E Coli present in recreational waters and the risk of gastrointeretional illness associated with swimming in the water (1).

The membrane filter method is used for determining the density of E Coli in water. This method involves the filtration of (3 different volumes) a water sample through a membrane which retains the bacteria. The filters (membranes) are then placed in petri dishes containing TEC, which is a media specifically for numeration of E Coli bacteria. The petri dishes containing the media and bacteria laden membranes are then incubated at 35°C for 2 hours. This 2 hour, 35°C serves to resuscitate injured or stressed bacteria. After the 2 hour 35°C incubation-they are transferred to a 44.5°C water bath incubator for 22 hours. After incubation the filters are transferred to a filter pad saturated with urea substrate. After 15 minutes colonies that are yellow or yellow-brown are counted.

Some known facts about E Coli that help us to identify and enumerate include:

1) It is thermotolerant at 44°C. Some bacteria cannot survive at this temperature but E Coli can function well at 44°C.

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°C and 44.5°C. Many bacteria cannot ferment lactose.

4) It can hydrolyze tryptophane 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°C we can easily confirm E Coli as other coliform will grow colonies but E Coli will not.

Equipment

Autoclave, dry oven or other sterilizer
Kraft paper
Aluminum foil
Autoclave indicator tape
Masking tap
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°C ±0.2°C.
Incubator - air, must be able to maintain a temperature of 35.0°C ±0.5°C.
+Thermometer - range of 20°C to 50°C with no larger than 0.2°C 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°C
Stirrer magnet - bar
Magnetic stirrer
Burner - alcohol or bunsen
Parafilm or volumetric flask covers
Millipore HA - 0.45 um Filters <u>or</u>
Gelman GN 6 Metricel - 0.45 um Filters <u>or</u>
MFS (Micro Filtration Systems) - 0.45 um

\+ 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°C high at 44.5°C. 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°C or a dry oven set at 170°C. 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 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 mls 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 minutes	2 hours
Pipets	30 minutes	2 hours
Funnels	30 minutes	2 hours
1 L dilution H_2O	35 minutes	-------
Used petri dishes	30 minutes	2 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.

Ethanol - methanol, or isopropanol in a small wide mouth container, for flame-sterilizing forceps

Buffered Dilution Water

NaH_2PO_4	-	Sodium Dihydrogen Phosphate	0.58 g
$NaHPO_4$	-	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 °C (15 lbs pressure) for 15 minutes. Final pH should be 7.2 ± 0.2.

*mTEC Agar (Difco 0334-15-0)

Composition:

Proteose Peptoe	5.0 g
Yeast Extract	3.0 g
Lactose	10.0 g
NaCl	7.5 g

mTEC prepared Agar plates and urea substitiate can be purchased from Northeast Laboratory, PO Box 788, Waterville Maine 04901. Telephone 207-873-7711.

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

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 water in flask and heat to boiling, until ingredients dissolve. Autoclave at 121°C (15 lb pressure) for 15 minutes and cool in a 44-46°C 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 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. Store at 6°C-8°C for 1 week.

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 dechlorination 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 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 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 6 hours. If immediate processing isn't possible, refrigerate the sample at 4°C. 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. Never mail a bacteria sample.

Calibration and Standardization

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

Check thermometers at least annually against an NBS certified thermometer or one traceable to NBS. Check mercury columns for breaks.

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 to 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 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 illustrated 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°C 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 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 aspectically 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°C for 2 hours.

After 2 hours incubation at 35 °C, transfer the plates to Whirl-Pak bags, seal and place inverted in a 44.5 °C 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 °C (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 °C 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 colonies per 100 mls.

The equation for calculating results is:

$$\text{E Coli/100 ml} = \frac{\text{\# of E Coli colonies x 100 x dilution factor}}{\text{volume of sample in mls}}$$

The desired number of colonies to be counted on a plate is 20-80.

The rules for calculating results are basically the same as those for counting Fecal Coliform. One important difference is that 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 on estimate based on a plate containing >80 colonies. For example:

> If sample volumes of 10, 30 and 50 mls produced results of 86, 120, 150 colonies respectively the result would be calculated as:
>
> $$\frac{86 \text{ x } 100 \text{ x } 1}{10} = \text{\underline{Estimated} 860 colonies/100 mls}$$
>
> 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 mls produce results of 5, 15 and 25 colonies respectively, the result would be calculated as:
>
> $$\frac{25 \text{ x } 100 \text{ x } 1}{50} = \text{50 colonies/100 mls}$$

If more than one plate produces colonies in the 20-80 range. A "weighted" 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 mls produce results of 20, 50 and 95 colonies respectively, the result would be calculated as:

$$\frac{(20 + 50) \times 100 \times 1}{(10 + 30)} = 175 \text{ colonies/100 mls}$$

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 mls produce results of 1, 3 and 5 mls respectively, the result is calculated:

$$\frac{(1 + 3 + 5) \times 100 \times 1}{(10 + 30 + 50)} = 10 \text{ colonies/100 mls}$$

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 mls all produce results of zero then the result would be calculated as:

$$\frac{1 \times 100 \times 1}{50} = <2 \text{ colonies/100 mls}$$

ESCHERICHIA COLIFORM
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 mls (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 COLIFORM
Membrane Filter Procedure

Document

*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: 6 hours at 4°C (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
- Waterbath incubator
  - Constant temp 44.5 °C ± 0.2 °C
  - Thermometer:

    must have 0.1 °C graduations
    must be calibrated (NIST)
  - Second incubator

    constant temp of 37°C ± 1 °C

Reagents

- Media
- NIST Traceability
- Preparation (received) date and expiration date
- Verify pH of prepared media 1/lot
- Dilution water - sterility
- Blank - dilution water only - 0 colonies
- Positive control - ≅ 1 ml primary effluent (etc.) - should be TNTC

Duplication Schedule

100% replication of single dilution
5% minimum duplication

Validity

- Count plates with 20-80 colonies after calculating colonies/100 mls
- Use weighted average of plates which were in 20-80 range (20-80 colonies on filter before applying dilution multiplication factor)

Reporting Escherichia Coliform Results

Escherichia Coliform Bench Sheet
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 colonies on plate: Sample Dilution #2 - # of colonies on plate: Sample Dilution #3 - # of colonies on plate:
Replicate Dilution - # of colonies on plate:
Show calculations to determine colonies/100 mls: NOTE: "TNTC" is <u>not</u> a valid result. An actual number must be calculated.

References

Fecal Coliform: Standard Methods for the Examination of Water and Wastewater, 18th Edition, Method 9222D, Pages 9-60 through 9-61.

Escherichia Coliform: EPA Method #1103.1, 1985, Test Methods for Escherichia Coli and Enterococci in Water by the Membrane Filter Procedure

Standard Methods for the Examination of Water and Wastewater, 18th Edition, Method #9213 D 3, pages 9-29 through 9-30.