SECTION IX. COLIPHAGE ASSAY

This Section outlines the procedures for coliphage detection by plaque assay. It should be noted that the samples to be analyzed may contain pathogenic human enteric viruses. Laboratories performing the coliphage analysis are responsible for establishing an adequate safety plan.

ASSAY COMPONENTS

Apparatus and Materials:

1. Sterilizing filter — 0.45 µm (Nuclepore Product No. 140667 or equivalent).

Always pass about 10 mL of 1.5% beef extract through the filter just prior to use to minimize phage adsorption to the filter.

2. Water bath set at 44.5 ± 1°C.

3. Incubator set at 36.5 ± 1°C.

Media and Reagents:

The amount of media prepared may be increased proportionally to the number of samples to be analyzed.

1. Saline-calcium solution — dissolve 8.5 g of NaCl and 0.22 g of $CaCl_2$ in a total of 1 L of dH_2O. Dispense in 9 mL aliquots in 16 × 150 mm screw-capped test tubes (Baxter Product No. T1356-6A or equivalent) and sterilize by autoclaving at 121°C for 15 min.

2. Tryptone agar slants — add 1.0 g tryptone (Difco Product No. 0123 or equivalent), 0.1 g yeast extract (Difco Product No. 0127 or equivalent), 0.1 g glucose, 0.8 g NaCl, 0.022 g $CaCl_2$, and 1.2 g of Bacto-agar (Difco Product No. 0140 or equivalent) to a total volume of 100 mL of dH_2O in a 250 mL flask. Dissolve by autoclaving at 121°C for 20 min and dispense 8 mL aliquots into 16 × 150 mm test tubes with tube closures (Baxter Product Nos. T1311-16XX and T1291-16 or equivalent). Prepare slants by allowing the agar to solidify with the tubes at about a 20° angle. Slants may be stored at 4°C for up to two months.

3. Tryptone bottom agar — Prepare one day prior to sample analysis using the ingredients and concentrations listed for tryptone agar slants, except use 1.5 g of Bacto-agar. After autoclaving, pipet 15 mL aliquots aseptically into sterile 100 × 15 mm petri plates and allow the agar to harden. Store the plates at 4°C overnight and warm to room temperature for 1 h before use.

4. Tryptone top agar — Prepare the day of sample analysis using the ingredients and concentrations listed for tryptone agar slants, except use 0.7 g of Bacto-agar. Autoclave and place in the 44.5 ± 1°C water bath.

5. Tryptone broth — Prepare on the day prior to sample analysis as for tryptone agar slants, except without agar.

6. Beef extract V powder (BBL Microbiology Systems Product No. 97531) — prepare buffered 1.5% beef extract by dissolving 1.5 g of beef extract powder and 0.375 g of glycine (final glycine concentration = 0.05 M) in 90 mL of dH_2O. Adjust the pH to 7.0 - 7.5, if necessary, and bring the final volume to 100 mL with dH_2O. Autoclave at 121°C for 15 min and use at room temperature.

Beef extract solutions may be stored for one week at 4°C or for longer periods at -20°C.

SAMPLE PROCESSING

Step 1. To measure the concentration of coliphage in water samples, use the coliphage sample prepared from the pH-adjusted 1MDS eluate as described in the **Elution Procedure** in **Part 2** of **Section VII. Virus Monitoring Protocol**.

Step 2. Filter the coliphage sample through a 0.45 µm sterilizing filter.

Step 3. Assay ten 1 mL volumes each for somatic and male-specific coliphage within 24 h. Store the remaining eluate at 4°C to serve as a reserve in the event of sample contamination or high coliphage densities. If the coliphage density is expected or demonstrated to be greater than 100 PFU/mL, dilute the original or remaining eluate with a serial 1:10 dilution series into saline-calcium solutions. Assay the dilutions which will result in plaque counts of 100 or less.

SOMATIC COLIPHAGE ASSAY

Storage of *E. coli* C Host Culture for Somatic Coliphage Assay:

1. For short term storage inoculate a *Escherichia coli* C (American Type Culture Collection Product No. 13706) host culture onto tryptone agar slants with a sterile inoculating loop by spreading the inoculum evenly over entire slant surface. Incubate the culture overnight at 36.5 ± 1°C. Store at 4°C for up to two weeks.

2. For long term storage inoculate a 5-10 mL tube of tryptone broth with the host culture. Incubate the broth culture overnight at 36.5 ± 1°C. Add 1/10th volume of sterile glycerol. Dispense into 1 mL aliquots in cryovials (Baxter Product No. T4050-8 or equivalent) and store at -70°C.

Preparation of Host for Somatic Coliphage Assay:

Step 1. Inoculate 5 mL of tryptone broth with *E. coli* C from a slant with an inoculating loop and incubate for 16 h at 36.5 ± 1°C.

Step 2. Transfer 1.5 mL of the 16 h culture to 30 mL of tryptone broth in a 125 mL flask and incubate for 4 h at 36.5 ± 1°C with gentle shaking. The amount of inoculum and broth used in this step can be proportionally altered according to need.

Preparation of ϕX174 Positive Control:

Step 1. Rehydrate a stock culture of ϕX174 (American Type Culture Collection Product No. 13706-B1) and store at 4°C.

Step 2. Prepare a 30 mL culture of *E. coli* C as described in section titled Preparation of Host for Somatic Coliphage Assay. Incubate for 2 h at 36.5 ± 1°C with shaking. Add 1 mL of rehydrated phage stock and incubate for an additional 4 h at 36.5 ± 1°C.

Step 3. Filter the culture through a 0.45 µm sterilizing filter.

Step 4. Prepare 10^{-7}, 10^{-8} and 10^{-9} dilutions of the filtrate using saline-calcium solution tubes.

These dilutions should be sufficient for most ϕX174 stocks. Some stocks may require higher or lower dilutions.

Step 5. Add 1 mL of the 10^{-9} dilution into each of five 16 × 150 mm test tubes. Using the same pipette, add 1 mL of the 10^{-8} dilution into each of five additional tubes and then 1 mL of the 10^{-7} dilution into five tubes. Label the tubes with the appropriate dilution.

Step 6. Add 0.1 mL of the host culture into each of the 15 test tubes from Step 5.

Step 7. Add 3 mL of the melted tryptone top agar held in the 44.5 ± 1°C water bath to one test tube at a time. Mix and immediately pour the contents of the tube over the bottom agar of a petri dish labeled with sample identification information. Rotate the dish to spread the suspension evenly over the surface of the bottom agar and place it onto a level surface to allow the agar to solidify.

Step 8. Incubate the inoculated plates at 36.5 ± 1°C overnight and examine for plaques the following day.

Step 9. Count the number of plaques on each of the 15 plates (don't count plates giving plaque counts significantly more than 100). The five plates from one of the dilutions should

give plaque counts of about 20 to 100 plaques. Average the plaque counts on these five plates and multiply the result by the reciprocal of the dilution to obtain the titer of the undiluted stock.

Step 10. Dilute the filtrate to 30 to 80 PFU/mL in tryptone broth for use in a positive control in the coliphage assay. Store the original filtrate and the diluted positive control at 4°C.

Before using the positive control for the 1st time, place 1 mL each into ten 16 × 150 mm test tubes and assay using Steps 6-8. Count the plaques on all plates and divide by 10. If the result is not 30 to 80, adjust the dilution of the positive control sample and assay again.

Procedure for Somatic Coliphage Assay:

Step 1. Sample preparation:

a. Add 1 mL of the water eluate sample to be tested to each of ten 16 × 150 mm test tubes.

b. Add 1 mL of buffered 1.5% beef extract to a 16 × 150 mm test tube for a negative control.

c. Add 1 mL of the diluted ϕX174 positive control to another 16 × 150 mm test tube.

Step 2. Add 0.1 mL of the host culture to each test tube containing eluate or positive control.

Step 3. Add 3 mL of the melted tryptone top agar held in the 44.5 ± 1°C water bath to one test tube at a time. Mix and immediately pour the contents of the tube over the bottom agar of a petri dish labeled with sample identification information. Tilt and rotate the dish to spread the suspension evenly over the surface of the bottom agar and place it onto a level surface to allow the agar to solidify.

Step 4. Incubate the inoculated plates at 36.5 ± 1°C overnight and examine for plaques the following day.

Step 5. Count the total number of plaques on the ten plates receiving the water eluate.

Step 6. Somatic coliphage enumeration.

a. Calculate the somatic coliphage titer (V_s) in PFU per 100 L according to the formula:

$$V_S = \frac{100 \times P \times D \times E}{I \times C}$$

where P is the total number of plaques from Step 5, D is the reciprocal of the dilution made on the inoculum before plating (D = 1 for undiluted samples) and E is the total volume of eluate recovered (from the Virus Data Sheet of the Total Culturable Virus Protocol). I is the total volume (in mL) of the eluate sample assayed on the ten plates. C is the amount of water sample filtered in liters (from the Sample Data Sheet of the Total Culturable Virus Protocol). Record the value of V_S in the ICR database.

b. Count the plaques on the positive control plate. Maintain a record of the plaque count as a check on the virus sensitivity of the *E. coli* C host. Assay any water eluate samples again where the positive control counts are more than one log below their normal average.

MALE-SPECIFIC COLIPHAGE ASSAY

Storage of *E. coli* Famp Host Culture for Male-Specific Coliphage Assay: [1]

1. For short term storage inoculate a *Escherichia coli* Famp host culture onto tryptone agar slants with a sterile inoculating loop by spreading the inoculum evenly over entire slant surface. Incubate the culture overnight at 36.5 ± 1°C. Store at 4°C for up to two weeks.

2. For long term storage inoculate a 5-10 mL tube of tryptone broth with the host culture. Incubate the broth culture overnight at 36.5 ± 1°C. Add 1/10th volume of sterile glycerol. Dispense into 1 mL aliquots in cryovials (Baxter Product No. T4050-8 or equivalent) and store at -70°C.

Preparation of Host for Male-Specific Coliphage Assay:

Step 1. Inoculate 5 mL of tryptone broth with *E. coli* Famp from a slant with an inoculating loop and incubate for 16 h at 36.5 ± 1°C.

[1]The term "male-specific coliphage" refers to coliphages whose receptor sites are located on the bacterial F-pilus. The *E. coli* Famp strain to be used for ICR monitoring will be provided to virus analytical laboratories by a U.S. EPA contractor.

Step 2. Transfer 1.5 mL of the 16 h culture to 30 mL of tryptone broth in a 125 mL flask and incubate for 4 h at 36.5 ± 1°C with gentle shaking. The amount of inoculum and broth used in this step can be proportionally altered according to need.

Preparation of MS2[2] Positive Control:

Step 1. Rehydrate a stock culture of MS2 (American Type Culture Collection Product No. 15597-B1) and store at 4°C.

Step 2. Prepare a 30 mL culture of *E. coli* Famp as described in section titled Preparation of Host for Male-Specific Coliphage Assay. Incubate for 2 h at 36.5 ± 1°C with shaking. Add 1 mL of rehydrated phage stock and incubate for an additional 4 h at 36.5 ± 1°C.

Step 3. Filter the culture through a 0.45 µm sterilizing filter.

Step 4. Prepare 10^{-7}, 10^{-8} and 10^{-9} dilutions of the filtrate using saline-calcium solution tubes.

These dilutions should be sufficient for most MS2 stocks. Some stocks may require higher or lower dilutions.

Step 5. Add 1 mL of the 10^{-9} dilution into each of five 16 × 150 mm test tubes. Using the same pipette, add 1 mL of the 10^{-8} dilution into each of five additional tubes and then 1 mL of the 10^{-7} dilution into five tubes. Label the tubes with the appropriate dilution.

Step 6. Add 0.1 mL of the host culture into each of the 15 test tubes from Step 5.

Step 7. Add 3 mL of the melted tryptone top agar held in the 44.5 ± 1°C water bath to one test tube at a time. Mix and immediately pour the contents of the tube over the bottom agar of a petri dish labeled with sample identification information. Rotate the dish to spread the suspension evenly over the surface of the bottom agar and place it onto a level surface to allow the agar to solidify.

Step 8. Incubate the inoculated plates at 36.5 ± 1°C overnight and examine for plaques the following day.

Step 9. Count the number of plaques on each of the 15 plates (don't count plates giving plaque counts significantly more than 100). The five plates from one of the dilutions should give plaque counts of about 20 to 100 plaques. Average the plaque counts on these five plates and multiply the result by the reciprocal of the dilution to obtain the titer of the undiluted stock.

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[2]The MS2 positive control strain or a mixture of male-specific coliphage strains to be used for positive or quality controls will be supplied to virus analytical laboratories by a U.S. EPA contractor.

Step 10. Dilute the filtrate to 30 to 80 PFU/mL in tryptone broth for use in a positive control in the coliphage assay. Store the original filtrate and the diluted positive control at 4°C.

Before using the positive control for the 1st time, place 1 mL each into ten 16 × 150 mm test tubes and assay using Steps 6-8. Count the plaques on all plates and divide by 10. If the result is not 30 to 80, adjust the dilution of the positive control sample and assay again.

Procedure for Male-Specific Coliphage Assay:

Step 1. Sample preparation:

a. Add 1 mL of the water eluate sample to be tested to each of ten 16 × 150 mm test tubes.

b. Add 1 mL of buffered 1.5% beef extract to a 16 × 150 mm test tube for a negative control.

c. Add 1 mL of the diluted MS2 positive control to another 16 × 150 mm test tube.

Step 2. Add 0.1 mL of the host culture to each test tube containing eluate or positive control.

Step 3. Add 3 mL of the melted tryptone top agar held in the 44.5 ± 1°C water bath to one test tube at a time. Mix and immediately pour the contents of the tube over the bottom agar of a petri dish labeled with sample identification information. Tilt and rotate the dish to spread the suspension evenly over the surface of the bottom agar and place it onto a level surface to allow the agar to solidify.

Step 4. Incubate the inoculated plates at 36.5 ± 1°C overnight and examine for plaques the following day.

Step 5. Count the total number of plaques on the ten plates receiving the water eluate.

Step 6. Male Specific coliphage enumeration.

a. Calculate the male specific coliphage titer (V_M) in PFU per 100 L according to the formula:

$$V_M = \frac{100 \times P \times D \times E}{I \times C}$$

where P is the total number of plaques from Step 5, D is the reciprocal of the dilution made on the inoculum before plating (D = 1 for undiluted samples) and E is the total volume of eluate recovered (from the Virus Data Sheet of the Total Culturable Virus Protocol). I is the total volume (in mL) of the eluate sample assayed on the ten plates. C is the amount of water sample filtered in liters (from the Sample Data Sheet of the Total Culturable Virus Protocol). Record the value of V_M in the ICR database.

b. Count the plaques on the positive control plate. Maintain a record of the plaque count as a check on the virus sensitivity of the bacterial host. Assay any water eluate samples again where the positive control counts are more than one log below their normal average.

SECTION X. MEMBRANE FILTER METHOD FOR *E. coli*

1. Citation: METHOD 1103.1, 1985

2. Scope

2.1 This method describes a membrane filter (MF) procedure for the detection and enumeration of *Escherichia coli* (*E. coli*). Because the bacterium is a natural inhabitant only of the intestinal tract of warm-blooded animals, its presence in water samples is an indication of fecal pollution and the possible presence of enteric pathogens.

2.2 The *E. coli* test is used as a measure of recreational water quality. Epidemiological studies have led to the development of criteria which can be used to promulgate recreational water standards based on established relationships between health effects and water quality. The significance of finding *E. coli* in recreational water samples is the direct relationship between the density of *E. coli* and the risk of gastrointestinal illness associated with swimming in the water (1).

2.3 The test for *E. coli* can be applied to fresh, estuarine and marine waters.

2.4 Since a wide range of sample volumes or dilutions thereof can be analyzed by the MF technique, a wide range of *E. coli* levels in water can be detected and enumerated.

3. Summary - The MF method provides a direct count of bacteria in water based on the development of colonies on the surface of the membrane filter (2). A water sample is filtered through the membrane which retains the bacteria. After filtration, the membrane containing the bacterial cells is placed on a selective and differential medium, M-TEC, incubated at 35°C for 2 h to resuscitate injured or stressed bacteria, and then incubated at 44.5°C for 22 h. Following incubation, the filter is transferred to a filter pad saturated with urea substrate. After 15 min, yellow or yellow-brown colonies are counted with the aid of a fluorescent lamp and a magnifying lens.

4. Definition - In this method, *E. coli* are those bacteria which produce yellow or yellow-brown colonies on a filter pad saturated with urea substrate broth after primary culturing on M-TEC medium.

5. Interferences - Water samples containing colloidal or suspended particulate material can clog the membrane filter and prevent filtration, or cause spreading of bacterial colonies which could interfere with identification of target colonies.

6. Safety Precautions

6.1 The analyst/technician must know and observe the normal safety procedures required in a microbiology laboratory while preparing, using, and disposing of cultures, reagents and materials and while operating sterilization equipment.

6.2 Mouth-pipetting is prohibited.

7. Apparatus and Equipment

7.1 Glass lens, 2-5X magnification, or stereoscopic microscope.

7.2 Lamp with cool, white fluorescent tube and diffuser.

7.3 Hand tally or electronic counting device.

7.4 Pipet container, stainless steel, aluminum, or borosilicate glass, for glass pipets.

7.5 Pipets, sterile, T.D. bacteriological or Mohr, glass or plastic, of appropriate volume.

7.6 Graduated cylinders, covered with aluminum foil or kraft paper and sterile.

7.7 Membrane filtration units (filter base and funnel), glass, plastic or stainless steel, wrapped with aluminum foil or kraft paper and sterile.

7.8 Ultraviolet unit for sterilizing the filter funnel between filtrations (optional).

7.9 Line vacuum, electric vacuum pump, or aspirator for use as a vacuum source. In an emergency, or in the field, a hand pump, or a syringe equipped with a check valve to prevent the return flow of air, can be used.

7.10 Flask, filter vacuum, usually 1 L, with appropriate tubing. A filter manifold to hold a number of filter bases is optional.

7.11 Flask for safety trap, placed between the filter flask and the vacuum source.

7.12 Forceps, straight or curved, with smooth tips to handle filters without damage.

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

7.14 Burner, Bunsen or Fisher type, or electric incinerator unit for sterilizing inoculation loops.

7.15 Thermometer, checked against a National Institute of Science & Technology (NIST) certified thermometer, or one traceable to an NIST thermometer.

7.16 Petri dishes, sterile, plastic, 50 × 12 mm, with tight-fitting lids, or 60 × 15 mm, glass or plastic, with loose-fitting lids. 100 × 15 mm dishes may also be used.

7.17 Bottles, milk dilution, borosilicate glass, screw-cap with neoprene liners, marked at 99 mL for 1-100 dilutions. Dilution bottles marked at 90 mL, or tubes marked at 9 mL may be used for 1-10 dilutions.

7.18 Flasks, borosilicate glass, screw-cap, 250-2000 mL volume.

7.19 Membrane filters, sterile, white grid marked, 47 mm diameter, with 0.45 ± 0.02 µm pore size.

7.20 Absorbent pads, sterile, 47 mm diameter (usually supplied with membrane filters).

7.21 Inoculation loops, at least 3 mm diameter, and needles, nichrome and platinum wire, 26 B & S gauge, in suitable holders. Disposable applicator sticks or plastic loops are alternatives to inoculation loops. **Note**: A platinum loop is required for the cytochrome oxidase test in 15.3.

7.22 Incubator maintained at 35 ± 0.5°C, with approximately 90 percent humidity if loose-lidded petri dishes are used.

7.23 Waterbath incubator maintained at 44.5 ± 0.2°C.

7.24 Waterbath maintained at 44-46°C for tempering agar.

7.25 Test tubes, 150 × 20 mm, borosilicate glass or plastic.

7.26 Test tubes, 75 × 10 mm, borosilicate glass.

7.27 Test tube caps, aluminum or autoclavable plastic, for 20 mm diameter test tubes.

7.28 Test tubes, screw-cap, 125 × 16 mm or other appropriate size.

7.29 Filter paper.

8. Reagents and Materials

8.1 **Purity of Reagents:** Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, reagents shall conform to the specifications of the **Committee on Analytical Reagents of the American Chemical Society** (3). The agar used in preparation of culture media must be of microbiological grade.

8.2 Whenever possible, use commercial culture media as a means of quality control.

8.3 **Purity of Water:** Reagent water conforming to Specification D1193, Type II water, **ASTM Annual Book of Standards** (4).

8.4 **Buffered Dilution Water**

8.4.1 **Composition:**

Sodium Dihydrogen Phosphate	0.58	g
Sodium Monohydrogen Phosphate	2.50	g
Sodium Chloride	8.50	g

8.4.2 **Preparation:** Dissolve the ingredients in 1 L of reagent water in a flask and dispense in appropriate amounts for dilutions in screw-cap bottles or culture tubes, and/or into containers for use as rinse water. Autoclave after preparation at 121°C (15 lb pressure) for 15 min. Final pH should be 7.4 ± 0.2.

8.5 **M-TEC Agar** (Difco 0334-15-0)

8.5.1 **Composition:**

Proteose Peptone #3	5.0	g
Yeast Extract	3.0	g
Lactose	10.0	g
NaCl	7.5	g
Dipotassium Phosphate	3.3	g
Monopotassium Phosphate	1.0	g
Sodium Lauryl Sulfate	0.2	g
Sodium Desoxycholate	0.1	g
Brom Cresol Purple	0.08	g
Brom Phenol Red	0.08	g
Agar	15.0	g

8.5.2 **Preparation:** Add 45.26 g of M-TEC medium to 1 L of reagent water in a flask and heat to boiling, until ingredients dissolve. Autoclave at 121°C (15 lb pressure) for 15 min. and cool in a 44-46°C waterbath. Pour the

medium into each 50 × 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 a refrigerator.

8.6 **Urea Substrate Medium**

8.6.1 **Composition:**

Urea	2.0	g
Phenol red	0.01	g

8.6.2 **Preparation:** Add dry ingredients to 100 mL reagent water in a flask. Stir to dissolve and adjust to pH 5.0 with a few drops of 1N HC1. The substrate solution should be a straw-yellow color at this pH.

8.7 **Nutrient Agar** (Difco 0001-02, BBL 11471)

8.7.1 **Composition:**

Peptone	5.0	g
Beef Extract	3.0	g
Agar	15.0	g

8.7.2 **Preparation:** Add 23 g of nutrient agar ingredients to 1 L of reagent water and mix well. Heat in boiling waterbath to dissolve the agar completely. Dispense in screw-cap tubes, bottles or flasks and autoclave at 121°C (15 lb pressure) for 15 min. Remove tubes and slant. The final pH should be 6.8 ± 0.2.

8.8 **Tryptic Soy Broth** (Difco 0370-02) or **Trypticase Soy Broth** (BBL 12464)

8.8.1 **Composition:**

Tryptone or Trypticase	17.0	g
Soytone or Phytone	3.0	g
Sodium Chloride	5.0	g
Dextrose	2.5	g
Dipotassium Phosphate	2.5	g

8.8.2 **Preparation:** Add 30 g of Tryptic (Trypticase) soy broth to 1 L of reagent water. Warm the broth and mix gently to dissolve the medium completely. Dispense in screw-cap tubes and autoclave at 121°C (15 lb pressure) for 15 min. The final pH should be 7.3 ± 0.2.

8.9 **Simmons' Citrate Agar** (BBL 11619, Difco 0091-02)

8.9.1 **Composition**

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

8.9.2 **Preparation:** Add 24.28 g of Simmons' citrate agar to 1 L of reagent water. Heat in boiling waterbath with mixing for complete solution. Dispense in screw-cap tubes and sterilize at 121°C (15 lb pressure) for 15 min. Cool tubes as slants. The final pH should be 6.8 ± 0.2.

8.10 **Tryptone** (Difco 0123-02) or **Trypticase Peptone** (BBL 11920) Broth

8.10.1 **Composition:**

Tryptone or Trypticase peptone	10.0	g

8.10.2 **Preparation:** Add 10 g of tryptone or trypticase peptone to 900 mL of reagent water and heat with mixing until dissolved. Bring solution to 1000 mL in a graduate or flask. Dispense in five mL volumes in tubes and autoclave at 121°C (15 lb pressure) for 15 min. The final pH should be 7.2 ± 0.2.

8.11 **EC Broth** (Difco 0314-02) or **EC Broth** (BBL 12432)

8.11.1 **Composition:**

Tryptose or Trypticase Peptone	20.0	g
Lactose	5.0	g
Bile Salts No. 3 or Bile Salts Mixture	1.5	g
Dipotassium Phosphate	4.0	g
Monopotassium Phosphate	1.5	g
Sodium Chloride	5.0	g

8.11.2 **Preparation:** Add 37 g of EC medium to 1 L of reagent water and warm to dissolve completely. Dispense into fermentation tubes (150 × 20 mm tubes containing inverted 75 × 10 mm vials). Sterilize at 121°C (15 lb pressure) for 15 min. The final pH should be 6.9 ± 0.2.

8.12 **Cytochrome Oxidase Reagent:** N, N, N^1, N^1 tetramethyl-p-phenylenediamine dihydrochloride, 1% aqueous solution.

8.13 **Kovacs' Indole Reagent:** Dissolve 10 g p-dimethylaminobenzaldehyde in 150 mL amyl or isoamyl alcohol and then slowly add 50 mL concentrated hydrochloric acid and mix.

9. Sample Collection, Preservation and Holding Times

9.1 Sampling procedures are described in detail in the **USEPA Microbiology Methods Manual**, Section II, A (5). Adherence to sample preservation procedures and holding time limits is critical to the production of valid data. Samples not collected according to these rules should not be analyzed.

9.1.1 **Storage Temperature and Handling Conditions:** Ice or refrigerate water samples at a temperature of 1-4°C during transit to the laboratory. Use insulated containers to assure proper maintenance of storage temperature. Take care that sample bottles are not totally immersed in water during transit or storage.

9.1.2 **Holding Time Limitations:** Examine samples as soon as possible after collection. Do not hold samples longer than 8 h between collection and initiation of analyses.

10. Calibration and Standardization

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

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

11. Quality Control

11.1 See recommendations on quality control for microbiological analyses in the **USEPA Microbiology Methods Manual**, Part IV, C (5).

12. Procedures

12.1 Prepare the M-TEC agar and urea substrate as directed in **Sections 8.5 and 8.6**.

12.2 Mark the petri dishes and report forms with sample identification and sample volumes.

12.3 Place a sterile membrane filter on the filter base, grid-side up and attach the funnel to the base; the membrane filter is now held between the funnel and the base.

12.4 Shake the sample bottle vigorously about 25 times to distribute the bacteria uniformly and measure the desired volume of sample or dilution into the funnel.

12.5 For ambient surface waters and waste waters, select sample volumes based on previous knowledge of pollution level, to produce 20-80 *E. coli* colonies on the membranes. Sample volumes of 1-100 mL are normally tested at half-log intervals.

12.6 Smaller sample size or sample dilutions can be used to minimize the interference of turbidity or high bacterial densities. Multiple volumes of the same sample dilution may be filtered and the results combined.

12.7 Filter the sample and rinse the sides of the funnel at least twice with 20-30 mL of sterile rinse water. Turn off the vacuum and remove the funnel from the filter base.

12.8 Use sterile forceps to aseptically remove the membrane filter from the filter base and roll it onto the M-TEC 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 h.

12.9 After 2 h incubation at 35°C, transfer the plates to Whirl-Pak bags, seal, and place inverted in a 44.5°C waterbath for 22-24 h.

12.10 After 22-24 h, remove the dishes from the waterbath. 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.

12.11 After 15-20 min. 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.

13. Calculation of Results

13.1 Select the membrane filter with the number of colonies within the acceptable range (20-80) and calculate the count per 100 mL according to the general formula:

$$\textit{E. coli}/100\ \text{mL} = \frac{\textit{No. E. coli Colonies Counted}}{\textit{Volume in mL of Sample Filtered}} \times 100\ \textit{mL}$$

13.2 See general counting rules in the **USEPA Microbiology Methods Manual**, Part II, C, 3.5 (5).

14. Reporting Results

14.1 Report the results as *E. coli* per 100 mL of sample.

15. Verification Procedure

15.1 Yellow or yellow-brown colonies from the urease test can be verified as *E. coli*. Verification of colonies may be required in evidence gathering, and is also recommended as a QC procedure with initial use of the test and with changes in sample sites, lots of commercial media or major ingredients in media compounded in the laboratory. The verification procedure follows:

15.1.1 Using a sterile inoculation loop, transfer growth from the centers of at least 10 well-isolated typical colonies to nutrient agar plates or slants and to Tryptic (Trypticase) soy broth. Incubate the agar and broth cultures for 24 h at 35°C.

15.1.2 After incubation remove a generous portion of material from the nutrient agar **with a platinum loop** and deposit on the surface of filter paper that has been saturated with cytochrome oxidase reagent prepared fresh that day. A positive test is indicated within 15 s by the development of a deep purple color where the bacteria were deposited.

15.1.3 Transfer growth from the Tryptic (Trypticase) soy broth to Simmons' citrate agar, Tryptone (Trypticase peptone) broth and EC broth in a fermentation tube. Incubate the Simmons' citrate agar for 24 h and Tryptone (Trypticase peptone) broth for 48 h at 35°C. Incubate the EC broth at 44.5°C in a waterbath for 24 h. The water level must be above the level of the EC broth in the tube. Add one-half mL of Kovacs' indole reagent to the 48 h Tryptone (Trypticase peptone) broth culture and shake the tube gently. A positive test for indole is indicated by a deep red color which develops in

the alcohol layer. *E. coli* is EC gas positive, indole positive, oxidase negative, and does not grow on citrate medium.

16. Precision and Bias

16.1 Performance Characteristics

16.1.1 **Precision** - The degree of agreement of repeated measurements of the same parameter expressed quantitatively as the standard deviation or as the 95% confidence limits of the mean computed from the results of a series of controlled determinations. The M-TEC method precision was found to be fairly representative of what would be expected from counts with a Poisson distribution (2).

16.1.2 **Bias** - The persistent positive or negative deviation of the average value of the method from the assumed or accepted true value. The bias of the M-TEC method has been reported to be -2% of the true value (2).

16.1.3 **Specificity** - The ability of a method to select and/or distinguish the target bacteria under test from other bacteria in the same water sample. The specificity characteristic of a method is usually reported as the percent of false-positive and false-negative results. The false-positive rate reported for M-TEC medium averaged 9% for marine and fresh water samples. Less than 1% of the *E. coli* colonies observed gave a false-negative reaction (2).

16.1.4 **Upper Counting Limit (UCL)** - That colony count above which there is an unacceptable counting error. The error may be due to overcrowding or antibiosis. The UCL for *E. coli* on M-TEC medium has been reported as 80 colonies per filter (2).

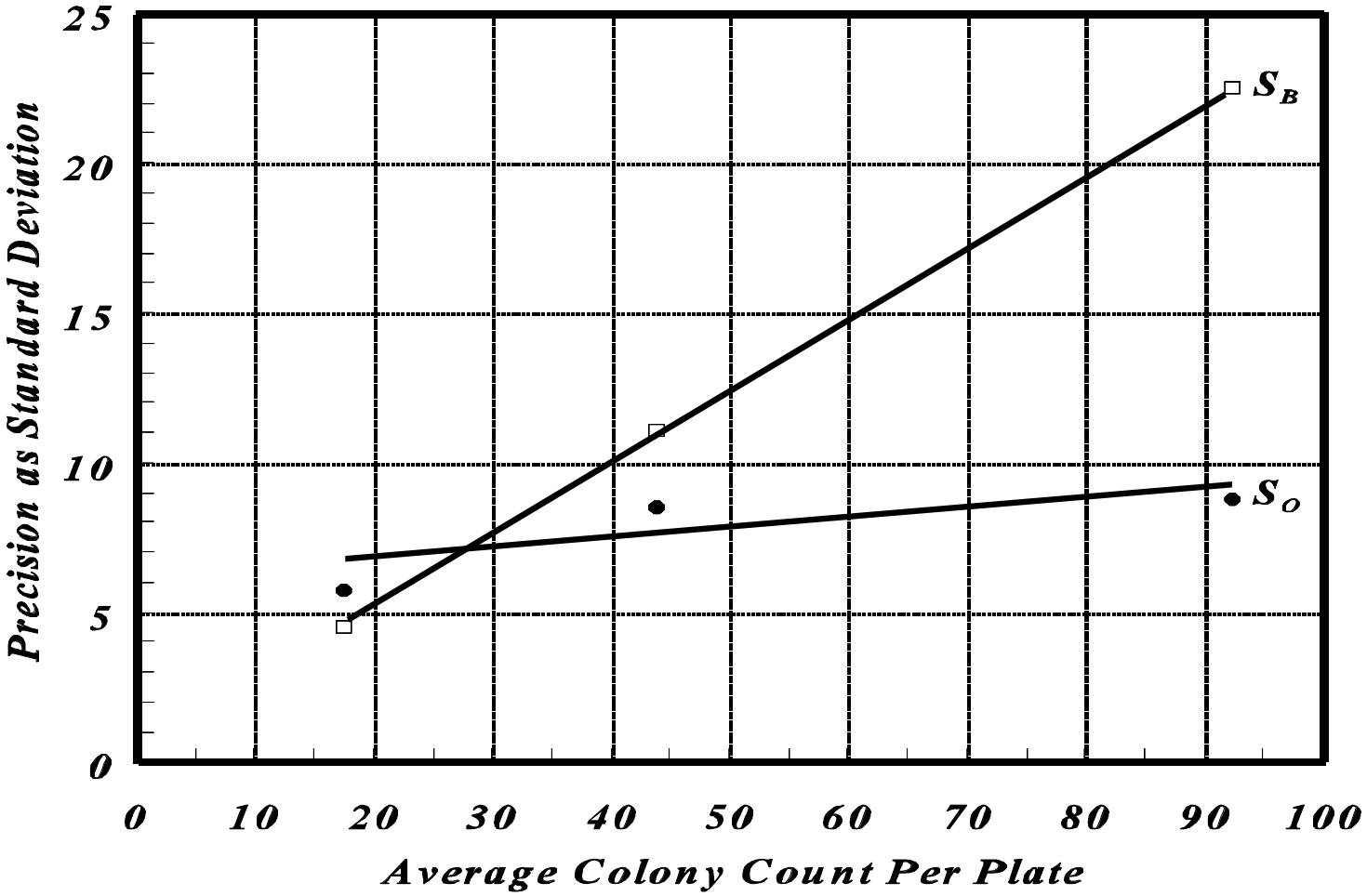
16.2 Collaborative Study Data

16.2.1 A collaborative study was conducted among eleven volunteer laboratories, each with two analysts who independently tested local fresh and marine recreational waters and sewage treatment plant effluent samples, in duplicate. The data were reported to the **Environmental Monitoring and Support Laboratory - Cincinnati, Ohio, U.S. Environmental Protection Agency**, for statistical calculations.

16.2.2 The results of the study are shown in **Figure X-1** where S_o equals standard deviation among replicate counts from a single analyst and S_b equals standard deviation between means of duplicates from analysts in the same

Figure X-1. Precision Estimates for *E. coli* in Water by the Membrane Filter M-TEC Method

S_O = Standard Deviation among Replicate Counts from a Single Analyst
S_B = Standard Deviation between the Means of Duplicate Counts by Analysts in the Same Laboratory



laboratory. The precision estimates from this study did not show any difference among the water types analyzed.

16.2.3 The precision of the method can be generalized as:
S_o = 0.028 count/100 mL + 6.11 (dilution factor) and
S_b = 0.233 count/100 mL + 0.82 (dilution factor), where the

$$\text{dilution factor} = \frac{100}{\textit{VOLUME OF ORIGINAL SAMPLE FILTERED}}$$

16.2.4 Because of the instability of microbial populations in water samples, each laboratory analyzed its own sample series and no full measure of recovery or bias was possible. However, all laboratories analyzed a single surrogate sample prepared from a freeze-dried culture of *E. coli*. The mean count (x) and the overall standard deviation of the counts (S_t) (which includes the variability among laboratories for this standardized *E. coli* sample) were 31.6 colonies/membrane and 7.61 colonies/membrane, respectively.

17. REFERENCES

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SECTION XI. MEMBRANE FILTER METHOD FOR *C. perfringens*

1. Scope and Application

1.1 This procedure enumerates *Clostridium perfringens* spores from surface and drinking water. Since *C. perfringens* is present in large numbers in human and animal wastes and its spores are resistant to wastewater treatment practices, extremes in temperature and environmental stress, it is an indicator of present fecal contamination as well as a conservative tracer of past fecal contamination. Some investigators have proposed *C. perfringens* as an indicator of the presence and the density of pathogenic viruses and possibly other microorganisms.

1.2 It is the user's responsibility to insure the validity of this method for untested matrices.

2. Summary of Method - An appropriate volume of water sample is passed through a membrane filter that retains the bacteria present in the sample. The membrane filter is placed on mCP agar and incubated anaerobically for 24 h at 44.5°C using a medium modified by Armon and Payment from Bisson and Cabelli (1,2). Upon exposure to ammonium hydroxide, the yellow straw-colored *C. perfringens* colonies turn dark pink to magenta and are counted as presumptive *C. perfringens*. Because of the selectivity of the mCP medium, a presumptive count is normally reported for routine monitoring purposes. Verification is not required for ICR monitoring, but if desired, colonies are confirmed by anaerobic growth in thioglycollate, a positive gram stain reaction and stormy fermentation of iron milk. The mCP counts are adjusted based on the percent confirmation. This method was originally prepared by Irwin Katz, U.S. EPA Region 2 for ASTM Subcommittee D19.24, Water Microbiology.

3. Definitions

3.1 *C. perfringens* - An obligate anaerobic gram-positive, spore forming, non-motile bacillus that ferments lactose with stormy gas production and ferments sucrose but does not ferment cellobiose. *C. perfringens* produces acid phosphatase and also produces exotoxins which cause gas gangrene and gastroenteritis.

3.2 Spores - *C. perfringens* produces single oval subterminal spores less than 1 µm in diameter during adverse conditions. Sporulation can also occur in the intestinal tract. The endospore that develops is a highly refractile body formed within the cell. Spores are resistant to heat, drying and chemical disinfectants, which would kill the vegetative cells of *C. perfringens*. This resistance to unfavorable conditions preserves the organisms for long periods of time.

4. Interferences

4.1 Waters containing sediment or large quantities of colloidal or suspended materials such as iron, manganese, alum floc or algae can clog the filter pores and prevent filtration, or can cause the development of spreading bacterial colonies that mask other colonies and prevent accurate counting.

4.2 When bacterial densities are high, a smaller sample volume or sample dilution can be filtered to minimize the interference of turbidity or high background (non-target) bacterial densities. Replicates of smaller sample volumes or dilutions of sample may be filtered and the results combined. However, the membrane filter technique may not be applicable to highly turbid waters with low *Clostridium* densities.

4.3 Toxic materials such as metals, phenols, acids, caustics, chloramines, and other disinfection by-products may also adversely affect recovery of *Clostridium* vegetative cells on the membrane filter. Although most probable number (MPN) methods are not usually expected to generate results comparable to membrane filter methods, an MPN method should be considered as an alternative procedure if the membrane filter method is not useable for these samples (3).

4.4 Some lots of membrane filters produce low recoveries or poor differentiation of target and non-target colonies due to toxicity, chemical composition, or structural defects. Quality control checks should be made on new lots of membranes (4).

5. Health and Safety

5.1 This method does not address all safety problems associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and determine regulatory limitations prior to use.

5.2 The analyst/technician must know and observe normal good laboratory practices and safety procedures required in a microbiology laboratory while preparing, using and disposing of cultures, reagents and materials and while operating sterilizers and other equipment and instrumentation.

5.3 Mouth-pipetting is not permitted.

6. Instruments, Equipment and Supplies

6.1 Sample container, sterile, non-toxic glass or rigid plastic with screw cap, or plastic bag, minimum of 125 mL capacity.

6.2 Pipet container, stainless steel, or aluminum, for sterilization and storage of glass pipets.

6.3 Pipets, sterile T.D. bacteriological or Mohr, glass or plastic, of appropriate volumes.

6.4 Graduated cylinders, 100 to 1000 mL, tops are covered with aluminum foil or kraft paper and sterilized.

6.5 Bottles, milk dilution, borosilicate glass or non-toxic heat stable plastic, screw-cap with neoprene liners, marked at 99 mL for 1:100 dilutions. Dilution bottles marked at 90 mL or tubes marked at 9 mL may be used for 1:10 dilutions.

6.6 Membrane filtration units, (filter base and funnel), glass, plastic or stainless steel, wrapped with aluminum foil or kraft paper and sterilized.

6.7 Membrane Filters - sterile, white, grid marked, 47 mm diameter, with 0.45 ± 0.02 µm pore size or other pore sizes for which the manufacturer provides data demonstrating equivalency.

6.8 Ultraviolet unit for disinfecting the filter funnel between filtrations in a series (optional).

6.9 Line vacuum, electric vacuum pump or aspirator as a vacuum source.

6.10 Flask, vacuum, usually 1 L, with appropriate tubing, to hold filter base. Filter manifolds to hold a number of filter bases are optional.

6.11 Flask, safety trap, placed between the filter flask and the vacuum source.

6.12 Forceps, straight or curved, with smooth tips to permit handling of filters without damage.

6.13 Petri plates, plastic or glass, 50 × 9 mm, with tight-fitting lids, or 60 × 12 mm, with loose fitting lids (dimensions are nominal).

6.14 Test Tubes, 20 × 150 mm, borosilicate glass or disposable plastic.

6.15 Caps, aluminum or autoclavable plastic, for 20 × 150 mm test tubes.

6.16 Test Tubes, screw cap, 16 × 125 mm or other appropriate size.

6.17 Inoculation loops, 3 mm diameter, and needles, nichrome or platinum wire, 26 B & S gauge, in suitable holders. Sterile disposable applicator sticks or plastic loops are acceptable alternatives to inoculation loops.

6.18 Thermometers, 0-50°C, graduated to 0.2 degrees, and 0-100°C for heat shock which has been checked against the appropriate National Institute of Standards and Technology (NIST) certified thermometer, or against a thermometer traceable to NIST.

6.19 Waterbath, that maintains 46-48°C for tempering agar.

6.20 Waterbath with gable cover that maintains 60°C ± 0.5°C for heat shocking samples.

6.21 Anaerobic system (anaerobic jar, reaction chamber, hydrogen/carbon dioxide disposable generator and anaerobic indicator), or any other system capable of producing the appropriate anaerobic conditions to support the growth of the organisms[1].

6.22 Filter Paper, circular, 11 cm, Whatman 40 or 110, or equivalent, for separation of mCP agar plates during anaerobic incubation.

6.23 Incubator, that maintains 44.5°C ± 0.2°C and is large enough to hold the anaerobic chamber.

6.24 Incubator, Water Bath, that maintains 44.5°C ± 0.2°C for incubation of Iron Milk Medium.

6.25 Microscope, stereoscopic, wide-field type, with magnification of 10 to 15X.

6.26 Microscope lamp, that produces diffuse light from a cool white fluorescent or tungsten lamp adjusted to give maximum visibility.

6.27 Counting device, hand tally or electronic.

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[1]BBL 60460 or BBL 60466 GASPAK Anaerobic System with BBL 70308 Disposable Hydrogen and Carbon Dioxide Generator Envelopes, BBL Microbiological Systems, Cockeysville, MD 21030, or equivalent.

6.28 Sonication unit, to aid in dissolving reagents.[2]

7. Reagents, Standards and Media

7.1 **Purity of Reagents** - Use reagent grade chemicals in all tests. Unless otherwise indicated, all reagents must conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society where such specifications are available (5). Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination. Use microbiological grade agar in preparation of culture media. Whenever possible, use commercial culture media as a means of improved quality control.

7.2 **Purity of Water** - Unless otherwise indicated, references to water mean reagent water as defined by Type II of Specification D1193 (6).

7.3 **Buffered Dilution and Rinse Water**

7.3.1 **Phosphate Buffer Dilution Water**

7.3.1.1 **Stock Phosphate Buffer Solution** - Dissolve 34.0 g of potassium dihydrogen phosphate (KH_2PO_4) in 500 mL of water. Adjust pH to 7.2 with 1 N NaOH and bring to 1000 mL with water. Dispense aseptically into screw-cap bottles and autoclave for 15 min at 121°C. Alternatively, sterilize by filtration through a 0.2 µm pore membrane filter and dispense aseptically into sterile screw-cap bottles. Store in refrigerator and handle aseptically. If cloudiness, a marked change in pH, or other evidence of contamination appears, discard the stock. Confirm that pH is 7.2 ± 0.5 before use.

7.3.1.2 **Magnesium Chloride Solution** - Dissolve 81.4 g of hexahydrate magnesium chloride ($MgCl_2 \cdot 6H_20$) in 1000 mL of water. Mix well and sterilize by filtration or autoclave for 15 min at 121°C. Store in refrigerator and handle aseptically. If cloudiness, or other evidence of contamination occurs, discard the stock solution.

7.3.1.3 **Phosphate Buffered Dilution Water** - Add 1.25 mL of stock phosphate buffer solution and 5 mL of magnesium chloride solution to 1000 mL of water in a volumetric flask and mix well. Dispense dilution water in amounts which will provide 99 ± 2 mL after sterili-

[2]Bronson Sonifier, 500 W, or Tekmar Sonic Disrupter, 500 W with 3 mm tip set at 18 W, or equivalent.

zation in screw-cap dilution bottles, or in larger volume containers for use as rinse water. Autoclave dilution bottles for 15 min at 121°C. Autoclave larger volumes for longer periods as appropriate. Alternatively, sterilize by filtration through a sterile 0.2 µm pore membrane filter unit and dispense aseptically into sterile screw-cap bottles.

7.3.2 **Peptone Dilution and Rinse Water** - Dissolve 1.0 g of peptone[3] in 100 mL of water, and bring to 1000 mL with water. Dispense in screw-cap bottles in volumes to produce 99 ± 2 mL after autoclaving. Autoclave for 15 min. at 121°C. Final pH should be 6.8 - 7.0. Adjust as necessary.

7.4 **Ethanol** - 95%, pure, for flame-sterilization of forceps and for preparation of acetone alcohol for gram stain.

7.5 **Ammonium Hydroxide Solution** (29.2% NH_4OH) - commercially available.

7.6 **Ferric Chloride Solution** - Weigh out 4.5 g of $FeC1_3 \cdot 6H_20$ and dissolve in 100 mL of water. Filter sterilize and store in refrigerator.

7.7 **Phenolphthalein diphosphate Solution** - Weigh out 0.5 g of phenolphthalein diphosphate and dissolve in 100 mL of water. Filter sterilize and store in refrigerator.

7.8 **Indoxyl β-D Glucoside Solution** - Weigh out 0.06 g of Indoxyl β-D Glucoside and dissolve in 80 mL of water (0.075 solution). Sonicator (item 6.28) can be used to speed dissolution. Filter-sterilize and use in 7.9.2.

7.9 **Modified mCP Agar** (1)

7.9.1 **Composition/L**

Tryptose	30.0	g
Yeast Extract	20.0	g
Sucrose	5.0	g
L-cysteine Hydrochloride	1.0	g
$MgSO_4 \cdot 7H_20$	0.1	g
Bromcresol Purple	0.04	g
Agar	15.0	g

7.9.2 **Preparation of Modified mCP Agar:** Add medium ingredients from 7.9.1 to 900 mL water in a liter Erlenmeyer flask. Stir and heat to dissolve in a boiling water bath. Bring the pH to 7.6 with 1 N NaOH. Autoclave for

[3]**Peptone** (Difco 0118), Difco Laboratories, Detroit, MI, or equivalent.

15 min at 121°C (15 lbs pressure). Cool to 50°C. Add the following reagents aseptically and mix well:

D-cycloserine	0.4	g
Polymyxin B sulfate	0.025	g
4.5% $FeCl_3 \cdot 6H_20$ solution	2.0	mL
0.5% Phenolphthalein diphosphate solution	20.0	mL
0.075% Indoxyl-β-D-Glucoside solution	80.0	mL

7.9.3 Dispense 4-4.5 mL into each petri plate using a sterile Cornwall syringe or Brewer pipette. Store agar plates inverted in a plastic bag in a refrigerator for no more than one month. It is recommended that the plates be stored in an anaerobic chamber in the refrigerator for optimal preservation.

7.10 **Modified Iron Milk Medium** (7)

7.10.1 **Composition/L**

Fresh pasteurized, homogenized milk (3.5% butterfat)	1.0	L
$FeSO_4 \cdot 7H_2O$	1.0	g

7.10.2 **Preparation:** Dissolve ferrous sulfate in 50 mL water. Add slowly to 1 L milk and mix with magnetic stirrer. Dispense 11 mL of medium into culture tubes. Cap and autoclave 12 min at 118°C. CAUTION: Do not exceed the recommended time and temperature limits to avoid coagulation.

7.11 **Fluid Thioglycollate Medium**[4]

7.11.1 **Composition/L**

L-Cystine	0.5	g
Agar (granulated)	0.75	g
NaCl	2.5	g
Dextrose (anhydrous)	5.0	g
Yeast extract	5.0	g
Tryptone	15.0	g
Sodium thioglycollate	0.5	g
Resazurin	0.001	g

[4]Fluid Thioglycollate Medium (BBL 12461), Becton-Dickinson Microbiology Systems, Cockeysville, MD; (Difco 0432-02-6) Difco Laboratories, Detroit, MI; or equivalent.

7.11.2 **Preparation:** Suspend 29.25 g of medium in 1 L of water. Mix thoroughly and heat to boil for 1-2 min or until solution is complete. Final pH is 7.1 ± 0.1. Dispense 15 mL portions into culture tubes. Cap and autoclave for 15 min at 121°C. Store tubes in the dark at room temperature. Do not refrigerate. If medium becomes oxidized (more than 30% of medium is pink), reheat once only in boiling water bath and cool before use.

7.12 **Gram Stain Reagents**

7.12.1 Gram stain reagent kits are commercially available and are recommended.

7.12.2 **Ammonium oxalate-crystal violet (Hucker's)**: Dissolve 2 g crystal violet (90% dye content) in 20 mL 95% ethyl alcohol. Dissolve 0.8 g $(NH_4)_2C_2O_4 \cdot H_2O$ in 80 mL water; mix the two solutions and age for 24 h before use. Filter through a 0.22 µm membrane filter. Store in a glass bottle.

7.12.3 **Lugol's solution, Gram's modification**: Grind 1 g iodine crystals and 2 g KI in a mortar. Add water, a few mL at time, and grind thoroughly after each addition until solution is complete. Filter solution through a 0.22 µm membrane filter, and rinse into an amber glass bottle with the remaining water (using a total of 300 mL).

7.12.4 **Counterstain**: Dissolve 2.5 g safranin dye in 100 mL 95% ethyl alcohol. Add 10 mL to 100 mL water. Filter through a 0.22 µm membrane filter.

7.12.5 **Acetone alcohol**: Mix equal volumes of ethyl alcohol (95%) with acetone.

8. Sample Collection, Preservation and Holding Times

8.1 **Collection** - Water samples are collected in sterile sample containers with leak-proof lids.

8.2 **Sample Preservation and Holding Conditions** - Hold water samples at a temperature below 10°C during transit to the laboratory by placing them on ice, surrounding them with blue ice or by refrigeration. Use insulated containers to maintain storage temperature during transit. Take care that sample bottle closures are not submerged in water during transit or storage.

8.3 **Holding Time** - Refrigerate samples upon arrival in the laboratory and analyze within 8 h after collection. *C. perfringens* spores can survive for extended periods

at 1-4°C. However, since a correlation is planned with other indicators, the holding time for *C. perfringens* must be limited to that of the other indicators.

9. Quality Control

9.1 Adherence to sampling procedures, preservation procedures and holding time limits is critical to the production of valid data. Reject samples if appropriate sampling, preservation and handling procedures have not been followed

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

9.3 Check thermometers at least annually against a National Institute of Standards and Technology (NIST) certified thermometer or one traceable to NIST and record the results. Examine mercury columns for separation and reunite before use. Adjust or post correction factors on equipment.

9.4 Use a loop to inoculate mCP agar plates with pure cultures of *C. perfringens* and *E. coli*. Carry these plates through the entire analytical procedure, as positive and negative controls.

9.5 For general quality control recommendations, see "Quality Assurance for Microbiological Analyses" in ASTM Special Technical Testing Publication 867 (8).

10. Procedure for Analyses of Water Samples for Spores

10.1 Prepare mCP Agar according to Section 7.9.

10.2 Mark the bottoms of the petri plates and laboratory data sheets with sample identities and volumes.

10.3 Grasp a sterile membrane filter by its edge using a sterile forceps and place on the filter base, grid side up. Attach the funnel to the base of the filter unit; the membrane filter is now held between the funnel and the base.

10.4. **Procedure for Inactivation of Vegetative Cells** - To obtain a count only of *C. perfringens* spores, hold water samples in a waterbath at 60°C for 15 min to kill all vegetative cells.

10.4.1 Equilibrate a waterbath at 60 C.

10.4.2 Determine the time necessary to bring a blank sample to 60°C. Use the same size container and volume as used for water samples.

10.4.3 Immerse the containers containing the water samples in the waterbath for the time necessary to warm sample to 60°C plus 15 min. Do not allow the container cap or container opening to become contaminated by water in the bath.

10.4.4 Cool the sample containers in cold tap water immediately after heat shock and proceed with the analyses in 10.3.

10.5 For greatest accuracy, it is necessary to filter a sample volume that will yield a countable plate. Select sample volumes based on previous knowledge, which will produce membrane filter plates with 20-80 *C. perfringens* colonies. A narrow range of dilution factors of 4 or 5 can usually be used to achieve the desired number of colonies. An example of such factors is shown in **Table XI-1**. However, if past analyses of specific samples have resulted in confluent growth or "too numerous to count" (TNTC) membranes from excessive turbidity, additional samples should be collected and filtration volumes adjusted to provide isolated colonies from one or more smaller volumes. The counts from smaller volumes can be combined for a final count/total volume filtered.

10.6 Shake the sample bottle vigorously about 25 times and measure the desired volume of sample into the funnel with the vacuum off. To measure the sample accurately and obtain good distribution of colonies on the filter surface, use the following procedures:

10.6.1 Sample volumes of 20 mL or more: Measure the sample in a sterile graduated cylinder and pour it into the funnel. Rinse the graduate twice with sterile dilution water, and add the rinse water to the funnel.

10.6.2 Sample volumes of 10-20 mL: Measure the sample with a sterile 10 mL or 20 mL pipet into the funnel.

10.6.3 Sample volumes of 1-10 mL: Pour about 10 mL of sterile dilution water into the funnel without vacuum. Add the sample to the sterile water using appropriate sterile pipet and filter the sample.

10.6.4 Sample volumes of less than 1.0 mL: Prepare appropriate dilutions in sterile dilution water and proceed as applicable in steps 10.6.1-10.6.3 above.

10.6.5 To reduce the chance for carryover, when analyzing a series of samples or dilutions, filter samples in the order of increasing volumes of original sample. The time elapsing between preparation of sample dilutions and filtration should be minimal and never more than 30 min.

Table XI-1. Sample Volumes to Obtain Colony Count on Membrane Filters * (Range of 20 - 80 Colonies)	
Sample Volume in mL	**Added as:**
0.05	5.0 mL of 10^{-2} dilution
0.20	2.0 mL of 10^{-1} dilution
0.80	8.0 mL of 10^{-1} dilution
3.20	3.2 mL of Undiluted Sample
15.00	15.0 mL of Undiluted Sample
60.00	60.0 mL of Undiluted Sample
*The range of volumes and dilutions selected for filtration of completely unknown samples can be broader, to provide a factor of 10 or more. Prepare at least three sample increments.	

10.7 After adding the sample to filter funnel, turn on vacuum and filter the sample. Rinse the sides of the funnel walls at least twice with 20-30 mL of sterile dilution water. Turn off vacuum and remove the funnel from the filter base.

10.8 Flame forceps, cool and aseptically remove the membrane filter from the filter base. Place the filter, grid side up, on the mCP agar using a rolling motion to prevent air bubbles. Reseat the filter if bubbles occur.

10.9 Remove the lids from mCP agar plates. Invert lids and nest them under the corresponding plate bottom for identification. Stack the plates in layers in the anaerobic chamber, separating each plate with sterile filter paper. Incubate the anaerobic chamber at 44.5°C for 24 h, maintaining anaerobic conditions through the use of a commercial anaerobic system. If visible condensation does not occur within 60 min after the BBL GasPak is activated, the reaction should be terminated by opening the jar, and removing the GasPak. Inspect the chamber seal for alignment and lubricant. Insert a new GasPak and seal the chamber. The disposable anaerobic indicator (moistened flat fiber wick impregnated with 0.35% methylene blue solution) is white to pale blue upon opening foil envelope. It turns blue upon exposure to air. Under anaerobic conditions the methylene blue indicator will decolorize (turn white) within 2 - 4 h. It should remain white through the incubation period.

10.10 After 24 h, remove one agar plate at a time from the chamber and reclose the chamber. Examine the mCP plate for straw-yellow colonies. If such colonies are

present, invert and expose the open agar plate 10-30 sec to the fumes from an open container of concentrated ammonium hydroxide.

10.11 If *C. perfringens* colonies are present, the phosphate in the phenolphthalein diphosphate will be cleaved from the substrate by acid phosphatase and typical colonies of *C. perfringens* will turn a dark pink or magenta after exposure to fumes of ammonium hydroxide.

10.12 Count pink or magenta colonies as presumptive *C. perfringens*.

10.13 Repeat steps 10.10 to 10.12 with the other culture plates.

11. Confirmation Tests

11.1 Pick at least 10 typical isolated *C. perfringens* colonies from the mCP plate and transfer each into a separate thioglycollate tube. Incubate at 35°C for 24 h. Examine by gram stain and for purity. *C. perfringens* are short gram-positive bacilli. Retain tubes for further testing.

11.2 Inoculate ten tubes of iron milk medium with 1 mL from the ten fluid thioglycollate tubes and incubate in a 44.5°C waterbath for two h. Examine hourly for stormy fermentation with rapid coagulation and fractured rising curd.

11.3 Those colonies which are gram-positive, non-motile, and produce stormy fermentation of milk in these confirmatory tests are considered confirmed *C. perfringens*.

12. Data Analyses, Calculations and Reporting Results

12.1 Pink or magenta colonies counted on mCP medium are adjusted to a count/100 mL and reported as: Presumptive *C. perfringens* colony forming units (CFU)/100 mL. The presumptive count is normally used for routine monitoring.

12.2 If confirmation tests are performed, original counts on mCP agar are adjusted based on the percent of colonies picked and confirmed. Report as confirmed *C. perfringens* CFU/100 mL of water sample.

13. Method Performance Characteristics

13.1 The detection limit is one *C. perfringens* CFU per sample volume or sample dilution tested.

13.2 The false positive rate is reported to be 7-9% by Bisson and Cabelli (2) and Fujioka and Shizumura (10). The false negative rate is reported to be 3% by Fujioka and Shizumura (10).

13.3 The single laboratory recovery is reported to be 79-90% by Bisson and Cabelli (2).

13.4 In a collaborative study, sixteen analysts from nine laboratories analyzed a sediment, a non-chlorinated wastewater and three spiked waters (marine water, lake water and a finished drinking water), as unknowns. Analysts were provided range values to reduce the number of dilutions necessary for the analyses.

13.4.1 The single operator precision as % Relative Standard Deviation (RSD) ranged from 14-28% while the overall precision (as % RSD) ranged from 24-41%, for S_t/S_o (overall precision/single operation precision) ratios of 1.13-1.80. The larger RSD values were not generated with the more difficult sample matrices of sediment and wastewater. Rather, they occurred with the seeded finished drinking water sample and are believed to have been caused by overestimates of the concentration of *C. perfringens*, which resulted in marginally low plate counts with inherently greater deviations. Overall, the S_t and S_o values were similar across sample types and concentration levels of *C. perfringens*.

13.4.2 Although there were no "standards" available for this RR study, sample 5, a seeded drinking water, had a reference count of 78 *C. perfringens* CFU/100 mL. The laboratories in this study achieved a mean recovery of 67 CFU from Sample 5 for an 86 percent recovery.

13.4.3 **Table XI-2** contains the statistical summary of the collaborative study results.

Table XI-2. Statistical Evaluation of Results (CFU/100 mL) (After Rejection of Outliers)

Sample	Initial n	Final n	X	S_0	S_t	%RSD (S_o)	%RSD (S_t)
1	30	30	2893.63	397.78	715.45	13.75	24.73
2	36	35	108.09	20.34	26.18	18.82	24.22
3	30	30	73.07	20.29	23.23	27.77	31.79
4	36	35	5985.71	1400.70	1585.80	23.40	26.49
5	27	27	67.22	18.64	27.60	27.73	41.06

14. Pollution Prevention

14.1 Pollution prevention is any technique that reduces or eliminates the quantity or toxicity of waste at the point of generation. It is the environmental management tool preferred over waste disposal or recycling. When feasible, laboratory staff should use a pollution prevention technique such as preparation of the smallest practical volumes of reagents, standards and media or downsizing of the test units in a method.

14.2 The laboratory staff should also review the procurement and use of equipment and supplies for other ways to reduce waste and prevent pollution. Recycling should be considered whenever practical.

15. Waste Management - The Environmental Protection Agency requires that laboratory waste management practices be conducted consistent with all applicable rules and regulations. The Agency urges laboratories to protect the air, water and land by minimizing and controlling releases from hoods and bench operations, complying with the letter and spirit of sewer discharge permits and regulations and by complying with solid and hazardous waste regulations, particularly the hazardous waste identification rules and land disposal restrictions.

16. Key Words - *Clostridium*, *Clostridium perfringens*, anaerobic bacteria, spore-forming bacteria, indicator organisms, pollution, water quality.

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