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 Soft-shelled Clams (M. arenaria) and American Oysters (C. virginica)
Name of the Method Developer	Thomas Howell, Spinney Creek Shellfish, Inc.
Developer Contact Information	Spinney Creek Shellfish, Inc. P.O. Box 310 Eliot, ME 03903 (207) 439-2719 tlhowell@spinneycreek.com

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 Soft-shelled Clams and American Oysters

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	Y	Matrix effects on the method were exhibited by soft-shelled clams. These, however, were eliminated by eluting with a 1:2(w/v) mixture of shellfish and growth broth and extending the blender time to 180 seconds.
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 Interstate Shellfish Sanitation Conference (ISSC)

For Method Approval

Section A. Justification for New Method

Name of the New Method -	Male-specific Coliphage (MSC) for Soft-shelled Clam and American Oyster Meats.
Specify the Type of Method -	Culture Method/Double Agar Overlay Method
Name of Method Developer -	Thomas Howell, Spinney Creek Shellfish, Inc.
Developer Contact Information –	Spinney Creek Shellfish, Inc. 27 Howell Lane P.O. Box 310 Eliot, Maine 03903 (207) 439-2719 (207) 439-7643 FAX tlhowell@spinneycreek.com
Date of Submission –	September 25, 2009

Purpose and Intended Use of the Method.

The primary purpose and intended use of this method in the NSSP is in conjunction with proposal 05-105; Re-Opening Criteria Based on New Indicator of Sewage-Borne Viral Pathogens. The modified method presented in this document addresses several problems and difficulties with the original method as provided in proposal 05-114 and its associated checklist, proposal 05-113. An additional use for this method of interest to the submitter is that it provides the potential to validate and optimize viral depuration/relay strategies used to reduce viral contamination in soft-shelled clams and American oysters harvested from growing areas impacted by wastewater treatment plant (WTP) outfall.

Need for the New Method in the NSSP, Noting Any Relationships to Existing Methods.

The need for an alternative viral indicator with respect to the NSSP is addressed in detail in the Public Health Significance text of proposal 05-105. (Please see Proposal 05-105).

Additionally, fecal coliforms (FC), a bacterial indicator, are used for process validation for conventional depuration processes. In growing areas impacted by moderate or low-level non-point source contamination, conventional depuration methods using FC for process validation are adequate, well proven, and widely accepted by the scientific and public health community.

Statistical analysis of FC samples, collected during water quality monitoring, are used to determine growing area classification. Limits on the geometric mean and 90th percentile are considered adequate to protect public health from the risks of viral contamination in areas not impacted by sewage and WTP pollution. However, in growing areas impacted by treated sewage, the relationship between bacterial and viral contamination can be substantially altered by the differential inactivation rates of chlorination and other disinfection methods on bacteria and viruses. This MSC method is needed in the NSSP to evaluate viral contamination in molluscan shellfish harvested from growing areas where FC levels in both water quality and shellfish meats may be misleading. MSC is a specialized indicator of viral contamination, which is substantially more meaningful than FC in evaluating the safety of shellstock harvested from growing areas potentially impacted by treated and partially treated wastewater. The MSC method is particularly useful and highly advantageous over FC for evaluating the efficacy of viral depuration and viral relay processes using soft-shelled clams and American oysters.

Method Limitations and Potential Indications of Cases Where the Method May Not Be Applicable to Specific Matrix Types.

The MSC method described here is limited to soft-shelled clams and American Oysters. Further SLV work is needed to evaluate different matrix types / other species of molluscan shellfish.

Other Comments.

This SLV work addresses the intended use of the applicant as well as related ISSC proposals 05-105, 05-114 and 05-113. The ISSC is concurrently dealing with ISSC proposal 05-105, 05-114 and 05-113. ISSC proposal 05-105 proposes using MSC to facilitate reopening of growing areas after a sewage spill. ISSC proposal 05-114 proposes the methodology for use of MSC and is one of several methods being considered by the LMR committee. ISSC proposal 05-113 proposes a laboratory checklist for the method. In this work, stemming from those 2005 ISSC proposal, problems were encountered with the viral extraction process. These have been resolved and SLV validation on the modified MSC method has been completed. **This validation work strongly suggests that this modified MSC method is appropriate (fit for purpose) for applications in Soft-shelled calms and American oysters where a regulatory limit of 50 PFU/100gram (proposal 05-105) is being recommended.**

Section B. Method Documentation

Modified Double Agar Overlay Method
for Determining Male-specific Coliphage
In Soft Shelled Clams and American Oysters
March 2009 revision

This method for determining levels of male-specific coliphage in soft shelled clam and American oyster meat is based on the method described by DeBartolomeis and Cabelli[1,2]. FDA has 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[3]. This original FDA (2004) method was submitted as ISSC Proposal 05-114.

Modification of the FDA (2004) Method

Spinney Creek Shellfish, Inc. (SCS) further refined these procedures for soft-shelled clam and oyster meat in work funded by the Maine Technology Institute in 2006. In this work and in parallel work conducted by Mercuria Cumbo of the Maine Department of Marine Resources, it was observed that the extraction protocol was inadequate. The supernatant produced when soft-shelled clams and some oysters were processed was opaque and creamy while the pellet was loose and indistinct. Subsequent re-washing of the pellets in growth broth, re-processing, and re-plating showed significant levels of MSC left in the pellet, indicating poor recovery. The problem was solved by; eluting the shellfish meats with growth broth (2:1), and increasing the blending time to 180 seconds. This modification, based on EU methodology (ISO 10705-4), resulted in a clear supernatant, a distinct, firm pellet. Further experimentation and subsequent validation work confirmed that this elution approach works very well. SLV validation work conducted by (SCS) in 2009 resulted in further modification of the method to increase the limit of quantitation/sensitivity (LOQ). This increase in LOQ was achieved by plating an increased amount of supernatant (25ml) and using 10 plates.

A. Apparatus and Materials.

Equipment and Materials for Collection and Transport of Shellfish Samples:
4 mil plastic bags
Labels
Cooler
Gel Packs
Temperature Control Blank

Laboratory Equipment:
Centrifuge with rotor for 50 ml conical (or larger) tubes, 9000 x *g* performance capability, 4°C
Water bath, 50-52°C
Air Incubator, 35-37°C
Balance
Stir plate and magnetic stirring bars, sterile
Mini vortexer
Blender

Autoclave, 121°C
Refrigerator, 0–4° C
Freezer, -20°C
Thermometers, range -20–121°C
pH meter
Erlenmeyer flasks, 1 L and 2 L
Graduated cylinders, 100 ml, 500 ml and 1000 ml
600 ml beaker
500 ml jars, autoclavable with caps
Inoculating loops (3 mm in diameter or 10 FL volume)
Bacti-cinerator
Sterile swabs
Sterile, disposable filters, 0.22 or 0.45 μm pore size
Syringes, sterile disposable; 5, 10 or 20 ml
Scrub brushes, sterile
Knives, sterile
Blender jars, sterile
Sterile plastic cups 250 ml
Pipets- 5 ml, 10 ml
Pipet-aid
Micro-Pipettors, 100 μL, 200 μL, 1000 μL, 2500 μL
Micro-Pipet tips 200 μL, 1000 μL, 2500 μL
Pipetor Stand
Centrifuge tubes, sterile disposable 50 ml or larger
Petri dishes, sterile disposable 100 x 15 mm
Petri dish racks
Test tubes 16 x 100 mm (for soft agar)
Test tubes 16 x 150 mm, with screw caps
Test tube racks--size to accommodate tubes
Freezer vials, sterile 30 ml with screw caps
Baskets with tops to hold freezer vials
Parafilm tape
Aluminum foil

Reagents:
Reagent water
Glycerol- sterile
Ethanol, 70% or laboratory disinfectant
Calcium chloride, 1M
Mineral oil

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:
Bottom Agar
DS Soft Agar
Growth Broth

Bacterial Host Strain:
E.coli F_{amp} . *E. coli* HS(pFamp)RR (selected by Dr. Victor J. Cabelli, University of Rhode Island, Kingston, RI, USA, frozen stock 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 to 990 ml of dH_2O in a 2000 ml flask. Dissolve, heat until clear.
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. Pipet (or pour) 15 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 through 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) for up to three months.
4. Sterilize prior to use at 121°C ± 2°C for 15 minutes, then temper to 50-52°C for no longer than 2 hours

1M $CaCl_2$ Solution:

1. Add 11.1 g of $CaCl_2$ anhydrous (FW 111.0, Dihydrate FW 147) to 100 ml
2. dH_2O in a screw top bottle and dissolve or use prepared from VWR.
3. Sterilize by autoclaving at 121°C for 15 minutes.
4. Store up to three months at 4°C.
5. Use at room temperature.

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.

Storage Slants: Tryptic Soy Agar.

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.
5. Short-term working stock culture – Grown in Growth broth and used within 6-12 hours (same day).

Glycerol Solution, 10%: Add 9 ml of distilled water to 1 ml of undiluted glycerol. Autoclave resulting 10% glycerol solution at 121°C for 15 minutes and use at room temperature. 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.

Propagation:
1. Vortex to aerate 10 ml of Growth Broth medium tempered to 35 – 37°C just prior to inoculation.
2. Transfer host strain to Growth Broth using sterile swab to collect material from several colonies off grown Bottom Agar streak plate and warmed to room temperature.
3. Gently shake to mix, then incubate at 35–37°C for 4-6 hours (turbidity=10^7cells/ml; O.D @ 540nm=0.4).
4. Once turbidity is observed, use of the host strain broth culture (log-phased growth) may commence

(following initial inoculation and mixing, do not shake or mix the host strain broth culture).

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 Soft Shelled Clam and American Oyster Tissues.

Sample Requirements. Samples of shellstock and shucked meats are held under dry refrigerated conditions at 1–4°C. Samples must be comprised of a representative number of animals (12 to 15). Samples are analyzed within 24 hours of collection. Animals with broken shells or animals that appear dead are discarded. Sample collection bags must be properly identified with lot #, date and time of collection, collection location and collector's initials.

Preparation of Shellfish for Analysis. Using soap and water, analyst's hands are thoroughly scrubbed and rinsed. Using a sterile brush, shells of whole animals are scrubbed under running potable water to remove loose material from the shells. Shellfish then are placed on a clean paper towel or in an open weave basket to dry. Scrubbed, drying animals should not come in contact with each other. Once the shells of washed shellfish are dry, analysts wash their hands thoroughly with soap and water, then rinse their hands with 70% alcohol and allow to air dry. Shellfish are shucked and the meats and liquors are saved into a sterile 250 ml cups.

Direct Analytical Technique for Soft Shelled Clams and American Oysters. For each soft shelled clam or American oyster sample ten (10) Bottom Agar plates and ten (10) 2.5 ml DS Soft Agar tubes are prepared. Use a 4 to 6 h culture of host strain, *E. coli* F_{amp}. Always begin analyses with a negative control (blank) plate and finish analyses with a positive control plate followed by a second negative control plate.

1. Shuck 12 soft shelled clams or American oysters into sterile 250 ml cup, tare and add to sterile blender. To make a 1:2 (wgt:vol) elution with growth broth eluent using twice the volume of the shellfish. Add to blender with sample. Homogenize by blending for 180 seconds at high speed.
2. Immediately weigh 33.0 g of homogenate from each sample into labeled sterile 50 ml centrifuge tubes after blender has stopped before foam separation can occur.
3. Centrifuge each sample for 15 min. @ 9,000-10,000 x g; 4°C.
4. Pipette off and weigh the supernatant in a new sterile 50 ml centrifuge tube.
5. Allow the supernatant to warm to RT (approximately 20-30 minutes).
6. Shake or vortex the supernatant.
7. Gently pipette 200 µL of log phase host strain, *E. coli* HS(pFamp)RR using 200 µL micro pipettor and a 200 µL pipet tip, then pipette 2500 µL aliquot of supernatant using the 2500 µL micro pipettor and a 2500 µL pipet tips, to 2.5 ml DS Soft agar tube (tempered to 52°C).
8. **Once E. coli F_{amp} is added to the mixture do not shake, only gently mix contents by rolling the tube between palms.**
9. Overlay the 5.2 ml onto a Bottom Agar plate containing Streptomycin and Ampicillin (50 g/ml final concentrations). Drag the mixture into a clear area and gently swirl the plates to spread sample and agar mixture.
10. Allow plates to set then inverted and incubated for 16 - 20 hours at 35- 37°C.

Calculations of Results

$$\frac{\text{Total number of MSC (N)}}{\text{Total supernatant plated (25gm)}} \times \frac{\text{Weight of supernatant extracted (Ws)}}{\text{grams of sample used (11gm)}} \times 100 =$$

$$\frac{N}{25\text{ gm}} \times \frac{Ws}{11\text{ gm}} \times 100 = (0.364)(N)(Ws) = \text{PFU of MSC/100 gm}$$

Example: Clam/Oyster plate counts - 13, 23, 12, 16, 12, 18, 17, 21, 19, 17 and 27.5 g supernatant.

Result = (0.364)(168MSC)(27.5gm) = 1681 PFU of MSC/100 gm*
**0.364=100/(25 x 11)*

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 shellfish samples is up to 24 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 100 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 >10,000 PFU of MSC/100gm.
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 micro-pipettors needs to be checked quarterly and records kept. Micro-pipettors 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 Coliphage 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
rpm - revolutions per minute
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
LOD - Limit of Detection
LOQ - Limit of Quantitation
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:

1. Cabelli, V.J. 1988. Microbial indicator levels in shellfish, water, and sediments from the upper Narragansett Bay conditional shellfish-growing area. Report to the Narragansett Bay Project, Providence, RI.
2. DeBartolomeis, J. and V.J. Cabelli. 1991. Evaluation of an *Escherichia coli* host strain for enumeration of F male-specific Coliphages. Appl. Environ. Microbiol. 57(4):1201-1205.
3. 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 soft-shelled clams and American oysters is inexpensive, easy to perform, and rapid, providing results within 24 hours. The cost of laboratory glassware, plastic-ware, agars, and reagents is approximately $25 per shellfish 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. The most expensive piece of equipment is a refrigerated centrifuge plus rotor, which costs approximately $10,000. There are no special skill sets required beyond those required to operate a state-approved shellfish laboratory under the NSSP.

C. Validation Criteria

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 by far the most involved and yields a database from which subsets of data can be use to generate other validation criteria. For this primary database ten trials were run for each soft-shelled clam and American oyster sample. Great effort was taken to use different shellstock from a variety of growing areas over a period of time and to find shellstock that had no detectable levels of MSC (<10PFU/100gm). Several trial batches of shellstock were held in depuration for several days to weeks prior to the validation trials to ensure no detectable levels of MSC. Table 1 below shows the trial #, growing area, harvest date, and date of analysis for shellstock used during these validation trials.

Table 1

Trial #	Growing Area	Harvest Date	Date of Analysis
Soft-shelled Clams			
1	Bremen	2/23/09	3/23/09
2	Bremen	2/23/09	3/26/09
3	Bremen	2/23/09	3/31/09
4	Broad Cove	3/22/09	4/2/09
5	John's Bay	3/22/09	4/7/09
6	Gaunt Neck	3/22/09	4/9/09
7	St. George River	4/6/09	4/14/09
8	Damariscotta River	4/27/09	5/1/09
9	Royal River	7/31/09	8/5/09
10	Cousins River	8/6/08	8/18/09
American Oysters			
1	CT-371	4/8/09	5/6/09
2	Sheepscot River	5/11/09	5/13/09
3	Sheepscot River	5/14/09	5/19/09
4	Sheepscot River	5/14/09	5/27/09
5	Westport Island	5/18/09	6/4/09
6	Piscataqua River	6/11/09	6/17/09
7	Piscataqua River	6/11/09	7/8/09
8	Spinney Creek	6/27/09	7/15/09
9	Stacy's Creek	7/15/09	7/22/09
10	Piscataqua River	7/22/09	7/29/09

For each of the 20 validation trials, 12-15 shellfish were homogenized in a 2:1 eluate of growth broth to shellfish meat in accordance with the method described above. The homogenate was evenly distributed to 5 sterile beakers with stir-plus bars, tared and weighed. 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 beakers were spiked with spike concentration 1 through 5 and three aliquots of 33 grams each were taken from each of the 5 beakers which were actively stirred to prevent separation. In this way, 3 true replicates were generated at each of the 5 spike concentrations. This methodology was consistently applied throughout the 2 sets of ten trials. The 5 sets of 3 aliquots were processed and plated according to the method description above.

Likewise, each spike concentration was determined. Clean supernatant was used instead of growth broth for the spike determination as a matrix effect was noticed during the initial trials. Initially, the spike determinations were underestimating the sample results. This problem was identified as clumping of the viral particles in the growth broth and was cured by using clean shellfish supernatant instead of growth broth and centrifuging them in a manner similar to the shellfish samples prior to plating. Table 2A and 2B below show the spike concentrations and MSC replicate plate count results in units of PFU of MSC/100gm. 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 1A and 1B for soft-shelled clams and American oysters, respectively.

Table 2A – Tabulated Results of the **Soft Shelled Clam** Validation Trials

Trial #	Spike Concentration (PFU/100gm)	MSC Replicate Plate Concentration (PFU/100gm)	Log of Replicate MSC Plates	RSD
1	9161	6568	3.817	0.0063
		6658	3.823	
		7274	3.862	
	1836	1554	3.191	0.0126
		1496	3.175	
		1785	3.252	
	498	385	2.585	0.0163
		324	2.511	
		380	2.580	
	155	68	1.833	0.0690
		98	1.991	
		127	2.104	
	48	20	1.301	0.1871
		10	1.000	
		29	1.462	
2	6802	5337	3.727	0.0009
		5316	3.726	
		5396	3.732	
	1902	1723	3.236	0.0072
		1918	3.283	
		1832	3.263	
	541	483	2.684	0.0096

		543	2.735	
		523	2.719	
	176	118	2.072	0.0358
		138	2.140	
		168	2.225	
	38	39	1.591	0.1198
		20	1.301	
		20	1.301	

3	12322	10579	4.024	0.0016
		10750	4.031	
		10438	4.019	
	3268	2642	3.422	0.0143
		2571	3.410	
		3166	3.501	
	828	828	2.918	0.0178
		708	2.850	
		658	2.818	
	211	150	2.176	0.0519
		259	2.413	
		209	2.320	
	94	70	1.845	0.1328
		30	1.477	
		30	1.477	

4	10392	6526	3.815	0.0070
		6370	3.804	
		7158	3.855	
	3106	2189	3.340	0.0102
		2106	3.323	
		1884	3.275	
	871	589	2.770	0.0217
		517	2.713	
		682	2.834	
	143	165	2.217	0.0282
		134	2.127	
		176	2.246	
	36	62	1.792	0.0396
		72	1.857	
		52	1.716	

5	7775	5737	3.759	0.0040
		5375	3.730	
		5666	3.753	
	1978	1129	3.053	0.0175
		1245	3.095	
		1447	3.160	

	535	410	2.613	0.0129
		470	2.672	
		470	2.672	
	192	109	2.037	0.0107
		100	2.000	
		100	2.000	
	111	60	1.778	0.1036
		30	1.477	
		60	1.778	

6	5625	3309	3.520	0.0101
		3289	3.517	
		2864	3.457	
	1455	708	2.850	0.0175
		640	2.806	
		805	2.906	
	334	210	2.322	0.0610
		181	2.258	
		342	2.534	
	103	80	1.903	0.0642
		80	1.903	
		50	1.699	
	30	40	1.602	0.1036
		30	1.477	
		20	1.301	

7	5012	3654	3.563	0.0089
		3468	3.540	
		3167	3.501	
	1382	1052	3.022	0.0100
		1073	3.031	
		944	2.975	
	450	230	2.362	0.0270
		311	2.493	
		271	2.433	
	72	80	1.903	0.1273
		30	1.477	
		60	1.778	
	41	10	1.000	0.1916
		20	1.301	
		30	1.477	

-

8	13875	13864	4.142	0.0088
		12483	4.096	
		11767	4.071	
	3713	3238	3.510	0.0052

		3511	3.545	
		3451	3.538	
	1009	778	2.891	0.0070
		821	2.914	
		748	2.874	
	237	160	2.204	0.1428
		290	2.462	
		70	1.845	
	97	109	2.037	0.0713
		60	1.778	
		70	1.845	

9	9409	6571	3.818	0.0110
		6404	3.806	
		7668	3.885	
	2513	1904	3.280	0.0035
		1988	3.298	
		1999	3.301	
	879	689	2.838	0.0224
		545	2.736	
		710	2.851	
	135	115	2.061	0.0364
		104	2.017	
		146	2.164	
	33	31	1.491	0.0923
		42	1.623	
		62	1.792	

10	7069	6667	3.824	0.0059
		6990	3.844	
		7406	3.870	
	2130	2240	3.350	0.0075
		2048	3.311	
		2013	3.304	
	427	478	2.679	0.0109
		454	2.657	
		519	2.715	
	147	74	1.869	0.0604
		128	2.107	
		106	2.025	
	19	21	1.322	0.1811
		32	1.505	
		11	1.041	

Table 2B - Tabulated Results of the **American Oyster** Validation Trials

Trial #	Spike Concentration (PFU/100gm)	MSC Replicate Plate Concentration (PFU/100gm)	Log of Replicate MSC Plates	RSD
1	15104	8338	3.921	0.0059
		9249	3.966	
		8610	3.935	
	4205	2221	3.347	0.0070
		2343	3.370	
		2476	3.394	
	1030	677	2.831	0.0153
		575	2.760	
		688	2.838	
	258	165	2.217	0.0419
		144	2.158	
		110	2.041	
	77	33	1.519	0.2026
		11	1.041	
		33	1.519	
2	14932	11590	4.064	0.0062
		11875	4.075	
		12952	4.112	
	4538	3498	3.544	0.0064
		3269	3.514	
		3623	3.559	
	901	926	2.967	0.0269
		713	2.853	
		1012	3.005	
	204	239	2.378	0.0214
		196	2.292	
		196	2.292	
	99	46	1.663	0.1275
		137	2.137	
		69	1.839	
3	9763	10288	4.012	0.0023
		10352	4.015	
		9955	3.998	
	2539	2766	3.442	0.0037
		2608	3.416	
		2674	3.427	
	683	513	2.710	0.0428
		886	2.947	
		621	2.793	
	136	251	2.400	0.1246
		91	1.959	
		310	2.491	

	25	46	1.663	0.0704
		34	1.531	
		58	1.763	

4	9015	7041	3.848	0.0023
		7280	3.862	
		7315	3.864	
	2281	2252	3.353	0.0046
		2270	3.356	
		2404	3.381	
	505	570	2.756	0.0068
		589	2.770	
		541	2.733	
	160	147	2.167	0.0313
		195	2.290	
		149	2.173	
	12	46	1.663	0.2604
		69	1.839	
		12	1.079	

5	13578	9068	3.958	0.0026
		9317	3.969	
		8881	3.948	
	3860	2317	3.365	0.0074
		2587	3.413	
		2524	3.402	
	924	790	2.898	0.0168
		677	2.831	
		638	2.805	
	227	146	2.164	0.0580
		170	2.230	
		262	2.418	
	34	33	1.519	0.0721
		22	1.342	
		34	1.531	

6	11610	11071	4.044	0.0039
		11790	4.072	
		11799	4.072	
	2906	2919	3.465	0.0062
		2933	3.467	
		3189	3.504	
	826	736	2.867	0.0079
		798	2.902	
		812	2.910	
	237	17	1.230	0.0507
		15	1.176	
		20	1.301	

	48	22	1.342	0.0737
		34	1.531	
		23	1.362	

7	5012	3654	3.563	0.0089
		3468	3.540	
		3167	3.501	
	1382	1052	3.022	0.0100
		1073	3.031	
		944	2.975	
	450	230	2.362	0.0270
		311	2.493	
		271	2.433	
	72	80	1.903	0.1273
		30	1.477	
		60	1.778	
	41	10	1.000	0.1916
		20	1.301	
		30	1.477	

-

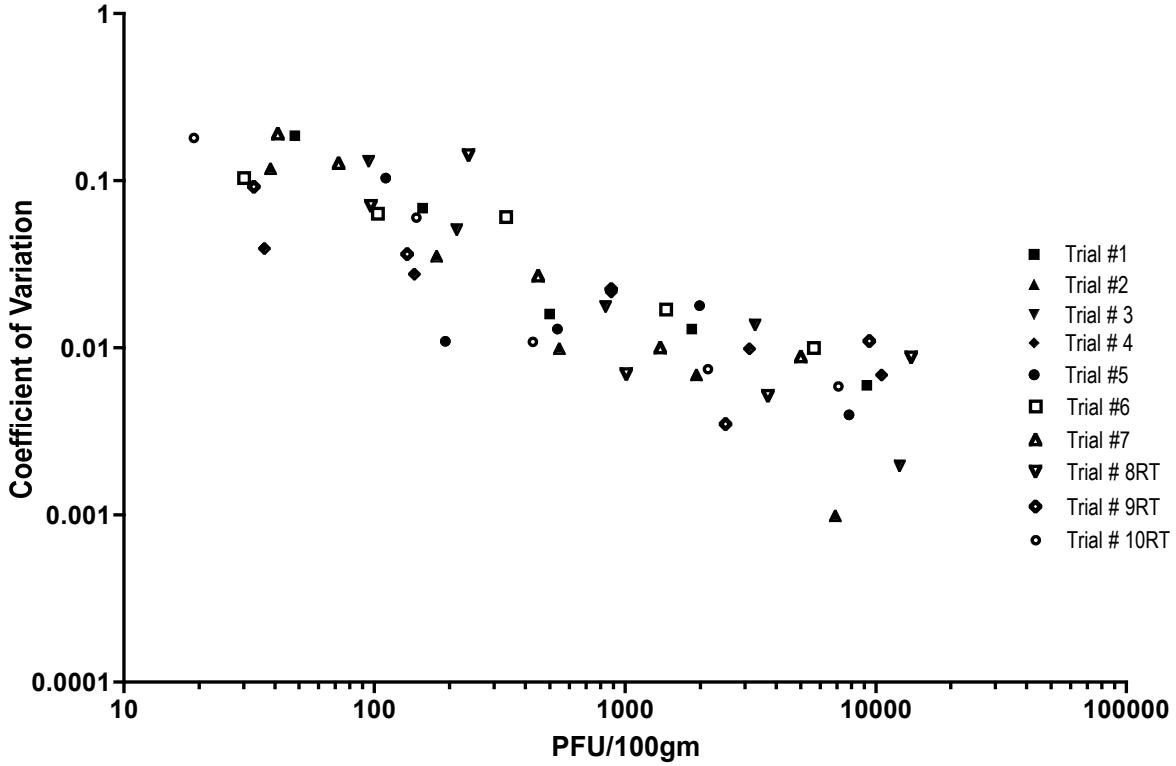
8	15524	12901	4.111	0.0031
		13110	4.118	
		13647	4.135	
	3719	3408	3.532	0.0078
		3030	3.481	
		3341	3.524	
	899	529	2.723	0.0273
		749	2.874	
		603	2.780	
	191	123	2.090	0.0202
		147	2.167	
		125	2.097	
	52	90	1.954	0.3080
		11	1.041	
		68	1.833	

9	12725	8598	3.934	0.0014
		8643	3.937	
		8431	3.926	
	3214	1848	3.267	0.0139
		2283	3.359	
		2101	3.322	
	751	539	2.732	0.0065
		555	2.744	
		512	2.709	
	136	198	2.297	0.0869
		177	2.248	
		89	1.949	
	32	22	1.342	0.0726

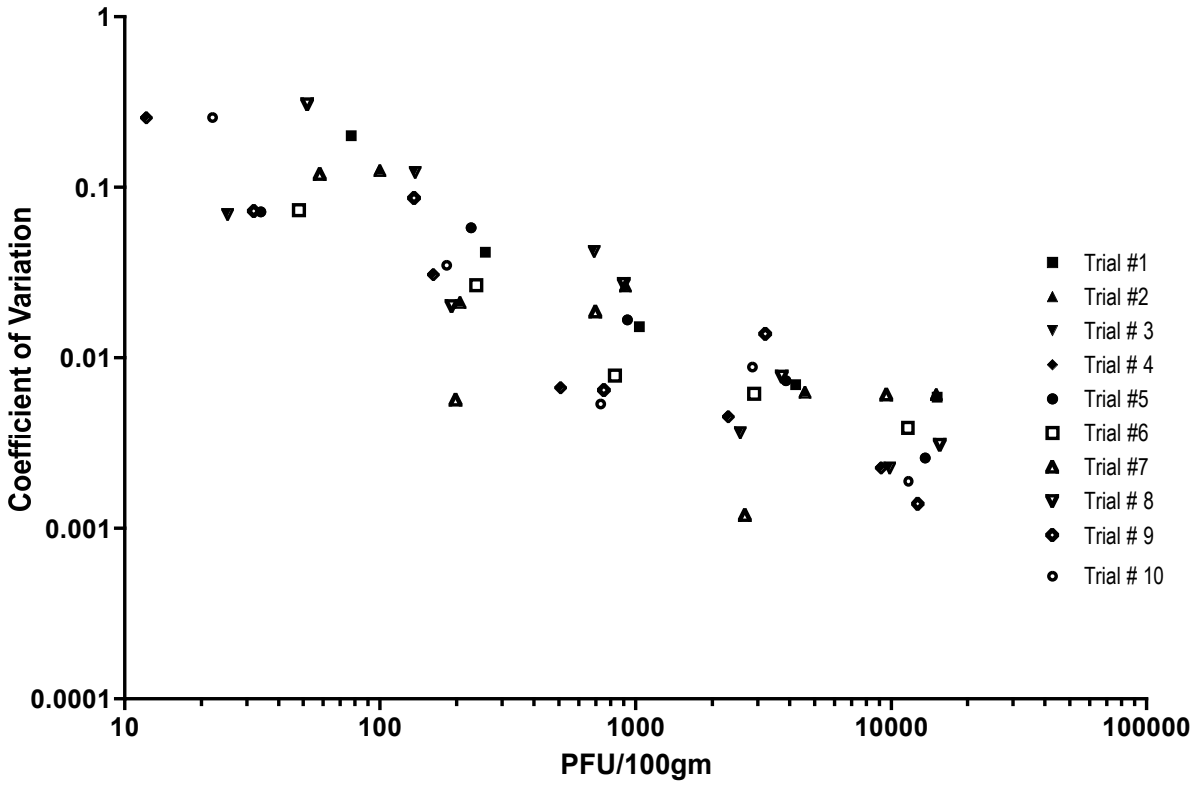
		33	1.519	
		22	1.342	

10	11636	8937	3.951	0.0019
		8841	3.947	
		9150	3.961	
	2848	2042	3.310	0.0089
		2340	3.369	
		2181	3.339	
	730	679	2.832	0.0054
		636	2.803	
		672	2.827	
	182	114	2.057	0.0350
		158	2.199	
		148	2.170	
	22	56	1.748	0.2590
		45	1.653	
		11	1.041	

Graph 1A - Coefficient of Variation verse Spike Concentration for **Soft-Shelled Clams**

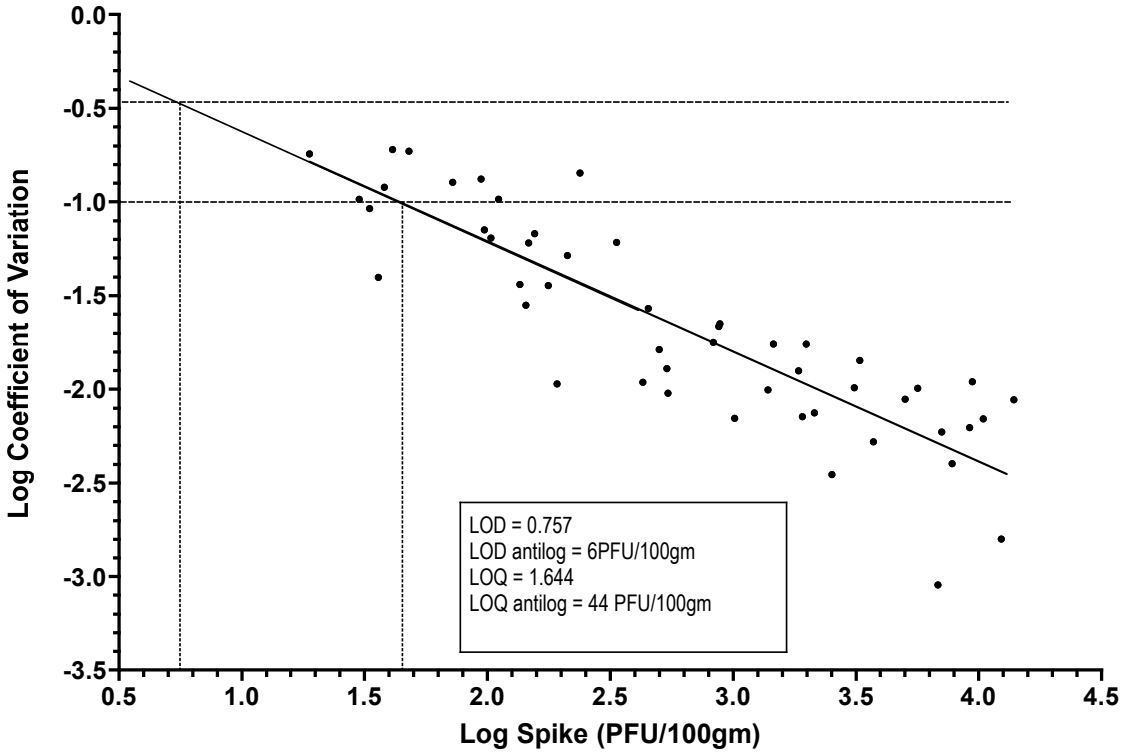


Graph 1B - Coefficient of Variation verse Spike Concentration for **American Oysters**

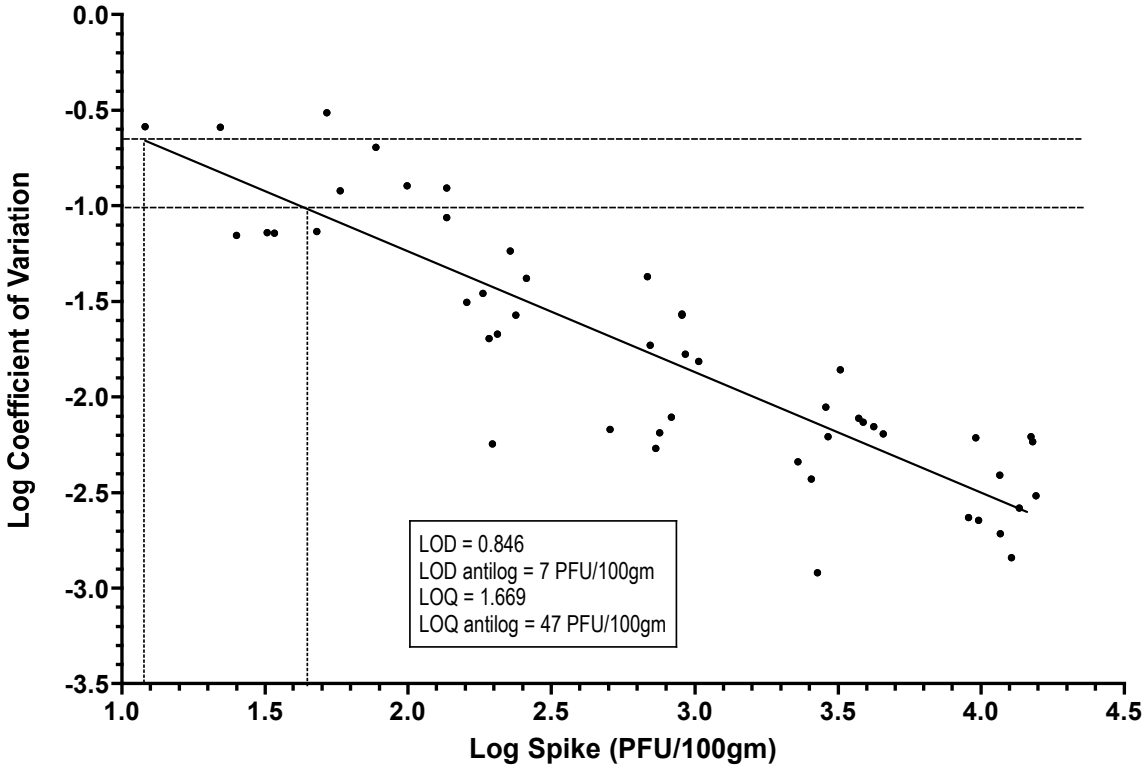


To precisely determine the LOD and LOQ graphically, it was necessary to take the Coefficient of Variation and the Spike Determinations and to re-plot these as log values. Graphs 2A and 2B below show the log/log plots of the soft-shelled clam and American oyster data. Graphically, the LOQ/sensitivity 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 2A indicates the LOQ and LOD for the soft-shelled clams to be 44 PFU/100gm and 6 PFU/100gm, respectively. Graph 2B indicates the LOQ and LOD for American oysters to be 47 PFU/100gm and 7 PFU/100gm, respectively. These values are consistent with the proposed action level of 50 PFU/100gm proposed for MSC.

Graph 2A - The LOQ/sensitivity and LOD of **Soft-shelled Clams**.

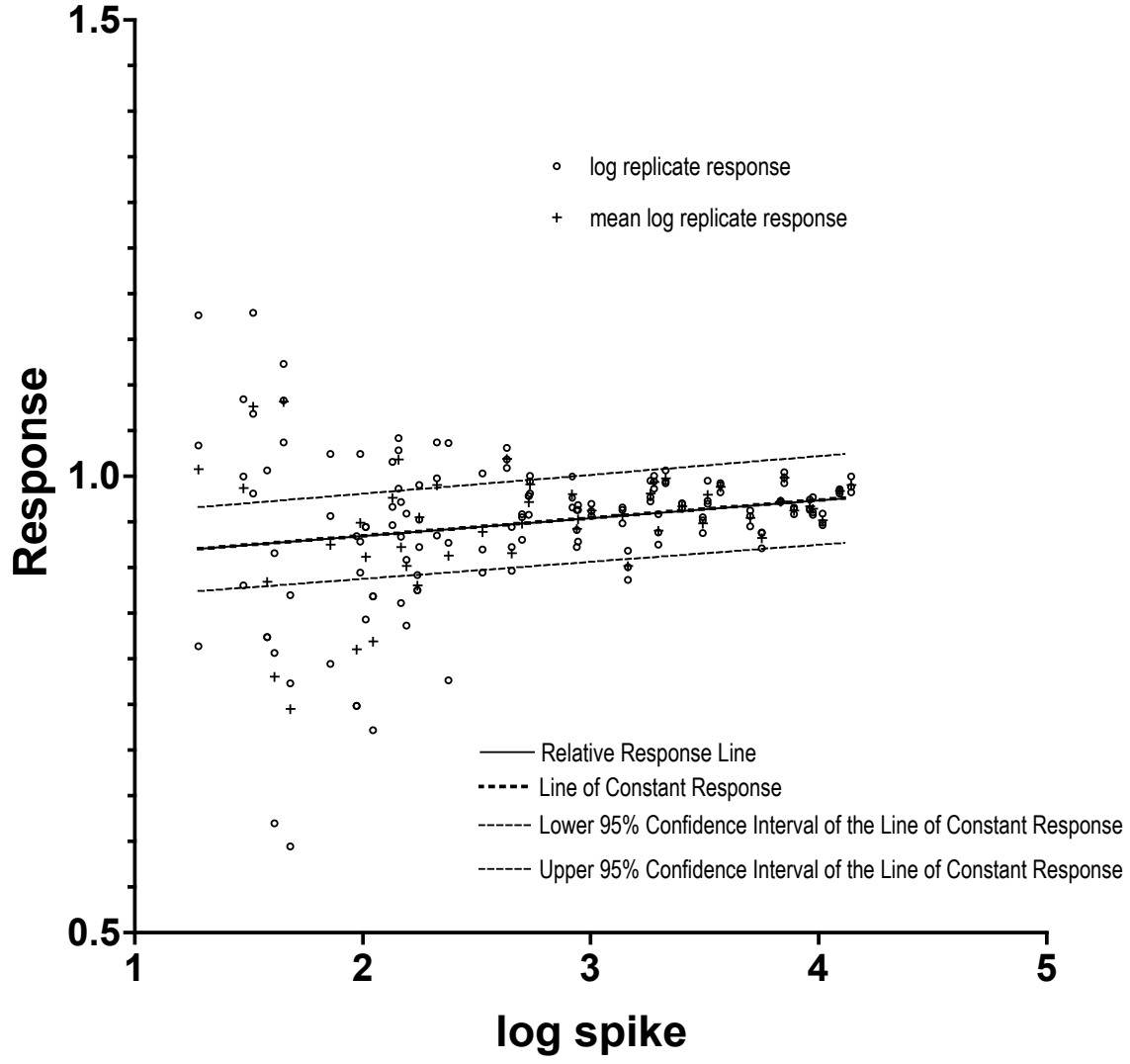


Graph 2B – The LOQ/Sensitivity and LOD of **American Oysters**.

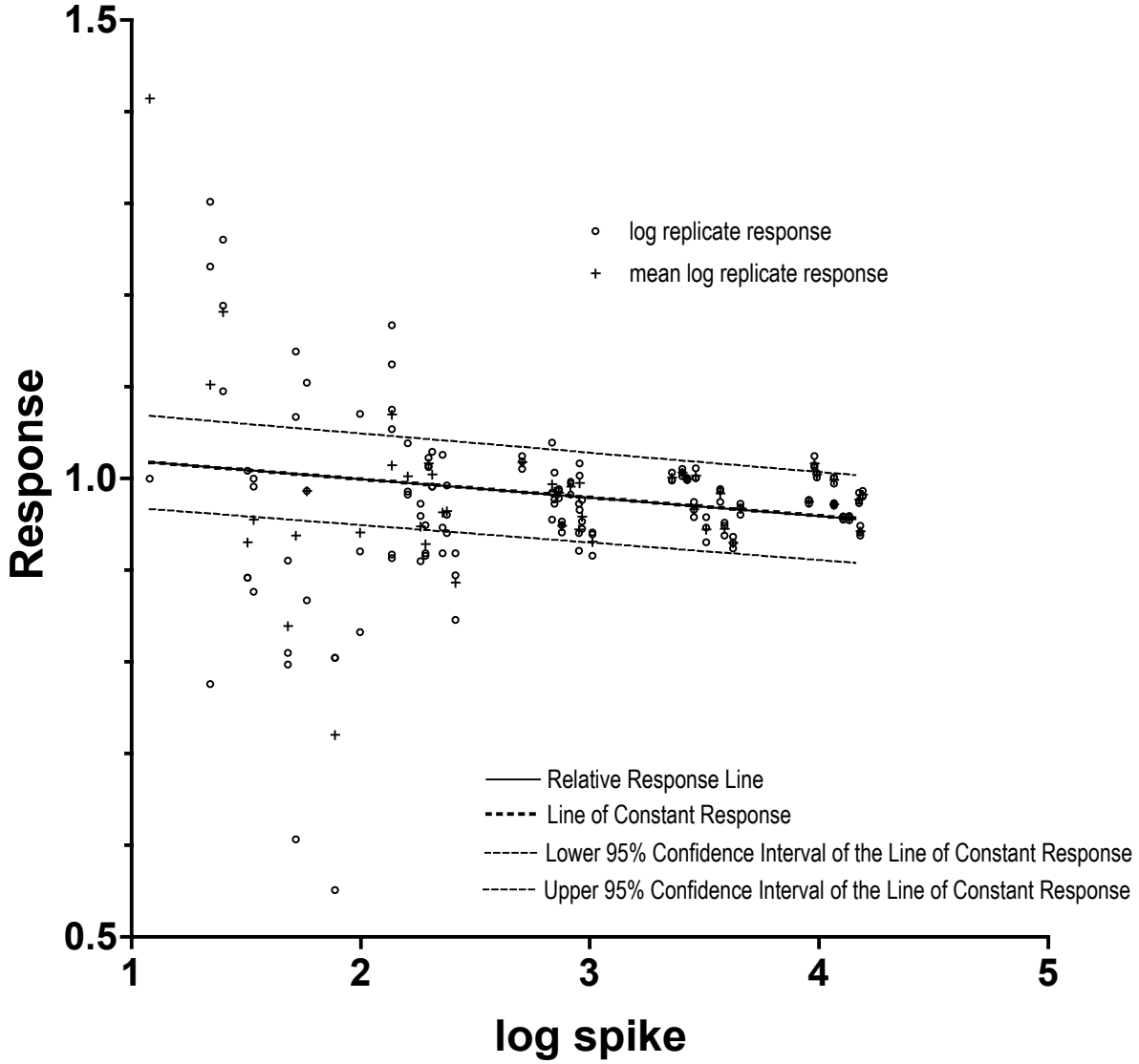


To determine the Linear Range, data from Table 2A and 2B was manipulated to construct the relative response line, the line of constant response and the upper and lower 95% confidence interval bracketing the line of constant response as instructed in the SOP. Graph 3A and 3B below both show that the upper (1.05) and the lower (.95) 95% confidence interval estimate are essentially parallel to the Relative Response line for both soft-shelled clams and American oysters. This suggests that the method is linear from the LOD (6 PFU/100gm for soft-shelled clams and 7 PFU/100gm for American oysters) through the working range of 10,000 PFU/100gm. The upper working range is estimated to be approximately 100 PFU per plate or 10,000 PFU/100gm. Beyond 100 colonies per plate, the plaques get difficult to count and differentiate. In summary, the method is linear within the working range of 6 PFU/100gm to 10,000PFU/100gm for soft-shelled clams (Graph 3A) and 7 PFU/100gm to 10,000PFU/100gm for American oysters (Graph 3B).

Graph 3A – Linear Range Determination for **Soft-shelled Clams**.



Graph 3B – Linear Range Determination for **American Oysters**.



Data Summary: Soft-shelled Clams
Linear range of the method as implemented 6 to 10,000 PFU/100gm
The limit of detection of the method as implemented 6 PFU/100gm
The limit of quantitation/sensitivity of the method as implemented 44 PFU/100gm

Data Summary: American Oysters
Linear range of the method as implemented 7 to 10,000 PFU/100gm
The limit of detection of the method as implemented 7 PFU/100gm
The limit of quantitation/sensitivity of the method as implemented 47 PFU/100gm

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 2A and 2B show the results for the Accuracy/Trueness of the method.

Table 2A – Calculation of the Accuracy/Trueness of the method for Soft-shelled Clams.

Average Log of Plates
2.655

Average Log of Spike
2.780

Accuracy	95.5%
Trueness	

Table 2B – Calculation of the Accuracy/Trueness of the method for American oysters.

Average Log of Plates
2.803

Average Log of Spike
2.869

Accuracy	97.7%
Trueness	

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 3A and 3B show the statistics for soft-shelled clams and American oysters, respectively.

Table 3A – Measurement Uncertainty for **Soft-shelled Clams**.

		antilog
Number of values	150	
Mean	0.1246	**1.332**
Std. Deviation	0.1500	
Std. Error	0.01225	
Lower 95% CI of mean	0.1004	**1.260**
Upper 95% CI of mean	0.1488	**1.409**

Table 3B – Measurement Uncertainty for **American Oysters**.

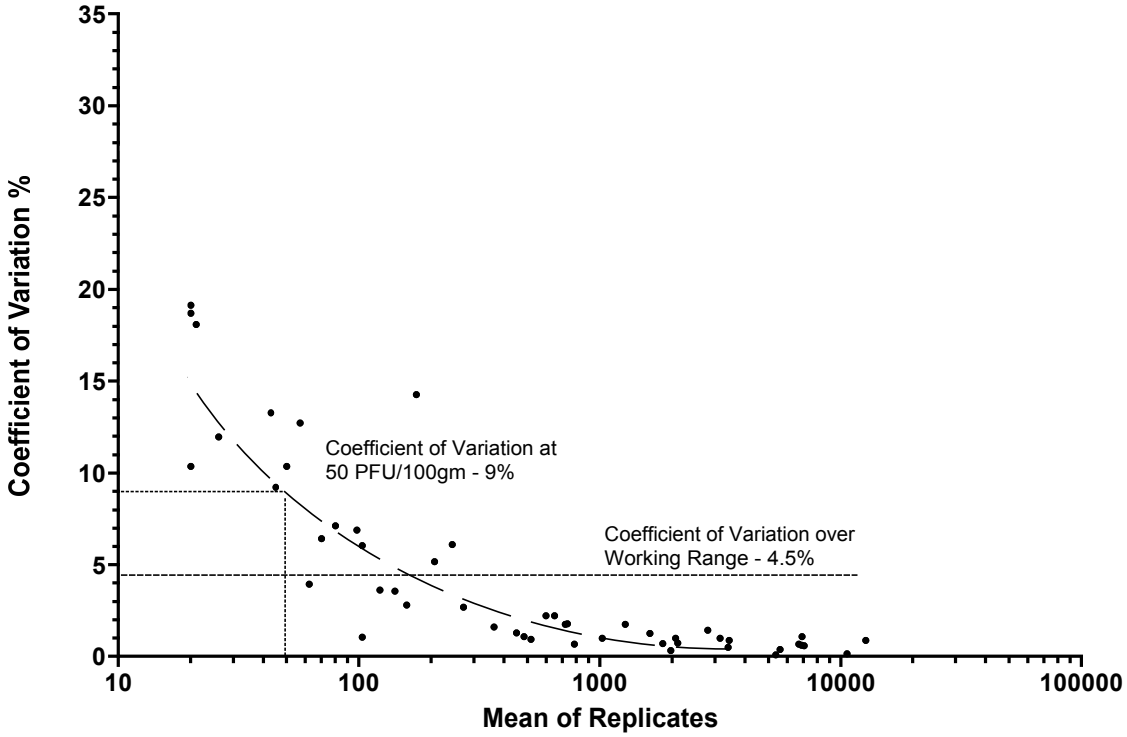
		antilog
Number of values	150	
Mean	0.0666	**1.166**
Std. Deviation	0.1849	
Std. Error	0.01509	
Lower 95% CI of mean	0.03677	**1.088**
Upper 95% CI of mean	0.09643	**1.249**

Data Summary: **Soft-shelled Clams**
Calculated % accuracy/trueness 95.5%
Calculated measurement uncertainty 1.260 to 1.409

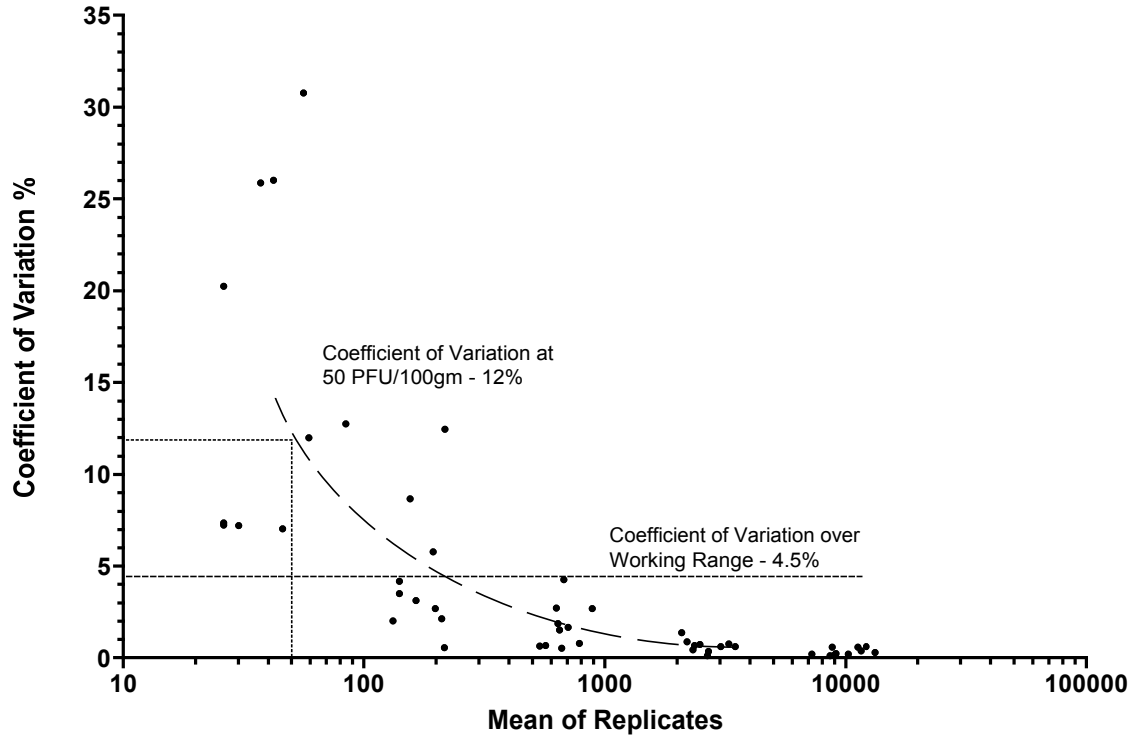
Data Summary: **American Oysters**
Calculated % accuracy/trueness 97.7%
Calculated measurement uncertainty 1.088 to 1.249

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 4A and 4B for soft-shelled clams and American oysters, respectively.

Graph 4A - Coefficient of Variability (%) of Replicate verses Mean of Replicate for **Soft-shelled clams**.



Graph 4B - Coefficient of Variability (%) of Replicate verses Mean of Replicate for **America Oysters**.



In Graph 4A and 4B above, the coefficient of variation at 50PFU/100gm level was determined graphically (approximately 9% for soft-shelled clams and 12% for American oysters) and shows the precision at this point of interest (proposal 05-105). As expected, 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 both soft-shelled clams and American oysters appear in Table 4 below.

Table 4 – Mean, Minimum, and Maximum Coefficient of Variation over the Working Ranges.

For Soft-shelled Clams

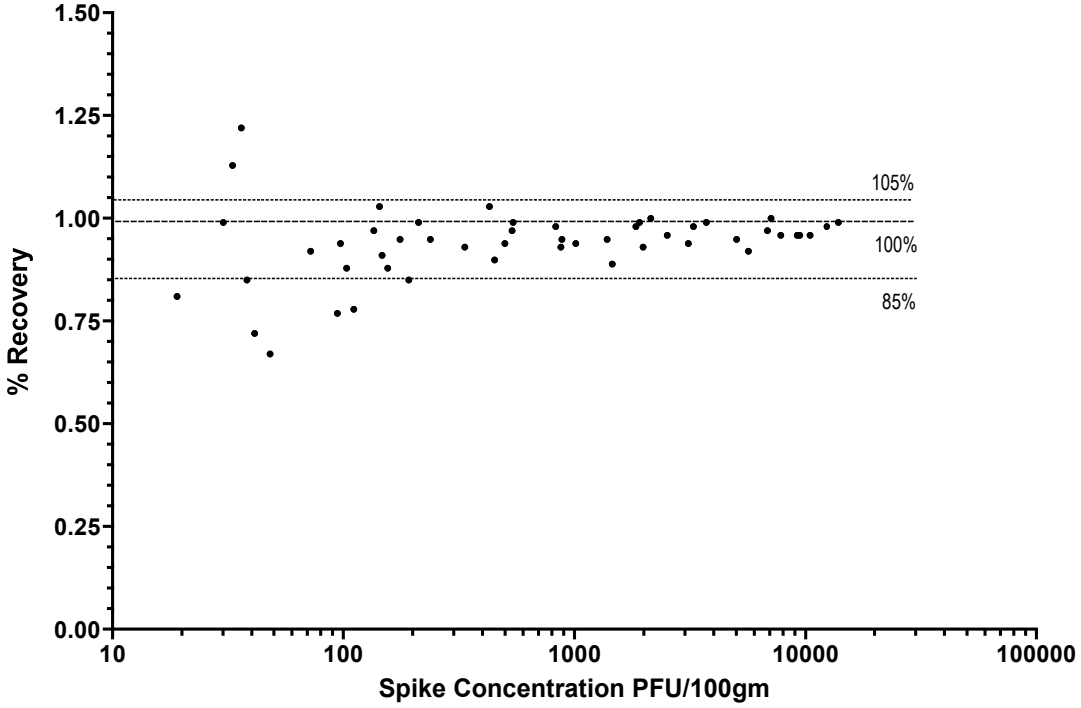
Average Coefficient of Variation = 4.5%
Minimum Coefficient of Variation = 0.09%
Maximum Coefficient of Variation = 19%

For American Oysters

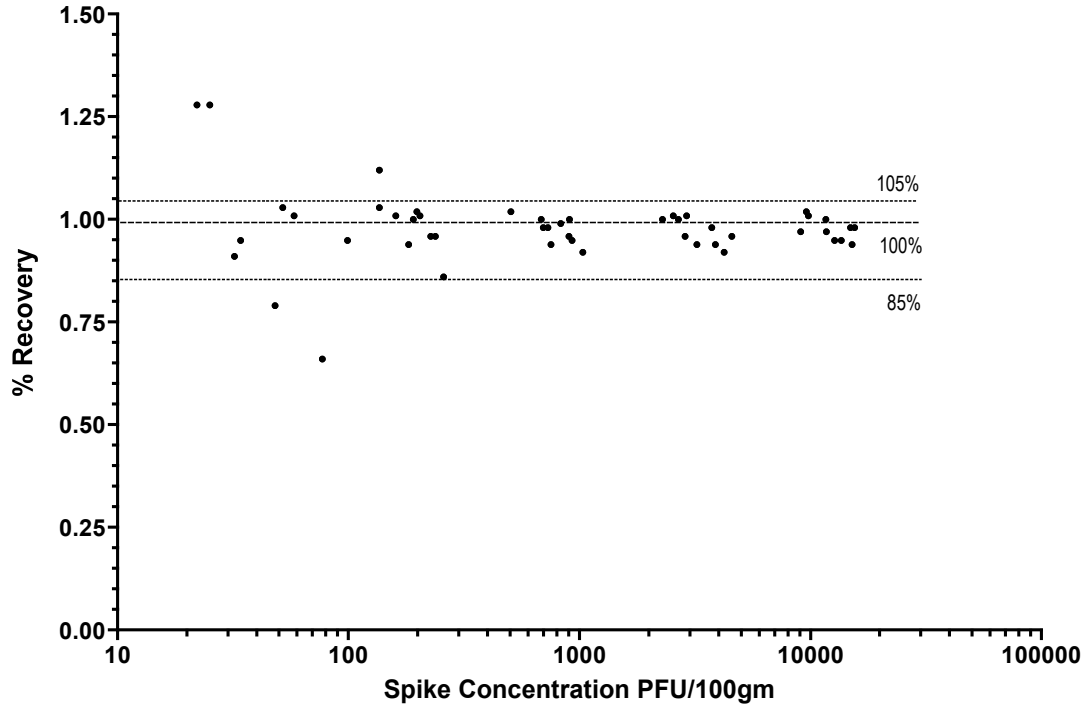
Average Coefficient of Variation = 4.5%
Minimum Coefficient of Variation = 0.12%
Maximum Coefficient of Variation = 30.8%

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 and multiplied by 100 to give a percentage. The percent recovery was then plotted against the spike concentrations. Graph 5A and 5 B show these recovery plots with the recoveries bracketed at 85% and 105% for both the soft-shelled clams (Graph 4A) and American oysters (Graph 4B). Recovery by the method is good for both the soft-shelled clams and American oysters. However, as expected the variability in recovery increases as you approach the LOD.

Graph 5A - Percent Recovery verse the Spike Concentration for **Soft-shelled Clams**.



Graph 5B - Percent Recovery verses the Spike Concentration for **American Oysters**.



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 5A and 5B show this calculation for soft-shelled clams and American oysters, respectively.

Table 5A – Method Recovery for the **Soft-shelled Clam**s.

Average Log of Spike	Average Log Replicates	% Recovery
2.780	2.6549	95.5%

Table 5B – Method Recovery for the **American Oysters.**

Average Log of Spike	Average Log Replicates	% Recovery
2.869	2.8028	97.7%

Data Summary: Soft-shelled Clams

- 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 **4.5%** .
- 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? **95.5%**

Data Summary: American Oysters

- 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 **4.5%** .
- 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? **97.7%**

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

Different lots of agar, tryptone, and host E-coli culture and were prepared well in advance of the trials. Ten different harvest lots of soft-shelled clams and 10 different harvest lots of American oysters were used for these analyses. Table 6A and 6B show the data, data analysis, and the results of the paired t-test for both soft-shelled clams and American oysters

Table 6A - Determination of the Method Ruggedness for **Soft-shelled Clams**.

Media A PFU/100gm	Media B PFU/100gm	Log Media A	Log Media B
8500	8226	3.9294	3.9152
2225	2528	3.3473	3.4028
529	571	2.7235	2.7566
123	144	2.0899	2.1584
5596	5829	3.7479	3.7656
1309	1531	3.1169	3.1850
385	342	2.5855	2.5340
155	17	2.1903	1.2304
6332	5850	3.8015	3.7672
1718	1811	3.2350	3.2579
	Skew	-0.2375	-1.0318
	Variance	0.4365	0.7101
	Ratio of Larger Variance to Lower Variance	1.6269	

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

Paired t-test (Media A verses Media B)

P value	0.4418
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.8046 df=9
Number of pairs	10

Table 6B - Determination of Method Ruggedness for the **American Oysters**.

Media A PFU/100gm	Media B PFU/100gm	Log Media A	Log Media B
6912	7143	3.8396	3.8539
1851	2051	3.2674	3.3120
404	538	2.6064	2.7308
77	78	1.8865	1.8921
7342	6895	3.8658	3.8385
1916	1916	3.2824	3.2824
448	370	2.6513	2.5682
68	79	1.8325	1.8976
7603	7195	3.8810	3.8570
2219	2067	3.3462	3.3153
	Skew	-0.5459	-0.5563
	Variance	0.5956	0.5683
	Ratio of Larger Variance to Lower Variance	1.0480	

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

Paired t-test (Media A verses Media B)

P value	0.6405
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.4832 df=9
Number of pairs	10

Data Summary: Soft-shelled Clams
Value for the test of symmetry of the distribution of Media A data -.2375
Value for the test of symmetry of the distribution of Media B data -1.0318
Variance of Media A data .4365
Variance of Media B data .7101
Ratio of the larger to the smaller of the variances of Media A and Media B 1.62
Is there a significant difference between Media A and Media N

Data Summary: American Oysters
Value for the test of symmetry of the distribution of Media A data -.5459
Value for the test of symmetry of the distribution of Media B data -.5563
Variance of Media A data .5956
Variance of Media B data .5683
Ratio of the larger to the smaller of the variances of Media A and Media B 1.05
Is there a significant difference between Media A and Media B N

Acknowledgement

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