APPENDIX O
OHIO WATER MICROBIOLOGY LABORATORY
COLIPHAGE DETECTION BY USEPA METHOD 1602: SINGLE-AGAR LAYER (SAL) PROCEDURE

Overview:

The following is a summary of USEPA Method 1602 (U.S. Environmental Protection Agency, 2001). The reader is encouraged to read the full method, available at http://www.epa.gov/microbes/

Method 1602 is a plaque assay method that detects and enumerates F-specific and somatic coliphages in water. A 100-mL water sample is supplemented with $MgCl_2$ (magnesium chloride), log-phase host bacteria (*E. coli* F-amp for F-specific coliphage and *E. coli* CN-13 for somatic coliphage), and double-strength molten agar. The total volume of the mixture is poured into plates and incubated overnight. Plates from a sample are examined for plaque formation (zones of bacterial host lawn clearing). The plaques are counted and summed for all plates from a single sample. The quantity of coliphage in a sample is expressed as plaque forming units (PFU)/100 mL.

Media and reagents:

4 M $MgCl_2$

- Make a 4 M stock solution:
  - 814 g $MgCl_2 \cdot 6H_20$
  - 300 mL reagent water
  - Stir to dissolve. Bring to a final volume of 1L, and mix thoroughly.
  - Autoclave for 15 minutes.
  - Store at 4°C.

Glycerol

- Used for freezing bacterial host stocks and coliphage stocks.
- Autoclave for 15 minutes. Remove promptly to avoid scorching.
- Store at room temperature.

Stock nalidixic acid

- Antibiotic solution used for *E. coli* CN-13.
  - 1.0 g nalidixic acid sodium salt
  - 100 mL reagent water
  - Combine ingredients and stir to dissolve.
  - Filter through a sterile, 0.22-µm membrane filter.
  - Dispense 100-mL aliquots into sterile 150-mL freezer bottles.

- *Mark bottle with the date prepared and store at -20°C for up to one year.*
- *On the day it will be used, thaw and mix well. Any leftover solution may be stored at 4°C for up to 24 hours.*
- *Nalidixic acid is considered toxic. Wear protective clothing, gloves, eye protection, and use in a fume hood.*

Stock Ampicillin/Streptomycin

- Antibiotic solution used for *E. coli* F-amp.

  0.15 g ampicillin sodium salt
  0.15 g streptomycin sulfate
  100 mL reagent water

  - Combine ingredients and stir to dissolve.
  - Filter through a sterile, 0.22µm membrane filter.
  - Dispense 100-mL aliquots into sterile 150-mL freezer bottles.
  - *Mark bottle with the date prepared and store at -20°C for up to one year.*
  - *On the day it will be used, thaw and mix well. Any leftover solution may be stored at 4°C for up to 24 hours.*

Tryptic Soy Broth (TSB)

- Used for preparing frozen, overnight, and 4-hour bacteria stock cultures, and TSA media.
- Follow the procedure as specified on the bottle of TSB (Difco, Detroit, MI).

  30 g powdered TSB
  1 L reagent water

  - Stir to dissolve powdered TSB.
  - Autoclave for 15 minutes.
  - Store TSB at 4°C in the refrigerator.

Double-strength TSA (2X TSA)

- 2X TSA must be used on the day of preparation.
  - Double all components of TSB (except reagent water) and add 18 g of agar per liter.
  - Heat to dissolve while stirring.
  - Autoclave for 15 minutes.
  - Cool to 48°C ± 1.0°C in a waterbath prior to use.

1.5% tryptic soy agar (TSA)

- To be used in streak plates for frozen cultures, and as bottom agar during the double agar layer procedure for enumerating spiking suspensions.

  15 g Bacto agar
  1 L tryptic soy broth - *(Make by adding 30 g powdered TSB to 1 L reagent water, and mixing until dissolved).*

  - While stirring, heat to dissolve the agar.
  - Dispense 100 mL into each dilution bottle.

    - Autoclave for 15 minutes.
    - *Agar can be stored in dilution bottles for up to 6 months in the refrigerator.*
- Prepare plates by adding appropriate antibiotics to molten tempered TSA before pouring plates.
    - Molten, tempered TSA is prepared by autoclaving a 100-mL dilution bottle briefly, then tempering in a 48°C water bath.
    - *E. coli* CN-13 plates require 1 ml stock nalidixic acid solution per 100 ml 1.5% TSA.
    - *E. coli* F-amp plates require 1 ml stock ampicillin/streptomycin solution per 100 ml 1.5% TSA.
    - Swirl until well mixed and dispense 17-18 mL per 100-mm plate.
    - *Plates can be stored inverted at 4°C for up to 2 weeks.*

0.7% tryptic soy agar (TSA)

- To be used as the top layer of agar during the double agar layer procedure.
    - 7 g Bacto agar
      1 L tryptic soy broth - *(Made by adding 30 g powdered TSB to 1L DI water, and mixing until dissolved).*
    - While stirring, heat to dissolve the agar.
    - Dispense 100 mL into each dilution bottle.
    - Autoclave for 15 minutes.
    - *Agar can be stored in dilution bottles for up to 6 months in the refrigerator.*
- Prepare top agar tubes by adding appropriate antibiotics to molten tempered TSA.
    - Molten, tempered TSA is prepared by autoclaving a 100-mL dilution bottle briefly, then tempering in a 48°C water bath.
    - *E. coli* CN-13 tubes require 1 ml stock nalidixic acid solution per 100 ml 0.7% TSA.
    - *E. coli* F-amp plates require 1 ml stock ampicillin/streptomycin solution per 100 ml 0.7% TSA.
    - Dispense 5 mL of 0.7% TSA with antibiotics per sterile 10-mL tube, label, and keep at 48°C in a waterbath until use.
    - *Tubes must be used the day they are prepared.*

Spot plates

Note: Condensation may accumulate at the edges of stored spot plates and may drip over agar surface if tilted, ruining the spot pattern. If the stored spot plates have condensation, incubate plates for approximately 10 minutes to reduce condensation prior to inoculation.

- To be used during the plaque confirmation procedure.
- Prepare 0.75% tryptic soy agar (TSA):
    - 7.5 g Bacto agar
      1 L tryptic soy broth *(Make by adding 30 g powdered TSB to 1 L reagent water, and mixing until dissolved).*
    - Heat and mix to dissolve.
    - Dispense 100 mL into each dilution bottle.

    - Autoclave for 15 minutes.
    - *Agar can be stored in dilution bottles for up to 6 months in the refrigerator.*
- Prepare spot plates when needed by adding 2 mL of the appropriate log-phase *E. coli* culture to 100 mL of molten tempered TSA. This will make six 85-mm plates. *Molten, tempered TSA is prepared by autoclaving a 100-mL dilution bottle briefly, then tempering in a 48°C water bath.*
- Add the appropriate antibiotic solution to the tempered TSA before pouring plates:
    - *E. coli* CN-13 plates require 1 mL of stock nalidixic acid solution per 100 mL TSA.
    - *E. coli* F-amp plates require 1 mL of stock ampicillin/streptomycin solution per 100 mL TSA.
- Swirl to mix and pour plates half full. Plates should be labeled with date of preparation and host stock. *Once poured, plates may be stored for up to 4 days at 4°C before use with environmental samples.*

Frozen host bacteria stock cultures

- Frozen stocks are used as inoculum for overnight host bacteria stock cultures.
    - Streak host bacteria onto 1.5% TSA plates with appropriate antibiotics (1mL of antibiotic per 100 mL of agar) to attain isolated colonies.
    - Incubate overnight at 36°C, pick an individual colony and inoculate into 25 mL sterile TSB with 0.25 mL of the appropriate antibiotic. Grow to log phase at 36°C ± 1°C for 4 hours in a shaking waterbath set at 70 rpm.
    - Add 6.25 mL sterile glycerol to the 25-mL log-phase culture and mix well.
    - Dispense 1-mL aliquots into sterile 1.7-mL microcentrifuge tubes and store at -70°C.
    - Label tubes with date they were frozen and the host.
    - **List stock culture information in coliphage log book.**
    - *Host bacteria stored at –70°C may be retained for up to one year. If stored at –20°C, the host bacteria may be retained for up to two months.*

Overnight host bacteria stock cultures

- Inoculum from an overnight bacterial host culture will reach log phase more rapidly than inoculum from frozen stock. *(For proper growth conditions, each flask of host bacteria should always contain 25 to 30 ml of medium.)*
    - To a sterile 125-ml shaker flask, add 25 ml fresh sterile TSB.
    - Add 0.25 ml of the appropriate antibiotic.
    - Inoculate the flask with a loopful of *E. coli* from the frozen stock cultures.
    - Incubate overnight (18-20 hours) at 36°C ± 1°C in a waterbath shaker set at 70 rpm.
    - Chill at 4°C until ready for use.

Log-phase host bacteria stock cultures

- Prepare log-phase cultures daily to allow 10 mL fresh culture per 100-mL sample. *(For proper growth conditions, each culture flask of host bacteria should contain 25 to 30 mL of medium.)*
    - Add 0.5 mL log-phase culture from the overnight host bacteria stock culture (or the previous days 4 hour culture) to 25 mL fresh sterile TSB in a sterile 125-ml shaker flask.
    - Add 0.25 mL of the appropriate antibiotic.
    - Incubate at 36ºC ± 1°C in a water bath shaker set at 70 rpm for approximately 4 hours, or until cultures are visibly turbid.
    - Chill at 4ºC to slow replication until ready for use. The suspension may be stored up to 48 hours. However, the best results occur when cultures are used immediately (within 6 hours).
    - *Store remaining bacterial host culture at 4ºC overnight to inoculate flasks for the preparation of new log-phase bacterial hosts.*

Coliphage stocks

- The coliphage stocks are as follows:
    - F-specific coliphage: MS2 stock coliphage (ATCC 15597-B1)
        - Used to spike positive controls, IPRs, OPRs, and MS, using *E. coli* F-amp as the bacterial host.
        - Stock may be stored at -70°C for long term storage.
        - Bulk working dilutions are stored at 4°C to 8°C for one month.
    - Somatic coliphage: phi-X 174 stock coliphage (ATCC 13706-B1)
        - Used to spike positive controls, IPRs, OPRs, and MS, using *E. coli* CN-13 as the bacterial host.
        - Stock may be stored at -70°C for long term storage.
        - Bulk working dilutions are stored at 4°C to 8°C for one month.

- Stock cultures (generation 2 stocks) were initially enumerated in October 2003. The 10^{-8} and 10^{-9} dilutions for MS2 coliphage and the 10^{-4} and 10^{-5} dilutions for phi-X 174 coliphage were most appropriate for use with QC samples. **Cultivation history is recorded in the coliphage QC log book, coliphage stock section.**
- For enumeration and analysis, bulk working dilutions of 10^{-7}, 10^{-8}, and 10^{-9} for MS2 coliphage and 10^{-3}, 10^{-4}, and 10^{-5} for phi-X 174 coliphage are prepared every month from the generation 2 coliphage stocks (stored in the -70°C freezer).

    For MS2:

    10^{-2}: 0.1 mL of frozen, gen.2 stock in 9.9 mL TSB
    10^{-4}: 0.1 mL of 10^{-2} dilution in 9.9 mL TSB
    10^{-6}: 0.1 mL of 10^{-4} dilution in 9.9 mL TSB
    10^{-7}: 10 mL of 10^{-6} dilution in 90 mL TSB
    10^{-8}: 10 mL of 10^{-7} dilution in 90 mL TSB
    10^{-9}: 10 mL of 10^{-8} dilution in 90 mL TSB

    For phi-X 174:

    10^{-2}: 0.1 mL of frozen, gen.2 stock in 9.9 mL TSB
    10^{-3}: 10 mL of 10^{-2} dilution in 90 mL TSB

10^{-4}: 10 mL of 10^{-3} dilution in 90 mL TSB
10^{-5}: 10 mL of 10^{-4} dilution in 90 mL TSB

Enumeration of coliphage QC spiking suspensions

- Stocks are re-enumerated every month to ensure coliphage are viable.
    - Dilutions to be analyzed, in duplicate, are 10^{-7}, 10^{-8}, and 10^{-9} for MS2 coliphage and 10^{-3}, 10^{-4}, and 10^{-5} for phi-X 174 coliphage.
    - One mL of the appropriate antibiotic (Nalidixic acid for *E. coli* CN-13 and Ampicillin/Streptomycin for *E. coli* F-amp) is added to a 100-mL bottle of melted, tempered 1.5% TSA, and is poured to make bottom agar plates. One bottle makes about six plates.
    - To prepare the top agar, aliquot 5 mL of the melted, tempered 0.7% TSA into test tubes that are kept in a 48°C waterbath until use. Add 50 µL of the appropriate antibiotic to each test tube. *Do not allow the agar to harden.*
    - Add 500 µL of the appropriate dilution and 100 µL of the appropriate bacterial host to the top agar tube.
    - Gently mix the tube, and pour all of the contents onto the appropriate bottom agar plate labeled with the corresponding dilution and bacterial host.
    - A method blank is also analyzed for each coliphage type by adding 500 µL of sterile TSB to the top agar tube.
    - After the top agar hardens, cover, invert, and incubate plates for 16 to 24 hours at 36°C ± 1°C.
    - **Plaque count results and calculations are recorded onto the "QC spiking suspension data and calculations bench sheet" in the coliphage QC log book.**
    - These calculations are used to determine spiking volumes for QC samples.
    - **Record coliphage/ mL in the coliphage QC log book, coliphage stock section.**

Method:

1) Preheat an incubator to 36 ± 1ºC, a waterbath to 36 ± 1ºC, and another waterbath to 48ºC ± 1ºC.

2) Aliquot 100 mL of 2X TSA into a 500-mL Erlenmeyer flask and place in a 48ºC ± 1ºC waterbath to equilibrate. Add 2 mL of the appropriate antibiotic (Nalidixic Acid for *E. coli* CN-13 or Ampicillin/Streptomycin for *E. coli* F-amp). The liquid in the waterbath must come up to the level of the media.

3) Dispense two, 100-mL aliquots of the environmental sample into separate, sterile, 250-mL Erlenmeyer flasks. Also dispense 100 mL of tap water into a third 250-mL Erlenmeyer flask to be used as the "temperature flask".

4) Add 0.5 mL of sterile stock magnesium chloride to each sample flask (not the temperature flask).

5) Place the flasks into the 36 ± 1ºC waterbath until the thermometer in the temperature flask reads 36 ± 1ºC.

6) Add 10 mL of log-phase *E. coli* to the appropriate flask of sample while still in the waterbath. Add an additional 10 mL of reagent water to the temperature flask.

7) Immediately transfer all of the flasks to the 48ºC waterbath. Monitor the temperature of the temperature flask, and when it reaches 43 ± 1ºC, remove the samples from the water bath.

8) *Note: the sample and host bacteria should not remain in contact with each other for more than 10 minutes prior to plating. After plating, the agar must harden within 10 minutes. Samples should also remain in contact with host for a minimum of three minutes before plates are poured.*

9) Add the sample/*E. coli* mixture to the 100 mL of 2X TSA with appropriate antibiotics. Mix gently by swirling.

10) Pour the contents into a series of 5 petri dishes (150-mm plates).

11) Allow the agar to harden, cover, invert, and incubate for 16-24 hours at 36 ± 1ºC.

12) Circular zones of clearing (typically 1 to 10 mm in diameter) in lawn of host bacteria in SAL plates after 16-24 hours are considered to be plaques. Count all plaques per plate series and record results as the total number of plaques per 100 mL sample = plaques/100 mL.

13) **Record results on results sheet (Appendix A).**

14) Optional step- use only when needed.
    - Spot plate plaque confirmation procedure:
      Use the spot plate procedure for confirmation of plaques if one or more such plaques on a single agar layer plate are questionable. Refer to section 12.6 in the method for details.

15) Disposal
    Samples, reference materials, and equipment known or suspected to have bacteriophage attached or contained must be sterilized prior to disposal.

Quality-Control Procedures:

Initial precision and recovery (IPR)

An IPR test is performed to demonstrate the ability to generate acceptable performance with this method. An IPR test is done before analyzing any field samples.

- A total of 4 spiked 100-mL reagent water samples for each coliphage type are needed for the IPR test. The target spike concentration is 80 PFU per 100-mL sample.
- Spike 400 mL of reagent water in bulk with the appropriate coliphage stock (MS2

for *E. coli* F-amp and phi-X 174 for *E. coli* CN-13). Spike approximately 320 F-specific or somatic coliphages per 400-mL bulk reagent water sample to achieve the target spike concentration of approximately 80 PFU/sample. To determine the exact volume of coliphage stock required to obtain 320 PFU, refer to the "QC spiking suspension data and calculations bench sheet" in the coliphage QC log book, coliphage stock section, for calculations.

- Mix the water by swirling the container.
- Aliquot each bulk sample into four, 100-mL samples.
- Include a method blank for each *E. coli* host. (100-mL sterile reagent water).
- Analyze each sample according to the single agar layer procedure.
- Compute the coliphage percent recovery in each sample.
- Using all four sample results for each coliphage type, compute the average percent recovery and the relative standard deviation of the recovery and compare with Section 14 of Method 1602 to determine if performance is acceptable.
- **Document results in the coliphage QC log book.**

Method blank

A method blank for each *E. coli* host must be done with every batch of samples (daily) to demonstrate freedom from contamination.

- Analyze 100 mL of sterile reagent water using the same procedure for the analysis of environmental samples, for each coliphage type.
- **Record results on QC results sheet (Appendix P).**

Matrix spike (MS)

MS samples are done to assess method performance in each matrix.

- An MS sample for each coliphage type is to be taken when a field sample has come from a source the lab has never analyzed before.
- For an ongoing basis, analyze one set of MS samples after every 20th field sample from that source.
- For the MS sample, additional water must be collected and analyzed at the same time as the unspiked field sample from the same source.
- For each coliphage type, analyze an unspiked, 100-mL field sample according to the SAL procedure.
- For each coliphage type, add approximately 80 PFU of the appropriate coliphage stock to a second, 100-mL aliquot of the same field sample. To determine the exact volume of coliphage stock required to obtain 80 PFU, refer to the coliphage stock enumeration chart in the coliphage QC log book, coliphage stock section, for calculations.
- Analyze the spiked samples using the SAL procedure.
- Compute the percent recovery of coliphage using the following equation:

$$R = 100 * (N_{sp} - N_{usp}) / T$$

Where: R = percent recovery
N_{sp} = number of coliphage detected in the spiked sample
N_{usp} = number of coliphage detected in the unspiked sample
T = number of coliphage spiked

- Compare the percent recovery with the corresponding limits below to determine if performance is acceptable.
    - The acceptance criteria for F-specific coliphage, is detect to 120%.
    - The acceptance criteria for somatic coliphage, is 48% to 291%.
- **Document results in coliphage QC log book.**

Ongoing precision and recovery (OPR)

The OPR serves as the positive control for this method.

- Analyze one OPR sample with every batch of samples (daily) per coliphage type.
- For each coliphage type, spike one, 100-mL reagent water sample with approximately 80 PFU of coliphage stock. To determine the exact volume of coliphage stock required to obtain 80 PFU, refer to the coliphage stock enumeration chart in the coliphage QC log book, coliphage stock section, for calculations.
- Analyze the spiked samples using the SAL procedure.
- Compute the percent recovery of coliphage in each OPR sample using the following equation:

$$R = 100 * (N/T)$$

Where: R = Percent recovery
N = number of coliphage detected
T = number of coliphage spiked

- Compare the percent recovery with the corresponding limits below to determine if performance is acceptable.
    - The acceptance criteria for F-specific coliphage, is 4% to 135%.
    - The acceptance criteria for somatic coliphage, is 79% to 183%, but also note additional criteria below (See **Appendix O1** for code definitions):

  If percent recovery is between 70% and 79%, results are reported, and no remark codes or value qualifiers are needed.

  If percent recovery is between 40% and 69%, rerun the OPR, and results are reported with Remark Code E, Value Qualifier s and c, and Lab Comment: Positive control results were slightly below USEPA acceptance criteria; results are acceptable but may be biased low.

  If percent recovery is less than 40%, no results are reported, and code the results with Null Value Qualifiers l and c, and Lab Comment Positive control results were considerably below USEPA acceptance criteria; results are not valid.
- **Record results on QC results sheet (Appendix P), in the coliphage QC log book, and in the LIMS.**
- Method precision should be assessed for each coliphage type. After the analysis of 5 OPR samples for which the recovery of coliphage is determined, calculate the average percent recovery (R) and the standard deviation of the percent recovery (s_r) for each coliphage type. Precision assessment is expressed as a percent recovery interval from $R - 2(s_r)$ to $R + 2(s_r)$.
- **Record precision assessment in the coliphage QC log book.**

Media sterility checks

- Each batch of medium will be checked for sterility by incubating one unit (tube or plate) of each batch of medium at 36°C ± 1°C for 48 to 72 hours and observe for growth.
- All media with growth shall be discarded.
- **Record results in the coliphage QC log book.**

Record maintenance

Records are maintained in the coliphage QC log book in order to define the quality of data that are generated.

- A record of the date and results of all QC samples must be maintained.
- A record of sterility check, precision assessment, IDC, ODC, and MS samples must be maintained.

Reference: U.S. Environmental Protection Agency, 2001, Method 1602: Male-specific (F+) and somatic coliphage in water by single agar layer (SAL) procedure: Washington D.C., EPA 821-R-01-029, 30 p.