APPENDIX Q
OHIO WATER MICROBIOLOGY LABORATORY
COLIPHAGE DETECTION BY USEPA METHOD 1601: TWO-STEP ENRICHMENT PROCEDURE

Overview:

The following is a summary of USEPA Method 1601 (U.S. Environmental Protection Agency, 2001). The reader is encouraged to read the full method, available at http://www.epa.gov/microbes/

Method 1601 determines the presence or absence of F-specific and somatic coliphages in ground water and other waters. A 1-L 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 tryptic soy broth (TSB) as an enrichment step for coliphage. After an overnight incubation, samples are "spotted" onto a lawn of host bacteria specific for each type of coliphage, incubated, and examined for circular lysis zones, which indicate the presence of coliphages.

Media and reagents:

10X TSB

- For use when analyzing environmental samples
  - 300g TSB (Difco, Detroit, MI)
  - 1 L reagent water
  - ○ While stirring, heat to dissolve.
  - ○ Autoclave for 15 minutes.
  - ○ Remove broth as soon as possible from the autoclave to prevent scorching.
  - ○ Store at 4°C ± 1°C until use.

4 M $MgCl_2$

- Make a 4 M stock solution:
  - 814 g $MgCl_2.6H_20$
  - 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.

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.*

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.

- Used on the second day of two-step enrichment procedure.
- Prepare 0.75% tryptic soy agar (TSA):

7.5 g Bacto agar
1 L tryptic soy broth

- Boil while stirring until dissolved.
- Dispense 100 mL into each dilution bottle.
- Autoclave for 15 minutes.
- *Agar can be stored in dilution bottles for up to 3 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℃ before use with environmental samples.*

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 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 3 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 TSA.
  - *E. coli* F-amp plates require 1 ml stock ampicillin/streptomycin solution per 100 ml TSA.
  - Swirl until well mixed and dispense 17-18 mL per 100-mm plate.
  - *Plates can be stored inverted at 4℃ 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 - *(Make by adding 30 g powdered TSB to 1 L 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 3 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 for 3 minutes, 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.*

Glycerol

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

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

- Used for the two-step enrichment procedure and is prepared the day before.
- 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

- Used on the first day of the two-step enrichment procedure.
- Prepare log-phase cultures daily to allow 5 mL fresh culture per 1-L 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 overnight 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, IDCs, ODCs, 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.
  - Somatic coliphage: phi-X 174 stock coliphage (ATCC 13706-B1)
    - Used to spike positive controls, IDCs, ODCs, 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.

- 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 ph-X 174 coliphage were most appropriate for use with QC samples. ***Cultivation history is recorded in the coliphage QC log book.***
- 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^{-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 500µL 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 incubator to 36 ± 1ºC.

2) Pour 1 L of sample into two sterile 1-L bottles (one for somatic and one for F-specific coliphage). One liter of sample is to the shoulder of the bottle. Leave ample head space for addition of reagents.

3) Add 50 mL 10X TSB, 12.5 mL stock magnesium chloride, 10 mL appropriate antibiotic (stock nalidixic acid solution for *E. coli* CN-13, and stock ampicillin/streptomycin solution for *E. coli* F-amp), and 5 mL log-phase *E. coli* to each sample bottle.

4) Mix well by inverting each bottle several times and incubate at 36ºC ± 1ºC for 16 to 24 hours with no further mixing.

5) After incubation, mix samples by inverting each bottle 25 times or more. Spot 10 µL from each bottle onto appropriate spot plates. Be sure to label each spot. Spot

QC and environmental samples on the same plate. One plate can be inoculated with up to a total of 15 spots.

6) Allow inocula to absorb into medium. This will take approximately 30 to 60 minutes. Cover, invert, and incubate plates at 36ºC ± 1ºC for 16 to 24 hours.

7) Lysis zone formation (circular zone of clearing) indicates a sample is positive for coliphages. If the spot contains an intact lawn of bacteria indistinguishable from the background lawn of bacteria, this indicates a negative result.

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

9) Other possible outcomes:

   a. A positive result may appear as one or more small plaques within the spot, despite the presence of some portion of the host bacteria lawn within the spot.
   b. A positive result may also appear as a zone of lysis containing small, discrete colonies of bacteria (phage–resistant mutants) within the spot.

10) Optional steps- use only when needed.

   a. How to remove interfering bacteria
      - It is possible that the spot contains bacterial growth or a very large number of bacterial colonies that are distinct from the background lawn of host bacteria. This could make it difficult to determine if lysis of host bacteria has occurred. In this case, the bacteria must be removed from the enrichment culture material before re-spotting onto a newly prepared spot plate. Removing the interfering bacteria can be done by filtration or by centrifugation. Refer to Section 12.2.11 in Method 1601.
      - To determine if there will be bacterial interference, a preliminary examination of the spot plates for lysis zones may be completed after 6 hours of incubation.

   b. Spot plate coliphage confirmation
      It may be used for confirmation of lysis zones if such zones on a spot plate are questionable. The lysis zones on a plate are removed and prepared for respotting onto new spot plates. Refer to Section 12.3 in Method 1601.

11) 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 demonstration of capability (IDC)

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

- A total of 10 spiked 1-L reagent water samples of each coliphage type are required for the IDC test.
- Spike 10 L of reagent water in bulk with one of the appropriate coliphage stock (MS2 for *E. coli* F-amp and phi-X 174 for *E. coli* CN-13) at a concentration of 12 PFU/10 L for F-specific coliphage, and 14 PFU/10 L for somatic coliphage. To determine the exact volume of coliphage stock required to obtain 12 PFU and 14 PFU, respectively, 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.
- For each coliphage type, aliquot each bulk sample into ten 1-L samples.
- Include one method blank and one positive control.
- Analyze each sample according to the two-step enrichment procedure.
- A minimum number of 3 samples for F-specific, and 5 samples for somatic must be positive for each set of 10 IDC samples.
- **Document results in coliphage 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.

- For each coliphage type, analyze 1-L sterile reagent water using the same procedure for the analysis of environmental samples.
- Spot the same as environmental samples, with one method blank spot per spot plate.
- **Record results on QC results sheet (Appendix R).**

Positive control

Positive controls are done to ensure that stock coliphage suspensions, host bacterial cultures, and growth media are performing properly.

- Add 1-L sterile reagent water to each of two sterile, 1-L bottles.
- Spike each bottle with one of the appropriate coliphage stock so that each bottle contains 20 PFU/L. To determine the exact volume of coliphage stock required to obtain 20 PFU, refer to the "QC spiking suspension data and calculations bench sheet" in the coliphage QC log book, coliphage stock section, for calculations.
- Analyze the spiked samples using the two-step enrichment procedure.
- Inoculate one positive control spot per spot plate.
- If multiple spot plates are inoculated with samples on the same day, a single enriched positive control sample may be used to inoculate multiple spot plates on that day.
- **Record results on QC results sheet (Appendix R).**

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.
- For the MS sample, additional water must be collected and analyzed at the same time as the unspiked field sample from the same source.
- Spike 3 L of field sample with one of the appropriate coliphage stocks at a concentration of 3.6 PFU/ 3 L for F-specific, or 4.2 PFU/ 3 L for somatic. To determine the exact volume of coliphage stock required to obtain 3.6 PFU and 4.2 PFU, respectively, refer to the "QC spiking suspension data and calculations bench sheet" in the coliphage QC log book, coliphage stock section, for calculations.
- For each coliphage type, dispense three 1-L aliquots into individual containers.
- Analyze according to the two-step enrichment procedure.
- One or more out of 3 samples must be positive to be considered acceptable.
- **Document results in coliphage log book.**

Ongoing demonstration of capability (ODC)
An ODC test is done to assure that results produced by the lab remain within the limits specified in the method.

- Analyze one set of ODC samples after every 20th field sample. (During busy times, ODC are done once a week).
- Spike 3 L of reagent water with one of the appropriate coliphage stocks at a concentration of 3.6 PFU/ 3 L for F-specific coliphage, or 4.2 PFU/3 L for somatic coliphage. To determine the exact volume of coliphage stock required to obtain 3.6 PFU and 4.2 PFU, respectively, refer to the "QC spiking suspension data and calculations bench sheet" in the coliphage QC log book, coliphage stock section, for calculations.
- Mix by swirling.
- For each coliphage type, dispense into three 1-L aliquots.
- Analyze according to the two-step enrichment procedure.
- One or more out of 3 samples must be positive for it to be acceptable.
- **Document results in the coliphage log book and in the LIMS.**

Media sterility checks

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

Record maintenance
Records are maintained in the coliphage 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, IDC, ODC, and MS samples must be maintained.

Reference: U.S. Environmental Protection Agency, 2001, Method 1601: Male-specific (F+) and somatic coliphage in water by two-step enrichment procedure: Washington D.C., EPA 821-R-01-030, 32 p