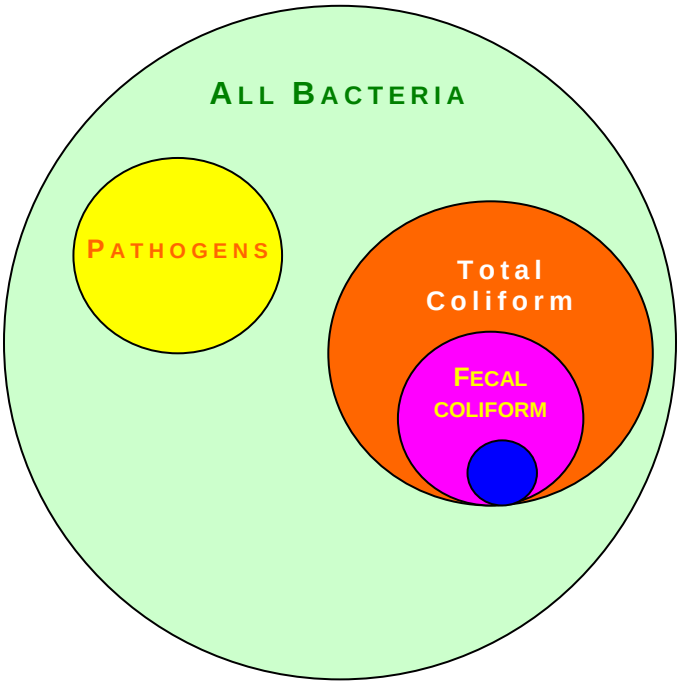
Chapter 15 Fecal Coliform

Microorganisms are an integral part of wastewater treatment. They are beneficial yet dangerous. Fecal coliforms originate in the intestines of warm-blooded animals, in this case, primarily humans. Fecal coliforms in the influent of domestic waste are usually several million per hundred milliliters. These bacteria function as decomposers in the secondary treatment process. They serve to metabolize the waste under aerobic conditions then flocculate and settle in the secondary clarifier. Those bacteria which are not trapped in the floc will flow over the secondary weir to the disinfection chamber. At this point, the bacteria are no longer needed and are killed by disinfection with chlorine, UV light, etc.

Coliforms are considered indicator organisms. In wastewater the primary coliform is the fecal coliform which originates in the intestines of warm blooded organisms. Since fecal coliforms are seldom pathogenic under normal circumstances and are easily cultured, their presence indicates the potential presence of pathogens. High levels of fecal coliforms discharged into the receiving stream would indicate that pathogens could also be discharged into the stream. On the other hand, if fecal coliforms are absent, pathogens are also absent since they die quickly outside the host. So why not add lots of chlorine to kill all the fecal coliforms and remove any chance that pathogens might survive? Besides being expensive, chlorine is carcinogenic and harms other organisms in the receiving stream. To reduce the possibility of over-chlorinating, sulfur dioxide has been used to remove excess chlorine prior to discharge. Many wastewater treatment systems have changed disinfection processes to avoid the headaches associated with chlorine. The common alternative is UV light which is very effective at inactivating fecal coliforms and pathogens.

Fecal coliforms belong to the group of bacteria called coliforms (orange circle). The coliform group of bacteria are defined as facultative anaerobic, gram-negative, non-spore forming, rod shaped bacteria that ferment lactose with gas and acid formation within 48 hours at 35°C.



Fecal coliforms (magenta circle) are members of the coliform group but have the added characteristic of being able to live at the elevated temperature of 44.5^{o}C. E. Coli (blue circle) is a specific type of fecal coliform and associated with human waste. Newer discharge permits are requiring analysis for E. Coli rather than the more general fecal coliform.

Test Theory

A sample is collected and analyzed using aseptic (sterile) technique. A measured volume of sample is filtered through a sterile 0.45µ membrane filter, transferred to an absorbent pad containing m-FC broth, then incubated at 44.5^{o}C for 24 hours. Blue/blue gray colonies are counted and reported as colony forming units (cfu) per 100 ml of sample. The method is limited by turbidity in the sample. Excessive

turbidity will reduce fecal coliform recovery, requiring the MPN method to be used instead of the membrane filter method.

Sample Collection

Unlike other wastewater tests, fecal coliforms must be collected in a clean, sterile borosilicate glass or plastic bottle containing sodium thiosulfate. Presterilized bags or bottles containing sodium thiosulfate can also be used. Sodium thiosulfate is added to remove residual chlorine which will kill fecal coliforms during transit. 0.1 ml of 10% sodium thiosulfate is added to a 120 ml sample bottle prior to sterilization. The minimum bottle size should be 120 ml to allow enough head space (1") for proper sample mixing.

Collection Procedure

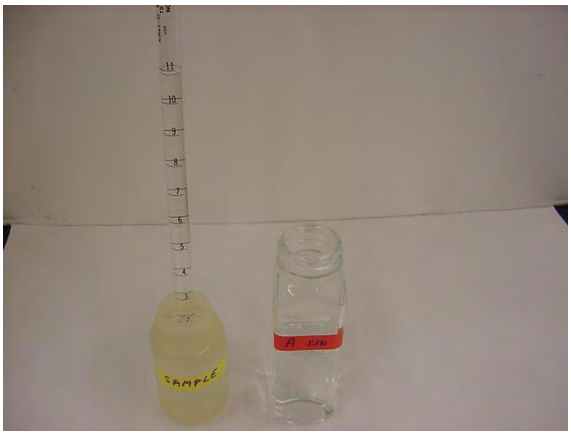
Select a site that will provide a representative sample. Fecal coliform samples are always grab samples and should be drawn directly from the flow stream without using collection devices such as unsterilized dippers or buckets. For example, do not collect a BOD sample then transfer some of the sample to the fecal coliform sample bottle. Keep the sample bottle lid closed tightly until it is to be filled. Remove the cap and do not contaminate the inner surface of the bottle, neck, threads or cap. Fill the container without rinsing, being sure to leave ample air space to allow mixing. Rinsing will remove the dechlorinating agent. All samples should be labeled properly with date and time of collection, sampler's name, and sample collection location. Leaking sample bottles allow for contamination of the sample and should be discarded and the sampling repeated.

Preservation

Fecal coliform samples should be analyzed as soon as possible after collection to prevent changes to the microorganism population. Fecal coliforms must be transported on ice, if they cannot be analyzed within 1 hour of collection. Fecal coliforms transported at ambient temperature may reproduce and higher bias to the numbers than desired or they may be killed off resulting in lower numbers, if handled poorly such as transport in sunlight. Fecal coliform samples should be stored by the laboratory in a refrigerator until time of analysis. The maximum holding time for state or federal permit reporting purposes is 6 hours.

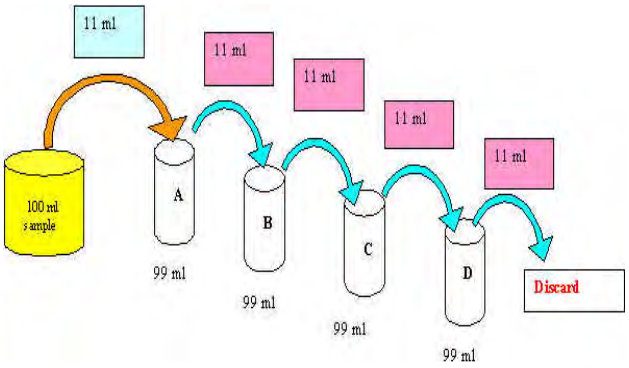
Dilutions

Ideally, sample volumes filtered should produce a countable plate with 20-60 colony forming units per plate. The lab technician may need to perform several dilutions to reach the desired colony count. If high levels of fecal coliforms are expected, the most common practice is to prepare a serial dilution. A serial dilution dilutes the sample concentration by a factor of 10. The most common dilution is prepared by transferring 11 ml of sample to 99 ml of sterile phosphate dilution water using a sterile serological pipet.



A 1:10 dilution (11ml :110 ml) will reduce the bacteria count in the sample by 10 times. For instance, if the expected sample answer is 4000 cfu/100 ml, a 1:10 dilution will produce a diluted sample containing 400 cfu/100 ml. Both of these samples will produce plates that are uncountable (TNTC) and outside the desired range of 20-60 cfu/100 ml. Therefore, a second serial dilution can be made by pipetting 11 ml of the diluted sample into a second 99 ml bottle of

sterile phosphate dilution water. This will now reduce the colony count from 400 to 40 cfu/100 ml. The 40 cfu/100 ml is a countable plate.



The serial dilution can continue as long as needed. In many instances, if the answer is truly unknown, the technician may make several dilutions to cover the anticipated colony range. Notice in the picture above that each time 11 ml is transferred; the remaining volume is approximately 100 ml which is then filtered.

Bottle	Volume original sample used	Volume filtered	Dilution	Cfu/100 ml
Original	100 ml	100 ml	none	4000
A	10 ml	100 ml	1:10	400
B	1 ml	100 ml	1:100	40
C	0.1 ml	100 ml	1:1000	4

The last bottle has 110 ml; so11 ml is discarded, leaving 100 ml. Each serial dilution reduces the amount of original sample by 10, so the colonies in bottle A actually come from 10 ml of sample. The colonies in dilution B come from 1 ml of sample, etc.

For most wastewater treatment plants with a well disinfected effluent, the colony count may be well below the state/federal permit limitations. In this instance, undiluted volumes of 10, 25, 50, and 100 ml will commonly be filtered.

Volume sample filtered	Cfu/100 ml
100 ml	92
50 ml	48
25 ml	26
10 ml	9

In this example, there are 2 countable plates (50 ml and 25 ml) that will be used in the calculations.

Test Preparation

Prior to analysis, there are number of preliminary steps that must be performed.

1. Obtain M-FC broth - the broth must be either prepared or purchased. Most labs purchase pre-sterilized 2 ml ampules. The broth sterility, growth characteristics, pH, purchase date, expiration date and lot number should be recorded. Each new lot of media should be tested against the previously acceptable broth to verify acceptable performance. Each lot should be tested for performance with positive and negative control cultures.

   A positive fecal coliform control culture is commonly E. Coli (ATCC 8739 or 25922) An E.Coli pure culture can be purchased, prepared, and stored in the refrigerator. A positive culture should produce blue colonies.

   A negative fecal coliform control culture is commonly Enterobacter aerogenes (ATCC 13048). A negative control culture should not grow.

2. Membrane filters and absorbent pads. Membrane filters are also purchased pre-

sterilized. They must be approved for membrane filtration based on data documenting toxicity, recovery, retention, and absence of growth promoting substances. They must be grid-marked, 47 mm in diameter, and 0.45 micron pore size. They should also be white and composed of cellulose ester. The lot number, date received and sterility must be recorded. Absorbent pads may be included with the membrane filter package or pre-loaded in the culture dish. In either case, the lot number, date received and sterility must be recorded.

3. Culture dishes (Petri dishes). Pre-sterilized disposable, plastic culture dishes (50 mm) are usually purchased. The lids must be tight-fitting. The lot number, date received, and sterility must be recorded.
4. Pipets, Graduated cylinders. Glass pipets should be placed in a stainless steel or aluminum container and sterilizing in a sterilizing oven at 170-180°C for 2 hours. Glass graduated cylinders should be wrapped with aluminum foil and sterilized. Pre-sterilized plastic pipets can be purchased in a package or wrapped individually. Reseal packages between each use. Pipets should be accurate to within 2.5% tolerance. Document lot number, date received, and sterility.
5. Sterile Phosphate Dilution/Rinse Water. Prepare or purchase phosphate dilution water. Prepared dilution water should be sterilized for a minimum of 15 minutes in the autoclave at 121°C. Volume, pH, and sterility should be checked after sterilization. pH should be 7.2 $\pm$ 0.5 and volume should be 99 $\pm$ 2 ml. Check sterility by adding 50 ml of water to 50 ml of sterile double strength tryptic soy broth (TSB). Incubate for 24-48 hours at 35.0 $\pm$ 0.5^{o}C. Record sterility. Measure and record the volume and pH on a second sterile bottle.
6. Membrane filtration apparatus. A plastic, glass or stainless steel, non-leaking, non-scratched, non-corroded and calibrated filtration apparatus should be sterilized for a minimum of 2 minutes in a germicidal (254 nm) UV lamp.
7. Waterbath The waterbath must be turned on and adjusted to 44.5 $\pm$ 0.2^{o}C. Waterbath incubators are used because they hold the temperature more accurately than dry air incubators and allow for better selection of fecal coliforms. Calibration-corrected temperatures should be recorded at least twice per day during each day the waterbath is in use, separated by at least 4 hours. Record the date, time, temperature, and technician initials.

Once the equipment and materials are satisfactory, the technician is ready to run the test.

Test Procedure

1. The lab bench should be disinfected with either alcohol or commercial lab disinfectant to reduce the potential for contamination. The technician should wear appropriate PPE
2. The filtration funnel apparatus is placed in the UV sterilizer and sterilizer for a minimum of 2 minutes. After sterilization, place the filtration apparatus on the filtration funnel until time of use. Record the sterilization time.

Safety: Do NOT look at the UV light. UV light may cause permanent eye damage.

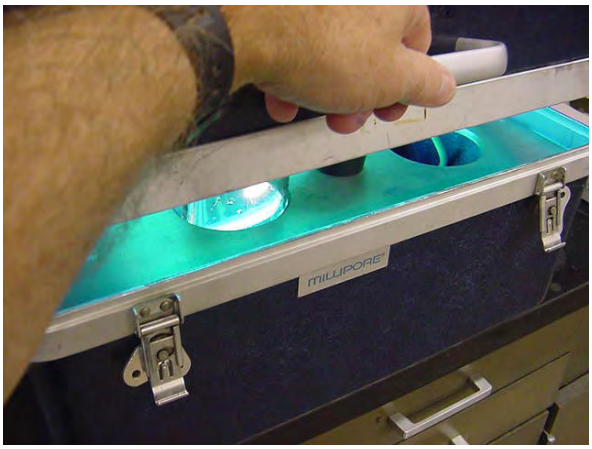


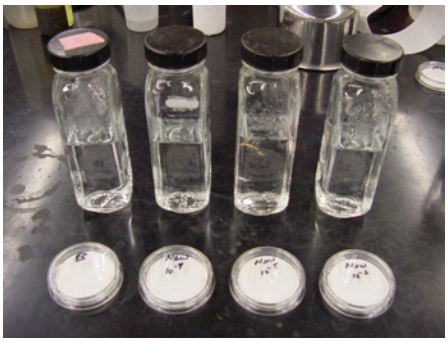
Figure: Sterilize for 2-3 minutes in UV sterilizer.

3. Obtain the number of Petri dishes needed and label. The first dish should be labeled pre-blank and the last dish should be labeled after blank or end control. The pre-blank will be 100 ml of sterile phosphate dilution water. The pre-blank is a sterility control and acts to document that all the equipment is sterile and the technician's technique is aseptic. The after blank is also 100 ml of sterile phosphate dilution water. The after blank helps confirm that filter rinsing has been adequate. Both blanks should show no growth. Any growth on either blank invalidates the test.

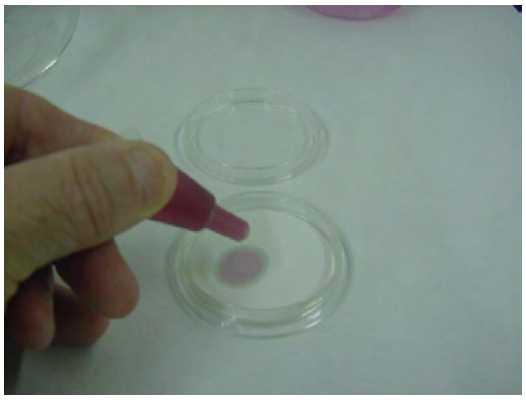
   The remainder of the samples and dilutions are run between the blanks. A test run is defined as an uninterrupted series of analyses.

   Prepare sample serial dilutions if necessary. If using less than 10 ml of sample, add the volume to sterile phosphate dilution water. This will aid in the uniform distribution of bacteria over the entire filter surface.

   For example, the Petri dishes could be labeled.
   1. Pre-blank
   2. 3 ml sample (in sterile phosphate dilution water)
   3. 10 ml sample
   4. 25 ml sample
   5. 50 ml sample
   6. 100 ml sample
   7. After blank



4. Aseptically add 2 ml of m-FC broth to each plate. Pour the broth directly onto the pad without touching the pad. Excessive media should be poured off into the sink. Re-cover each dish.



5. Light a Bunsen burner. Use flat, blunt, non-corrugated forceps. Sterilize the forceps by dipping in 95% ethyl alcohol and igniting in the Bunsen burner flame.

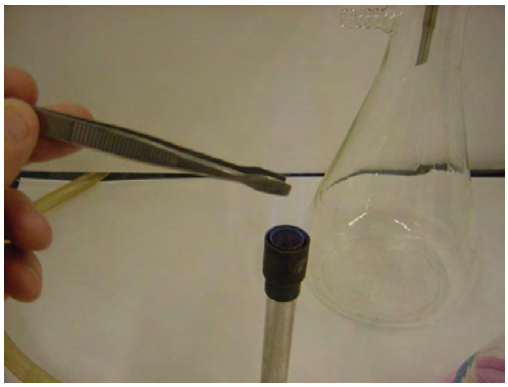


Figure: Do not overheat the tip of the forceps. The membrane will stick to hot forceps and tear.

Safety: Hold the tips of the forceps down to avoid burning your fingers with burning alcohol. Do not shake flaming alcohol off the forceps, let it burn off.

6. Aseptically remove a membrane filter and place it grid side up on the filter funnel support.

Figure: Carefully peel back the edges of the membrane filter. Sterile forceps can be used to help separate the filter from its blue backing. The blue backing is discarded.

It is best to hold the upper portion of the funnel in one hand while doing this in order to avoid contamination of the funnel.

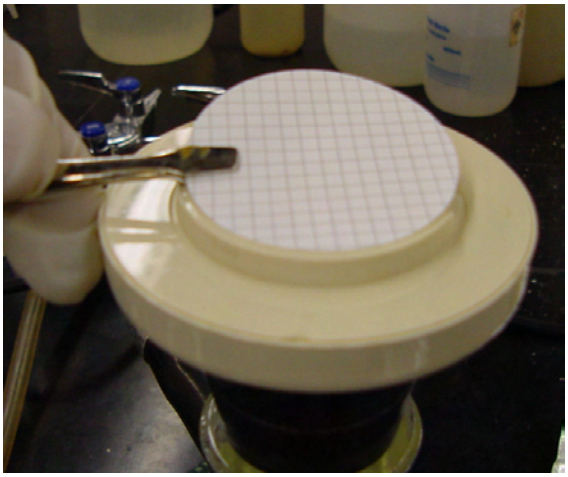


Figure: Place the filter grid side up. Discard if the filter chips, cracks or tears.

Replace the upper portion of the funnel making sure the membrane filter is still centered on the funnel support.

7. Shake a bottle of sterile phosphate dilution water vigorously (the pre-blank). Remove the cap and pour the dilution water into the filter funnel.
8. Turn the vacuum on. After the dilution water has passed <u>completely</u> through the filter, rinse the funnel 2 times with 20 - 30 ml of sterile phosphate rinse water to assure the sample has been rinsed from the funnel.

   Stop between <u>each</u> rinse to allow organisms to get caught on the filter. A rinse bottle can be used. Start rinsing at the top of the funnel and rinse down the sides. Repeat after all the rinse water has been filtered.

9. Turn the vacuum off. <u>TIP</u>: Carefully break the residual vacuum in the flask to prevent tearing the filter during removal.
10. Re-sterilize the forceps by dipping in alcohol and igniting in a Bunsen burner flame.
11. Remove the membrane filter using sterile forceps. Remove the petri dish cover and transfer the filter, grid side up, into the prepared petri dish by gently allowing it to role over the edge onto the m-FC saturated pad.

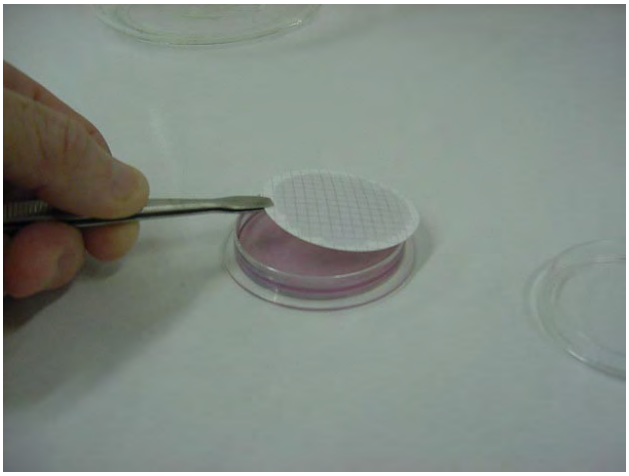


Figure: Set the filter on the back edge and pull forward until the membrane falls onto the absorbent pad.

TIP: Light patches on the filter indicate air is trapped beneath the filter. Using sterile forceps, gently lift the membrane filter edge and replace it.

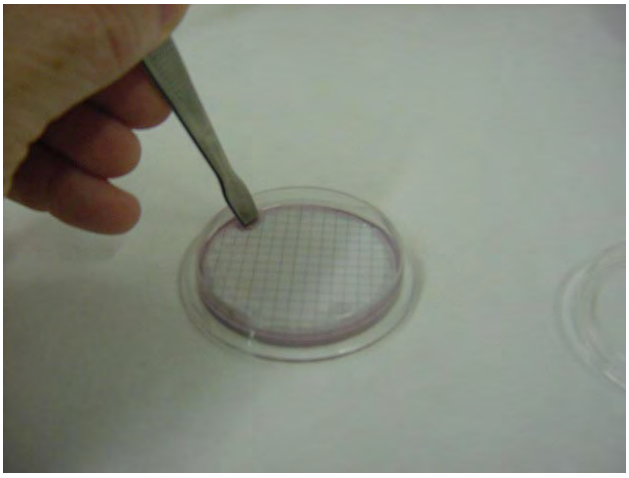


Figure: Gently secure the filter edges to the absorbent pad by running forceps around the perimeter of the Petri dish. Do not touch the area of the filter that contains bacteria.

12. Replace the petri dish cover, making sure it fits snuggly. Label the dish as blank.
13. Repeat the procedure for the remaining samples and after blank. If highly contaminated samples are run, decontaminate the filter funnel after each sample by exposing to UV light again. Alternatively, run an additional sterile phosphate dilution water blank after the filter has been removed. This will prevent carryover between samples.
14. Place the inverted dishes in a Whirlpack bag. All filtered culture plates should be in the waterbath within 30 minutes after filtration.
15. Immerse in the waterbath at 44.5 $\pm$ 0.2 ^{o}C for 24 $\pm$ 2 hours.

Figure: Squeeze out the air from the bag, roll the bag tightly, and then fold over the edges to seal the bag.

Colony Counting

After 24 $\pm$ 2 hours, remove the Whirlpack bag from the waterbath. Count all the colonies on each plate with various shades of blue.

The pre-blank must have no growth, indicating reagents, equipment and technique are acceptable. Data is rejected if growth occurs on the pre-blank.

Corrective Action: If growth occurs on the pre-blank, first examine aseptic technique then equipment, media sterility.

The after-blank must also have no growth. Growth on the after-blank usually indicates rinsing technique is poor. Data is rejected if growth occurs on the after blank.

Corrective Action: If growth occurs on the after-blank, examine rinse technique, increase the number or volume of rinses.

The technician should make enough dilutions to have a least 1 plate with a countable number of 20-60 blue colonies. Plates are <u>not</u> rejected if there is <u>not</u> a countable number.

Reporting

Confluent Growth is defined as a plate having growth covering the entire plate with no distinct colonies.

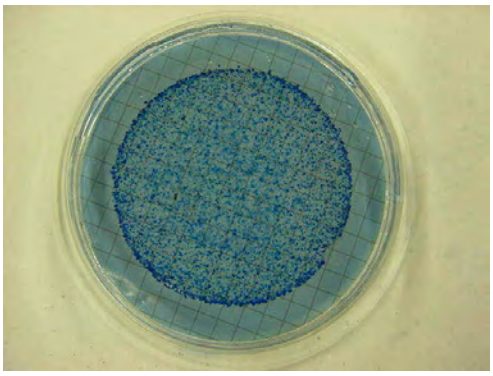


Figure: Confluent growth, distinct colonies are not visible.

Corrective Action: Confluent growth cannot be used for DMR purposes. A resample must be requested.

TNTC is defined as Too Numerous to Count. Plates having excessive growth on the entire plate with distinct colonies are too numerous to count accurately. Plates with greater than 200 colonies are considered TNTC.

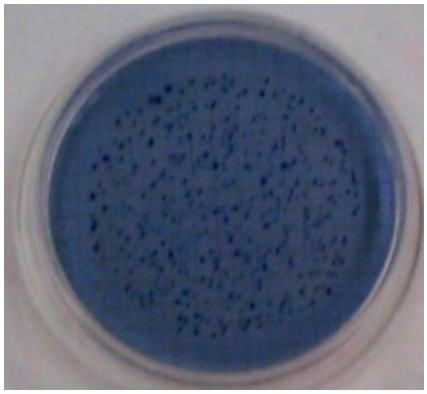


Figure: TNTC, too numerous to count. Individual colonies are visible but accurate counting is unlikely.

Corrective Action: TNTC growth cannot be used for DMR purposes. A resample must be requested and different dilutions selected.

A countable plate is defined as having between 20-60 colonies on the entire plate. These plates are acceptable.

Plates having no colonies are reported as <1 cfu/100 ml.

If no plate has between 20-60 colonies, all the counts are added from the other plates and divided by the total volume of **sample** filtered.

Fecal Coliform Calculations

$$\text{Fecal coliform} = \text{coliform} \frac{\text{colonies counted} \times 100}{\text{ml sample filtered}}$$

Example 1:	ml sample filtered	Colonies
Pre-Blank		<1
Effluent undiluted	100	94
Effluent 1:10 dil.	10	33
Effluent 1:100 dil.	1	1
After blank		<1

First, examine the blanks. If either blank shows growth, all sample data is invalid and must be rejected. The analysis must be repeated.
If the blanks show no growth, identify the plate(s) with a countable number. In this case, the plate with 33 is the only plate with a countable number. Identify the volume of sample which contained the 33 colonies (10 ml)

Calculate: $\frac{33 \text{ cfu} \times 100}{10 \text{ ml}} = 330 \text{ cfu/100 ml}$

Example 2:	ml sample filtered	Colonies
Pre-Blank		<1
Effluent	5 ml	6
Effluent	15 ml	28
Effluent	25 ml	45
Effluent	50 ml	87
Effluent	100 ml	TNTC
After blank		<1

In this example, there are 2 countable plates (28 and 45).

Calculate: $\frac{(28 + 45) \text{ cfu} \times 100}{(15 + 25) \text{ ml}}$ = 182 cfu/100 ml

Example 3	ml sample filtered	Colonies
Pre-Blank		<1
Effluent 1:10000	0.01 ml	<1
Effluent 1:1000	0.1 ml	15
Effluent 1:100	1 ml	95
Effluent 1:10	10 ml	TNTC
Effluent	100 ml	TNTC
After blank		<1

Since there is no countable plate, total all the coliform counts on **all** filters and report as cfu/100 ml.

Calculate: $\frac{(95+15+ 0) \text{ cfu} \times 100}{(1 + 0.1 + 0.01) \text{ ml}}$ = 9909 cfu/100 ml

Report to 2 significant figures, i.e. 9900 cfu/100 ml

Example 4:	ml sample filtered	Colonies
Pre-Blank		<1
Effluent	5 ml	<1
Effluent	15 ml	<1
Effluent	25 ml	<1
Effluent	50 ml	<1
Effluent	100 ml	<1
After blank		<1

There are no countable plates, so report the answer as <1 cfu/100 ml. Report as 1 cfu/100 ml on the DMR.

Example 5:	ml sample filtered	Colonies
Pre-Blank		<1
Effluent 1:10 dil	10 ml	<1
Effluent 1:100 dil.	1 ml	<1
Effluent 1:1000 dil.	0.1 ml	<1
After blank		<1

In this example, there are also no countable plates. Because 100 ml of sample was not analyzed, the result is reported as <10 cfu/100 ml

Calculate: $\frac{(<1) \text{ cfu} \times 100}{10 \text{ ml}}$ = <10 cfu/100 ml

Quality Control

Fecal coliforms are not predominant in most environments but are certainly predominant in a wastewater treatment plant. Poor lab hygiene can result in erratic laboratory results. Disinfecting lab benches frequently and maintaining general lab cleanliness will help reduce contamination and provide a safe working environment. Be sure to autoclave contaminated materials before disposal in the trash.

In spite of the simplicity of the method, the quality control required for this test is enormous because of the sterility requirements.

Sample QC

 Sample bottles. Sterile sample bottles containing 0.1 ml of sodium thiosulfate should be available. Each bottle must be able to contain a minimum of 120 ml and be autoclavable. Sample bottles are sterilized and checked for sterility using tryptic soy broth.

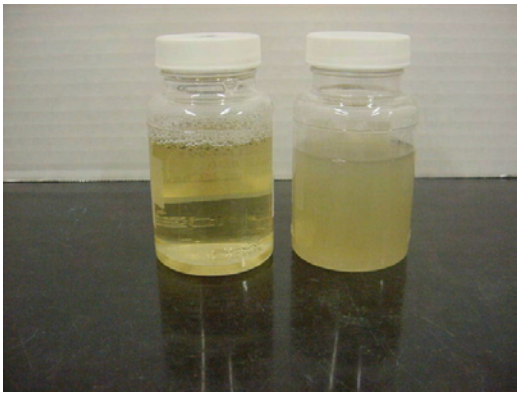


Figure: Growth in TSB indicates a contaminated sample bottle.

If using commercially available sterile bottles, confirm sterility using tryptic soy broth.

QC- record date/time sterilized

QC- record sterility after adding 50 ml of TSB and incubating for 24 hours at 35.0°C

QC- record lot number, sterility, date of purchase, and expiration date for commercially purchased bottles.

Corrective action- If sample bottle shows growth, repeat. If still positive, reject the entire batch and re-sterilize.

 Sample holding time exceeds 6 hours.

QC- Record

- date and time of sample collection
- sample type (grab)
- sampler
- date and time of arrival
- date and time of analysis
- analyst

Corrective Action: Reject samples and request a resample if:

1. holding time exceeded
2. disinfectant present in sample (odor)
3. non-sterile container was used
4. insufficient sample volume

 Samples that will take longer than an hour to analyze must be preserved on ice during transport and refrigerated until time of analysis

QC- record preservation, refrigeration temperature.

Corrective Action: Adjust refrigerator to below 6°C. Service the refrigerator if the temperature does not adjust properly. Reject any sample that shows evidence of freezing. (ice)

 Samples should be run in duplicate 10% of the time.

 Samples must be shaken vigorously to reduce bacterial clumping.

 Sample dilutions are run to obtain a countable plate of 20-60 colonies per plate.

Equipment QC

 pH meter calibrated. The pH meter is used to measure the pH of the various bacterial broths.

QC- record buffer lot numbers, purchase date, expiration date, slope

Corrective action- discard expired buffers, recalibrate if pH meter slope is below 95% or above 105% efficiency.

 Top loading balance calibrated. The top loading balance is used to weigh bacterial media and potentially sample volumes. 100 ml of sample can be weighed if a sterile graduated cylinder is not available. Bacterial media is very light and easily spilled. Be sure to clean the balance pan after each use to prevent cross-contamination.

QC- record monthly calibration and sensitivity of 0.1 gm

QC- record annual calibration by service contractor.

Corrective action: service as needed.

 All thermometers calibrated

QC- calibrate annually, record

- serial number of thermometer
- serial number of NIST thermometer
- temperature of lab thermometer
- temperature of NIST thermometer
- temperature correction factor
- date of check
- analyst

Corrective action- discard thermometer if correction factor >1.0°C. Dispose of mercury thermometers properly.

 Waterbath must be 44.5°C ± 0.2°C. The waterbath is used to provide a tighter temperature control with only minor variations.

QC - record twice per day at least 4 hours

QC- record date, time, temperature, and analyst

Corrective action- Waterbath outside the control limits must be adjusted prior to use. Service the waterbath if temperature cannot be adjusted or varies excessively. Be sure the thermometer is immersed to the correct depth in the waterbath to prevent inaccurate reading.

 Autoclave. The autoclave is usually used to sterilize bacterial media, phosphate dilution water, samples bottles, and contaminated materials prior to cleaning or disposal. Be sure materials placed in the autoclave can withstand the temperature. Use autoclavable plastics such as polypropylene.

Safety: Contaminated materials may contain billions of bacteria and a slight cut or poor hygiene when cleaning can have potentially fatal consequences.

QC - Record for each autoclave run

- date/time sterilized
- contents in autoclave
- sterilization temperature
- sterilization time in/out
- spore strip/integrator results
  - -lot number
  - -expiration date
- analyst

Corrective action- discard any prepared media if the sterilization time exceeds 15 minutes or the complete sterilization cycle exceeds 45 minutes. Excessive heating will denature the carbohydrates in the media. Use spore strips or integrator strips for each sterilization cycle to document effective sterilization.

Service the autoclave if spore strip results indicate inadequate sterilization. Re-sterilize contaminated materials if sterilization time is less than 30 minutes. Contaminated materials that have been sterilized (gloves, Petri dishes, etc.) can be disposed of in the general trash.

 Sterilizing oven. The sterilizing oven is usually used to sterilize glassware (pipets and graduated cylinders) and wooden applicator sticks.

QC - Record for each run

- date/time sterilized
- contents in sterilizing oven
- sterilization temperature 170 $\pm$ 10°C
- sterilization time in/out (2 hours)
- spore strip/integrator results
  - -lot number
  - -expiration date
- analyst

Corrective action- If spore strip results are invalid, repeat the sterilization process after adjusting the temperature.

 Ultraviolet Sterilizer. The UV sterilizer is primarily used to sterilize the membrane filtration funnel. The UV lamps have a limited shelf life and must be replaced periodically. They should be tested quarterly with a UV light meter or agar spread plate. The lamp should be replaced when the UV intensity is less than 70% of its initial output or if an agar spread plate containing 200-300 colonies, exposed to the UV light for 2 minutes, does not show a count reduction of 99%.

Figure: Pre-UV heterotrophic plate count

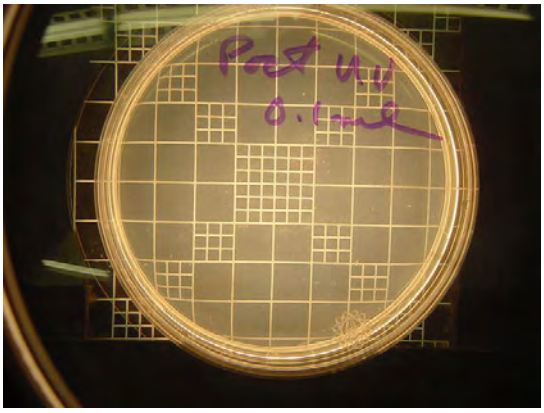
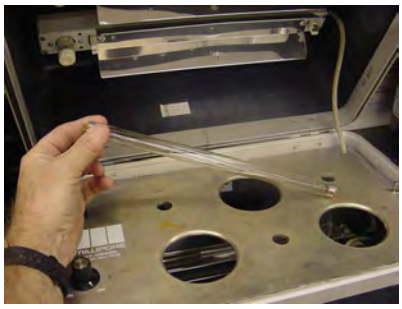


Figure: Post-UV heterotrophic plate count

The UV lamps should be cleaned monthly using ethyl or isopropyl alcohol. Do not touch the glass surface with fingers.



Corrective action- If the lamps do not show 99% kill, replace lamps. Lamps contain mercury, discard safely.

 Sterile phosphate dilution water. The phosphate dilution water used for dilutions, blanks, controls, and rinsing must be sterilized.

Commercially purchased dilution water must also be checked for sterility.
For dilution water bottles,
QC- Record for each batch

- pH after sterilization (7.2 $\pm$ 0.5)
- Volume of dilution water (99 $\pm$ 2 ml)
- Sterility

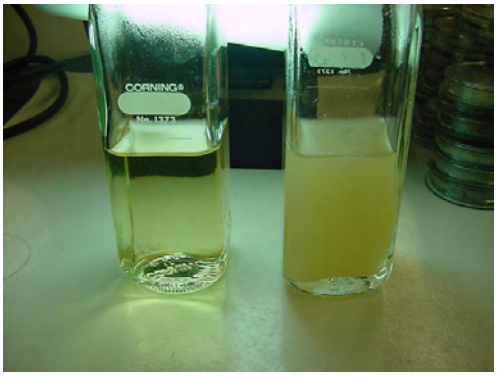


Figure: Phosphate dilution water sterility check.
TSB is added and checked for growth after 24 hours. Left bottle is sterile. Right bottle shows growth, indicating batch should be rejected.

Corrective action- If the pH, volume, or sterility is incorrect, reject the batch. If sterilizing large loads of dilution water in the autoclave, a longer sterilization time may be needed.

 Petri dishes, absorbent pads, membrane filters. Each lot purchased should be verified for sterility. Purchase a 6 month supply and rotate stock.
QC- Record for each batch of Petri dishes and absorbent pads.

- Lot number
- Sterility
- Expiration date

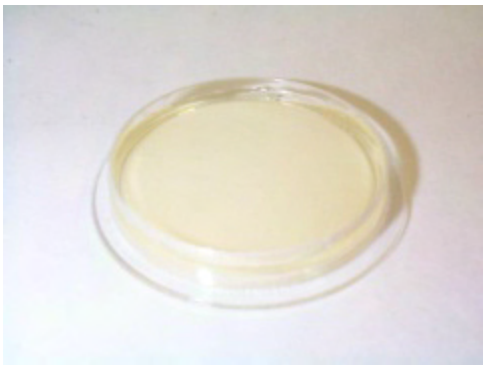


Figure: Sterility is checked by adding 5 ml TSB, incubating and checking for growth.

Corrective action- repeat with a second test. If positive, check with manufacturer.

QC- Record for each batch of m-FC broth

- Date received
- Lot number
- pH
- Expiration date
- Sterility
- Growth characteristics

Each lot of m-FC broth should be shown to be able to differentiate between fecal coliforms and total coliforms. The new lot of m-FC broth should show a comparable recovery to the current lot. Perform a parallel study to document recovery.

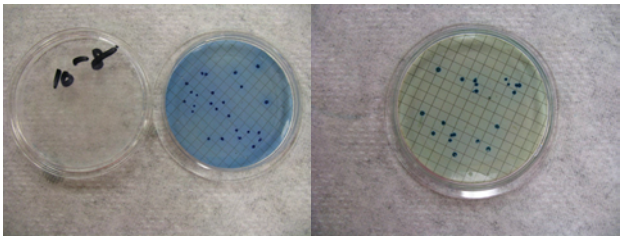


Figure: old broth, new broth at same dilution

 Dehydrated culture media

Dehydrated culture media such as tryptic soy broth should purchased in 1/4 pound bottles. Store is a cool, dry place away from sunlight. The desiccator is a good place to store opened bottles. Opening the factory seal only slightly will allow the seal to be reused and help prevent moisture absorption. Store opened bottles upside down to minimize water absorption. Use opened bottles of media within 6 months, discard media that is caked or discolored.

QC Record for each media

- Date received
- Date opened
- Date expiration
- Lot number

 Filtration apparatus.

The filtration apparatus, even if pre-marked by the manufacturer must be calibrated. Place the blue backing paper from a membrane filter on the apparatus. Measure 97.5 ml of water and pour into the filtration funnel. Mark the lower control value on the side of the funnel. Pipet 5 ml into the funnel giving a total volume of 102.5 ml. Mark the upper control value on the funnel. Recheck annually.

 Reagent Grade water.

The water used to prepare media, blanks, controls, and rinse filters must be of the highest quality and be shown to not contain toxic materials. The reagent grade water must be checked monthly for

QC - conductivity,
heterotrophic plate count
chlorine residual.

QC - It must also be checked annually for heavy metals - chromium, cadmium, copper, zinc, lead, and nickel. Heavy metals may inhibit bacterial growth.

 Inhibitory residue test

The inhibitory residue test is a test that shows the detergent used to wash glassware does not effect the growth of bacteria. Some laboratory

detergents contain bacteriostatic substances which are difficult to rinse out. This test should be performed annually or prior to using a new supply of detergent. The test involves performing heterotrophic plate counts on 3 sets of dishes. The first set follows the normal laboratory glassware washing procedure. The second set follows the normal procedure with additional rinsing to remove residual detergent. The third set is washed but not rinsed. The plate counts are run and the result compared. If an inhibitory effect cannot be eliminated by routine washing, a different detergent should be used.

Detergent residue on glassware can be spot checked using 0.04% bromthymol blue and checking the color reaction. The color should be blue-green. Purple usually indicates detergent has not been rinsed completely, yellow indicates acid has not been rinsed completely.

Fecal Coliform Test QC

 Pre-blank sterility control is run for each sample series. A sterility control should be run between each different sample set (influent, effluent) to reduce chances of cross-contamination.

Corrective Action: Any growth on the pre-blank indicates contamination and invalidates the test. There are a number of variables that can contribute to contamination of the pre-blank. Evaluate each source of error.

- Petri dish
- Absorbent pad
- Membrane filter
- UV light
- Phosphate dilution water
- Phosphate rinse water
- Technician technique

 After-blank rinse control run after each sample series.

Corrective action: Any growth on the after-blank also indicates contamination and invalidates the test. If the pre-blank was sterile, the equipment was not a problem. The most likely source of error is carry-over of a contaminated sample due to poor rinsing technique. Re-evaluate the rinsing procedure.

 Petri plates placed in the waterbath within 30 minutes of filtration.

 Negative Controls

Negative controls should be run for each new lot of m-FC broth. A negative control should show no growth of blue colonies. The negative control shows the media will differentiate between fecal and total coliforms. Enterobacter aerogenes is a total coliform and will not produce blue colored colonies on m-FC broth.

Corrective action: If blue colonies are present, either contamination of the Enterobacter aerogenes has occurred or the m-FC broth is defective. Repeat with a second culture of Enterobacter. If still positive, check with the media manufacturer.

 Positive Controls

A positive control should show growth of blue colonies. E. Coli is a fecal coliform and will produce blue colonies. Failure to produce blue colonies may indicate the E. Coli culture is bad or that toxic materials are present which inhibit growth.

Corrective action: If no blue colonies are present, repeat with a second culture of E. Coli. or use raw wastewater. If still negative, check with the media manufacturer.

MPN (Most Probable Number)

The MPN method (also called Multiple Tube Fermentation Technique) for fecal coliform detection is often used when the sample has excessive turbidity. Excessive turbidity in the

sample will plug the membrane filter, causing poor bacteria recovery and slow filtration times.

The MPN method involves adding the wastewater sample to a series of 5 sets of tubes, each of which contains either lactose broth or lauryl tryptose broth and an inverted tube. The tubes are then incubated at 35 $\pm$ 0.5^{o}C for 24 to 48 hours. Each tube is then observed for growth and gas production. If growth and gas production is observed in an inverted tube, the result is positive and the bacteria are **presumed** to be coliform bacteria. A sterile applicator stick or inoculating needle is used to transfer a small portion from the positive tube to a second tube containing EC broth. Each EC tube is incubated in a water bath at 44.5 $\pm$ 0.2C for 24 hours. Tubes which again show growth and gas are now **confirmed** as fecal coliforms. The coliform density is then calculated from statistical probability formulas that predict the most probable number, MPN, of coliforms necessary to produce certain combinations of gas-positive and gas-negative tube in the series of inoculated tubes.

E. Coli

E. Coli are a more specific indicator of human pollution and possible pathogen presence. Newer discharge permits are requiring measurement of E. Coli rather than fecal coliform. Methods for E. Coli in wastewater are not yet approved and will be added later.