Annex B
(normative)

Filtration water treatment systems microbial reduction

B.1 Summary

Bacteriophage fr, and Bacteriophage MS2 are used as a viral surrogates to determine the average reduction of mechanical water treatment systems. Presented in this Annex are the methods that are used for suspension preparation, negative control and analysis of the challenge organisms for use testing are presented in this Annex.

B.2 Equipment

— autoclave;
— radiometer (International light IL-700);
— incubator, 35 ± 1 °C (95 ± 1 °F);
— refrigerator, 5 ± 3 °C (41 ± 3 °F);
— water bath 50 ± 1 °C (122 ± 1 °F);
— freezer;
— microwave;
— vortex mixer;
— pH meter;
— hemocytometer;
— Quebec colony counter; and
— centrifuge.

B.3 Microorganisms

All organisms shall be obtained from: American Type Culture Collection, 19301 Parklawn Drive, Rockville, Maryland 20852-1776
— fr coliphage (ATCC #15767-B1);
— fr Escherichia coli host strain (ATCC #19853);
— MS2 coliphage (ATCC #15597-B1); and
— MS2 Escherichia coli host strain (ATCC #15597).

B.4 Supplies

— sterile petri dishes, 20 x 60 mm and 15 x 100 mm;
— sterile pipettes, 1 mL and 10 mL;
— sterile centrifuge tubes, 10 mL and 50 mL;
— sample bottles, 125 mL sterile screw cap;
— test tubes, 16 x 125 mm;
— sterile inoculating loop;
— sterile filtration apparatus;
— sterile 0.22 μm mixed cellulose membrane filters;
— sterile 0.45 μm mixed cellulose membrane filters; and
— disposable sterile 250 mL polypropylene container.

B.5 Reagents

— sterile buffered dilution water (SBDW) shall be prepared according to the Standard Methods for the Examination of Water and Wastewater (dilution water: buffered water);
— Ethylenediaminetetraacetic acid (EDTA), Sigma #ED2SS; and
— Lysozyme, Boehringer Mannheim, #1 243004. Store at 2 to 8 °C (35 to 46 °F).

B.6 Safety precautions and hazards

B.6.1 Steam sterilized samples and equipment are to be handled with protective gloves when being removed from the autoclave.

B.6.2 Cryogenic culture vials are handled with cryoprotective gloves.

B.6.3 All microbiological samples and contaminated test supplies are steam sterilized to 121 ± 1 °C (250 ± 1 °F) at 15 psi for a minimum of 20 min prior to being discarded.

B.6.4 Ultraviolet light is used to expose the organism during calibration. This light can result in skin cancer and retinal damage; hence, personnel must be protected from exposure.

B.7 Growth medium

NOTE 1 — Common bacteriological mediums may be purchased from bacteriological medium manufacturers and prepared according to the manufacturer’s instructions. Formulations should match formulas shown below.

NOTE 2 — The quality of the growth media should be monitored by examining growth promotion and sterility prior to use.

B.7.1 TSB (tryptic soy broth)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>tryptone</td>
<td>1.7 g</td>
</tr>
<tr>
<td>soytone</td>
<td>0.3 g</td>
</tr>
<tr>
<td>dextrose</td>
<td>0.25 g</td>
</tr>
<tr>
<td>sodium chloride</td>
<td>0.5 g</td>
</tr>
<tr>
<td>dipotassium phosphate</td>
<td>0.25 g</td>
</tr>
<tr>
<td>DI water</td>
<td>100 mL</td>
</tr>
<tr>
<td>pH</td>
<td>7.3 ± 0.2</td>
</tr>
</tbody>
</table>

Dissolve by boiling, adjust to final pH, and dispense 8 mL aliquots into 16 x 150 mm test tubes. Autoclave at 121 ± 1 °C (250 ± 1 °F) at 15 psi for 20 min. Store cooled broth at 5 ± 3 °C (41 ± 1 °F).

B.7.2 1.5% TSA (tryptic soy agar)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>tryptone</td>
<td>7.5 g</td>
</tr>
<tr>
<td>soytone</td>
<td>2.5 g</td>
</tr>
<tr>
<td>sodium chloride</td>
<td>2.5 g</td>
</tr>
<tr>
<td>bacto-agar</td>
<td>7.5 g</td>
</tr>
<tr>
<td>DI water</td>
<td>500 mL</td>
</tr>
<tr>
<td>pH</td>
<td>7.3 ± 0.2</td>
</tr>
</tbody>
</table>

Dissolve by boiling, adjust to final pH and autoclave at 121 ± 1 °C (250 ± 1 °F) at 15 psi for 20 min. Pour tempered media into sterile petri dishes. Store agar plates at 5 ± 3 °C (41 ± 1 °F). Allow plates to room temperature before use.

B.7.3 Phage top agar 1% TSA (tryptic soy agar)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>tryptone</td>
<td>7.5 g</td>
</tr>
</tbody>
</table>
### Dissolve by boiling, adjust to final pH, and autoclave at 121 ± 1 °C (250 ± 1 °F) at 15 psi for 20 min. Store agar at 5 ± 3 °C (41 ± 1 °F). On the day of testing, liquefy and place in the 45 ± 1 °C (113 ± 1 °F) water bath. It is very important to keep the phage top agar at 45 ± 1 °C (113 ± 1 °F) to prevent agar solidification.

### B.8 Preparation of challenge organisms

#### B.8.1 fr coliphage

**B.8.1.1 Stock culture preparation of fr coliphage**

NOTE — This Section describes the propagation and harvesting methods for stock suspensions of fr coliphage for use as a challenge suspension for low flow (< 1 GPM) water treatment units. If units possessing a flow rate greater than 1 GPM are to be tested, the stock preparation procedure may have to be repeated multiple times to achieve the required volume. This method should also be repeated when cryogenic stocks are low.

a) One day prior to preparing fr coliphage stock, thaw a cryogenically frozen E. coli host strain (ATCC #19853) and inoculate one TSB tube with 0.1 mL of the stock suspension. Incubate at 35 ± 1 °C (95 ± 1 °F) for 4 to 18 h.

b) On the day of preparing fr coliphage stock, liquefy 1% TSA and temper the media in a 45 ± 1 °C (113 ± 1 °F) water bath. 1.5% TSA plates shall be room temperature prior to use.

c) Make serial dilutions of fr coliphage suspension (10^-1 to 10^-12) using sterile SBDW. Plate 10^-5 to 10^-12 dilutions in triplicate on 1.5% TSA plates. In a sterile tube add 0.1 mL of E. coli host and 1.0 mL (or 0.1 mL) of diluted fr coliphage and vortex. Add ~ 4 mL of melted 1% TSA to each tube and quickly pour onto 1.5% TSA plates. Rock the plate to spread inoculum evenly. After the 1% TSA layer has solidified, invert and incubate at 35 ± 1 °C (95 ± 1 °F) for 18 ± 2 h.

d) Select the plates, which show complete lysis of host cells by the fr coliphage. Flood the surface of each plate with 3 mL of TSB and gently remove the 1% TSA layer using a cell scraper. Pour the contents into two sterile 50 mL centrifuge tubes and bring the total volume to 40 mL with TSB. Add 0.2 g EDTA and 0.026 g lysozyme to each tube. Incubate at room temperature for 2 h, mixing every 15 min.

e) After the 2 h incubation, centrifuge the tubes at 5250 ×g for 20 min, at 20 ± 1 °C (68 ± 1 °F). Remove the resulting supernatant while avoiding the pellet. Aseptically construct a sterile 47 mm filtration assembly using a 0.45 μm mixed cellulose filter. Pretreat the filter with 10 mL of TSB broth just prior to the filtration to minimize fr coliphage adsorption to the filter. Filter the supernatant. Lastly, aseptically set up a sterile 47 mm filtration assembly using a 0.2 μm membrane and filter the resulting supernatant using this set up.

f) For long-term storage (greater than 28 d), add 1/10 volume of sterile glycerol to suspension, dispense into 1 mL and 3 mL aliquots in cryovials, and store at -70 ± 1 °C (-94 ± 1 °F).

The concentration of fr coliphage should be 10^{10} to 10^{12} plaque forming units (PFU)/mL. Alternate equivalent enumeration methodologies may be utilized if they have been validated according to the guidelines specified by AOAC (Association of Analytical Chemists). The document from Feldstine et al. (Journal of AOAC International vol. 85, No. 5, 2002) entitled “AOAC
International Methods Committee Guidelines for Validation of Qualitative and Quantitative Food Microbiological Official Methods of Analysis" details that statistical evaluation for determining method validation and equivalency. This article can be found at <www.aoac.org/vmeth/micguide.pdf>.

B.8.1.2 Enumeration of fr coliphage plaques

a) Thaw a cryogenically frozen E. coli host strain and inoculate one TSB tube with 0.1 mL of the stock suspension. Incubate at 35 ± 1 °C (95 ± 1 °F) for 4 to 18 h.

b) Liquefy 1% TSA and temper the media in a 45 ± 1 °C (113 ± 1 °F) water bath. Allow 1.5% TSA plates to warm to room temperature.

c) Make serial dilutions of fr coliphage suspension (10^{-1} to 10^{-12}) using sterile SBDW. Plate 10^{-7} to 10^{-12} dilutions in triplicate on 1.5% TSA plates. In a sterile tube add 0.1 mL of E. coli host and 1 mL (or 0.1 mL) of diluted fr coliphage suspension and vortex. Add ~ 4 mL of 1% TSA to each tube and quickly pour onto 1.5% TSA plates. Rock the plate to spread inoculum evenly. After the 1% TSA layer has solidified, invert and incubate at 35 ± 1 °C (95 ± 1 °F) for 18 ± 2 h.

c) After incubation, enumerate plates containing 25 to 250 distinct plaque forming units (PFU) using a colony counter. Calculate the titer of the fr coliphage suspension by multiplying the number of PFU obtained by the inverse of the dilution factor. Express results as the number of PFU/mL. The concentration of fr coliphage should be 10^{10} to 10^{12} PFU/mL. Alternate equivalent enumeration methodologies may be utilized if they have been validated according to the guidelines specified by AOAC (Association of Analytical Chemists). The document from Feldstine et al. (Journal of AOAC International vol. 85, No. 5, 2002) entitled "AOAC International Methods Committee Guidelines for Validation of Qualitative and Quantitative Food Microbiological Official Methods of Analysis" details that statistical evaluation for determining method validation and equivalency. This article can be found at <www.aoac.org/vmeth/micguide.pdf>.

B.8.2 MS2 coliphage

B.8.2.1 Stock culture preparation of MS2 coliphage

NOTE — This Section describes the propagation and harvesting methods for stock suspensions of MS2 coliphage for use as a challenge suspension for low flow (< 1 GPM) water treatment units. If units possessing a flow rate greater than 1 GPM are to be tested, the stock preparation procedure may have to be repeated multiple times to achieve the required volume of MS2 coliphage. This method should also be repeated when cryogenic stocks are low.

a) One day prior to preparing MS2 coliphage stock, thaw a cryogenically frozen E. coli host strain and inoculate one TSB tube with 0.1 mL of the stock suspension. Incubate at 35 ± 1 °C (95 ± 1 °F) for 4 to 18 h.

b) On the day of preparing MS2 coliphage stock, liquefy 1% TSA and temper the media in a 45 ± 1 °C (113 ± 1 °F) water bath. 1.5% TSA plates shall be room temperature prior to use.

c) Make serial dilutions of MS2 coliphage suspension (10^{-1} to 10^{-12}) using sterile SBDW. Plate 10^{-5} to 10^{-12} dilutions in triplicate on 1.5% TSA plates. In a sterile tube add 0.1 mL of E. coli host and diluted MS2 coliphage suspension and vortex. Add ~ 4 mL of melted 1% TSA to each tube and pour onto 1.5% TSA plate. Rock the plate to spread inoculum evenly. After the 1% TSA layer has solidified, invert and incubate at 35 ± 1 °C (95 ± 1 °F) for 18 ± 2 h.

d) Select the plates, which show complete lysis of host cells by the MS2 coliphage. Flood the surface of each plate with 3 mL of TSB and gently remove the 1% TSA layer using a cell scraper. Pour the contents into two sterile 50 mL centrifuge tubes and bring the total volume to 40 mL with TSB. Add 0.2 g EDTA and 0.026 g lysozyme to each tube. Incubate at room temperature for 2 h, mixing every 15 min.
e) After the 2 h incubation, centrifuge the tubes at 5250 ×g for 20 min at 20 ± 1 °C (68 ± 1 °F). Remove the resulting supernatant while avoiding the pellet. Aseptically construct a sterile 47 mm filtration assembly using a 0.45 μm mixed cellulose filter. Pretreat the filter with 10 mL of TSB broth just prior to the filtration to minimize MS2 coliphage adsorption to the filter. Filter the supernatant. Lastly, aseptically set up a sterile 47 mm filtration assembly using a 0.2 μm membrane and filter the resulting supernatant using this set up.

f) For long-term storage (greater than 28 d), add 1/10 volume of sterile glycerol to suspension, dispense into 1 mL and 3 mL aliquots in cryovials, and store at -70 ± 1 °C (-94 ± 1 °F).

g) Titer the MS2 coliphage suspension as in B.8.2.2. The concentration of MS2 coliphage should be $1 \times 10^{10}$ to $1 \times 10^{12}$ PFU/mL. Alternate equivalent enumeration methodologies may be utilized if they have been validated according to the guidelines specified by AOAC (Association of Analytical Chemists). The document from Feldstine et al. (Journal of AOAC International vol. 85, No. 5, 2002) entitled “AOAC International Methods Committee Guidelines for Validation of Qualitative and Quantitative Food Microbiological Official Methods of Analysis” details that statistical evaluation for determining method validation and equivalency. This article can be found at <www.aoac.org/vmeth/micguide.pdf>.

B.8.2.2 Enumeration of MS2 coliphage plaques

a) Thaw a cryogenically frozen E. coli host strain and inoculate one TSB tube with 0.1 mL of the stock suspension. Incubate at 35 ± 1 °C (95 ± 1 °F) for 4 to 18 h.

b) Liquefy 1% TSA and temper the media in a 45 ± 1 °C (113 ± 1 °F) water bath. Allow 1.5% TSA plates to warm to room temperature.

c) Make serial dilutions of MS2 coliphage suspension ($10^{-1}$ to $10^{-12}$) using sterile SBDW. Plate $10^{-7}$ to $10^{-12}$ dilutions in triplicate on 1.5% TSA plates. In a sterile tube add 0.1 mL of E. coli host and diluted MS2 coliphage suspension and vortex. Add ~ 4 mL of 1% TSA to each tube and quickly pour onto 1.5% TSA plate. Rock the plate to spread inoculum evenly. After the 1% TSA layer has solidified, invert and incubate at 35 ± 1 °C (95 ± 1 °F) for 18 ± 2 h.

d) After incubation, enumerate plates containing 25 to 250 distinct plaque forming units (PFU) using a colony counter. Calculate the titer of the MS2 coliphage suspension by multiplying the number of PFU obtained by the inverse of the dilution factor. Express results as the number of PFU/mL. The concentration of MS2 coliphage should be $10^{10}$ to $10^{12}$ PFU/mL. Alternate equivalent enumeration methodologies may be utilized if they have been validated according to the guidelines specified by AOAC (Association of Analytical Chemists). The document from Feldstine et al. (Journal of AOAC International vol. 85, No. 5, 2002) entitled “AOAC International Methods Committee Guidelines for Validation of Qualitative and Quantitative Food Microbiological Official Methods of Analysis” details that statistical evaluation for determining method validation and equivalency. This article can be found at <www.aoac.org/vmeth/micguide.pdf>.

B.9 Drinking water treatment unit challenge organism suspension preparation

B.9.1 Determination of the concentration of challenge organism

This determination will be based upon the unit flow rates, injection feed pump rate, suspension density, and the final challenge organism concentration for the unit challenge. The suspension will have to be of adequate volume to deliver the challenge organism to two complete on/off cycles at each sample point (see 6.5).

Example:

— unit flow rate: 1.0 gal/min (GPM); duplicate units tested so total of 2.0 GPM (7560 mL/min);
— injection rate: 10 mL/min;
— suspension density: 1 x 10⁹/mL;
— final concentration: 7.0 x 10⁹/mL; and
— on/off cycle: 10 min / 10 min (20 min on for two complete cycles).

a) To challenge for 20 min at two 10 min intervals, a total of 200mL of suspension is needed to challenge 151,200 mL of water (7560 min x 20 min).

— (7.0 x10⁹/mL) (151,200 mL) = (injection feed concentration) (200 mL); where
— injection feed concentration = 5.3 x 10⁷/mL.

b) To prepare this from the stock suspension:

— (200 mL) (5.3 x 10⁷/mL) = (mL of suspension density) (1.0 x 10⁹); where
— mL of suspension density = 10.6 mL; and
— add 10.6 mL of suspension to 189.4 mL of PBS.

Once suspension has been made, mix the suspension using a magnetic stirrer. Remove a 10 mL aliquot from the challenge suspension and set aside for density verification according to Standard Methods for the Examination of Water and Wastewater.

B.10 Analysis of negative control, influent and effluent samples

B.10.1 Negative control (Flush samples)

Plate 10⁰ in duplicate on TSA plates. Invert and incubate at 35 ± 0.5 °C (95 ± 1 °F) for 24 h.

B.10.2 Enumeration of phage

B.10.2.1 Enumeration of fr plaques

a) Make serial dilutions of the influent and effluent samples (10⁰ to 10⁻⁵) using sterile SBDW. Plate 10⁰ to 10⁻⁵ dilutions in duplicate on 1.5% TSA plates. In a sterile tube, add 0.1 mL of E. coli host and 1.0 mL (or 0.1 mL) of diluted sample and vortex. Add ~ 4 mL of melted 1% TSA to each tube and pour onto 1.5% TSA plates. Rock the plate to spread inoculum evenly. After the 1% TSA layer has solidified, invert and incubate at 35 ± 1 °C (95 ± 1 °F) for 18 ± 2 h.

b) After incubation, enumerate plates containing 25 to 250 distinct plaque forming units (PFU) using a colony counter. Calculate the titer of the fr coliphage suspension by multiplying the number of PFU obtained by the inverse of the dilution factor. Express results as the number of PFU/mL.

B.10.2.2 Enumeration of MS2 coliphage plaques

a) Make serial dilutions of the influent and effluent samples (10⁰ to 10⁻⁵) using sterile SBDW. Plate 10⁰ to 10⁻⁵ dilutions in duplicate on 1.5% TSA plates. In a sterile tube, add 0.1 mL of E. coli host and 1.0 mL (or 0.1 mL) of diluted sample and vortex. Add ~ 4 mL of melted 1% TSA to each tube and pour onto 1.5% TSA plates. Rock the plate to spread inoculum evenly. After the 1% TSA layer has solidified, invert and incubate at 35 ± 1 °C (95 ± 1 °F) for 18 ± 2 h.

b) After incubation, enumerate plates containing 25 to 250 distinct plaque forming units (PFU) using a colony counter. Calculate the titer of the MS2 coliphage suspension by multiplying the number of PFU obtained by the inverse of the dilution factor. Express results as the number of PFU/mL.

B.11 Results
a) Calculate the geometric mean of plaque forming units (PFU) per mL for the influent sample plates with 25 to 250 colonies in Annex AB, Section AB.10.2. This is the $N_0$ value in the equation below. Take precaution to match the influent samples with each corresponding effluent sample to determine each sample point pair log reduction (LR) value.

b) Calculate the geometric mean of PFU/mL for the effluent sample plates with 25 to 250 colonies in Annex BA, Section BA.10.2. This is the $N_s$ value in the equation below.

c) Determine the log reduction (LR) for each sample point pair for each test filter. If there are no colonies on the filter(s) corresponding effluent sample(s) plate, then this sample point pair LR is recorded as $< 1$ PFU/mL.

If there are one or more colonies on the effluent sample plate, this sample point pair LR is calculated from the equation:

$$LR = \log_{10} \left( \frac{N_s \text{ (from b)}}{N_0 \text{ (from a)}} \right)$$

Example:

$N_0 = 5 \times 10^7$ cfu PFU / 100 mL

$N_s = < 1$ cfu PFU / 100 mL (use 1 cfu PFU / 100 mL for LR calculation est.)

$$LR = \log_{10} \left( \frac{1}{5 \times 10^7} \right) = 7$$

If the effluent sample plate has confluent growth, the LR cannot be determined and is recorded as such.