APPENDIX I

OHIO WATER MICROBIOLOGY LABORATORY

ANALYSIS OF *CLOSTRIDIUM PERFRINGENS* IN ENVIRONMENTAL WATER SAMPLES

1. Prepare mCP basal agar as follows (or order Oxoid CM0992):
   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 $MgSO_4 \cdot 7H_2O$
   0.04 g bromcresol purple
   15.0 g Bacto agar

2. pH adjust agar to 7.6 (with 1 N NaOH)

3. Label each batch of media with an assigned number and record in the media QC log book.

4. 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.

5. 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% $FeCl_3 \bullet 6H_2O$ solution
      (filter-sterilize 0.45 g $FeCl_3$ in 10 mL reagent water, store at 4°C for up to 2 months)
   2.0 mL 0.5% Phenolphthalein diphosphate solution*
      (filter-sterilize 0.5 g phenolphthalein diphosphate in 100 mL reagent water, store at 4°C)
   8.0 mL 0.075% indoxyl β-D glucoside solution**
      (dissolve 0.006 g indoxyl β-D glucoside in 8.0 mL sterile reagent water)

   *Alternatively, 0.01 g phenolphthalein diphosphate can be added directly without making the solution.
   **Alternatively, 0.006 g indoxyl β-D glucoside can be added directly without making the solution.

   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.

6. Label each sub-batch with an assigned number and record in media logbook.

7. Store plates inverted in the refrigerator for up to 1 month.

8. 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.

Note: To quantify the concentration of spores only, heat treat the sample at 60°C for 15 min prior to filtration.

9. Run a filter blank using buffer water before filtering each sample.

10. Plate a positive control (10 and 30 mL of 10^{-2} dilution raw sewage) for every 20th sample or when a new analyst is processing or reading plates.

11. Incubate plates anaerobically in a GasPak bag or chamber at 44.5°C for 24 hours for the analysis of vegetative bacteria or at 41°C for 24 hours for the analysis of spores.

12. In the fume hood using protective gloves and forceps, place membrane filters with straw-yellow colonies onto cellulose pads saturated with NH_4OH. Wait for approximately 15 seconds, then examine for positive colonies.

13. 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.

14. Calculate the number of colony-forming units per 100-mL (CFU/100 mL) sample using the following equation:

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