

**APPENDIX I**  
**OHIO DISTRICT MICROBIOLOGY LABORATORY**  
**ANALYSIS OF *CLOSTRIDIUM PERFRINGENS* IN ENVIRONMENTAL WATER SAMPLES**

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1. Prepare mCP basal agar as follows:
  - 900 mL reagent water
  - 30.0 g tryptose
  - 20.0 g yeast extract
  - 5.0 g sucrose
  - 1.0 g L-cysteine hydrochloride
  - 0.1 g MgSO<sub>4</sub>·7H<sub>2</sub>O
  - 0.04 g bromcresol purple
  - 15.0 g Bacto agar
2. Label each batch of media with an assigned number and record in the media QC log book.
3. Dispense 100-mL aliquots into dilution bottles and autoclave for 15 minutes at 121°C. Store bottles in the refrigerator for up to 6 months.
4. To prepare plates, melt the agar using a beaker with water on a hot plate or by placing in the autoclave for a 5-minute cycle. After the agar is tempered and before pouring plates, add the following ingredients to each 100-mL bottle of agar:
  - 0.04 g D-cycloserine
  - 0.0025 g polymyxin-B sulfate
  - 0.2 mL 4.5% FeCl<sub>3</sub>·6H<sub>2</sub>O solution  
(filter-sterilize 4.5 g FeCl<sub>3</sub> in 100 mL reagent water, store at 4°C)
  - 2.0 mL 0.5% Phenolphthalein diphosphate solution  
(filter-sterilize 0.5 g phenolphthalein diphosphate in 100 mL reagent water, store at 4°C)
  - 0.006 g indoxyl β-D glucoside

To make a large batch of agar, combine the 100-mL bottles into a sterile flask, calculate the amounts of additives for the total volume of agar, and add each additive in one batch.
5. Label each sub-batch with an assigned number and record in media logbook.
6. Store plates inverted in the refrigerator for up to 1 month.
7. Analyze samples by membrane filtration using appropriate volumes. For turbid surface water, use 3-, 10-, and 30-mL volumes. For clean surface water, use 10-, 30-, and 50-mL volumes. Record media batch and sub-batch numbers on the Results Worksheet.
8. Run a filter blank using PB water before filtering each sample.

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9. Plate a positive control (10 and 30 mL of 10<sup>-2</sup> dilution raw sewage) for every 20<sup>th</sup> sample or when a new analyst is processing or reading plates.
10. Incubate plates anaerobically in a GasPak bag or chamber at 42°C for 24 hours.
11. In the fume hood using protective gloves and forceps, place membrane filters with straw-yellow colonies onto cellulose pads saturated with NH<sub>4</sub>OH. Wait for approximately 15 seconds, then examine for positive colonies.
12. Magenta colonies that are approximately 1 to 2 mm in diameter are enumerated as *C. perfringens*. New analysts may compare size and color of colonies to those in the positive control.
13. Calculate the number of colony-forming units per 100-mL (CFU/100 mL) sample using the following equation:

$$CFU/100mL = \frac{Colony\ count * 100}{Volume\ plated\ (mL)}$$