Bacteriological Examination of Waters: Membrane Filtration Protocol

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Information

History

One component of potable water quality analysis is the presence or absence of human pathogenic bacteria that are transmitted through the fecal-oral route, i.e., mainly intestinal pathogens. Since it is difficult and expensive to routinely examine waters for the presence of every type of pathogen, it is more practical to screen the water for the presence of fecal contamination by testing for the presence of an **indicator microorganism.** Indicator microorganisms are ones that have the following properties:
a) the microorganism is not found in water and will be present in the water only when a contamination event has occurred; and
b) the density of the microorganisms present should be proportional to the degree of contamination.
In the 1890's, it was suggested that *Escherichia coli* should be used as an indicator microorganism to detect the presence of pathogenic bacteria through the fecal-oral route (4). This bacterium was selected due to the work of Theodore Escherich in the 1880s (2). Escherich found that *Bacillus coli*, (now known today as *E. coli*) was distributed in the intestines (i.e., an enteric bacterium) and feces of animals and thus meets the properties of the indicator microorganism described above. Today, some water quality standards are still based on the detection of *E. coli* and/or related bacteria termed "coliforms" (1). Many different techniques can be used to detect the presence of these indicator microorganisms. Such techniques are ones that should have the following properties:

- The technique should be sensitive to detect the presence of the indicator, even at low concentrations.
- The technique needs to be able to process large amounts of water.
- The technique should be easy, cheap and can detect the presence of the indicator quickly.

In 1951, Goetz and Tsuneishi (5) published a technique that used cellulose nitrate and cellulose acetate membranes as a means of capturing any bacterium present in a sample of water during filtration. This technique is still employed today.

Purpose

The membrane filtration technique is used to examine water samples from different sources. The membrane is incubated on an agar

plate. Bacterial (and other) cells trapped on the membrane will grow into colonies that can be counted, and a bacterial density of the water samples can be calculated. (1)

Theory

Total Coliforms & Fecal Coliforms

Total coliforms are indicator microorganisms that can be detected by membrane filtration. The total coliforms belong to the family *Enterobacteriaceae*, but the definition of the group is more operational than phylogenetic.

The definition of coliforms is not completely specific to bacteria of fecal origin. In addition, the definition of total coliforms can vary on country and public health organizations (7).

To be considered "total coliform" in the United States (1), a bacterium should exhibit the following characteristics:

- Gram-negative rod;
- aerobe or facultative anaerobe;
- not a spore former; and
- ferment lactose with the production of acid within 24 hours at 35°C (if using the membrane filtration technique) or acid and gas within 48 hours at 35°C (for multiple-tube fermentation technique, not described in this protocol).

Coliforms may include bacteria of the following genera: *Escherichia, Enterobacter, Klebsiella*, *Citrobacter* and *Serratia*. Not all total coliforms are pathogenic. A subset of total coliforms are the **fecal coliforms**, which are found within the digestive tract and shed through feces. These indicator microorganisms have shown a better correlation with the occurrence of fecal contamination. This group is characterized by its ability to ferment lactose with the production of acid (and gas, depending on the method) at 44.5°C within 24 hours. Since they can grow at a higher temperature, they are also said to be thermotolerant coliforms.

Some fecal coliforms can be pathogenic, while others are not. Bacteria belonging to the genera*Escherichia* and *Enterobacter* can be considered as fecal coliforms.

Membrane Filtration

The membrane filtration technique is used to examine water samples from different sources. An appropriate volume of the sample is filtered through a membrane with a pore size of 0.45 mm. The membrane is incubated on an agar plate. Bacterial (and other) cells trapped on the membrane will grow into colonies that can be counted, and a bacterial density can be calculated. When using the membrane filtration technique to test for the presence of indicator microorganisms, different filtration volumes are suggested depending on the source of the water sample (Tables 1 and 2) (1)

Table 1. Suggested sample volumes for membrane filtration to detect total coliforms (1). Note that filtering of 0.01 ml of sample is the same as filtering 1 ml of a 1/100 dilution of the original sample.

	Volume to be filtered (ml)							
Source	100	50	10	1	0.1	0.01	0.001	0.0001
Drinking water	X							
Swimming pool	X							
Wells, springs	X	X	X					
Lakes, reservoirs	X	X	X					
Water supply intakes			X	X	X			
Bathing beaches			X	X	X			
River water				X	X	X	X	
Chlorinated sewage				X	X	X		
Raw sewage					X	X	X	X

Table 2. Suggested sample volumes for membrane filtration to detect fecal coliforms (1). Note that filtering of 0.01 ml of sample is the same as filtering 1 ml of a 1/100 dilution of the original sample.

Source	Volume to be filtered (ml)							
	100	50	10	1	0.1	0.01	0.001	0.0001
Lakes, reservoirs	X	X						
Wells, springs	X	X						
Water supply intakes		X	X	X				
Natural Bathing waters		X	X	X				
Sewage treatment plant			X	X	X			
Farm ponds, rivers				X	X	X		
Stormwater runoff				X	X	X		
Raw sewage					X	X	X	
Feedlot runoff					X	X	X	
Sewage sludge						X	X	X

It is suggested that duplicate volumes are filtered for drinking water, and three different volumes (or dilutions) are filtered for all other sample sources. The membrane filtration technique exhibits a high degree of reproducibility and may be used to detect other types of organisms when in combination with an appropriate medium. It has the potential of having a very low detection limit, since large volumes of sample can be

filtered. However, the turbidity of the sample may limit the volume of sample that is practical to filter. High numbers of background bacteria or toxic substances may interfere with the test and result in underestimation of the density of coliforms.

Detection of total coliforms and fecal coliforms (1)

The characteristics that define the total coliform and fecal coliform groups are easily tested in the laboratory using culture media and incubation conditions that are selective for Gram-negative rods and/or differential for lactose fermenters. Growth media that are used include:

- Detection of total coliforms – **mEndo agar LES**
  This growth medium contains lactose and a pH indicator that changes color when acid is produced (from lactose fermentation). Coliforms typically will produce metallic (golden) sheen, which is due to the extensive production of aldehydes and acid from the fermentation of lactose. Some total coliforms may also be dark red, mucoid or have a dark center but without a metallic sheen; these are considered to be atypical total coliform colonies (7). *E. coli* will form colonies with a metallic sheen. It is important to note that some non-coliforms may exhibit red colonies. Filters used to detect the presence of total coliforms in a sample of water should be incubated at 35°C for 22 - 24 hours.
- Detection of fecal coliforms - **mFC agar.**
  This growth medium contains bile salts. Enteric bacteria can grow in the presence of bile salts, while others cannot. mFC agar also contains rosolic acid, which inhibits bacteria other than fecal coliforms. Aniline blue, a pH indicator, turns dark blue upon acid production, helping in the identification of lactose-fermenting bacteria (7). Fecal coliforms form blue colonies on this medium;*E. coli* will form flat dark blue colonies. While rosolic acid is commonly used as part of mFC agar, it has been suggested that rosolic acid is not a necessary component of this medium (1, 6). Filters used to detect the presence of fecal coliforms in a sample of water should be incubated at 44.5°C for 22 – 26 hours.
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Either liquid broth or a solid medium may be used for these assays. Solid media are commonly used and are specified in this protocol.

When reporting total and fecal coliforms in water samples, it is standard to use colony-forming units (CFU)/100 ml of sample.

RECIPE

Nitrocellulose membrane

47mm membrane filters with a pore size of 0.45 mm (Fig. 1)

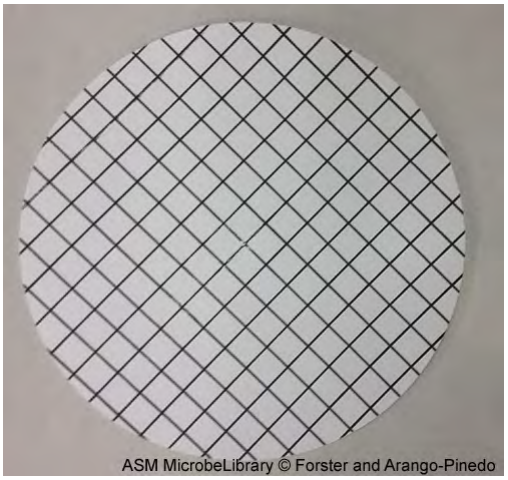

FIG 1 Nitrocellulose membrane. This membrane is used for the detection of total coliform and fecal coliform bacteria from samples of water. It has a 47 mm diameter and a pore size of 0.45 µm. The small pore size in the membrane filter will capture bacterial cells present in a sample of water during filtration. A grid is printed on the membrane to assist with counting colonies after incubation.

Membrane Filtration Device

You may use either a Buchner Funnel or a Filtration column (Fig. 2 & 3) to filter the water.

FIG 2 Filtration Column used in membrane-filtration. This filtration column can be used for the detection of total coliform and fecal coliform bacteria from samples of water. The column is first connected to a

vacuum pump. A nitrocellulose membrane is placed between the chamber and catchment vessels. The water sample is then poured into the chamber of the column.

Vacuum pump. (Fig. 3)

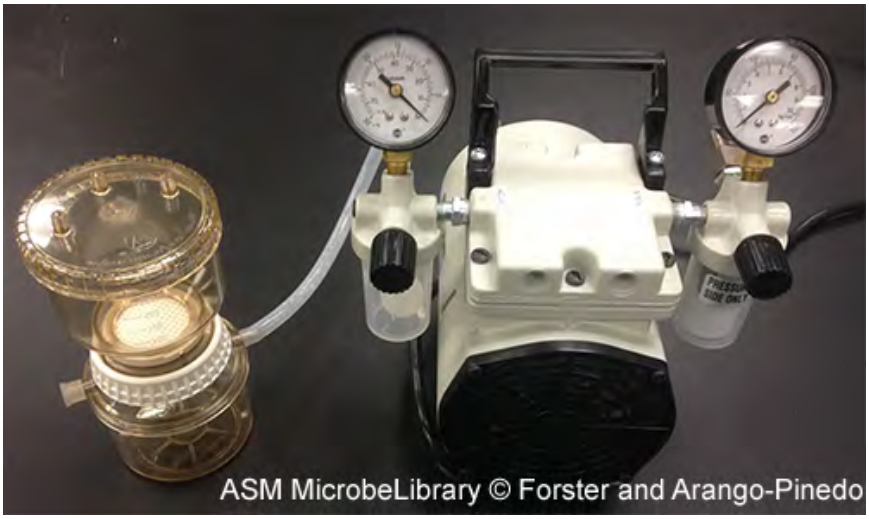

FIG 3 Vacuum set-up for membrane-filtration. This figure shows the filtration column with filter paper assembled and connected to a vacuum pump. When the vacuum is turned on, the water will filter through the nitrocellulose membrane into the catchment vessel. Any bacteria present in the water will be trapped on the nitrocellulose membrane.

Media

The formulations for mEndo Agar LES and mFC agar are provided for reference purposes. However, commercially prepared dehydrated media are available from numerous sources. It is strongly recommended (1) that these prepared media be used, following the manufacturers' instructions.

mENDO agar (DIFCO) (3)

Lactose .....9.4 g
Tryptose.....7.5 g
Casitone.....7g
Thiopeptone.....3.7 g
Sodium Chloride.....3.7 g
Potassium Phosphate, dibasic.....3.3 g
Sodium Sulfite.....1.6 g
Yeast Extract.....1.2 g
Potassium Phosphate, monobasic.....1.0 g
Basic Fuchsin.....0.8 g
SodiumDeoxycholate.....0.1 g
Sodium Lauryl Sulfate..... ....0.05 g
Agar.....15 g

Suspend ingredients in 1 liter distilled water containing 20 mL of 95% ethanol. Mix thoroughly. Heat with frequent agitation and bring to a boil to completely dissolve the powder; remove from heat immediately. DO

NOT AUTOCLAVE. Let cool to just above the point where the agar will start to solidify (45 to 50ºC). Pour about 4 to 6 mL aseptically into prepared in 50 mm petri dishes that will fit the nitrocellulose paper (Fig. 4A).

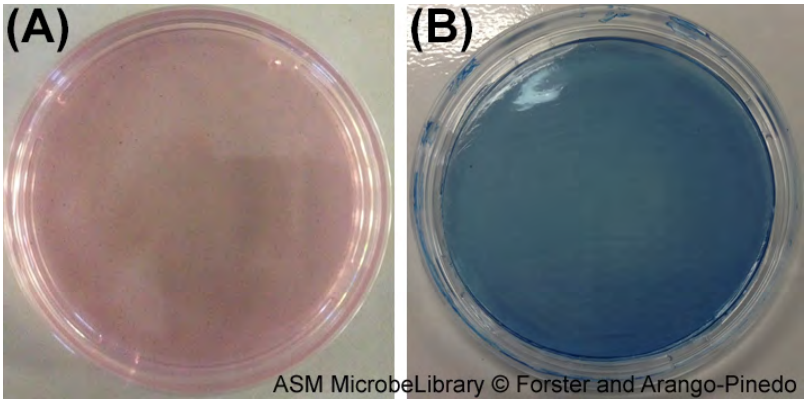

FIG 4 mENDO agar (A) and mFC agar (B) prior to incubation. These growth media are selective and differential media used in the detection of total coliforms and fecal coliforms, respectively. On mENDO agar, coliforms will form red colonies with a metallic sheen. On mFC media, fecal coliforms will form dark blue colonies.

mFC agar – (DIFCO) (3)

Tryptose.....10.0 g
Proteose Peptone No. 3.....5.0 g
Yeast Extract .....3.0 g
Lactose 12.5g
Bile Salts No. 3.....1.5 g
Sodium Chloride .....5.0 g
Agar.....15.0 g
Aniline Blue..... 0.1 g

Suspend ingredients in 1 liter of distilled water. Mix thoroughly. Heat with frequent agitation until boiling and boil for one minute. Remove from heat immediately. Add 10 ml of a 1% solution of rosolic acid in 0.2N NaOH. Continue heating for 1 minute. DO NOT AUTOCLAVE. If necessary, adjust to pH 7.4 with 1N HCl. Let cool to just above the point where the agar will start to solidify (45 to 50ºC). Pour about 4 to 6 mL aseptically into prepared in 50 mm petri dishes that will fit the nitrocellulose paper (Fig. 4B).

Forceps, Ethanol, Bunsen Burner

These materials should be used for aseptic technique when placing the membrane on either the filtration column or the agar plates.

Sterile water (in squirt bottle)

Sterile water is used to make sure that the vacuum chamber is set properly before the addition of the water sample to be tested, and to

rinse bacteria and sample remnants from the walls of the filter tower or funnel.

Positive Control Sample

100 ml of water with a 5 ml of a 1:100 dilution of an overnight culture of *E. coli*

This sample is to be used as a positive control to ensure that you are able to detect *E. coli* from a water sample using the membrane filtration technique.

Negative Control Sample

Sterile Water

This sample is to be used as a negative control when using the membrane filtration technique.

Water Samples to be tested

Volumes of water needed for testing are indicated in Table 1.

Protocol

NOTES:

- Use aseptic technique throughout the procedure.
- Buchner Funnel or a Filtration column should be autoclaved prior to use.

Day 1:

1. Decide on the volume of water to be filtered (or the appropriate dilution to be prepared) as described in Table 1 and/or 2. Calculate and write down all the details that are relevant (which are the dilutions to be made, what are the volumes of each dilution to be filtered). If applicable, prepare the dilutions and gently mix the sample (or dilution) by inverting the tube or bottle several times before you obtain an aliquot.
2. Put together the filtration apparatus.
    a. If using a Buchner funnel (with cover), insert the stopper into a flask with a side arm. Make sure the tubing of the vacuum pump is inserted into the side arm of the flask.
    b. If using a filtration column, make sure the tubing of the vacuum pump is inserted into the side arm of the flask.
3. Turn on Bunsen burner. Dip forceps in ethanol and pass through the flame. This will sterilize your forceps.
    a. If using a Buchner funnel (with cover), remove the cover. With sterile forceps, take a sterile filter membrane and place it on the filter holder of either the funnel.
    b. If using a filtration column, remove the top chamber from the column. With sterile forceps, take a sterile filter membrane and place it on top of the catchment vessel.
4. Using the squirt bottle, pour a small volume of sterile water in the filtration set-up, turn the pump on and apply vacuum for a few seconds to get the membrane wet. Always place the lid back on the column before applying vacuum!

5. If filtering a small volume (less than 10 ml), add 10-20 ml of sterile water to the filtration tower.Beginning with the smallest volume (or the highest dilution), add the volume of sample that you want to filter. Gently mix the sample (or dilution) by inverting the tube or bottle several times before obtaining the volume.
6. Turn the pump on and filter the sample into the flask. Be careful not to allow the flask or catchment chamber of the column to fill with water, or the water may be sucked into the pump, damaging the vacuum system.
7. Using a squirt bottle with sterile water, rinse the walls of the tower. Turn the vacuum on to filter the water.
8. Using sterile forceps (step 3), transfer the filter membrane to a properly labeled mENDO agard LES plate, avoiding the creation of air pockets between membrane and agar (Fig. 5).

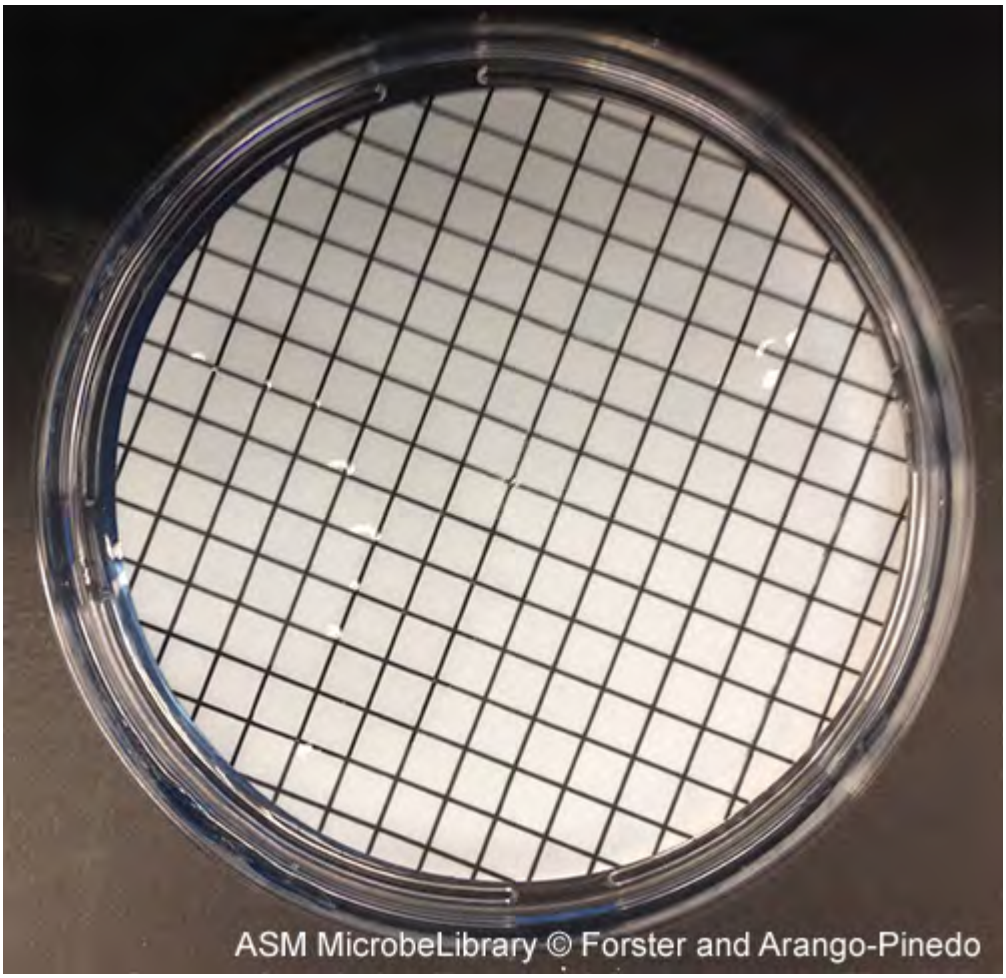

FIG 5 Placement of membrane onto agar following vacuum filtration. After filtering the water sample using the filtration column, the filter is aseptically transferred onto the plate, grid side up. The grid should be gently pressed onto the agar such that the nutrient agar can be absorbed into the membrane, allowing bacterial colonies to develop.

9. Repeat the procedure (steps 3-8) with the same volume (or dilution).
10. Repeat procedure two more times with the same volume, but place membranes on mFC plates.
11. Repeat the procedure (steps 3-10) with the next volume (or dilution).

12. Repeat the procedure (steps 3-10) with the largest volume (or lowest dilution)
13. Incubate mENDO agar LES plates at 35 ± 0.5 °C for 22 – 24 hours and mFC plates at 44.5 ± 0.2°C for 22 to 26 hours, all lid side down. In order to maintain the temperature within such a narrow range, a water bath is typically used for incubation of the mFC agar plates. These plates are placed in watertight plastic bags and then submerged in the water bath.

Day 2 (24 hours later):

14. After 22 – 24 hours, remove the mEndo agar LES plates from the 35°C incubator and count the colonies that are dark red, mucoid, have a dark center or (more typically) produce a metallic sheen (Fig. 6). These are considered to be total coliform colonies.

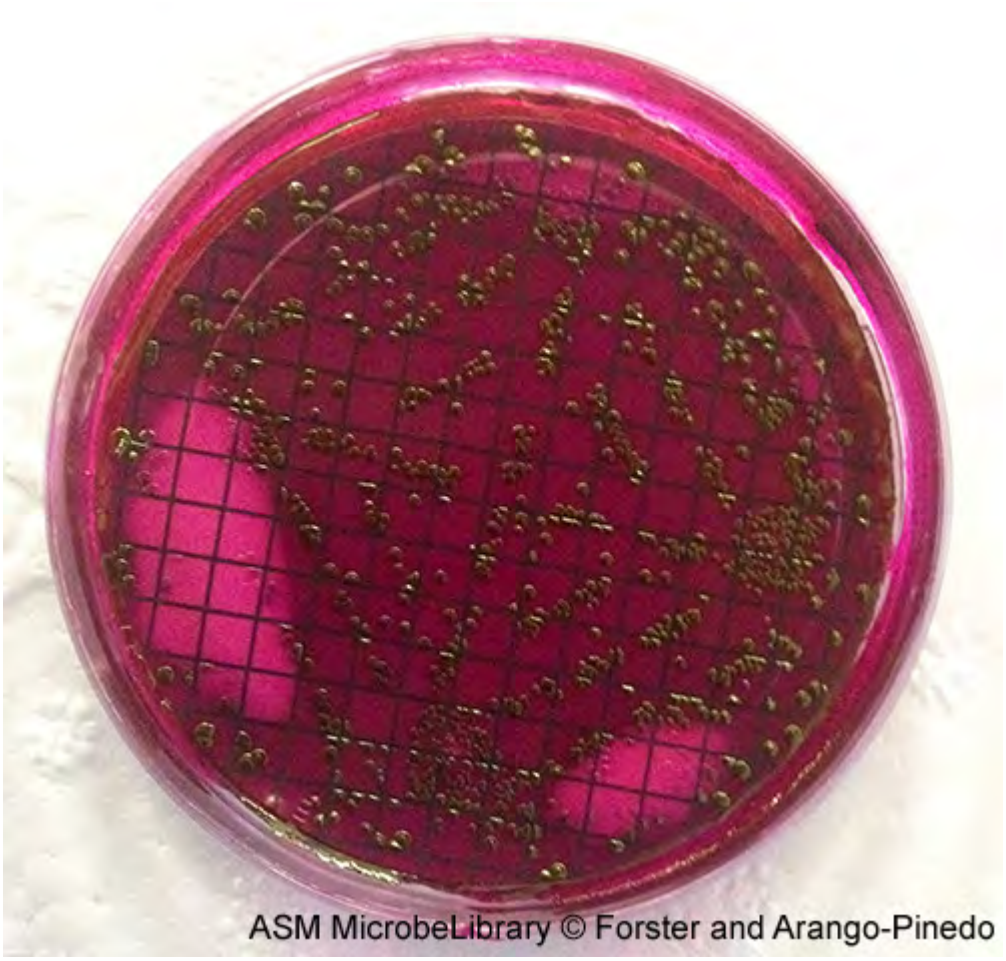

FIG 6 Detection of total coliforms following 24 hour incubation at 35°C. Metallic green colonies indicate the presence of *E. coli* in a water sample.

15. From the mEndo agar LES plates, choose two total coliform colonies that are isolated on the membrane and perform a gram-stain to confirm that they are Gram-negative rods. Use phase-contrast microscopy or Schaffer-Fulton stain to confirm that these colonies contain bacteria that are non-spore formers.
16. After 22 to 26 hours, remove the mFC agar plates from the 44.5°C

incubator and count the colonies that have any blue color. These are considered to be fecal coliform colonies (Fig. 7 & 8).

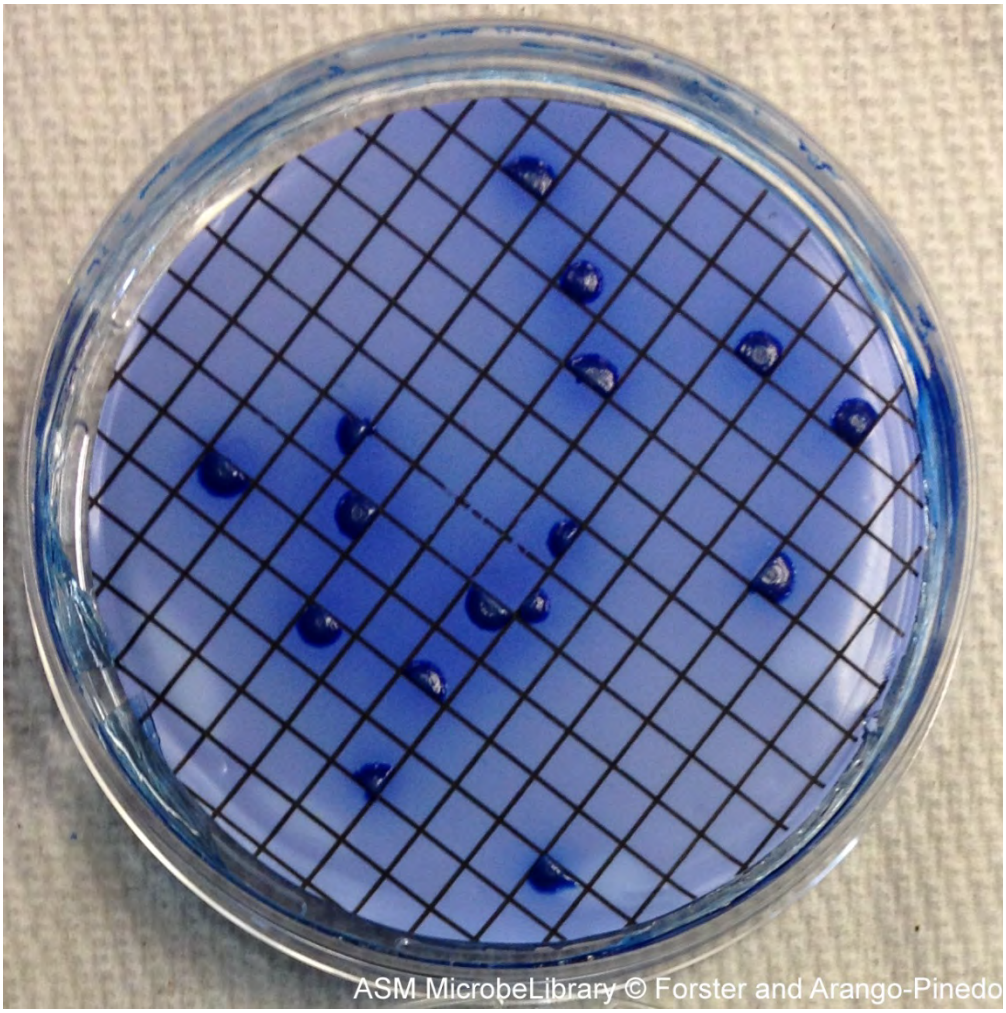

FIG 7 Detection of fecal coliforms following 24 hour incubation at 44.5°C. Blue colonies are indicative of fecal coliform bacteria.

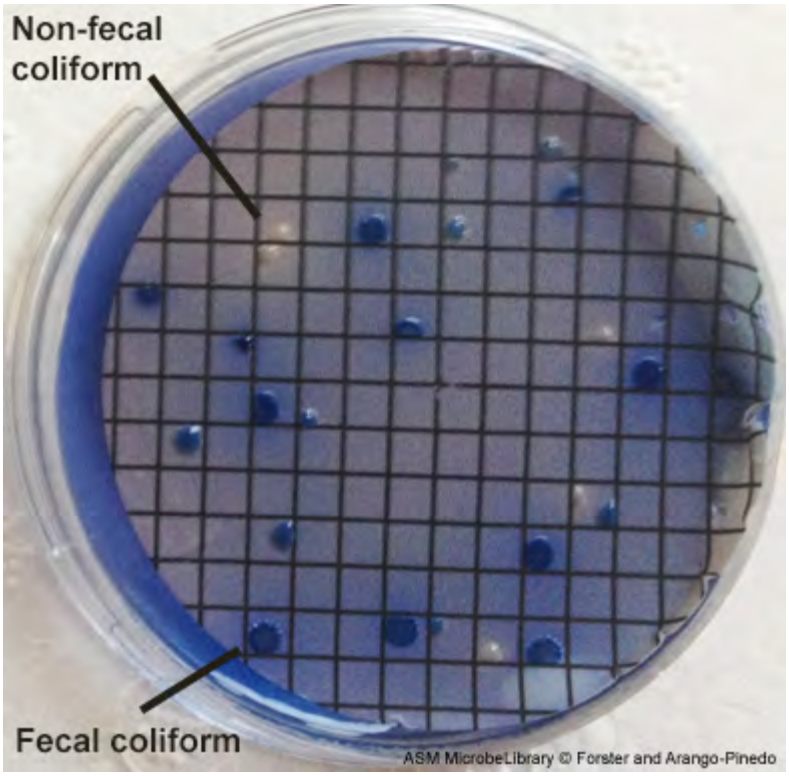

FIG 8 Detection of fecal coliforms following 24 hour incubation at 44.5°C. This plate shows two different types of colonies. The blue colonies are indicative of fecal coliform bacteria. The yellow colonies are indicative of non-fecal coliform bacteria. When determining cell density of fecal coliform bacteria present in water samples, only blue colonies should be counted.

17. From the mFC agar plates, choose two fecal coliform colonies that are isolated on the membrane. Confirm that they are Gram-negative rods and non-spore formers.
18. Choose two non-total coliform colonies from the mEndo agar LES plates and two non-fecal coliform colonies from the mFC agar plates. The bacteria from these colonies may or may not be Gram-negative.
19. Calculate the total and fecal coliform CFU per 100 ml for each sample (as described below).

Calculating the cell density of the original sample:

20. The original density is estimated from the volume of sample filtered (or the volume of dilution and the dilution factor), and the number of colonies counted on the membrane. As counts are reported per 100 ml of sample (not per ml), the per ml values must be multiplied by a factor of 100.
21. Counts between 20 and 80 total coliforms (with not higher than 200 total colonies) per plate are optimal for calculations. Counts between 20 and 60 fecal coliforms per plate are optimal for calculations.

To calculate density (CFU per 100 ml):

$$\text{Density} = \frac{\text{number of colonies on membrane} \times 100}{\text{volume (ml) of undiluted sample filtered}}$$

Or if the sample was diluted and a volume of the dilution was filtered:

$$\text{Original Density} = \frac{\text{number of colonies on membrane} \times 100}{(\text{volume (ml) filtered} \times \text{dilution})}$$

SAFETY

The ASM advocates that students must successfully demonstrate the ability to explain and practice safe laboratory techniques. For more information, read the laboratory safety section of the ASM Curriculum Recommendations: Introductory Course in Microbiology and the Guidelines for Biosafety in Teaching Laboratories.
Care should be taken while working near a Bunsen burner. Ethanol and nitrocellulose membranes are flammable. Proper laboratory procedures should be followed when working with materials that may harbor microorganisms.

COMMENTS AND TIPS

It is recommend that the dilutions (step 1) be performed. Otherwise it may become difficult to determine the accurate cell density of coliforms present in water samples.
Demonstration that some bacteria in water can, indeed pass through a 0.45 µm pore-size filter can readily be demonstrated. Simply remove 5 – 10 mL of filtrate from the catchment vessel and inoculate a flask or test tube with a general purpose liquid growth medium, such as tryptic soy broth. Incubate the inoculated media at 35°C for 24 to 48 hours. The growth medium will be turbid, indicating that small but viable bacterial cells passed through the filter. A smaller pore-size filer (0.22 µm) is typically used to "filter-sterilize" water samples. A comparison between removals by these two types of filters could also be conducted.
It is recommended that you make your students aware of some of the EPA standards for testing that can be found at the following URLs:
http://water.epa.gov/scitech/methods/cwa/bioindicators/upload/method_1603.pdf
http://water.epa.gov/type/oceb/beaches/upload/2006_06_19_beaches_rvsdman.pdf

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