Method Application and Single Lab Validation Checklist For Acceptance of a Method for Use in the NSSP

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the ISSC LMR Committee for acceptance will require at a minimum 6 months for review from the date of submission.

Name of the New Method	Male-specific Coliphage for Wastewater
Name of the Method Developer	USFDA Gulf Coast Seafood Laboratory
Developer Contact Information	USFDA Gulf Coast Seafood Laboratory, 1 Iberville Drive, Dauphin Island, AL 36 kevin.calci@fda.hhs.gov

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
Clearly define the need for which the method has been developed.	Y	
What is the intended purpose of the method?	Y	
Is there an acknowledged need for this method in the NSSP?	Y	
What type of method? i.e. chemical, molecular, culture, etc.	Y	Culture method for Male-specific Coliphage in Wastewater Influent, Mid-process Samples, and Effluent

B. Method Documentation		
1. Method documentation includes the following information:		
Method Title	Y	
Method Scope	Y	
References	Y	
Principle	Y	
Any proprietary aspects	N	
Equipment required	Y	
Reagents required	Y	
Sample collection, preservation and storage requirements	Y	

Safety requirements	Y	
Clear and easy to follow step-by-step procedure	Y	
Quality control steps specific for this method	Y	

C. Validation Criteria		
1. Accuracy / Trueness	Y	
2. Measurement uncertainty	Y	
3. Precision characteristics (repeatability)	Y	
4. Recovery	Y	
5. Specificity	NA	
6. Working and Linear ranges	Y	
7. Limit of detection	Y	
8. Limit of quantitation / Sensitivity	Y	
9. Ruggedness	Y	
10. Matrix effects	N	
11. Comparability (if intended as a substitute for an established method accepted by the NSSP)	NA	

D. Other Information		
1. Cost of the method	Y	
2. Special technical skills required to perform the method	Y	
3. Special equipment required and associated cost	Y	
4. Abbreviations and acronyms defined	Y	
5. Details of turn around times (time involved to complete the method)	Y	
6. Provide brief overview of the quality systems used in the lab	Y	

Submitters Signature	Date:
Submission of validation data and draft method to committee	Date:
Reviewing members:	
Accepted	Date:
Recommendations for further work	Date:

Comments:

Single Laboratory Validation (SLV) Protocol For Submission to the ISSC For Method Approval

Name of the New Method -	A Culture Method/Double Agar Overlay Method for the Determination of Male-specific Coliphage (MSC) for Wastewater
Name of Method Developer -	Kevin Calci, USFDA-GCSL
Developer Contact Information -	USFDA Gulf Coast Seafood Laboratory, 1 Iberville Drive, Dauphin Island, AL 36 kevin.calci@fda.hhs.gov
Date of Interim Submission -	May 15, 2017

Section A. Need for the New Method

FDA has long been using Male-Specific Coliphage (MSC) to evaluate the potential viral contamination of shellfish growing water by wastewater treatement plant (WTP) outfalls. Methods using MSC as an indicator of viral contamination have been successful in evaluation of viral persistence in molluscan shellfish impacted by WTP outfalls (Daskin et al, 2008)(ISSC MSC Workshop). Studies continue to show a significant inverse relationship between decreasing MSC levels in shellfish and increasing wastewater dilution, which is in turn strongly associated with increasing distance from the WTP disharges (Goblic et al, 2011). The relationship between the level of viral contamination in shellfish and dilution of treated wastewater is really contingent on the viral reduction efficientcy of the WTP impacting the area.

The purpose of this method is to assess the $\log_{10}$ reduction of MSC, as a process indicator for enteric viruses,namely Human Norovirus, in wastewater samples including raw influent, pre-disinfected effluent and final effluent. By comparing $\log_{10}$ values of these results, the viral reduction performance of a WTP can be assessed under different environmental and operational conditions (Amarasiri et al, 2017) (Pouillot et al, 2015). Understanding the viral reduction performance at different stages in a wastewater treatment process is a valuable assessment tool to determine growing area classsification and management options for shellfish growing areas adjacent to and downstream from the WTP outfall. This newly configured FDA method for the determination of MSC in wastewater samples has been adapted from previous methods so that it may be more readily implimented at NSSP Laboratories.

The recognized need for an alternative viral indicator is addressed in detail in the newly accepted 2015 Revision of the NSSP Guide for the Control of Molluscan Shellfish, Section IV Guidance Documents, Chapter II, @ .19, Determining Appropriately Sized Prohibited Areas Associated with Wastewater Treatment Plants, page 292. The need and utility for this method was likewise address at the MSC Informational Meeting of the Growing Area Committee (MSC Summit) in

Charlotte, NC in August 2014. A pre-proposal was reveiwed at the 2015 ISSC Meeting and given the Proposal Number of 15-114. The LMRC agreed that the pre-proposal was sufficient and that there is a need for the method. The LMRC recommended to Task Force I that Proposal 15-114 be referred to an appropriate committee as determined by the Conference Chair and await the SLV data.

Section B. Method Documentation

Modified Double Agar Overlay Method for Determination of Male-specific Coliphage in Wastewater

May 2017 Revision

This method for determining levels of male-specific coliphage in wastewater is based on the method described by Cabelli in work on the Narragansett Bay Project. (Cabelli, 1998) The development of an *Escherichia coli* host cell that consitutively expresses the F plasmid and is resistent to 95% of the somatic phage in wastewater was described in a subsequent paper. (DeBartolomeis and Cabelli,1991) FDA refined the method for oyster and hard clam meats as described in the workshop instructions, *Male-specific Bacteriophage (MSB) Workshop*, conducted in Gloucester, Massachusetts on March 9-12, 2004. (US Food and Drug Administration, 2004) This original FDA (2004) method was submitted as ISSC Proposal 05-114. Spinney Creek Shellfish, Inc. (SCS) further refined these procedures for soft-shelled clams and American Oysters in work funded by the Maine Technology Institute in 2006 with the assistance of Mercuria Cumbo of the Maine Department of Marine Resources. This method was approved for limited use by the 2009 ISSC in Manchester, NH. In work funded by UNH Sea Grant, SLV work continued for species extension to quahogs, which was approved for limited use by the 2013 ISSC in San Antonio, Texas. Method development and preliminary SLV trials were conducted in 2015 by Kevin Calci and Ashley Cooper at USFDA-GCSL. Additional SLV trials were conducted at the Spinney Creek Shellfish Laboratory in collaboration with Kevin Calci, the method developer in work supported by UNH Sea Grant.

A. Apparatus and Materials.

Equipment and Materials for Collection and Transport of Wastewater Samples:
250 or 500 ml Sterile Sample Containers
Sealable Bio-hazard Bags (used when shipping)
Labels
Cooler
Gel Packs
Sampling Device
10% Sodium Thiosulfate Solution (for effluent samples)

Laboratory Equipment:
Water bath, 50-52°C
Air Incubator, 35-37°C
Balance
Stir plate and magnetic stirring bars, sterile
Mini vortexer
Autoclave, 119°C - 121°C
Refrigerator, 0–4° C
Freezer, -20°C

pH meter
Erlenmeyer flasks, 2L and 4L
Graduated cylinders, 1000 ml
500 ml jars, autoclavable with caps
Inoculating loops (3 mm in diameter or 10 □L volume)
Bacti-cinerator or flame
Sterile swabs
Sterile, disposable filters, 0.22 or 0.45µm pore size
Syringes, sterile disposable; 5ml
Serological Pipets- 1 ml, 2 ml, 5 ml, 10 ml
Pipet-aid, or
(Micropipette option; 100 µL and 1000 µL (marked with red tape for positive controls), 200 µL (for aliquots of host cells), 2500 µL (for sample aliquots),
Micropipette tips, sterile 100 µL, 1000 µL, 2500 µL
Micropipette Stand)
Petri dishes, sterile disposable 100 x 15 mm
Petri dish racks
Test tubes 16 x 100 mm (for soft agar)
Dilution tubes, 16 x 150 mm, sterile with screw caps
50ml conical tubes, sterile with screw caps
Test tube racks--sizes to accommodate tubes
Freezer vials, sterile 30 ml with screw caps
Baskets with tops to hold freezer vials
Parafilm tape
Aluminum foil
Counter-pen, digital

Reagents:
Reagent water
Glycerol- sterile
Ethanol, 70% or laboratory disinfectant
Calcium chloride, 1M
Mineral oil
Sodium Thiosulfate (for effluent sample bottles to eliminate chlorine residual)

Antibiotic stocks:
Ampicillin sodium salt (Sigma A9518)
Streptomycin sulfate (Sigma S6501)
Streptomycin and Ampicillin stock solutions (50 µg/ml each).
Note: Antibiotics must always be added to liquids and media after these have been autoclaved and cooled.

Media Components:
Agar, Granulated
Dextrose
NaCL

$CaCl_2$
DI water

Media:
Bottom Agar
DS Soft Agar
Growth Broth

Bacterial Host Strain:
E.coli F_{amp} □ *E. coli* HS(pF_{amp})RR (ATCC # 700891).

MSC (Coliphage) Stock:
Type Strain - MS2, ATCC # 15597

B. Media Composition.

Bottom Agar:

Tryptone	10.0 g
Dextrose	1.0 g
NaCl	5.0 g
Agar	15.0 g
DI water	990 ml
Final pH	6.7 ± 0.2 at 25°C

1. With gentle mixing, add all the components, except antibiotics, to 990ml of dH2O in a 1000ml flask (increase flask size to make larger volumes). Dissolve, heat until clear, bringing to a boil.

2. Sterilize at 121°C ± 2°C for 15 minutes.

3. Temper to 50°C in the water bath.

4. Add 5 ml of Streptomycin sulfate/Ampicillin solution, aseptically to the flask (50 µg/ml each in final) and mix. Transfer to 2 – 500ml sterile jars (easier to pour plates from jars).

5. Pour 15-17 ml aliquots aseptically into sterile 100 x 15 mm Petri dishes and allow the agar to harden. Tip Petri dish lids off slightly to reduce condensation.

6. Store bottom agar plates inverted at 4°C and warm to room temperature for 1 hour before use.

7. Plates stored sealed at 4°C can be used up to 3 months.

Streptomycin sulfate/Ampicillin Solution:

1. Dissolve 0.5g of streptomycin sulfate and 0.5g of ampicillin in 50 ml of dH_2O with a sterile 100 ml graduated cylinder in sterile 600 ml beaker with sterile stir bar.

2. Stir for 2 to 3 minutes, no heat.

3. Filter by injecting through a sterile 0.22 µm filter.

4. Store in 5 ml aliquots in sterile 30 ml capped freezer vials at -20°C for up to one year. Label and date.

5. Allow to come to room temperature before adding and mixing in tempered bottom agar at 50°C.

DS Soft Agar:

Tryptone	10.0 g
Dextrose	1.0 g
NaCl	5.0 g
1M $CaCl_2$	0.5 ml
Agar	7.0 g
DI water	500 ml
Final pH	6.7 ± 0.2

1. With gentle mixing, add all the components to 500 ml of dH_2O in a 1000 ml flask.

2. Bring flask contents to a boil.

3. Dispense in 2.5 ml aliquots into 16 x 100 ml tubes, cover and freeze (-20°C)

4. Sterilize prior to use at 121°C ± 2°C for 15 minutes, then temper to 50-52°C in a water bath set to 50 ºC ± 2 ºC for no longer than 2 hours.

5. Store up to 3 months at -20 °C.

1M $CaCl_2$ Solution:

1. Add 11.1 g of $CaCl_2$ anhydrous (FW 111.0, Dihydrate FW 147) to 100 ml dH_2O in a screw top bottle and dissolve or use prepared from VWR.

2. Sterilize by autoclaving at 121°C for 15 minutes.

3. Store up to three months at 4°C.

Growth Broth:

Tryptone	10.0 g
Dextrose	1.0 g
NaCl	5.0 g
DI water	1000 ml

1. With gentle mixing, add all the components to 1000 ml of dH_2O water in a 2000 ml flask.

2. Dissolve and dispense into sterile screw top containers.

3. Sterilize at 121°C ± 2°C for 15 minutes.

4. Store for up to three months at 4°C.

C. Storage and Propagation of Host Strain, E. coli F_{amp}:

Storage:

1. Lab stock culture – Frozen at – 80°C indefinitely (most desirable method) in broth culture containing 10% glycerol under no selective pressure. Selective pressure is reapplied when the culture is retrieved, by streaking onto Bottom Agar plates containing the two antibiotics.

2. Long-term working stock culture – Grown tryptic soy agar slant with sterile mineral oil overlay under no selective pressure and stored at room temperature in the dark for up to 2 years.

3 Long-term working stock – 6-hour grown tryptic soy agar slant and deep stab with sterile mineral oil overlay containing the two antibiotics, Ampicillin and Streptomycin (least desirable method).

4. Short-term working stock culture - Grown Bottom Agar streak plate stored at 4°C up to 3 weeks.

Glycerol Solution, 10%:

1. Add 9 ml of distilled water to 1 ml of undiluted glycerol.

2. Autoclave resulting 10% glycerol solution at 121°C for 15 minutes and use at room temperature.

3. For storage, add 1/5th volume of 10% glycerol solution, let stand for 30 minutes, dispense 1 ml aliquots in 2 ml cryo-vials and store at -70 to –80°C (best) or at –20°C.

D. Control Plates.

1. Negative Control - Add 2.5 ml of Growth Broth and 0.2 ml host to the 2.5 ml DS Soft Agar tube.

2. Positive Control - Make serial dilutions using growth broth of the concentrated MS2 control (to grow approximately 50-100 PFU per 2.5 ml), and add 2.5 ml of appropriate MS2 dilution and 0.2 ml of host to 2.5 ml DS Soft agar.

E. MSC Density Determinations in wastewater Treatment Plant (WWTP) Samples.

Sample Requirements:

1. Sterile 250 or 500 ml Nalgene bottles (or comparable bottle) with a permanent fill mark at the approximate 200 or 400 ml level are recommended for wastewater samples including influent, pre-treated effluent, and effluent.

2. Sample collection bottles must be properly labeled with sample number, location, sample type, date and time.

3. Sample bottles are filled to the 200 or 400 ml line. Effluent sample bottles must contain 1.0 ml of 10% sodium thiosulfate solution for 200 ml or 2.0 ml of 10% sodium thiosulfate solution to for 400 ml to inactivate any residual chlorine.

4. Wastewater samples are held under refrigerated conditions at 1-4ºC.

Note - A sealed bio-hazard bag is recommended for the samples bottles containing sewage samples going into an insulated shipping box when using overnight carriers.

Propagation of Host Cells:

1. Allow grown Bottom Agar streak plate and Growth Broth to temper to room temperature.

2. Vortex to aerate 20 ml of Growth Broth in a 16 x 150 mm tube, with screw cap.

3. Transfer host strain to Growth Broth using sterile swab to collect material from three colonies off grown Bottom Agar streak plate.

4. Gently shake to mix, then incubate at 35–37°C for 4-6 hours.

5. Once turbidity is observed, use of the host strain broth culture (log-phased growth) may commence.

Note - Following initial inoculation and mixing, do not shake or mix the host strain broth culture (to avoid mixing of cell debris at bottom with log-phase E. coli with pili)

Preparation of Wastewater samples for Analysis:

1. Analyst must wear gloves during handling of stir bars and sample bottle.

2. Water samples are removed from 1 - 4º C.

3. Sample bottle is shaken vigorously for 20 seconds (ensure cap is tightened), and a sterile magnetic stir bar is aseptically transferred to bottle.

4. Sample bottle is placed on stir plate set to medium for five minutes prior to analysis.

5. For the **high range** of this method a 10^{-2} decimal dilution is prepared by transferring 1ml of sample with a sterile 2 ml pipette (using a pipette aid) to a sterile 16x150mm screw cap tube containing 9 ml of growth broth. Sample tube is then vortexed for 10 seconds. For the second decimal dilution, 2ml are transferred from the first tube to a sterile 50ml conical tube with cap containing 18ml of growth broth using a second sterile 2ml pipette. The appropriately labeled 50 ml conical tube is then vortexed for 10 seconds.

6. For the **low range** of this method, 30ml of sample is transferred to a sterile 50 mm conical tube with cap using a sterile 10 ml pipette. The appropriately labeled 50 ml conical tube is vortexed for 10 seconds.

7. Prepped samples in labeled 50ml conical tube are stored in a test tube rack which can be stored short term at 0-4°C.

8. Return sample bottles to refrigeration and clean the work surface with disinfectant.

Note: The samples bottles containing wastewater samples should be autoclaved prior to disposal. Sample bottles must be washed and sterilized for re-use.

Direct Analytical Technique for WWTP samples:

This MSC method for wastewater has both a **high range** and a **low range** routine. Combined, the working range is from 5 to 1,200,000 PFU/100ml. The **high range** routine is adequate for enumeration of MSC in WWTP influent and has a working range from 1,000 to 1,200,000 PFU/100ml. The **low range** routine is generally adequate for enumeration of MSC in final effluent and has a working range from 5 to 12,000 PFU/100ml. When testing for pre-treatment effluent (before disinfection) or at times when the effluent is questionable, both high and low ranges routines should be used together.

1. In the morning, propagate host cells as described above.

2. Tubes may be inoculated on a staggered time schedule:

Tubes in incubator at 7:00am	Ready at 11:00am
Tubes in incubator at 8:00am	Ready at 12:00pm
Tubes in incubator at 10:00am	Ready at 2:00pm
Tubes in incubator at 11:00am	Ready at 3:00pm

3. Before experimentation, prepare the wastewater samples for analysis as described above.

4. One hour before experimentation (at 3 hours of host growth), autoclave required number of soft agar tubes at 121ºC for 15min. Temper soft agar tubes in water bath set to 50-52ºC.

High Range Routine:

For each high range (influent) sample, four (4) Bottom Agar plates and four (4) 2.5 ml DS Soft Agar tubes are prepared. Always begin the day's analyses with a negative control (blank) plate and finish analyses with a positive control plate and a negative control plate.

1. Allow prepared samples (50ml conical tubes, racked and labeled) to warm to room temperature immediately before analysis (20-30 minutes)

2. Remove tubes containing appropriate timed host cells from incubator immediately before analysis.

3. Vortex sample in 50ml conical tube for 10 seconds.

4. Moving quickly and smoothly, gently pipette 200µL of host cells into each of 4 soft agar tubes using a 1 ml serological pipet or 200µL micropipette with sterile tip.

5. Immediately thereafter, pipette 2500µL aliquot of sample into each of the 4 soft tubes using a 10 ml serological pipet and pipet aid or 2500µL micropipette with sterile tip.

6. Mix sample, host and soft agar by gently rolling test tube between hands.

Note: *Once E. coli F_{amp} is added to the mixture do not shake, only gently mix contents by rolling the tube between palms.*

7. Then overlay the contents of the soft agar tubes onto a Bottom Agar plates. Drag the mixture into a clear area and gently swirl the plates to spread sample and agar

mixture.

8. Allow plates to set then inverted and incubated for 16 - 20 hours at 35- 37°C.

9. Circular zones of clearing (typically 1 to 10 mm in diameter) in lawn of host bacteria after 16 – 20 hours of incubation are counted as plaques. PFUs on each plate are counted and totaled using a digital counter pen and adequate light

10. Calculations of **High Range** Routine Results;

N = Total number of PFUs counted on 4 the plates,

The maximum readable limit on PFUs count is 1000 on the four plate,

PFU count exceeding 1,000/4 plate is considered TNTC or >1,000,000 PFU/100gm

$$\text{Result} = \frac{(\text{N PFUs}) * 100}{.1 \text{ ml}} = \text{N} * 1{,}000 \text{ PFU/100ml}$$

Example: High range version plate counts - 13, 23, 12, and 16 PFUs

Result = (64)(1000) = 64,000 PFU/100ml*

Low Range Routine:

For each low range (effluent) sample, eight (8) Bottom Agar plates and eight (8) 2.5 ml DS Soft Agar tubes are prepared. Always begin the day's analyses with a negative control (blank) plate and finish analyses with a positive control plate and a negative control plate.

1. Allow prepared samples (50ml conical tubes, racked and labeled) to warm to room temperature immediately before analysis (20-30 minutes)

2. Remove tubes containing appropriate timed host cells from incubator immediately before analysis.

3. Vortex sample in 50ml conical tube for 10 seconds.

4. Moving quickly and smoothly, gently pipette 200µL of host cells into each of 8 soft agar tubes using a 1 ml serological pipet or 200µL micropipette with sterile tip.

5. Immediately thereafter, pipette 2500µL aliquot of sample into each of the 8 soft tubes using a 10 ml serological pipet and pipet aid or 2500µL micropipette with sterile tip.

6. Mix sample, host and soft agar by gently rolling test tube between hands.

Note: *Once E. coli F_{amp} is added to the mixture do not shake, only gently mix contents by rolling the tube between palms.*

7. Then overlay the contents of the soft agar tubes onto a Bottom Agar plates. Drag the mixture into a clear area and gently swirl the plates to spread sample and agar mixture.

8. Allow plates to set then inverted and incubated for 16 - 20 hours at 35- 37°C.

9. Circular zones of clearing (typically 1 to 10 mm in diameter) in lawn of host bacteria after 16 – 20 hours of incubation are counted as plaques. PFUs on each plate are counted and totaled using a digital counter pen and adequate light

10. Calculations of **Low Range** Routine Results;

N = Total number of Plaque forming units (PFUs) counted on 8 the plates,

The maximum readable limit on PFUs count is 2000 on the eight plate,

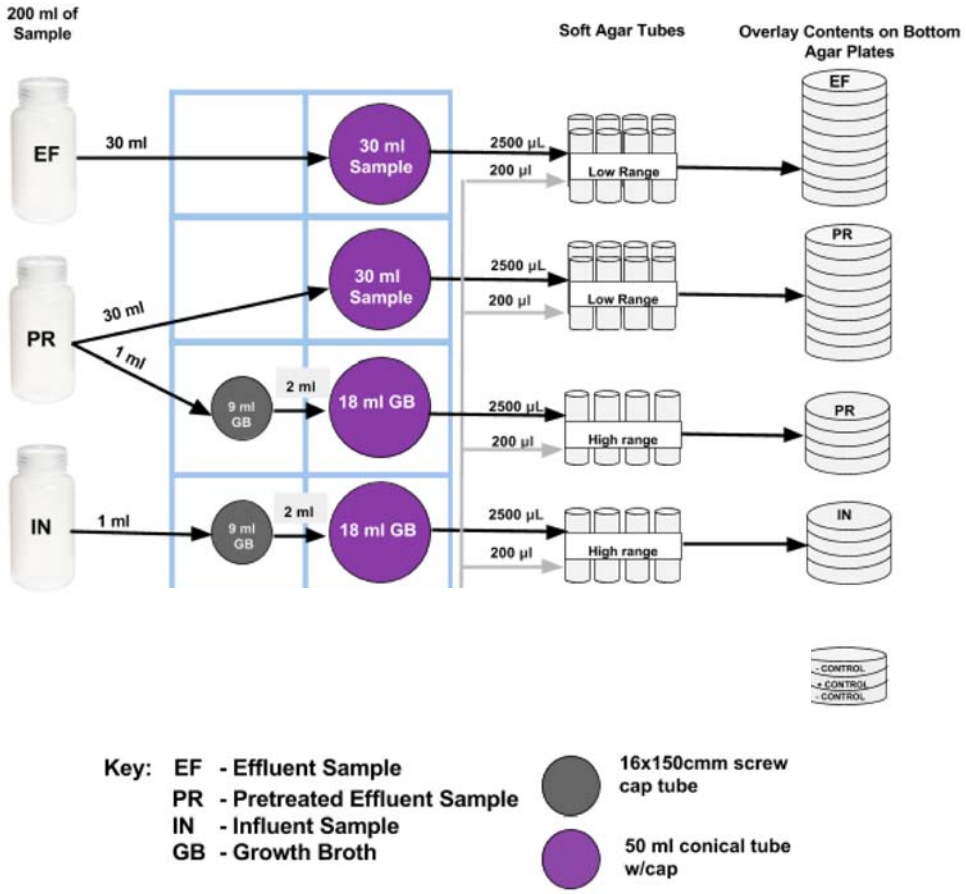
PFU count exceeding 2,000/8 plates is considered TNTC or >10,000 PFU/100gm

$$\text{Result} = \frac{(\text{N PFUs}) * 100}{20 \text{ ml}} = \text{N} * 5 \text{ PFU/100ml}$$

Example: High range version plate counts - 21, 17, 20, 19, 13, 23, 12, and 16 PFUs

Result = (141)(5) = 702 PFU/100ml. Rounding off result to nearest 10s... Result = 700PFU/100gm*

MSC Method for Wastewater Schematic:



Samples Bench Sheet:

Sample Bench Sheet
For use with the NSSP Method for Determination of MSC in Wastewater
2016 Edition

Initiation Analysts: Jerry Jone Date: 10/10/16 Time: 1:00PM
Completing Analysts: Bill Bellicheck Date: 10/11/16 Time: 8:00AM

Low Range Routine

Sample#:1EF	WWTP: Hopetown, NE				Type: Effluent					Date/Time:	10/9/16
PFU Counts	3	2	0	1	0	2	0	1	=	9	45 PFU/100ml

Sample#:3Pre	WWTP: Hopetown, NE				Type: Pre-treatment					Date/Time:	10/9/16
PFU Counts	199	215	203	170	233	210	206	188	=	1,624	8,120 PFU/100ml

Sample#:	WWTP:				Type:					Date/Time:	
PFU Counts									=		

Hign Range Routine

Sample#:2In	WWTP: Hopetown, NE				Type: Influent		Date/Time:	10/9/16
PFU Counts	171	193	201	177	= 742			177,000 PFU/100ml

Sample#:	WWTP: Hopetown, NE				Type: Pre-treatment		Date/Time:	10/9/16
PFU Counts	3	1	2	0	= 6			6,000 PFU/100ml

Sample#:	WWTP:				Type:		Date/Time:	
PFU Counts					=			

For unknown or mid-range sample, run both rountines above on the sample

Controls:	Start Neg Contol:	count	End Pos Control:	count
		0	43	
			End Neg Control:	0

F. Sample Collection and Storage.

1. Record all pertinent information on the collection form.

2. During transportation store samples in a cooler at 0 to 10°C

3. At laboratory, store samples in a refrigerator at 0 to 4 °C.

4. Maximum holding times for wastewater samples is up to 72 hours.

G. Quality Assurance.

1. Positive and negative control plates are run with MSC analyses each day.

2. Media sterility checks are made per batch and records are maintained.

3. Media log book is maintained (pH, volume, weights of each components, lot numbers, etc.).

4. An intra- and inter-laboratory performance program is developed.

5. Circular zones of clearing (typically 1 to 10 mm in diameter) in lawn of host bacteria after 16- 20 hours of incubation are counted as plaques. (Count the number of plaques on each plate.)

6. MSC determinations are reported as plaque forming unit (PFU) per 100 grams.

7. The desired range for counting is 0 to 300 PFU per plate. If the count exceeds the upper range or if the plaques are not discrete, results should be recorded as "too numerous to count" (TNTC) or >12,000 PFU/100ml for **Low Range** Routine and >1,200,000 PFU/100ml for **High Range** Routine.

8. Temperatures incubators are checked twice daily (at least 4 hours apart) to ensure operation within the stated limits of the method, and results are recorded in a logbook.

9. Check thermometers at least annually against a NIST-certified thermometer.

10. Calibrate the balance monthly using ASTM-certified Class 1 or 2 or NIST Class S reference weights.

11. Laboratory analysts adhere to all applicable quality control requirements set forth in the most recent version of FDA's *Shellfish Laboratory Evaluation Checklist*.

12. Calibration of micropipettes needs to be checked quarterly and records kept. Micropipettes used for handling MSC control and transferring host cells need to have a barrier tip or be dedicated to the specific use to prevent contamination

H. Safety.

Samples, reference materials, and equipment known or suspected to have sewage, coliphage and/or *E.coli* attached or contained must be sterilized prior to disposal.

I. Technical Terms.

°C	-	degrees Celsius
µL	-	microliter
g	-	gram
L	-	liter
M	-	molar
ml	-	milliliter
Ave.	-	average
MSC	-	Male-specific Coliphage, Male-specific Bacteriophage, F+ Bacteriophage
NIST	-	National Institute of Standards and Technology
PFU	-	plaque forming units
RT	-	room temperature
TNTC	-	too numerous to count

Host Strain:	*E.coli* F_{amp} bacteria (*E.coli* HS(pFamp)RR)
Male-specific Coliphage:	Viruses that infect coliform bacteria only via the F-pili.
Plaque :	Clear circular zones (typically 1 to 10 mm in diameter) in lawn of host cells after incubation.

References:

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Pouillot, R., J.M. van Doren, , J. Woods, , D. Plante, M. Smith, G. Goblick, C. Roberts, A. Locas, W. Hajen, J. Stobo, J. White, J. Holtzman, E. Buenaventura, W. Burkhardt III, A. Catford, R. Edwards, A. DePaola, and K.R. Calci, 2015. Meta-analysis of the reduction of norovirus and male-specific coliphage concentrations in wastewater treatment plants. Appl. Environ. Microbiol. 81, 4669-4681.

U.S. Food and Drug Administration. 2004. Male-specific Coliphage (MSC) Workshop, conducted in Gloucester, Massachusetts on March 9-12, 2004.

Other Information:

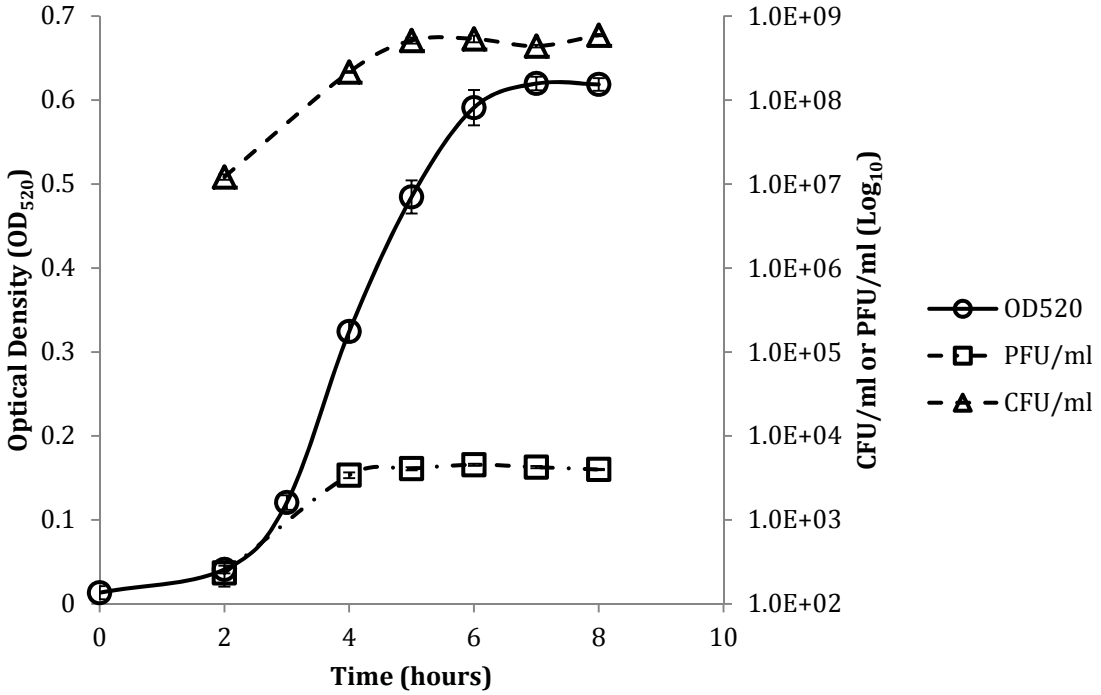
This method for the enumeration of male-specific coliphage in wastewater samples is inexpensive, easy to perform, and rapid, providing results within 24 hours. The cost of laboratory glassware, plastic-ware, agars, and reagents is approximately $18 per series of samples (an influent, a pre-disinfection effluent, and a final effluent sample). In a well set-up laboratory, the method requires 6 hours of time from initiating host to pouring plates. Hands on technician time to perform this test is significantly less on the order of 1-4 hours per test depending upon how many tests are done per day. There are no special skill sets required beyond those required to operate a state-approved shellfish laboratory under the NSSP.

To standardize these assessments, an index of viral performance for use in the NSSP to determine classification options adjacent to WWTP outfall can be estimated. Subtracting the log value of final effluent score from the log value of the corresponding raw influent score will yield an index of viral performance ranging from 0 to 5. A viral index of 4 to 5 indicates 99.99% to 99.999% reduction of enteric viruses and would be consider high performance. A viral index of 1 to 2 indicates 90% to 95% reduction of enteric viruses through the treatment process and would be considered poor performance. A viral index <1 would be considered ineffective and should lead the SSCA to consider 10,000:1 rather than 1000:1 for the determination of the size of the prohibited zone adjacent to the outfall.

C. Validation Criteria

Determination of Optimal Optical Density of Famp Host (OD)

Procedures for enumeration of double-agar overlay method for male-specific coliphage provide different ranges for OD of host growth. Effort was taken to determine the working range of the host *E. coli* at 520nm, which is the current EPA measurement. Graph 1 shows that at approximately 4 to 6 hours growth the OD520 of host is in the range of 0.35 to 0.6, during which time the MS2 plaquing efficiency of the host *E. coli* is optimal and consistent. Therefore, we conclude that a host OD520 of 0.35 to 0.7, or approximately 4 to 6 hours of growth, is ideal for MSC enumeration.



Graph 1. Optical Density (OD520) of *E. coli* HS(pFamp)RR in tryptone broth compared to plaque forming units (PFU) of MS2 coliphage. 10 ml of tryptone broth was inoculated with *E. coli* and incubated at 35ºC. OD was measured every hour starting at t = 2 hours. At these intervals 100 µL of host was serially diluted and 100 µL of pre-determined dilutions were plated to determine CFU/mL. 200 µL of the same host sample was used to determine PFU/ml of stock MS2 controls.

The Determination of LOD, LOQ, and Linear Range using the NSSP SOP for the Single Laboratory Validation of Marine Biotoxin and Non-MPN Based Microbiological Methods.

The SOP for the determination of LOQ, LOD, and the Linear Range is the most robust of the SOPs and yields a database from which subsets of data can be use to generate other validation criteria. For this database ten trials were run using clean effluent samples. Great effort was taken to find and verify clean effluent. The Dover, NH WWTP and the Hampton, NH WWTP were identified as high performing plants of different design capable of consistently producing clean effluent. Table 1 list the metadata for the effluent samples collected for these trials

Table 1. Effluent Samples used for the Determination of LOD, LOQ, Linear Range

Trial #	Date Sampled	WWTP	Treatment Process
1	4/11/17	Dover	Tertiary, UV Dis-infection
2	4/11/17	Hampton	Secondary, Chlorine
3	4/18/17	Dover	Tertiary, UV Dis-infection
4	4/18/17	Hampton	Secondary, Chlorine
5	4/24/17	Dover	Tertiary, UV Dis-infection
6	4/24/17	Hampton	Secondary, Chlorine
7	5/1/17	Dover	Tertiary, UV Dis-infection
8	5/1/17	Hampton	Secondary, Chlorine
9	5/8/17	Dover	Tertiary, UV Dis-infection
10	5/8/17	Hampton	Secondary, Chlorine

For each of the 10 validation trials, 150 ml of clean effluent sample was aseptically transferred into 5-200ml sterile dilution bottles. A master spike solution was prepared in growth broth and was varied in concentration during the trials. The master spike solution was on the order of 10^3 MSC/ml. Four subsequent serial dilutions were made for each trial from the master spike at a 3:1 dilutions. This represented different spike concentrations over the working range of the method. The 5 dilution bottles were asceptically spiked with 5 ml of spike concentration 1 through 5, shaken vigourously and then 4 aliquots of 30 ml were transferred into 4-50 ml sterile conical tubes for each spike concentration (3 conical tubes for the replicates and a 4th tube for spike determination). In this way, 3 true replicates were generated at each of the 5 spike concentrations. This methodology was consistently applied throughout the ten trials. The 5 sets of 3 aliquots were processed and plated according to the method description above. Clean effluent was used instead of growth broth for the spike determination. Spike determinations using growth broth were underestimating the sample results. This problem was solved by using the same clean effluent similiarly spiked. This strategy was employed as there is no standard method available for a truly independant spike determinations.

Table 2 below shows the Spiking Study Database for MSC Method in Wastewater SLV. Spike Concentrations and MSC replicate plate count results are in units of PFU of MSC/100ml.

Table 2. Spiking Study Database for MSC Method in Wastewater SLV

WW Spiking Database 8 plate -

Date	X Value Measured Spike (PFU/100ml)	Repicate Plates (PFU/100ml)	Log of plates	Y Value RSD	Log RSD	X Value log of spike
	3400	4105	3.613	0.0025	-2.603	3.531
		4140	3.617			
		3980	3.600			
	1050	1025	3.011	0.0134	-1.874	3.021
		1225	3.088			
		1180	3.072			
Trial 1						
Dover	315	245	2.389	0.0352	-1.453	2.498
4/11/17		330	2.519			
		360	2.556			
	60	75	1.875	0.0629	-1.202	1.778
		60	1.778			
		45	1.653			
	10	30	1.477	0.2090	-0.680	1.000
		10	1.000			
		30	1.477			

	5950	5490	3.740	0.0046	-2.340	3.775
		5110	3.708			
		5155	3.712			
	1515	1355	3.132	0.0051	-2.289	3.180
		1365	3.135			
		1450	3.161			
Trial 2						
Hampton	410	225	2.352	0.0304	-1.517	2.613
4/11/17		225	2.352			
		170	2.230			
	70	65	1.813	0.0204	-1.690	1.845
		55	1.740			
		60	1.778			
	25	25	1.398	0.0321	-1.494	1.398
		30	1.477			
		25	1.398			

	5135	4315	3.635	0.0063	-2.199	3.711
		4800	3.681			
		4550	3.658			
	980	1085	3.035	0.0139	-1.856	2.991
		1005	3.002			
Trial 3		1220	3.086			
Dover						
4/18/17	405	385	2.585	0.0241	-1.618	2.607
		315	2.498			
		415	2.618			
	75	65	1.813	0.0379	-1.422	1.875
		90	1.954			
		80	1.903			
	25	20	1.301	0.1072	-0.970	1.398
		25	1.398			
		40	1.602			

	5175	4925	3.692	0.0065	-2.189	3.714
		5300	3.724			
		5490	3.740			
	1130	1280	3.107	0.0103	-1.986	3.053
		1160	3.064			
Trial 4		1340	3.127			
Hampton						
4/18/17	355	280	2.447	0.0317	-1.499	2.550
		335	2.525			
		405	2.607			
	40	60	1.778	0.0590	-1.229	1.602
		100	2.000			
		75	1.875			
	20	25	1.398	0.0634	-1.198	1.653
		30	1.477			
		20	1.301			

	11575	10655	4.028	0.0128	-1.891	4.064
		12800	4.107			
		10220	4.009			
	2080	2025	3.306	0.0212	-1.674	3.318
		2650	3.423			
		2735	3.437			
Trial 5						
Dover	525	680	2.833	0.0360	-1.444	2.720
4/24/17		705	2.848			
		465	2.667			
	190	205	2.312	0.0179	-1.746	2.279
		185	2.267			
		170	2.230			
	20	45	1.653	0.2069	-0.684	1.301
		60	1.778			
		15	1.176			

	12210	11140	4.047	0.0076	-2.121	4.087
		12165	4.085			
		10580	4.024			
	2555	2720	3.435	0.0058	-2.239	3.407
		2510	3.400			
		2520	3.401			
Trial 6						
Hampton	495	555	2.744	0.0395	-1.403	2.695
4/24/17		350	2.544			
		395	2.597			
	85	90	1.954	0.1082	-0.966	1.929
		110	2.041			
		45	1.653			
	20	20	1.301	0.1396	-0.855	1.301
		35	1.544			
		15	1.176			

	4430	3530	3.548	0.0131	-1.882	3.646
		4370	3.640			
		4075	3.610			
	1035	1100	3.041	0.0256	-1.592	3.015
		780	2.892			
		880	2.944			
Trial 7	240	275	2.439	0.0188	-1.727	2.380
Dover		230	2.362			
5/1/17		230	2.362			
	65	80	1.903	0.1126	-0.949	1.813
		75	1.875			
		35	1.544			
	10	20	1.301	0.1305	-0.884	1.000
		15	1.176			
		10	1.000			

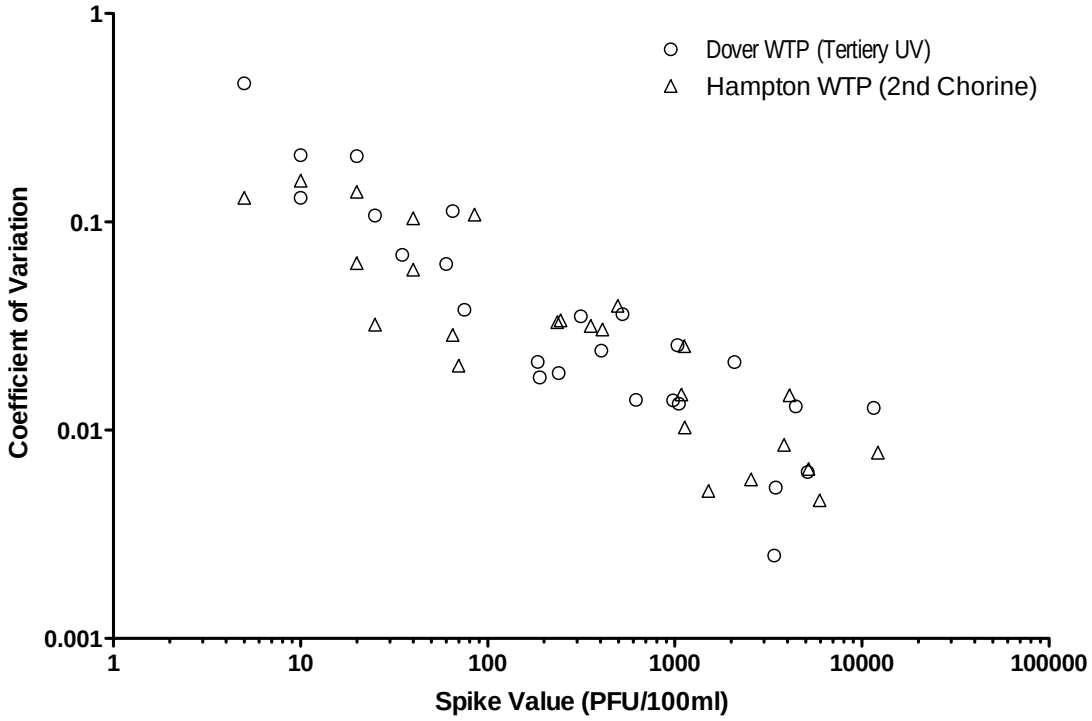
	4110	4415	3.645	0.0147	-1.833	3.614
		5630	3.751			
		5260	3.721			
	1125	955	2.980	0.0253	-1.596	3.051
		1060	3.025			
		1350	3.130			
Trial 8	245	315	2.498	0.0336	-1.474	2.389
Hampton		450	2.653			
5/1/17		325	2.512			
	40	35	1.544	0.1040	-0.983	1.602
		55	1.740			
		80	1.903			
	10	10	1.000	0.1580	-0.801	1.000
		10	1.000			
		20	1.301			

	3460	2765	3.442	0.0053	-2.273	3.539
		2940	3.468			
		3000	3.477			
	620	605	2.782	0.0140	-1.853	2.792
		725	2.860			
		650	2.813			
Trial 9						
Dover	185	210	2.322	0.0213	-1.672	2.267
5/8/17		175	2.243			
		215	2.332			
	35	35	1.544	0.0694	-1.158	1.544
		25	1.398			
		40	1.602			
	5	10	1.000	0.3618	-0.442	0.699
		5	0.699			
		3	0.477			

	3840	3490	3.543	0.0085	-2.073	3.584
		3675	3.565			
		4005	3.603			
	1085	825	2.916	0.0148	-1.831	3.035
		710	2.851			
Trial 10		855	2.932			
Hampton						
5/8/17	235	175	2.243	0.0330	-1.482	2.371
		135	2.130			
		185	2.267			
	65	60	1.778	0.0386	-1.413	1.813
		55	1.740			
		75	1.875			
	5	10	1.000	0.1305	-0.884	0.699
		15	1.176			
		20	1.301			

The replicate plate count results were log transformed and the relative standard deviation (RSD) were calculated. The RSD or coefficient of variation was plotted against the spike concentration and appears in Graph 2 below.

Graph 2. Coefficient of Variation verses Spike Concentration for Clean Effluent



To accurately determine the LOD and LOQ graphically, it was necessary to take the Coefficient of Variation and the Spike Determinations and to re-plot these as log values. Graph 3 below show the linear regression of the log transformed replicate and spiking data. Graphically, the LOQ/sensitivity of the method may be found at the point of intersection of the log spike concentration and the log coefficient of variation of –1.0 (or its antilog, 10%). The LOD may be found at the point of intersection of the log spike concentration and the log coefficient of variation of –0.477 (or its antilog of, 33%). Taking the antilog of the spike concentrations at these points of intersection gives the LOQ and LOD, respectively. Graph 3 indicates the LOQ and LOD for clean effluent to be 5.8 PFU/100gm and 0.9 PFU/100ml, respectively. The biostatistics program Prism 5.0 for Mac OS was used linear regression analysis and plots. The statistical summary of the linear regression from the log coefficient of variation verses log spike for the clean effluent data is presented in Table 3.

Graph 3. The LOD and LOQ/Sensitivity for Clean Effluent Samples

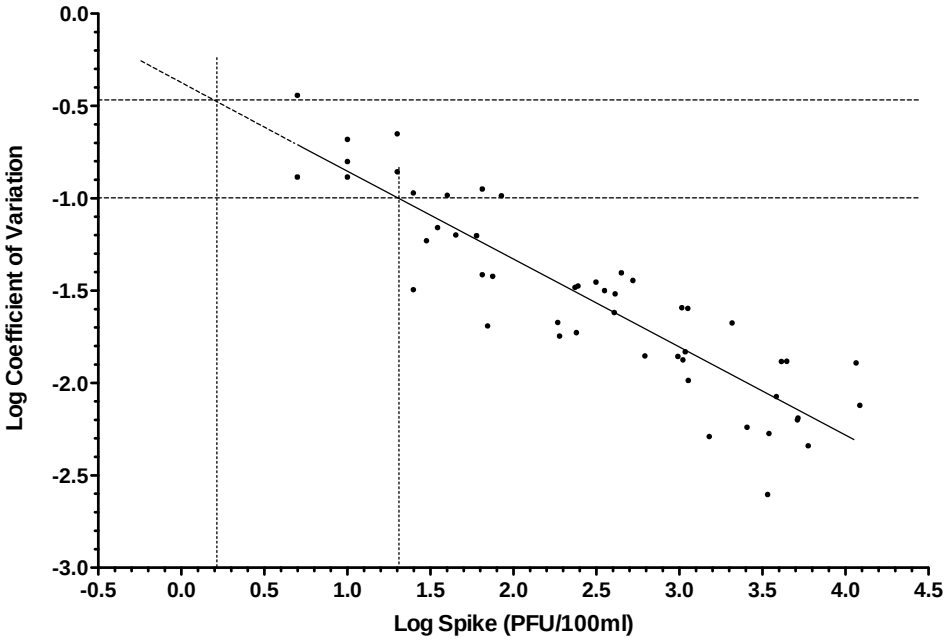


Table 3. Linear Regression Statistics for the Effluent Spiking Trials

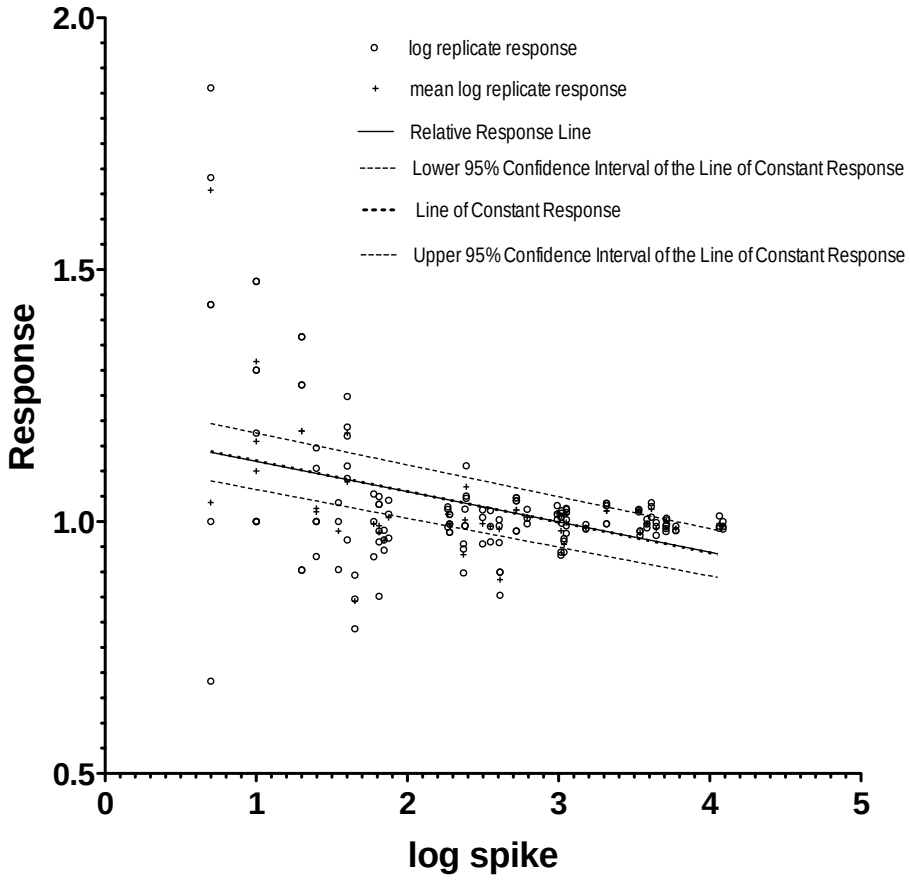
Best-fit values	
Slope	-0.4767 ± 0.03387
Y-intercept when X=0.0	-0.3746 ± 0.08890
X-intercept when Y=0.0	-0.7859
1/slope	-2.098
95% Confidence Intervals	
Slope	-0.5449 to -0.4085
Y-intercept when X=0.0	-0.5536 to -0.1957
X-intercept when Y=0.0	-1.345 to -0.3618
Goodness of Fit	
R square	0.8049
Sy.x	0.2244
Is slope significantly non-zero?	
F	198.1
DFn, DFd	1.000, 48.00
P value	< 0.0001
Deviation from zero?	Significant
Data	
Number of X values	50
Maximum number of Y replicates	1
Total number of values	50
Number of missing values	0

LOQ = Antilog [-2.098 (-1.0 + 0.375)] = 20.42 PFU/100ml
LOD = Antilog [-2.098 (-0.477 + 0.375)] = 1.63 PFU/100ml

The correlation coefficient (R square value) of this linear regression is 0.8049 which is above the threshold level of 0.64 and indicates a good fit. The LOD and LOQ as determined by the spiking trials shows LOD and LOQ of 1.63 PFU/100ml and 20.42 PFU/100ml, respectively. Rounding up, the LOD and LOQ are 2 PFU/100ml and 21 PFU/100ml, respectively.

To determine the **Linear Range**, data from Table 2 was manipulated to construct the relative response line, the line of constant response and the upper and lower 95% confidence interval bracketing the line of constant response as instructed in the SOP. Graph 4 below show that the upper (1.05) and the lower (.95) 95% confidence interval estimates are essentially parallel to the Relative Response line. This suggests that the method is linear through the working range of 5 PFU/100ml to 12,000 PFU/100ml.

Graph 4. Linear Range Determination



Data Summary:

Linear range of the method as implemented 5 to 12,000 PFU/100ml
The limit of detection of the method as implemented 2 PFU/100ml
The limit of quantitation/sensitivity of the method as implemented 21 PFU/100ml
Indeterminates <2 PFU/100ml to >12,000 PFU/100ml

The Determination of Accuracy/Trueness and Measurement Uncertainty is based upon the NSSP SOP for the Single Laboratory Validation of Marine Biotoxin and Non-MPN Based Microbiological Methods using the more robust databases acquired from the determination of the LOQ/LOD/Linear Range. The Accuracy/Trueness is calculated by dividing the log average of the plates by the log average of the spike concentrations, then multiplying the result by 100 to get a percent value. Table 4 shows the results for the Accuracy/Trueness of the method.

Table 4. Calculation of the Accuracy/Trueness of the MSC Method for Wastewater (Low Range Routine).

Average log of plates (2.473 PFU/100ml)/Average log of spike (2.455 PFU/100ml)
= Accuracy/Trueness of 100.7 %.

The Measurement Uncertainty is determined by subtracting the log mean replicate plate values from the reference or log spike values, then calculating the 95% confidence limits of the mean difference. Table 5 show the results of statistical analyis for Method Uncertainty.

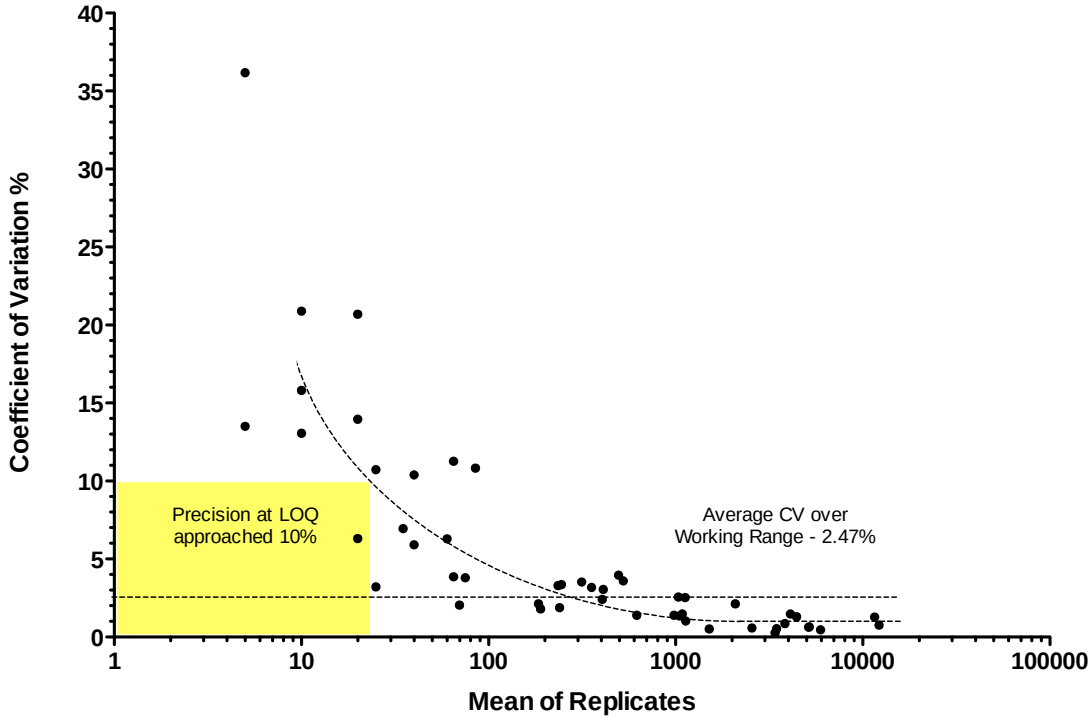
Table 5 – Measurement Uncertainty in wastewater using low range routine.

		Antilog
Number of values	150	
Mean	-0.01787	**0.960**
Std. Deviation	0.1566	
Std. Error	0.01278	
Lower 95% CI of mean	-0.04314	**0.905**
Upper 95% CI of mean	0.00739	**1.017**

Data Summary: Wastewater using Low Range Routine
Calculated % accuracy/trueness 100.7 %
Calculated measurement uncertainty 0.905 to 1.017

The Determination of the Precision and Recovery is based upon the NSSP SOP for the Single Laboratory Validation of Marine Biotoxin and Non-MPN Based Microbiological Methods using the more robust data set acquired from the determination of the LOQ/LOD/Linear Range. To examine the precision over the working range of the method, a simple graphical approach was followed. The coefficients of variation were determined from the log transformed replicate data (50 sets of three true replicates) and were plotted verses the mean of the triplicate results (non log transformed data). The results are shown in Graph 5 for effluent using the low range routine.

Graph 5 - Coefficient of Variability (%) of Replicate verses Mean of Replicate for Effluent Samples using the Low Range Routine of the Method.



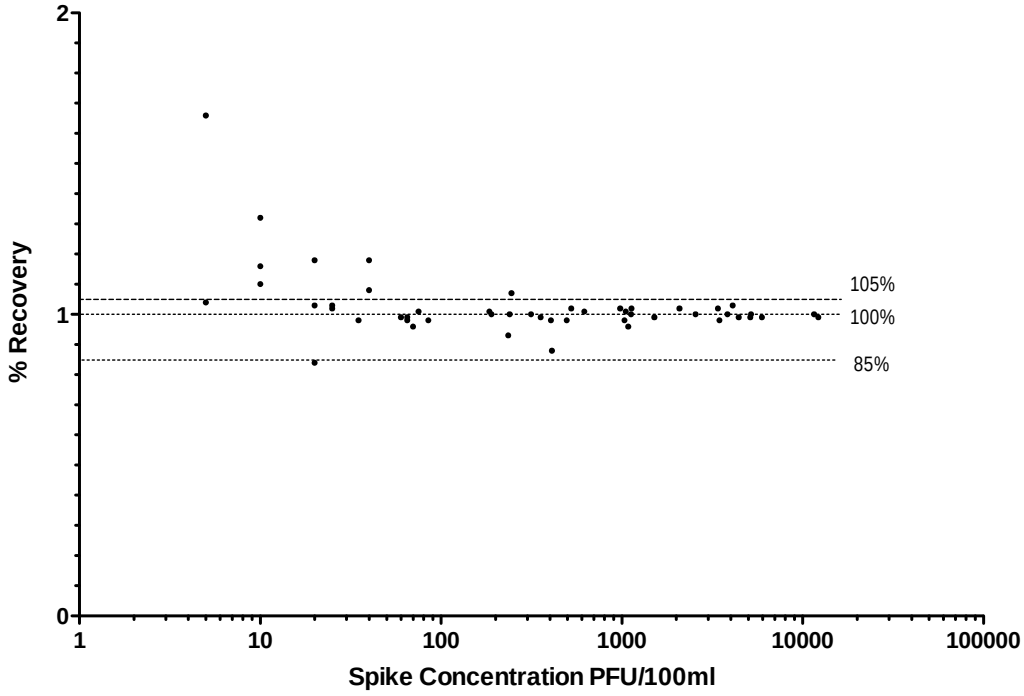
In Graph 4 above, the precision decreases as the LOQ and LOD are approached. The mean, minimum, and maximum coefficient of variations as determined over the working range for effluent samples appear in Table 6 below.

Table 6 – Mean, Minimum, and Maximum Coefficient of Variation over the Working Ranges.

Average Coefficient of Variation = 2.47%
Minimum Coefficient of Variation = 0.25%
Maximum Coefficient of Variation = 36.2%

To examine the **Recovery** over the working range of the method, a simple graphical approach was followed. The data from the LOD/LOQ/Linear Range was used for this determination. The mean of replicates was divided by the spike concentration. The percent recovery was then plotted against the spike concentrations. Graph 6 show these recovery plots with the recoveries bracketed at 85% and 105% for clean wastewater samples using the low range method routine. Recovery by the method is highly variable due to the problems associated with spike determinations. However, recovery for the method over all is high at 98.8%. (see Table 7)

Graph 6 - Percent Recovery verse the Spike Concentration for effluent using the low range method routine



As indicated above, the percent recovery of the method as implemented by this laboratory was calculated by dividing the log average of the replicates by the log spike concentration and multiplying by 100 to get a percent. Table 6 below shows this calculation from effluent trials for the low range method routine

Table 7 – Method Recovery from effluent trials.

Average Log of Spike	Average Log Replicates	% Recovery
2.455	2.473	100.7%

Data Summary:

- Is the precision of the method under study consistent through the working range? **N, It varies as expected as the method approaches the LOD**
- The coefficient of variation of the test method as implemented is **2.5%**
- Is the recovery of the method under study consistent through the working range? **N, It varies as expected as the method approaches the LOD**
- What is the overall percent recovery of the method under study? **100.7%**

Ruggedness was determined using the NSSP SOP for the Single Laboratory Validation of Marine Biotoxin and Non-MPN Based Microbiological Methods.

Two batches of bottom plates, soft agar tubes, and growth browths were prepare using two different lots of granulate agar (Media A and Media B), well in advance of the trials. Ten samples of clean effluent from the Dover and Hampton WTPs were similarly spiked and plated using media A and media B batches. The spike level was varied throughout the experiment. Table 8 show the data, data analysis, and the results of the paired t-test for effluent

Table 8 - Determination of the Method Ruggedness for Effluent

Media A PFU/100gm	Media B PFU/100gm	Log Media A	Log Media B
3000	3575	3.48	3.55
5130	5055	3.71	3.70
5335	5465	3.73	3.74
14055	14980	4.15	4.18
13370	15955	4.13	4.20
12275	12200	4.09	4.09
5545	4845	3.74	3.69
5340	4495	3.73	3.65
210	235	2.32	2.37
190	175	2.28	2.24
	Skew	-1.35	-1.29
	Variance	0.47	0.48
	Ratio of Larger Var to Lower Var	0.01	

skew between -2 and 2 indicates symmetry
Ratio of Varieances < 2 indicates homogeneity of variance

Paired t-test (Media A verses Media B)

P value	0.0.7648
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.3084 df=9
Number of pairs	10

Data Summary:

Value for the test of symmetry of the distribution of Media A data -1.35
Value for the test of symmetry of the distribution of Media B data -1.29
Variance of Media A data .0.47
Variance of Media B data .0.48
Ratio of the larger to the smaller of the variances of Media A and Media B 0.01
Is there a significant difference between Media A and Media N