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

Name of the New Method	Male-Specific Coliphage Quantification from 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 36528 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	FDA has long been using Male-Specific Coliphage (MSC) to evaluate the potential viral contamination of shellfish growing water by wastewater treatment plant (WWTP) outfalls. Methods using MSC as an indicator of viral contamination have been successful in evaluation of viral persistence in molluscan shellfish impacted by WWTP outfalls. 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 WWTP discharges. This method provides the necessary tools 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 WWTP can be assessed under different environmental and operational conditions. Understanding the viral reduction performance at different stages

		in a wastewater treatment process is a valuable assessment tool to aid in the determination of growing area classification and management options for shellfish growing areas adjacent to and downstream from the WWTP outfall.
What is the intended purpose of the method?	Y	The purpose of this method is to enumerate MSC from wastewater, including influent, pre-treatment effluent and final effluent.
Is there an acknowledged need for this method in the NSSP?	Y	The recognized need for a viral indicator, and a method to test for it, is addressed in the 2019. 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. The need and utility for this method was likewise addressed at the MSC Informational Meeting of the Growing Area Committee (MSC Summit) in Charlotte, NC in August 2014.
What type of method? i.e. chemical, molecular, culture, etc.	Y	Culture method for Male-Specific Coliphage (MSC) in wastewater utilizing a double agar overlay for viable viral plaque production.

B. Method Documentation		
1. Method documentation includes the following information:		
Method Title	Y	Male-specific Coliphage (MSC) Quantification from Wastewater
Method Scope	Y	The method is intended to be used to quantify MSC in raw influent, pre-disinfected, and post-disinfected (final effluent) wastewater.
References	Y	References for the method and its application are provided in Appendix A.
Principle	Y	A double agar overlay is used with *E. coli* Famp host strain that constitutively expresses pili for MSC binding and subsequent infection. Viral plaques are visualized on a confluent host lawn. The

		amount of sample inoculated is used to quantify the plaque forming units/100 mL of wastewater.
Any proprietary aspects	N	
Equipment required	Y	Provided in Appendix B.
Reagents required	Y	Provided in Appendix C.
Sample collection, preservation and storage requirements	Y	Provided in Appendix D.
Safety requirements	Y	Sewage can contain bacterial, viral, and protozoan pathogens. Standard biological laboratory safety protocols should be followed. No specific requirements are needed for this method.
Clear and easy to follow step-by-step procedure	Y	Appendix E.
Quality control steps specific for this method	Y	Standard laboratory quality control procedures should be followed.

C. Validation Criteria		
1. Accuracy / Trueness	Y	**101%**, data in Appendix I.
2. Measurement uncertainty	Y	**-0.049 to -0.0006**, data in Appendix I.
3. Precision characteristics (repeatability)	Y	Average Coefficient of Variation = **5.4%**, Appendix I.
4. Recovery	Y	**101%**, Figure 1.
5. Specificity	Y	**SI_{avg}=1**, Table 1.
6. Working and Linear ranges	Y	**1 to 3.5 log PFU/100 mL**, Figure 3.
7. Limit of detection	Y	**1.6 PFU/100 mL**, Figure 2.
8. Limit of quantitation / Sensitivity	Y	**20 PFU/100 mL**, Figure 2.
9. Ruggedness	Y	**No effect** of media batches (Table 2). Optimal host density after **4-6 h growth** (Figure 4). Optimal host volume between **150-250 µL** (Figure 5). Optimal water bath temperature of **50 ºC** (Figure 6).
10. Matrix effects	Y	No matrix effects were observed by wastewater type or source (Figure 7).
11. Comparability (if intended as a substitute for an established method accepted by the NSSP)	Y	This method is not intended as a substitute for an established NSSP method; however, it was compared to the EPA 1602 SAL (Table 3, Figure 8).

D. Other Information		
1. Cost of the method	Y	Consumables and media are ~$18 per sample. Laboratory costs will vary,

		depending on operational overhead and analyst salary.
2. Special technical skills required to perform the method	Y	Basic microbiological laboratory skills.
3. Special equipment required and associated cost	N	Tempering water bath (~$3000).
4. Abbreviations and acronyms defined	Y	Listed in Appendix J.
5. Details of turnaround times (time involved to complete the method)	Y	The method can be performed, and results read within 24 h.
6. Provide brief overview of the quality systems used in the lab	Y	The laboratory adheres to the quality system standards of FDA/CFSAN, as well as those of the NSSP.

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

Comments:

Validation Criteria

As it is challenging to find wastewater matrix that is free of MSC, final effluent sample without detectable MSC was used to generate spiked sample data (Appendix H and I). MS-2 was used as the inoculum and enumerated using the double-agar overlay method. Unless otherwise noted, statistical analysis was conducted as recommended in the SLV Documents for Non-MPN Based Microbiological Methods on the ISSC website.

Accuracy/Trueness:

The Accuracy/Trueness was determined using spiked samples (data in Appendix I). The average of all spiked samples was 2.47 log PFU/100 mL, and the average of all the spikes was 2.45 log PFU/100 mL, resulting in an Accuracy/Trueness of 101%.

Measurement Uncertainty:

The Measurement Uncertainty was determined using spiked samples (data in Appendix I). The differences between measurement and reference values were calculated after log transformation of all 150 sample measurements, to increase statistical confidence. The mean difference was -0.025 log PFU/100 mL. The 95% CI of the mean difference was -0.049 to -0.0006, calculated as the mean plus or minus twice the standard error.

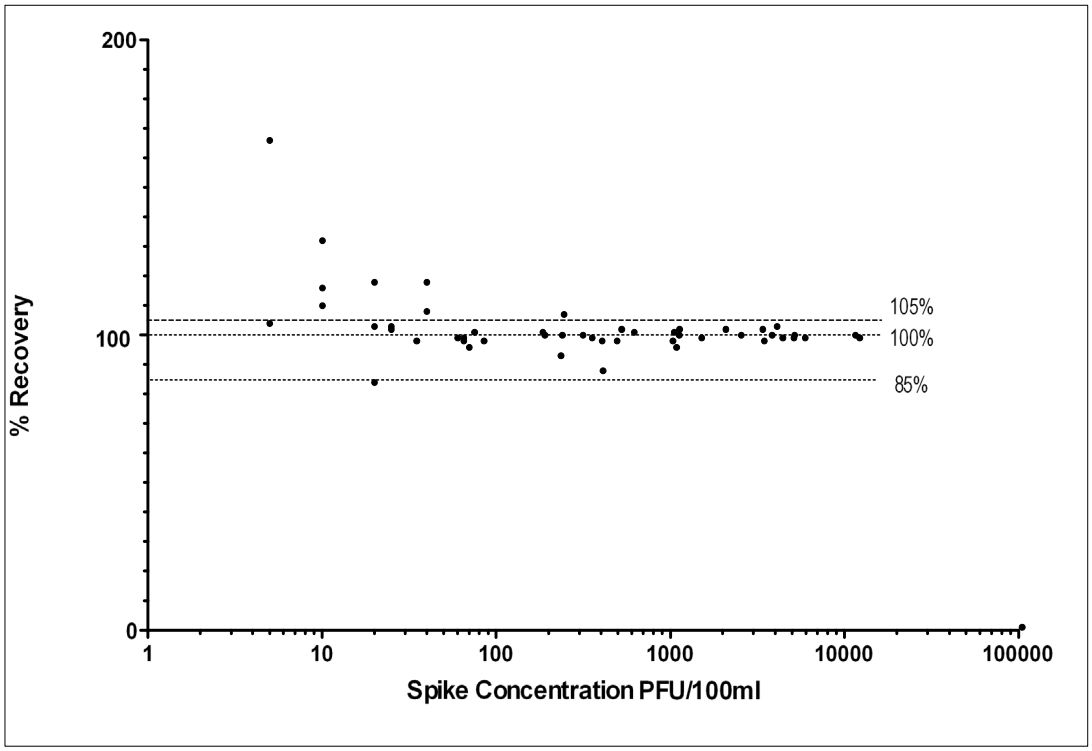
Precision:

To examine the precision of the method, log transformed data from 50 sets of three true spiked replicates (Appendix I) were tabularized against the mean of the triplicate results. The average Coefficient of Variation was determined to be 5.4% over the range, with a minimum of 0.25% and a maximum of 36.2% near the limit of detection.

Recovery:

To examine recovery of the method, spike data (Appendix I) was used. The mean of replicate data was divided by the spike concentration. The percent recovery was then plotted against the spike concentrations (Figure 1). The overall percent recovery of the method is 101%. A wider variation is observed below the limit of quantification, as would be expected.

Figure 1. Percent recovery verses the concentration in spiked samples.



Specificity:

The specificity of the method is dependent on the bacterial host strains ability to produce pili and not be susceptible to somatic phage. The *E. coli* Famp host strain was genetically selected to constitutively express the pili while being resistant to somatic bacteriophage. The specificity of the host is well-documented in the scientific literature, including recent work by Stewart *et. al*., which demonstrated all propagatable phage from sewage using the Famp host strain were male-specific coliphage. In further support, a prototypical T-even (somatic phage) was selected to test the specificity of the double-agar overlay method. The average log PFU of MS-2 was 1.9 and the average log MS-2 in the presence of 2.15 log T-even phage was 1.9, providing an SI_{avg}=1.

Table 1. Enumeration of MS-2 in the presence of a T-even phage.

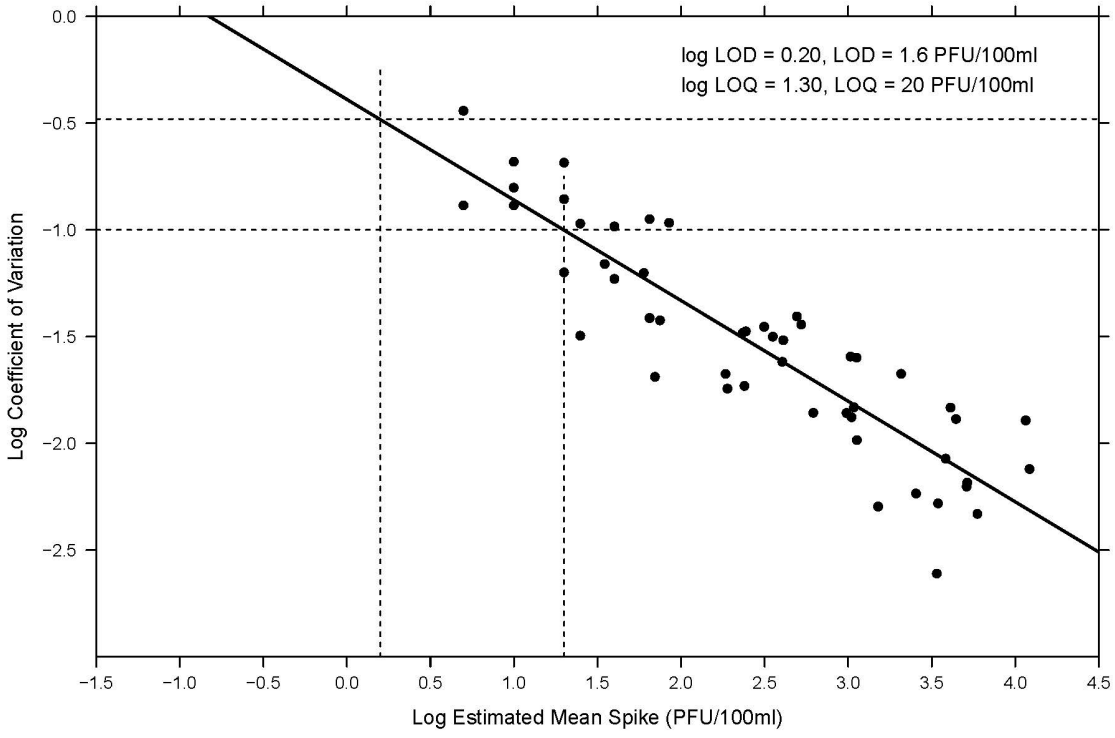
Replicate	MS-2 (PFU/plate)	MS-2 and T-4 (PFU/plate)	MS-2 (log PFU/plate)	MS-2 and T-4 (log PFU/plate)
1	88	93	1.9	2.0
2	82	68	1.9	1.8

3	72	66	1.9	1.8
4	74	58	1.9	1.8
5	92	95	2.0	2.0

LOD, LOQ, and Linear Range:

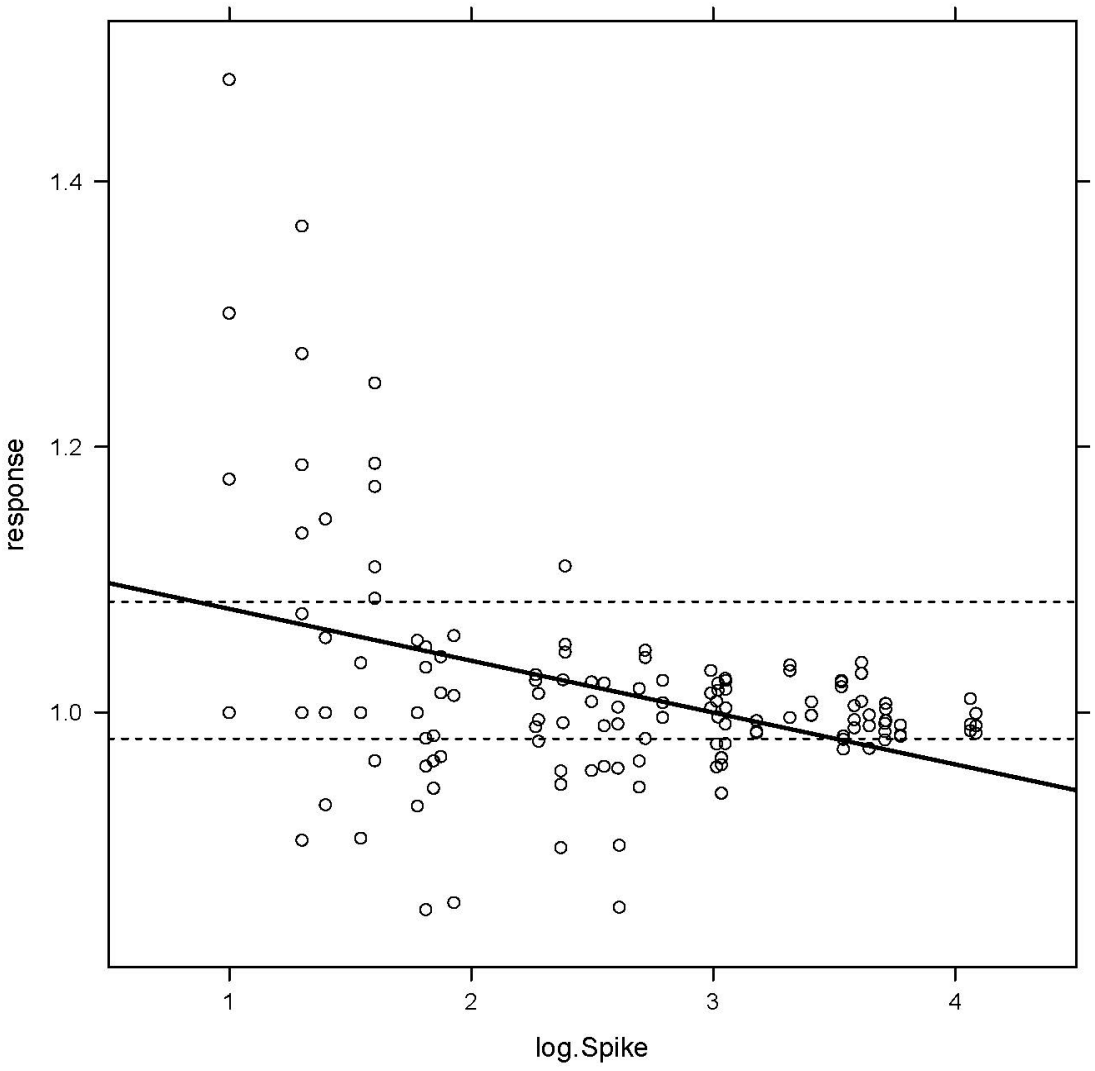
Spike data (Appendix I) was used to determine the LOD and LOQ. The replicate plate count results were log transformed and the log coefficient of variation was plotted against the log spike concentration (Figure 2). The LOQ of the method is the point of intersection of the log spike concentration and the log coefficient of variation of –1.0 (or its antilog, 10%). The LOD is the point of intersection of the log spike concentration and the log coefficient of variation of –0.477 (or its antilog of, 33%). The correlation coefficient (R square value) of this linear regression is 0.80 which indicates a good fit. The LOD and LOQ as determined by the spiking trials shows LOD and LOQ are 1.6 PFU/100 mL and 20 PFU/100 mL, respectively.

Figure 2. Log coefficient of variation verses log spike concentration.



To determine the Linear Range, spike data greater or equal to 1 log PFU/100 mL (Appendix I) was used 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 (Figure 3). The line of constant response falls between the upper (1.05) and the lower (.95) confidence interval estimates through the working range of the data. This suggests that the method is linear through the range of 1 Log PFU/100 mL to 3.5 Log PFU/100 mL.

Figure 3. Relative response line of spiked effluent samples.



Ruggedness:

Multiple variations of the method were examined to evaluate ruggedness: different lots of media (as described in the NSSP SOP for the Single Laboratory Validation of Marine Biotoxin and Non-MPN Based Microbiological Methods), host growth time, host volume, and temperature of the tempering water bath.

Ruggedness – Media: Two batches of bottom agar plates, soft agar tubes, and growth broth were prepared (Media A and Media B). Ten samples of spiked effluent (Appendix H) were plated using both Media A and Media B. The test of symmetry of the distribution of Media A was -1.29 and Media B was -1.35. The variance of Media A was 0.47 and Media B was 0.48. Normality Test (Shapiro-Wilk) passed at P=0.705. Paired T Test was used on data in Table 2. and the change in concentration of MSC that occurred with between the batches is not enough to exclude the possibility that the difference is due to chance (P=0.765).

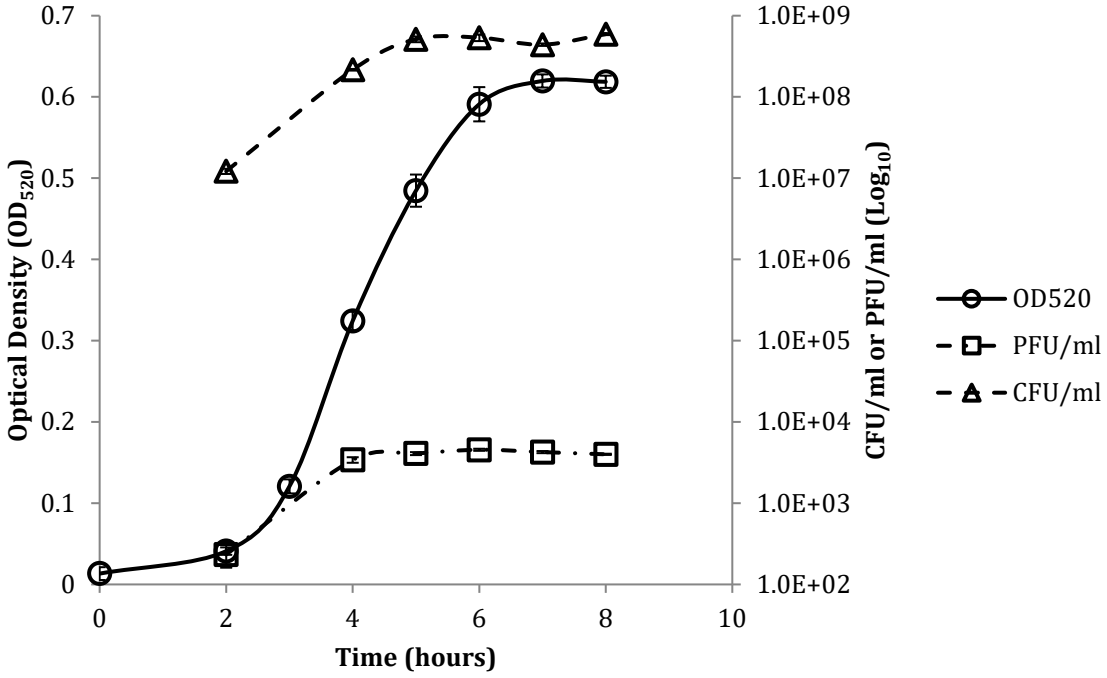
Table 2. Determination of method ruggedness – media effects.

Trial #	Log PFU/100 mL	
	Media A	Media B
1	3.48	3.55
2	3.71	3.70
3	3.73	3.74
4	4.15	4.18
5	4.13	4.20
6	4.09	4.09
7	3.74	3.69
8	3.73	3.65
9	2.32	2.37
10	2.28	2.24

Ruggedness – Host Growth Time: Effect of the growth time of was taken to determine the host *E. coli* was examined by measuring the Optical Density (OD) at 520 nm over time and comparing the enumeration of a known amount of MS2 at each time point. Tryptone broth was inoculated with *E. coli* and incubated at 35 ºC. OD_{520} was measured every hour starting at t = 2 hours. At each time point, 100 µL of host was serially diluted and plated to determine CFU/mL. 200 µL of the same host sample was used to determine PFU/mL of stock MS2 controls. After approximately 4 to 6 hours of growth the OD_{520} of

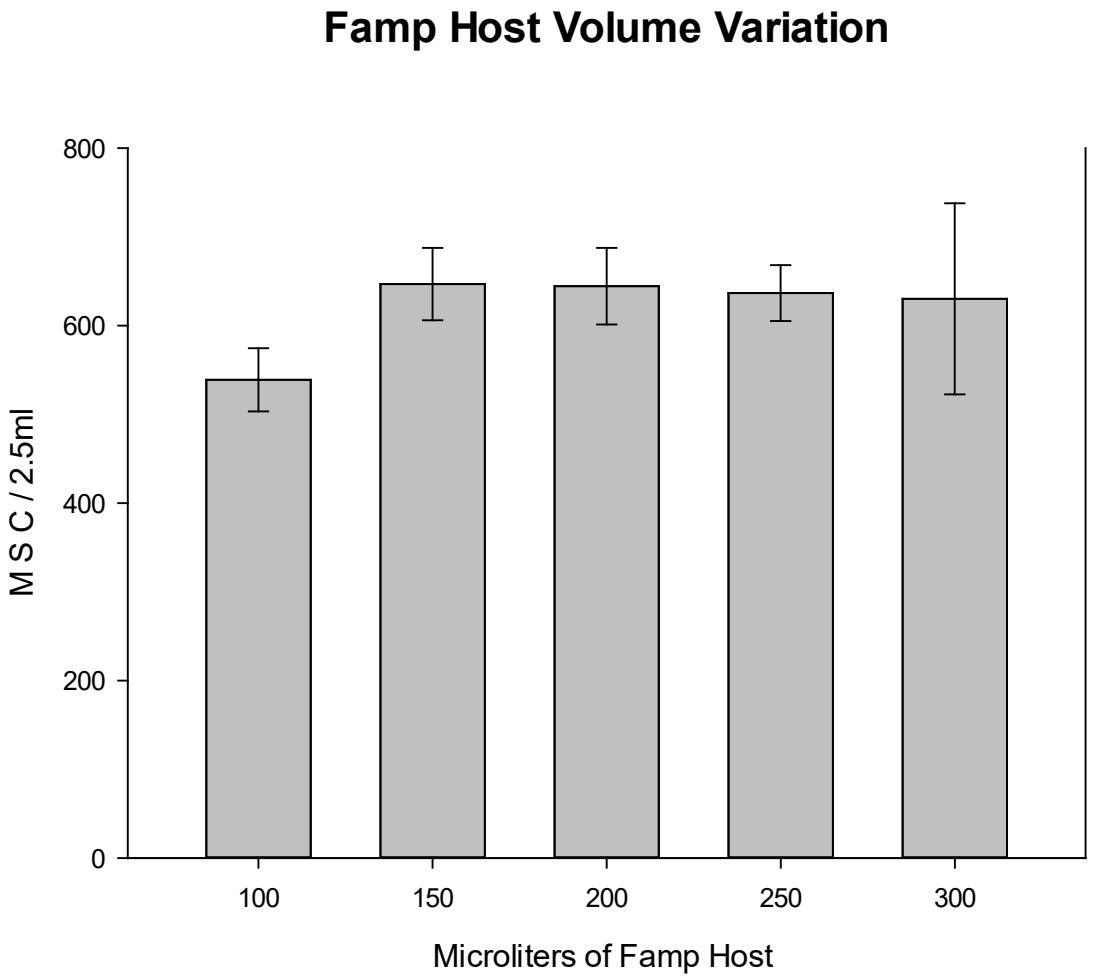
host is in the range of 0.35 to 0.6 (Figure 4), during which time the MS2 plaquing efficiency of the host *E. coli* is optimal and consistent. Therefore, we conclude that a host OD520 of 0.35 to 0.7, or approximately 4 to 6 hours of growth, is ideal for MSC enumeration.

Figure 4. Determination of method ruggedness – host growth time.



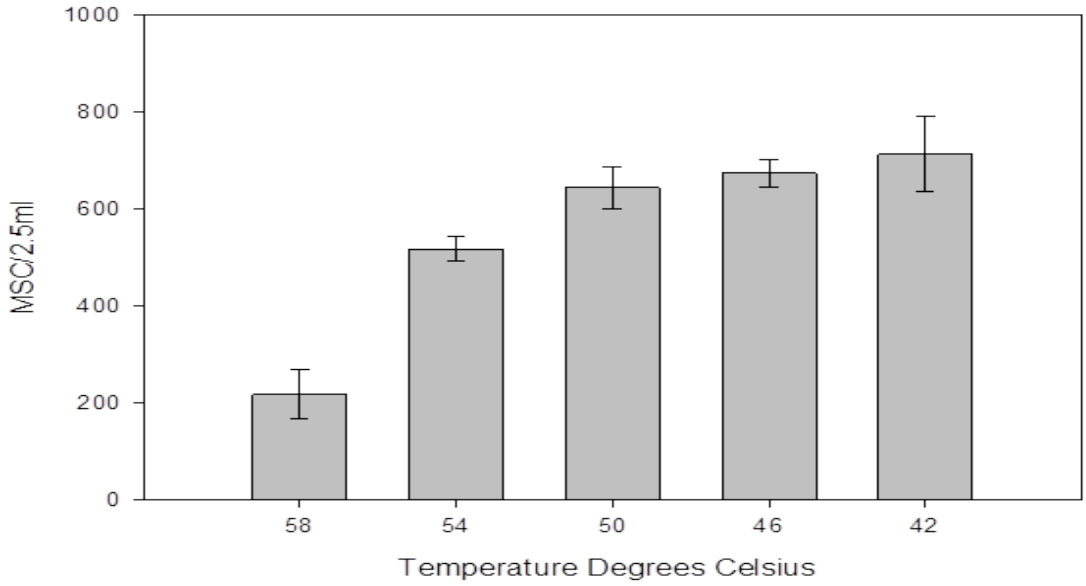
Ruggedness – Host Volume: The effect of host volume was examined using a working stock of MS-2 for comparison. The host was grown according to the protocol and an appropriate dilution of the MS-2 stock was run, in triplicate, using various volumes of host culture. The results demonstrate that 100 µl of Famp host produces fewer MSC plaques than 150, 200, 250 or 300 µl of Famp host (Figure 5). Although 300 µl of host produces a similar mean there was more variability, indicated by the increased standard deviation. This data supports the use of 200 µl of host, as stated in the protocol.

Figure 5. Determination of method ruggedness - effect of *E. coli* host volume.



Ruggedness – Water Bath Temperature: The effect of water bath temperature was examined using a working stock of MS-2 for comparison. An appropriate dilution of the MS-2 stock was tested, in triplicate, according to the protocol, but with varying tempering temperatures of the water bath holding the soft agar tubes. The results demonstrate that the two cooler temperatures (42 and 46 ºC) do not show a significant difference in the mean recovery of MSC (Figure 6). Elevated temperatures (54-58 ºC) caused a decrease in MSC recovery. This data supports the use of 50-52 ºC, as stated in the protocol.

Figure 6. Determination of method ruggedness - effect of water bath temperature.

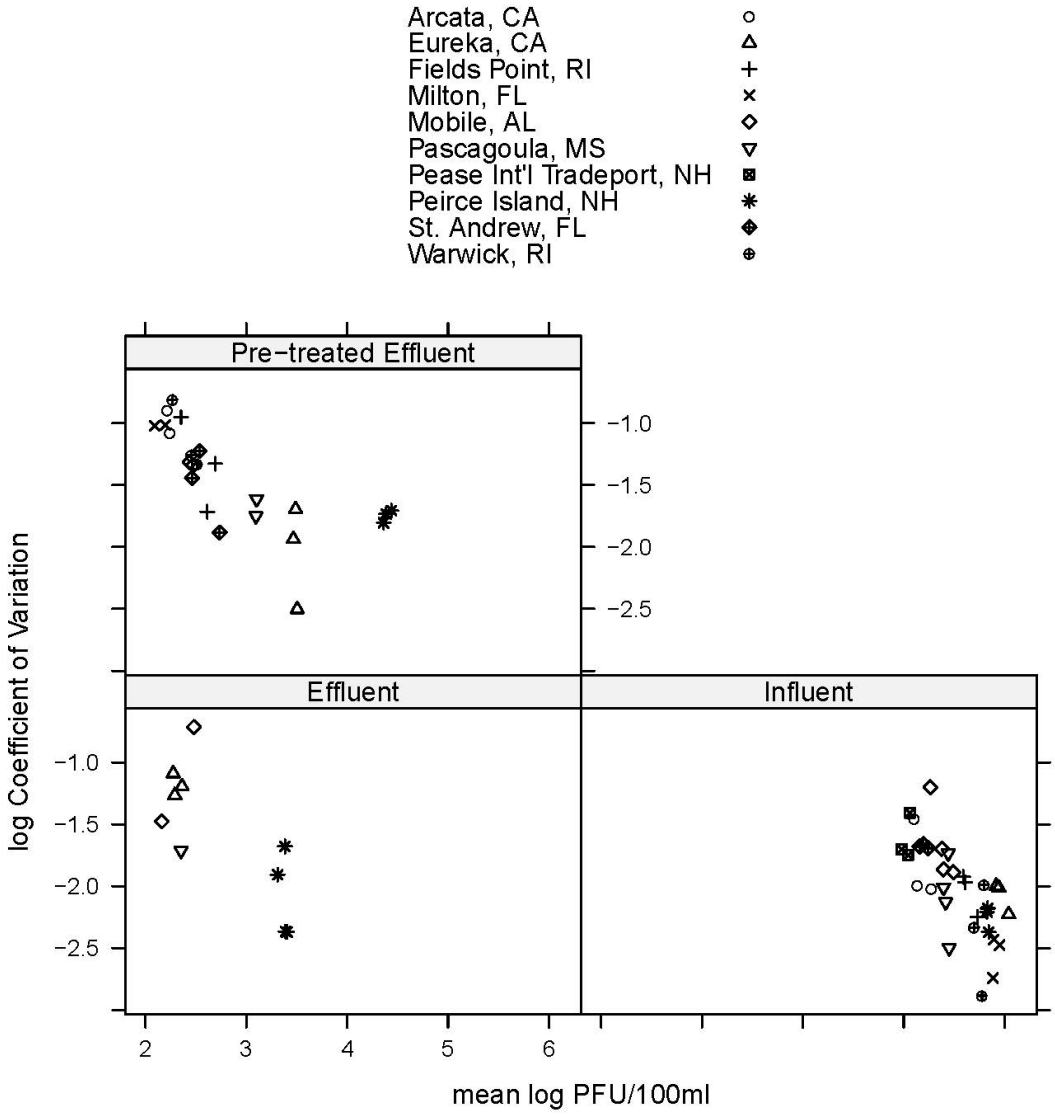


Matrix Effects:

MSC are ubiquitous to all influent and pre-disinfected effluent, so these types of wastewater cannot be utilized for the spike samples required under an SLV. Some highly treated effluent with UV disinfection can reduce MSC to non-detectable levels using the proposed direct plate double agar overlay. These disinfected effluents were used in the spiked analysis. To test the matrix effects of method on a diverse source and type of wastewater, 10 geographically unique WWTP influent, pre-disinfected effluent and post-disinfected effluent were collected and analyzed using the double agar overlay method (Appendix F and G). Influent, under the prescribed protocol, is diluted 1:100 in PBS prior to plating, which would dilute any compounds that may potentially interfere with the assay.

The coefficient of variation did not differ across the wastewater types, nor did they cluster by location (Figure 7), indicating no noticeable effect of the wastewater types or source on the performance of the method.

Figure 7. Log CV vs. mean Log PFU/100 mL for 10 WWTPs.



Comparability:

Although there is currently no accepted NSSP method for wastewater, there is a single agar layer (SAL) method developed by EPA for ground and other waters. Method 1602 has only been validated for use in ground water. The method is a single agar layer procedure for the direct enumeration of MSC when using the Famp host strain. Briefly, a 100 mL water sample is assayed by adding magnesium chloride, log phase host bacteria, and 100 mL of double strength molten tryptic soy agar to the sample. The sample is thoroughly mixed, and the total volume is poured into 10 – 100 mm petri dishes. After an overnight incubation, circular zones of clearing (plaques) are counted and summed for all plates from a single sample. This SAL EPA method was performed in parallel with the Modified Double Agar Overlay Method for Determination of Male-Specific Coliphage in Wastewater submitted under this SLV. Three trials of ten different target concentrations of MSC in wastewater were analyzed for a total of 30 comparisons. To obtain the different target concentrations, Mobile WWTP final effluent was mixed in various ratios with pre-treated influent from the same treatment plant.

The test of symmetry for the distribution of the data (Table 3) generated by the SAL method was -0.0963, and the Double Agar Overlay method was -0.5230. The variance of the SAL method was 0.1233, and the Double Agar Overlay method was 0.1442. The ratio of variances is 1.17, indicating a significant difference between the methods. The sample mean of the Double Agar Overlay exceeds the sample mean of the Single Agar Layer by an amount that is greater than would be expected by chance. This seems to be primarily due to the Double Agar Overlay producing consistently more plaques in each sample compared to the Single Agar Layer at all concentrations. This appears to be a limitation of the upper detection limit of the EPA SAL method.

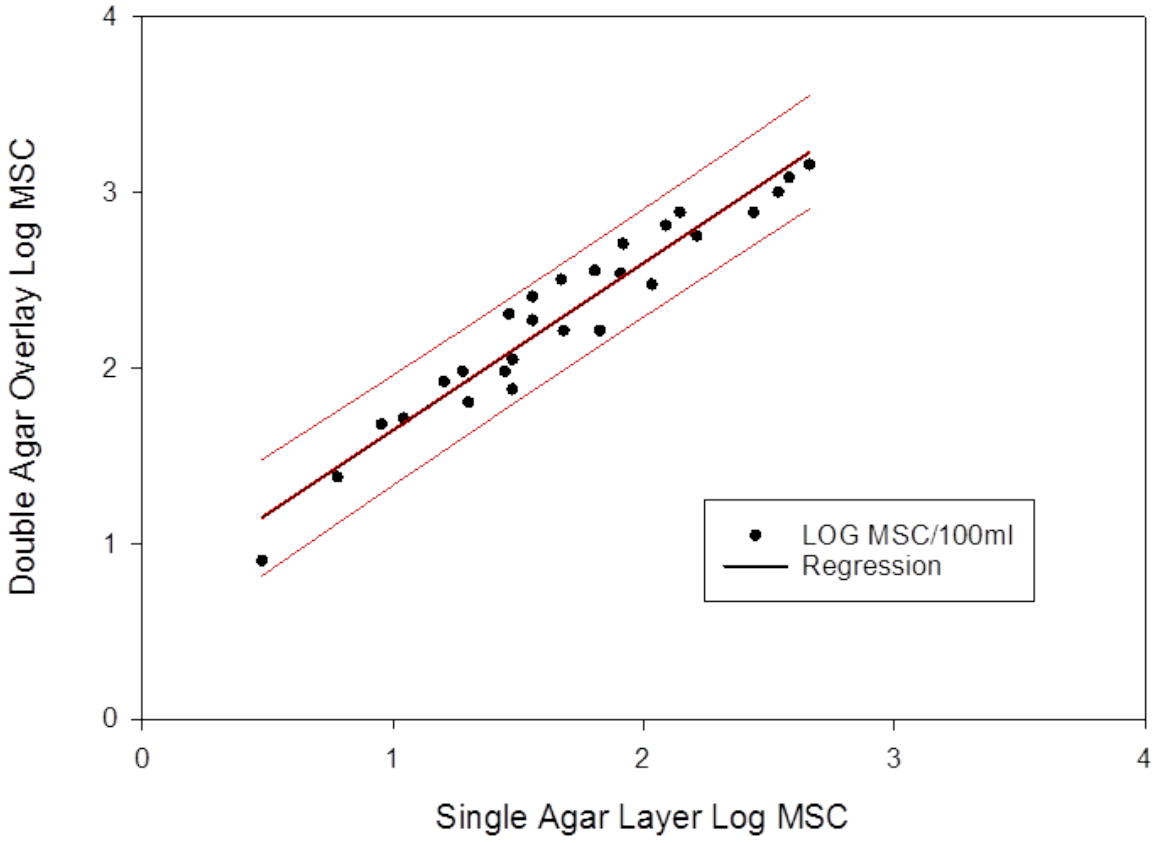
The parallel run of the EPA 1602 SAL and the Modified Double Agar Overlay produced data which demonstrated the Modified Double Agar Overlay quantified more virus than the EPA 1602 SAL. The data fell well inside of the 95% prediction interval (Figure 8). In addition, the data does not show the cumbersome nature of the SAL method. The need for media preparation each day of analysis and the high volume of molten agar transfers make it less user-friendly.

In summary, in head-to-head comparison, the Modified Double Agar Overlay performs better than the EPA Single Agar Overlay. This lack of comparability, as defined by the NSSP single laboratory methods criteria, should be of little consequence because the EPA SAL is not currently used in the NSSP. This comparison should help bolster the confidence in the method.

Table 3. Data for determination of comparability of the Double Agar Overlay and Single Agar Layer methods.

	Single	Single	Double	Double
Sample	PFU/100 mL	log PFU/100 mL	PFU/100 mL	log PFU/100 mL
1	<	N/A	<	N/A
2	67	1.83	164	2.21
3	108	2.03	300	2.48
4	123	2.09	652	2.81
5	163	2.21	568	2.75
6	140	2.15	776	2.89
7	275	2.44	772	2.89
8	345	2.54	1008	3.00
9	381	2.58	1224	3.09
10	458	2.66	1448	3.16
11	<	N/A	<	N/A
12	9	0.95	48	1.68
13	30	1.48	76	1.88
14	36	1.56	188	2.27
15	29	1.46	204	2.31
16	36	1.56	256	2.41
17	47	1.67	320	2.51
18	64	1.81	360	2.56
19	81	1.91	348	2.54
20	83	1.92	512	2.71
21	<	N/A	<	N/A
22	3	0.48	8	0.90
23	6	0.78	24	1.38
24	11	1.04	52	1.72
25	20	1.30	64	1.81
26	16	1.20	84	1.92
27	19	1.28	96	1.98
28	28	1.45	96	1.98
29	30	1.48	112	2.05
30	48	1.68	164	2.21

Figure 8. Log MSC values generated by the Single Agar Overlay versus Double Agar Overlay. The 95 % prediction interval is shown in red.



Appendix A. 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.

Stewart-Pullaro, J., J.W Daugomah, D.E. Chestnut, D.A. Graves, M.D. Sobsey and G.I. Scott. 2006. F+RNA coliphage typing for microbial source tracking in surface waters. J. Applied Microbiology. 101, 1015-1026 doi: 10.111/j.1365-2672.2006.03011.x

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

Appendix B. Equipment and Materials:

Equipment and Materials for Collection and Transport of Wastewater Samples:
250 or 500 ml Sterile Sample Containers
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
Sterile inoculating loops (3 mm in diameter or 10 µL volume)
Sterile swabs
Sterile filters, 0.22µm, and sterile syringes, or other means of filter sterilization
Serological Pipets- 1 ml, 2 ml, 5 ml, 10 ml
Pipet-aid, or appropriate volume micropipettes
Petri dishes, sterile disposable 100 x 15 mm
Test tubes, of appropriate sizes
50ml conical tubes, sterile with screw caps
Test tube racks--sizes to accommodate tubes
Freezer vials, sterile 30 ml with screw caps
Aluminum foil
Counter-pen, digital (optional)

Appendix C. Reagents and Media

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

Antibiotics:
Ampicillin sodium salt (Sigma A9518)
Streptomycin sulfate (Sigma S6501)

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

MSC (Coliphage) Stock:
Type Strain - MS2, ATCC # 15597-B1

Media Types:
Bottom Agar
DS Soft Agar
Growth Broth

Media Composition:

Bottom Agar:

Tryptone	10.0 g
Dextrose	1.0 g
NaCl	5.0 g
Agar	15.0 g
DI water	990 ml
pH prior to autoclaving	6.7 ± 0.2 at 25°C
Streptomycin sulfate	0.05 g
Ampicillin	0.05 g

1. With gentle mixing, add all the components, except antibiotics, to dH2O. Dissolve.
2. Adjust pH as needed.
3. Heat until clear and bring to a boil.
4. Autoclave at 121°C ± 2°C for 15 minutes.
5. Temper to 50-55°C.
6. Add 0.22 µm filter sterilized antibiotic solution such that the final conc. of each antibiotic is 0.05g/L. Mix gently.
7. Aseptically fill sterile petri dishes.
8. Store bottom agar plates inverted under refrigeration.

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
pH prior to autoclaving	6.7 ± 0.2 at 25°C

1. With gentle mixing, add all the components to the dH_2O.
2. Bring to a boil.
3. Dispense in 2.5 ml aliquots into glass culture tubes.
4. Cover and store frozen (-20°C) for up to 3 months.
5. Autoclave prior to use at 121°C ± 2°C for 15 minutes, and then temper to 50-52°C in a water bath for no longer than 2 hours.

*1M $CaCl_2$ Solution:

1. Dissolve 11.1 g of $CaCl_2$ anhydrous (FW 111.0, Dihydrate FW 147) in 100 ml dH_2O.
2. Sterilize by autoclaving at 121°C for 15 minutes.
3. Store up to three months at 4°C.
4. Alternatively, use commercially available 1M solution.

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 dH_2O water.
2. Dissolve and dispense into screw top containers.
3. Sterilize at 121°C ± 2°C for 15 minutes.
4. Store for up to three months at 4°C.

Appendix D. Sample Collection, Preservation and Storage Requirements:

Sample Collection:

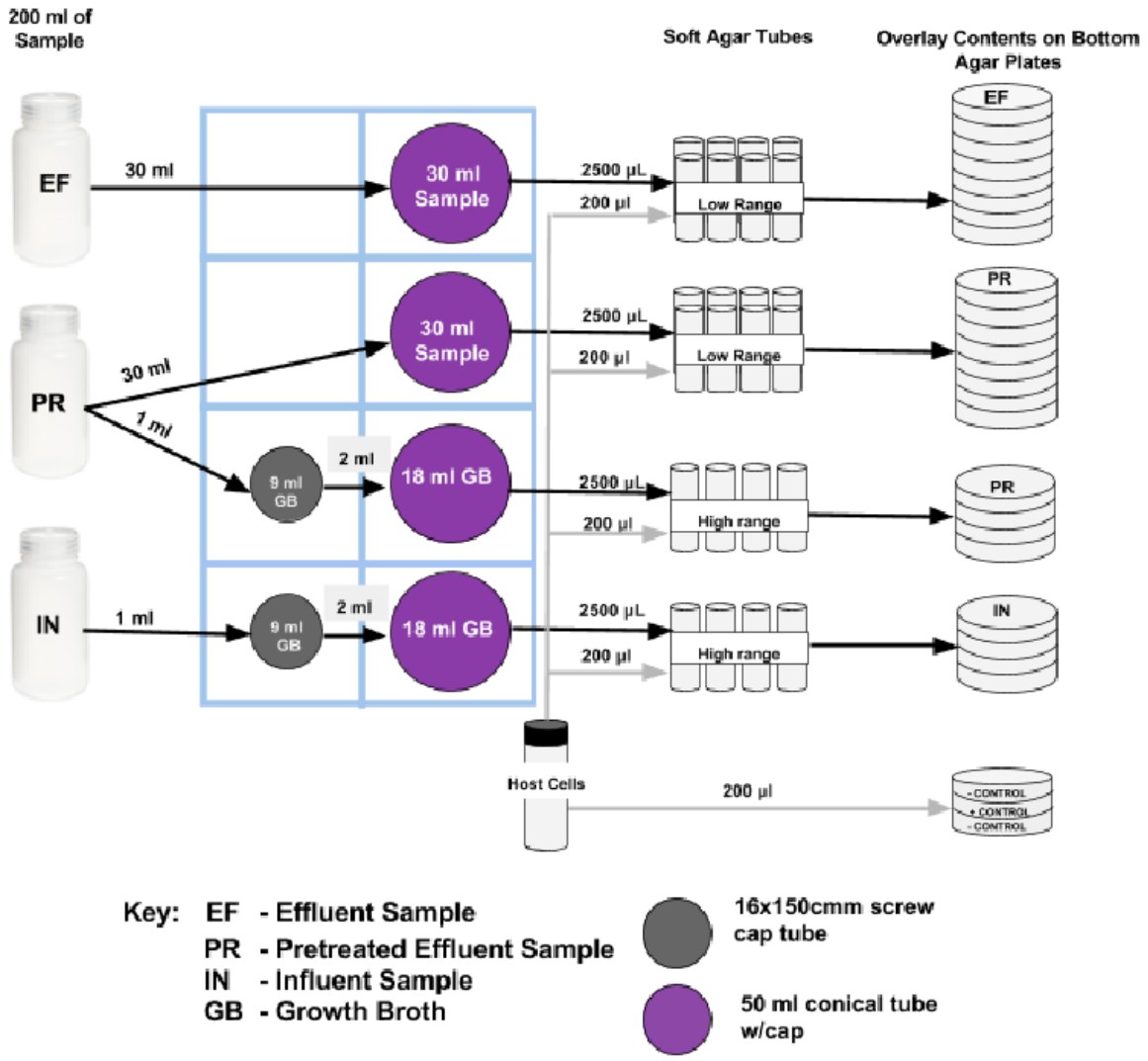
1. Sterile 250 or 500 ml Nalgene bottles (or comparable bottle) with a permanent fill mark at the approximate 200 or 400 ml level are recommended for pre-treated effluent, and final effluent.
2. Sample collection bottles or whirl Pak bags must be properly labeled with sample number, location, sample type, date and time.
3. When the WWTP uses chlorination, final effluent sample bottles must contain 0.2 ml of 10% sodium thiosulfate solution for every 200 ml to inactivate any residual chlorine.
4. Influent bottles must be suitable for volumes collected.

Sample 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 time for wastewater samples is 30 hours.

Appendix E. Protocol:

MSC Method for Wastewater Schematic:



Key: EF - Effluent Sample
PR - Pretreated Effluent Sample
IN - Influent Sample
GB - Growth Broth

16x150cmm screw cap tube

50 ml conical tube w/cap

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

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 sterile tube.

3. Aseptically transfer host strain growth from Bottom Agar streak plate to Growth Broth.

4. Gently invert 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-phase 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 0 - 4º C.

3. Sample bottle is shaken vigorously (25 times in a 12" arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) (ensure cap is tightened).

4. For the **high range** of this method a 10^{-2} decimal dilution is prepared by transferring 1ml of sample to 9 ml of growth broth. Dilution tube is then vortexed for 10 seconds. For the second decimal dilution, 2ml are transferred from the original sample tube to 18ml of growth broth. The dilution is then vortexed for 10 seconds.

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

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

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. 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.

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 to warm to room temperature immediately before analysis.

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

3. Vortex samples 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. 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 invert and incubate 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 under 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 300 for each plate,

PFU count exceeding 1,200/4 plate is considered TNTC or >1,200,000 PFU/100ml

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

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 to warm to room temperature immediately before analysis.

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 invert and incubate 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 under 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 2400 on the eight plates,

PFU count exceeding 2,400/8 plates is considered TNTC or >12,000 PFU/100ml

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

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

Result = (141)(5) = 705 PFU/100ml.*

Positive control plates are run with MSC analyses by adding MS2 for predictable plaque formation. Negative control plates are run with sterile growth broth at the start and end of each group of sample analysis.

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

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.

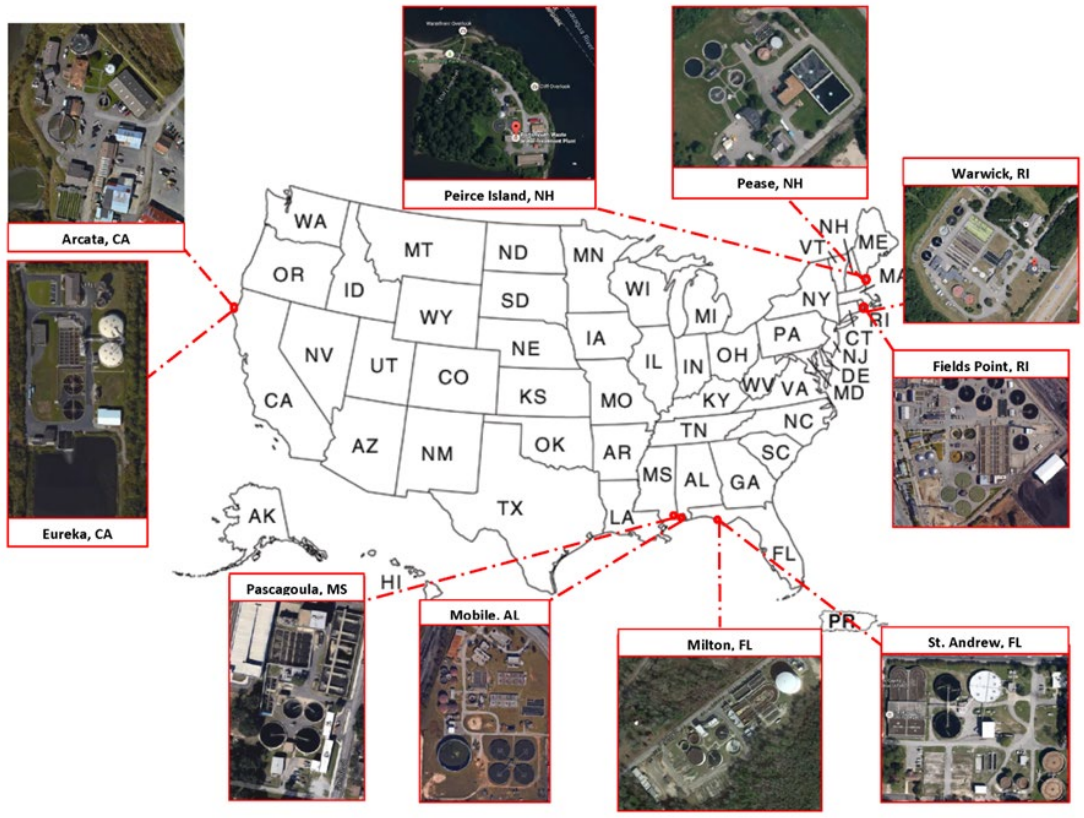
Glycerol Solution, 10%:

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.

Appendix F. Source of naturally contaminated samples:

For some experiments in the validation study, naturally contaminated samples were used. A geographic distribution of samples was obtained to represent possible matrix variability and/or interference. The table below provides the relevant metadata associated with each collection. The figure below provides a visual representation of the geographic distribution of samples.

Trial #	Location	Date	Plant Treatment	Plant Disinfection
1	Mobile, AL	07/01/2015	2°, mechanical	Chlorine
2	Pascagoula, MS	07/01/2015	2°, mechanical	Chlorine
3	Arcata, CA	07/13/2015	1° mechanical 2°, lagoon	Chlorine
4	Eureka, CA	07/13/2015	2°, mechanical	Chlorine
5	Milton, FL	07/21/2015	2°, mechanical	Chlorine
6	St. Andrew, FL	07/21/2015	2°, mechanical	Chlorine
7	Fields Point, RI	08/06/2015	2°, mechanical	Chlorine
8	Warwick, RI	08/06/2015	2°, mechanical	Chlorine
9	Peirce Island, NH	08/11/2015	1°, mechanical	Chlorine
10	Pease, NH	08/10/2015	2°, mechanical	Chlorine



Appendix G. Raw data from naturally contaminated samples:

Plant Location	Sample Type	Replicate #	MSC (PFU/100mL)	MSC (Log PFU/100mL)	RSD
Mobile, AL	Influent	1	304000	5.483	0.0630
			264000	5.422	
			76000	4.881	
		2	360000	5.556	0.0130
			260000	5.415	
			320000	5.505	
		3	284000	5.453	0.0200
			268000	5.428	
			180000	5.255	
		4	292000	5.465	0.0137
			252000	5.401	
			208000	5.318	
	Pre-treated Effluent	1	240	2.380	0.0459
			400	2.602	
			280	2.447	
		2	320	2.505	0.0484
			200	2.301	
			320	2.505	
	Effluent	1	120	2.079	0.0334
			160	2.204	
			160	2.204	
		2	160	2.204	0.1930
			160	2.204	
			1080	3.033	
Pascagoula, MS	Influent	1	284000	5.453	0.0075
			236000	5.373	
			264000	5.422	
		2	276000	5.441	0.0032
			296000	5.471	
			276000	5.441	
		3	320000	5.505	0.0184
			212000	5.326	
			312000	5.494	
		4	264000	5.422	0.0097
			216000	5.334	
			268000	5.428	
	Pre-treated Effluent	1	1360	3.134	0.0243
			1440	3.158	

			1040	3.017	
		2	1360	3.134	0.0176
			1320	3.121	
			1080	3.033	
	Effluent	1	240	2.380	0.0194
			240	2.380	
			200	2.301	
		2	400	2.602	0.0000
			400	2.602	
Arcata, CA	Influent	1	212000	5.326	0.0096
			188000	5.274	
			168000	5.225	
		2	140000	5.146	0.0101
			152000	5.182	
			120000	5.079	
		3	192000	5.283	0.0352
			84000	4.924	
			128000	5.107	
	Pre-treated Effluent	1	80	1.903	0.1270
			200	2.301	
			280	2.447	
		2	280	2.447	0.0834
			160	2.204	
			120	2.079	
		3	200	2.301	0.0000
			200	2.301	
			200	2.301	
Eureka, CA	Influent	1	1184000	6.073	0.0060
			1112000	6.046	
			1004000	6.002	
		2	820000	5.914	0.0101
			724000	5.860	
			952000	5.979	
		3	952000	5.979	0.0097
			752000	5.876	
			940000	5.973	
	Pre-treated Effluent	1	2560	3.408	0.0201
			3320	3.521	
			3440	3.537	
		2	3280	3.516	0.0031
			3120	3.494	

			3200	3.505	
		3	3000	3.477	0.0116
			3160	3.500	
			2640	3.422	
	Effluent	1	280	2.447	0.0814
			200	2.301	
			120	2.079	
		2	320	2.505	0.0640
			160	2.204	
			240	2.380	
		3	160	2.204	0.0543
			240	2.380	
Milton, FL	Influent	1	800000	5.903	0.0037
			812000	5.910	
			740000	5.869	
		2	940000	5.973	0.0033
			860000	5.934	
			884000	5.946	
		3	764000	5.883	0.0018
			788000	5.897	
			752000	5.876	
	Pre-treated Effluent	1	120	2.079	0.0000
			120	2.079	
			120	2.079	
		2	120	2.079	0.0952
			80	1.903	
			200	2.301	
		3	120	2.079	0.0965
			120	2.079	
			280	2.447	
St. Andrew, FL	Influent	1	144000	5.158	0.0209
			184000	5.265	
			112000	5.049	
		2	116000	5.064	0.0219
			176000	5.246	
			188000	5.274	
		3	212000	5.326	0.0204
			188000	5.274	
			132000	5.121	
	Pre-treated Effluent	1	360	2.556	0.0361
			280	2.447	

			240	2.380	
		2	480	2.681	0.0596
			240	2.380	
			360	2.556	
		3	600	2.778	0.0131
			520	2.716	
			520	2.716	
Fields Point, RI	Influent	1	552000	5.742	0.0056
			496000	5.695	
			572000	5.757	
		2	464000	5.667	0.0107
			412000	5.615	
			352000	5.547	
		3	328000	5.516	0.0120
			440000	5.643	
			412000	5.615	
	Pre-treated Effluent	1	120	2.079	0.1115
			240	2.380	
			400	2.602	
		2	520	2.716	0.0470
			360	2.556	
			640	2.806	
		3	440	2.643	0.0192
			440	2.643	
			360	2.556	
Warwick, RI	Influent	1	588000	5.769	0.0013
			596000	5.775	
			608000	5.784	
		2	552000	5.742	0.0103
			612000	5.787	
			724000	5.860	
		3	520000	5.716	0.0047
			512000	5.709	
			464000	5.667	
	Pre-treated Effluent	1	80	1.903	0.1545
			200	2.301	
			400	2.602	
		2	360	2.556	0.0550
			320	2.505	
			200	2.301	
		3	360	2.556	0.0466

			400	2.602	
			240	2.380	
Pease Int'l Tradeport, NH	Influent	1	188000	5.274	0.0392
			108000	5.033	
			76000	4.881	
		2	76000	4.881	0.0199
			96000	4.982	
			120000	5.079	
		3	140000	5.146	0.0178
			96000	4.982	
			100000	5.000	
Peirce Island, NH	Influent	1	728000	5.862	0.0043
			708000	5.850	
			652000	5.814	
		2	688000	5.838	0.0066
			620000	5.792	
			740000	5.869	
		3	640000	5.806	0.0062
			736000	5.867	
			636000	5.803	
	Pre-treated Effluent	1	22000	4.342	0.0196
			32400	4.511	
			29200	4.465	
		2	30000	4.477	0.0185
			21200	4.326	
			22400	4.350	
		3	19200	4.283	0.0157
			25600	4.408	
			24800	4.394	
	Effluent	1	2280	3.358	0.0160
			1960	3.292	
			1920	3.283	
			2480	3.394	
		2	2520	3.401	0.0053
			2360	3.373	
			2440	3.387	
			2600	3.415	
		3	2560	3.408	0.0175
			2080	3.318	
			2880	3.459	
			2360	3.373	

Appendix H. Spiked sample preparation:

For each of 10 validation trials, 150 ml of effluent sample free of target analyte (see Table below for origin) 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 ~10^3 MSC/ml. Four subsequent serial dilutions were made for each trial from the master spike at a 3:1 dilutions. The 5 dilution bottles were aseptically spiked with 5 ml of spike concentrations 1 through 5, shaken vigorously and then 4 aliquots of 30 ml were transferred into 4-50 ml sterile conical tubes for each spike concentration (three conical tubes for the replicates and a fourth tube for spike determination). In this way, three 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 as detailed in Appendix I. Effluent free of target analyte was used instead of growth broth for the spike determination. This strategy was employed as there is no standard method available for a truly independent spike determination.

Trial #	Date Sampled	WWTP	Treatment Process
1	4/11/17	Dover	Tertiary, UV Disinfection
2	4/11/17	Hampton	Secondary, Chlorine
3	4/18/17	Dover	Tertiary, UV Disinfection
4	4/18/17	Hampton	Secondary, Chlorine
5	4/24/17	Dover	Tertiary, UV Dis-infection
6	4/24/17	Hampton	Secondary, Chlorine
7	5/01/17	Dover	Tertiary, UV Disinfection
8	5/01/17	Hampton	Secondary, Chlorine
9	5/08/17	Dover	Tertiary, UV Disinfection
10	5/08/17	Hampton	Secondary, Chlorine

Appendix I. Spiked sample raw data:

Trial & Date	log of spike	Log of replicate plates	RSD	Log RSD
Trial 1 Dover 4/11/2017	3.531	3.613	0.0025	-2.603
		3.617		
		3.600		
	3.021	3.011	0.0134	-1.874
		3.088		
		3.072		
	2.498	2.389	0.0352	-1.453
		2.519		
		2.556		
	1.778	1.875	0.0629	-1.202
		1.778		
		1.653		
	1.000	1.477	0.2090	-0.680
		1.000		
		1.477		
Trial 2 Hampton 4/11/2017	3.775	3.740	0.0046	-2.340
		3.708		
		3.712		
	3.180	3.132	0.0051	-2.289
		3.135		
		3.161		
	2.613	2.352	0.0304	-1.517
		2.352		
		2.230		
	1.845	1.813	0.0204	-1.690
		1.740		
		1.778		
	1.398	1.398	0.0321	-1.494
		1.477		
		1.398		
Trial 3 Dover 4/18/2017	3.711	3.635	0.0063	-2.199
		3.681		
		3.658		
	2.991	3.035	0.0139	-1.856

		3.002		
		3.086		
	2.607	2.585	0.0241	-1.618
		2.498		
		2.618		
	1.875	1.813	0.0379	-1.422
		1.954		
		1.903		
	1.398	1.301	0.1072	-0.970
		1.398		
		1.602		
Trial 4 Hampton 4/18/2017	3.714	3.692	0.0065	-2.189
		3.724		
		3.740		
	3.053	3.107	0.0103	-1.986
		3.064		
		3.127		
	2.550	2.447	0.0317	-1.499
		2.525		
		2.607		
	1.602	1.778	0.0590	-1.229
		2.000		
		1.875		
	1.301	1.398	0.0634	-1.198
		1.477		
		1.301		
Trial 5 Dover 4/24/2017	4.064	4.028	0.0128	-1.891
		4.107		
		4.009		
	3.318	3.306	0.0212	-1.674
		3.423		
		3.437		
	2.720	2.833	0.0360	-1.444
		2.848		
		2.667		
	2.279	2.312	0.0179	-1.746
		2.267		

		2.230		
	1.301	1.653	0.2069	-0.684
		1.778		
		1.176		
Trial 6 Hampton 4/24/2017	4.087	4.047	0.0076	-2.121
		4.085		
		4.024		
	3.407	3.435	0.0058	-2.239
		3.400		
		3.401		
	2.695	2.744	0.0395	-1.403
		2.544		
		2.597		
	1.929	1.954	0.1082	-0.966
		2.041		
		1.653		
	1.301	1.301	0.1396	-0.855
		1.544		
		1.176		
Trial 7 Dover 5/1/2017	3.646	3.548	0.0131	-1.882
		3.640		
		3.610		
	3.015	3.041	0.0256	-1.592
		2.892		
		2.944		
	2.380	2.439	0.0188	-1.727
		2.362		
		2.362		
	1.813	1.903	0.1126	-0.949
		1.875		
		1.544		
	1.000	1.301	0.1305	-0.884
		1.176		
		1.000		
Trial 8 Hampton 5/1/2017	3.614	3.645	0.0147	-1.833
		3.751		
		3.721		

	3.051	2.980	0.0253	-1.596
		3.025		
		3.130		
	2.389	2.498	0.0336	-1.474
		2.653		
		2.512		
	1.602	1.544	0.1040	-0.983
		1.740		
		1.903		
	1.000	1.000	0.1580	-0.801
		1.000		
		1.301		
Trial 9 Dover 5/8/2017	3.539	3.442	0.0053	-2.273
		3.468		
		3.477		
	2.792	2.782	0.0140	-1.853
		2.860		
		2.813		
	2.267	2.322	0.0213	-1.672
		2.243		
		2.332		
	1.544	1.544	0.0694	-1.158
		1.398		
		1.602		
	0.699	1.000	0.3618	-0.442
		0.699		
		0.477		
Trial 10 Hampton 5/8/2017	3.584	3.543	0.0085	-2.073
		3.565		
		3.603		
	3.035	2.916	0.0148	-1.831
		2.851		
		2.932		
	2.371	2.243	0.0330	-1.482
		2.130		
		2.267		
	1.813	1.778	0.0386	-1.413

		1.740		
		1.875		
	0.699	1.000	0.1305	-0.884
		1.176		
		1.301		

Appendix J. Abbreviations and Technical Definitions:

°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 in lawn of host cells after incubation.

Attachment 1

PART IV – MSC Wastewater Analysis			
		4.1 Collection and Transportation of Samples	
C	**11**	☐	**4.1.1 Sample containers are of a suitable size to contain sample and to allow adequate headspace for proper shaking. Wastewater samples are collected in clean, sterile, watertight, properly labeled sample containers.**
C	**34**	☐	**4.1.2 ~~Effluent sample bottles must contain 0.2 ml of 10% sodium thiosulfate solution for 200 ml or 0.4 ml of 10% sodium thiosulfate solution for 400 ml to inactivate any residual chlorine~~Effluent sample bottles must contain 0.2 mL of 10% sodium thiosulfate solution for every 200 mL of sample to inactivate any residual chlorine.**
K	1	☐	4.1.3 Samples are identified with collector's name, sampling location, time and date of collection.
C	**9**	☐	**4.1.4 Immediately after collection, wastewater samples are placed in dry storage ice chest or equivalent) capable of maintaining a temperature of 0 to 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed in the refrigerator unless processed immediately.**
O	1		4.1.5 A temperature blank is used to represent the temperature of samples upon receipt at the laboratory. Temperature should be equivalent or less than that of the growing area waters. Results are recorded and maintained.
C	**9**	☐	**4.1.6 Analysis of the sample is initiated as soon as possible after collection. Wastewater samples are not tested if they have been held for more than 30 hours from the time of collection.**
		4.2 MSC Equipment and Supplies	
K	33	☐	~~4.2.1~~4.2.1 ~~50ml conical tubes are sterile and records maintained.~~ Wastewater samples or appropriately diluted samples are transferred into a sterile vessel.~~50 mL conical tubes.~~
K	9	☐	4.2.2~~2~~ The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
~~C~~K	~~27, 28~~1	☐	**4.2.3 The sterility of each batch/lot of pre-sterilized or reusable syringes, filters, and/or filter units is determined. Results are recorded and records maintained.~~4.2.3 Sterile 0.22 µm pore size syringe filters and pre-sterilized plastic or sterile glass syringes are used to sterilize the antibiotic solutions.~~**
K	1	☐	~~4.2.4 The sterility of each lot of pre-sterilized syringes and syringe filters is determined. Results are recorded and records maintained.~~
~~K~~	~~1~~	☐	~~4.2.5 The sterility of each batch of reusable glass syringes is determined. Results are recorded and records maintained.~~
C	**27, 28**	☐	**4.2.~~4~~6 The balance used provides a sensitivity of at least ~~mg (~~0.01g~~.~~).**
C	**27, 28**		**4.2.~~5~~7 The temperature of the incubator used is maintained at 36 ± 1°C.**
K	**2**		**4.2.6 T~~t~~he temperature of the freezer is maintained at <-15°C**
~~K~~	~~1~~		~~4.2.3 The sterility of each batch/lot of pre-sterilized or reusable syringes, filters, and/or filter units is determined. Results are recorded and records maintained.~~

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C	**28**	☐	**4.2.~~1~~78 The sterility of the transfer vessel utilized ~~Sterile~~ ~~disposable 50 mL centrifuge tubes~~ ~~are used, and their sterility~~ is determined with each lot. Results are recorded and records maintained.**
			4.3 MSC Media Preparation
K	~~28~~	☐	4.3.1 Media preparation and sterilization is according to the validated method.
K	27, 28		4.3.2 ~~Bottom agar, double strength soft agar and growth broth are prepared from their individual components.~~Antibiotic solutions are filter sterilized using sterile 0.22 um pore size filters.
~~K~~	~~27, 28~~	☐	~~4.3.3 Soft agar is prepared double strength in volumes of 2.5 mL.~~
~~C~~	~~27, 28~~		~~**4.3.4 The streptomycin and ampicillin solutions are added to tempered bottom agar and vortex mix for 2 minutes on stir plate.**~~
O	27, 28	☐	4.3.5 Storage of the bottom agar under refrigeration does not exceed ~~1 month~~. Six (6) weeks
K	27, 28	☐	4.3.6 Unsterilized soft agar is stored at ~~-20 °C~~ <-15°C for up to three (3) months.
K	27, 28 add ref?		4.3.7 The soft agar is ~~removed from the freezer and autoclave~~ sterilized ~~for 15 minutes at 121°C~~ for 15 minutes at 119°C to 124°C ~~121°C~~ before use.
K	27, 28	☐	4.3.8 Storage under refrigeration of prepared ~~of~~ growth broth ~~in the refrigerator~~ with screw-cap closures shall not exceed three (3) months, and with loose fitting closures shall not exceed one (1) month.~~in loosely capped tubes/bottles does not exceed 1 month and in screw capped tubes/bottles does not exceed 3 months.~~
K	27, 28	☐	4.3.9 Bottom agar plates stored under refrigeration are allowed to reach room temperature before use.
			4.4 ~~MSC Sample Analysis~~Preparation of Host Culture for MSC Analysis
C	**28**	☐	**4.4.1 E.coli *Famp* ATCC 700891 is the bacterial host strain ~~used in this procedure.~~**
K	27, 28	☐	4.4.2 Host cell growth broth is tempered at 36 ± 1°C ~~and vortexed (or shaken) to aerate prior to inoculation with host cells.~~ p~~P~~rior to inoculation with host cells.
K	27, 28	☐	4.4.3 Several host cell colonies are transferred to a tube of tempered~~, aerated~~ growth broth and incubated at 36 ± 1°C for 4-6 hours to provide host cells in log phase growth for sample analysis.
C	**27, 28**	☐	**4.4.4 After inoculation, the host cell growth broth culture is not shaken.**
			4.5 MSC Sample Analysis
C	**33**	☐	**4.~~4.5~~5.1 Wastewater sample is ~~first shaken vigorously for 20 seconds~~shaken vigorously (25 times in a 12" arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) and then diluted to the high range (10^{-2}) and/or low range (no dilution) with sterile room temperature growth broth based on sample type.**

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K	27, 28	☐	4.~~4.6~~5.2 The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28	☐	4.~~4.7~~5.3 Two hundred microliters (0.2 mL) of log phase host strain *E coli* is added to the ~~tempering~~ tempered soft agar immediately prior to adding the sample. ~~supernatant.~~
C	**27, 28**	☐	**4.~~4.8~~5.4 2.5 mL of sample is added to ~~each~~ a tube of ~~tempering~~ tempered soft agar.**
C	**27, 28**		**4.~~4.9~~5.5 The ~~soft agar/sample/host cell mixture~~ tube contents is gently rolled between the palms of the hands to mix.**
C	**27, 28**	☐	**4.~~4.10~~5.6 The ~~soft agar/sample/host cell mixture~~ tube contents ~~is~~ is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**
C			4.4.115.7 Four (4) plates are used for the analysis of Influent samples and high range Pretreated Effluent samples. Eight (8) plates are used for the analysis low range Pretreated Effluent and Effluent samples.
K	27, 28	☐	4.~~4.11~~25.8 Negative and positive control plates are prepared and accompany each set of samples analyzed. The results are recorded, and records maintained.
K	27, 28	☐	4.~~4.12~~35.9 Room Temperature ~~Sterile~~ Growth broth is used as the negative control or blank.
K	27, 28	☐	4.~~4.13~~45.10 Type strain MS2 (ATCC 15597-B1) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.
K	2	☐	4.~~4.14~~55.11 A negative control plate is plated at the ~~beginning and~~ end of each set of samples analyzed.
K	27, 28	☐	4.~~4.15~~6 5.12 The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	**27, 28**	☐	**4.~~4.16~~75.13 All plates are incubated at 36 ± 1°C for 18 ± 2 hours.**
		4.~~5~~ 6 Computation of Results - MSC	
C	**33**	☐	**4.~~5~~6.1 Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted. The countable plaque range of the method is 1 to 300 PFU per plate.**
C	**33**	☐	**4.~~5~~6.2 When there are no plaques on all 4 plates of high range, the count is <1000 PFU/100 mL. When there are no plaques on all 8 plates of low range, the count is <5 PFU/100 mL.**
K	28	☐	4.~~5~~6.3 The formula used for determining the density of MSC in PFU/100 mL is: High Range: N * 1000 = PFU/100mL; Low Range: N * 5 = PFU/100mL; Where N is the total number of plaques counted on all plates.
O	9	☐	4.~~5~~6.4 The MSC count is rounded off conventionally to give a whole number.

Attachment 1

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PUBLIC HEALTH SERVICE

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SHELLFISH LABORATORY EVALUATION CHECKLIST

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	**EMAIL:**
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**
OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K - Key O - Other NA- Not Applicable

Check the applicable analytical methods:

☐	Preparation of Samples for the Alkaline Phosphatase Probe Method: Direct Plating [PART III]
☐	Preparation of Samples for the Alkaline Phosphatase Probe Method: APW Enrichment and Colony Isolation [PART III]
☐	Alkaline Phosphatase Probe Hybridization [PART III] **- Note: Temperature ranges for hybridization can be expanded with an appropriate study as described in the method.**

PART I – QUALITY ASSURANCE			
			ITEM
Code	REF		
			1.1 Quality Assurance (QA) Plan
K	4, 6	☐	1.1.1 Written Plan (check those items which apply).
		☐	a. Organization of the laboratory.
		☐	b. Staff training requirements.
		☐	c. Standard operating procedures.
		☐	d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance and rejection criteria established.
		☐	e. Laboratory safety.
		☐	f. Internal performance assessment.
		☐	g. External performance assessment.
C	**4**	☐	**1.1.2 The QA plan is implemented.**
K	6	☐	1.1.3 The Laboratory participates in a Vibrio proficiency testing program annually. Specify the program(s): _____
			1.2 Educational/Experience Requirements
C	**State's Human Resources Department**	☐	**1.2.1 In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department	☐	1.2.2 In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**	☐	**1.2.3 In commercial laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology or equivalent discipline with at least two (2) years of laboratory experience.**
K	USDA Microbiology & EELAP	☐	1.2.4 In commercial laboratories, the analyst(s) must have at least a high school diploma and shall have at least three (3) months of experience in laboratory sciences.
			1.3 Work Area
O	4, 6	☐	1.3.1 Adequate for workload and storage.
K	6	☐	1.3.2 Clean, well-lighted.
K	6	☐	1.3.3 Adequate temperature control.
O	6	☐	1.3.4 All work surfaces are nonporous, easily cleaned and disinfected.
K	6	☐	1.3.5 Microbiological quality of the air is fewer than 15 colonies for a 15 minute exposure and determined monthly. The results are recorded and records maintained
			1.4 Laboratory Equipment
K	5	☐	1.4.1 To determine the pH of prepared media and reagents, the pH meter has a standard accuracy of at least 0.1 pH units.

K	9	☐	1.4.2 The pH electrodes being used consist of a pH half-cell and reference half-cell or equivalent combination electrode free from Ag/AgCl or contains an ion exchange barrier preventing passage of Ag ions into the solution which may affect the accuracy of the pH reading.
K	6	☐	1.4.3 The effect of temperature on the pH is compensated for by an internal/external ATC probe or by manual adjustment.
K	4	☐	1.4.4 The pH meter is calibrated daily or with each use. Results are recorded and records maintained.
K	6	☐	1.4.5 A minimum of two (2) standard buffer solutions is used to calibrate the pH meter. The first must be near the electrode isopotential point (pH 7). The second is near the expected sample pH (i.e., pH 4 or pH 10). Standard buffer solutions are used once and discarded.
K	4, 17	☐	1.4.6 Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope *(Circle the method used).*
K	5, 15	☐	1.4.7 The balances used provide a sensitivity of at least 0.01 g at the weights of use for direct plating and 0.1 g for MPN.
K	6	☐	1.4.8 Balance calibrations are checked monthly according to manufacturer specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance calibrations is verified at the weight range of use. Results are recorded and records maintained.
K	6	☐	1.4.9 Refrigerator temperatures are monitored at least once daily on workdays. Results are recorded and records maintained.
C	**12, 15**	☐	**1.4.10 Refrigerator temperatures in which AP-probes are stored are maintained between 2 and 8 ºC.**
K	1	☐	1.4.11 The temperature of general purpose refrigerators, those not containing AP-probes, are maintained between 0 and 4 ºC.
C	**2**	☐	**1.4.12 Freezer temperatures are maintained at -15 ºC or below.**
K	6	☐	1.4.13 Freezer temperature is monitored at least once daily on workdays. Results are recorded and records maintained.
C	**12**		**1.4.14 The temperature of the incubator is maintained at 35 ± 2.0 ºC.**
C	**6**	☐	**1.4.15 Working thermometers used in the air incubators are graduated in at least 0.5 ºC increments.**
K	5, 8	☐	1.4.16 Working thermometers are located on top and bottom shelves of use in the air incubator or appropriately placed based on the results of spatial temperature checks.
C	**6**	☐	**1.4.17 Temperature of the water bath is maintained appropriately under all loading conditions.**
C	**5**	☐	**1.4.18 Working thermometers used in the water bath are graduated in at least 0.1 ºC increments.**
K	4, 6	☐	1.4.19 Air incubator/water bath temperatures are taken twice daily on workdays. Results are recorded and records maintained.
C	**3**	☐	**1.4.20 All working thermometers are appropriately immersed.**

C	**5**	☐	**1.4.21 Working thermometers are either: calibrated mercury-in-glass thermometers, calibrated non-mercury-in-glass thermometers, or appropriately calibrated electronic devices, including Resistance Temperature Devises (RTDs) and Platinum Resistance Devices (PTDs).**
C	**5, 6**	☐	**1.4.22 A standards thermometer has been calibrated by NIST or a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at the points 0, 35, 42, 54 and/or 55 ºC (54 ºC for *Vibrio parahaemolyticus* and 55 ºC for *Vibrio vulnificus*). These calibration records (certificates of calibration) are maintained.**
K	3	☐	1.4.23 Standards thermometers are checked annually for accuracy by ice point determination. Results are recorded and maintained. Date of most recent determination: _____.
C	**5**	☐	**1.4.24 Either mercury-in-glass thermometers, non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury or low drift electronic resistance thermometers with at least an accuracy of ±0.05 ºC are used as the laboratory standards thermometer (*Circle the thermometer type used*).**
K	3, 8	☐	1.4.25 ~~All working thermometers are checked annually against the standards thermometer at the temperature(s) of use. Results for are recorded and records maintained.~~ The accuracy of working thermometers is checked annually against the standards thermometer either at the temperatures at which they are used or by ice point determination. Results are recorded and records maintained.
O	8	☐	1.4.26 Appropriate pipet aids are available and used to inoculate samples.
K	7	☐	1.4.27 Micropipettors are calibrated annually and checked for accuracy quarterly at volumes of use. Results are recorded and records maintained.
			1.5 Labware and Glassware Washing
K	5		1.5.1 Utensils and containers are clean borosilicate glass, stainless steel or other noncorroding material.
K	5	☐	1.5.2 Culture tubes are of a suitable size to accommodate the volume for nutritive ingredients and sample.
O	5	☐	1.5.3 Dilution bottles and tubes are made of borosilicate glass or plastic and closed with secure caps or screw caps with nontoxic liners.
K	5	☐	1.5.4 Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method of preparation is used to ensure the appropriate volumes of diluent.
C	**5**	☐	**1.5.5 Pipettes used to inoculate the sample deliver accurate aliquots, have unbroken tips and are appropriately graduated. Pipettes larger than 10 mL are not used to deliver 1 mL aliquots; nor, are pipettes larger than 1.1 mL used to deliver 0.1 mL aliquots.**

K	5	☐	1.5.6 In washing reusable pipets, glassware and labware, a succession of at least three (3) fresh water rinses plus a final rinse of deionized water is used to thoroughly rinse off all detergent.
C	**8**	☐	**1.5.7 An alkaline or acidic detergent is used for washing glassware/labware.**
C	**6**	☐	**1.5.8 With each load of labware/glassware washed, the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali) with aqueous 0.04% bromothymol blue (BTB) solution. Results are recorded, and records maintained.**
			1.6 Sterilization and Decontamination
K	5	☐	1.6.1 The autoclave is of sufficient size to accommodate the workload.
K	4	☐	1.6.2 Routine autoclave maintenance is performed, and the records are maintained.
C	**19, 20, 21 ~~6, 8~~**	☐	**1.6.3 The autoclave provides sterilization conditions suitable to the load contents. Sterilization temperature range may be 119ºC - 124ºC as determined by the lab's equipment Quality Assurance Verification Testing and recommended practices from the media manufacturer. Sterilization is determined for each load using a working-maximum registering thermometer, or an appropriate working temperature monitoring device.**
K	2, 5, 6	☐	1.6.4 An autoclave standards thermometer (or data logger) has been calibrated by a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at 121 °C. If in-house checks for accuracy of the standards thermometer will be conducted at the steam point, calibration of the autoclave standards thermometer at 100 °C is also recommended, but not required.
K	2, 10, 18	☐	1.6.5 The autoclave standards thermometer (or data logger) is checked every five (5) years for accuracy at either 121 °C by a qualified calibration laboratory; or, is checked in-house at the steam point (100 °C) if it has been previously calibrated at both 100 °C and 121 °C. Any change in temperature at the steam point changes the calibrated temperature at 121 °C by the same magnitude. Date of most recent determination: _____
K	2, 8	☐	1.6.6 Working autoclave thermometers (or data loggers) are checked against the autoclave standards thermometer at 121 ºC yearly. Date of last check: _____ Method: _____
K	6	☐	1.6.7 Spore strips/suspensions appropriate for use in an autoclave media cycle are used monthly according to manufacturer's instructions to evaluate the effectiveness of the sterilization process. Results are recorded, and the records maintained.
O	6	☐	1.6.8 Heat sensitive tape is used with each autoclave batch.

K	6, 8	☐	1.6.9 Autoclave sterilization records including the length of sterilization cycle, total heat exposure time and chamber temperature are maintained. Type of record: Autoclave log, computer printout or chart recorder tracings. (*Circle the appropriate type or types*)
K	5, 8	☐	1.6.10 For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperatures in the range of 160 to 180 ºC.

K	8	☐	1.6.11 Records of temperature and exposure times are maintained for the operation of the hot-air sterilizing oven during use.
K	8	☐	1.6.12 Spore strips/suspensions are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. Results are recorded, and records maintained.
K	5	☐	1.6.13 Reusable pipets are stored and sterilized in aluminum or stainless-steel containers.
K	5	☐	1.6.14 Reusable pipets (in canisters) are sterilized in a hot-air oven at 170 ºC for two (2) hours.
C	**2**	☐	**1.6.15 The sterility of reusable pipets is determined with each load sterilized. Results are recorded, and records maintained.**
C	**2**	☐	**1.6.16 The sterility of autoclave sterilized disposable pipet tips and microcentrifuge tubes is determined with each load sterilized. Results are recorded, and records maintained.**
C	**2**	☐	**1.6.17 The sterility of pre-sterilized disposable pipettes, pipet tips and microcentrifuge tubes is determined with each lot received. Results are recorded, and records maintained.**
K	8	☐	1.6.18 Spent broth cultures and agar plates are decontaminated by autoclaving for at least 30 minutes before conventional disposal.
			1.7 Media and Reagent Preparation
C	**12, 15**	☐	**1.7.1 Media and reagents are prepared from the individual components and pH adjusted appropriately, except in the case of TCBS, which is commercially dehydrated.**
K	1, 5, 8	☐	1.7.2 Dehydrated media, and media and reagent components are properly stored in a cool, clean, dry place.
K	1	☐	1.7.3 Media and components are labeled with the analyst's initials, date of receipt, date opened or date of preparation, if applicable (dye solutions).
C	**1, 2, 7**	☐	**1.7.4 Caked or expired media or components are discarded.**
C	**6**	☐	**1.7.5 Reagent water is distilled or deionized (*circle appropriate choice*), tested monthly and exceeds 0.5 megohms-cm resistivity (2 megohms-cm in-line) or is less than 2.0 µSiemens/cm conductivity at 25 ºC. (*Circle the appropriate water quality descriptor determined*). Results are recorded and the records maintained.**
C	**6**	☐	**1.7.6 Reagent water for media and diluent preparation is analyzed for residual chlorine monthly and is at a non-detectable level (≤0.1 mg/L). Results are recorded, and records maintained.** **Specify method of determination: _____**
K	6	☐	1.7.7 Reagent water for media and diluent preparation contains <100 CFU/mL as determined monthly using the heterotropic plate count method. Results are recorded, and records maintained.
K	12	☐	1.7.8 The volume and concentration of media (APW) in the tube is suitable for the amount of sample inoculated.
C	**2**	☐	**1.7.9 The total time of exposure of the sugar containing agar VVA to autoclave temperatures does not exceed 45 minutes. Total exposure time of APW and T1N3 agar does not exceed 60 minutes. TCBS, CC and mCPC are not autoclaved.**

C	**1**	☐	**1.7.10 Media and diluent sterility is determined for each load sterilized. Results are recorded, and records maintained.**
C	**1**	☐	**1.7.11 Media productivity is determined using media-appropriate positive and negative control cultures for each lot of dehydrated media received or with each batch of media prepared when the medium is made from its individual components.** **Positive *Vibrio parahaemolyticus* productivity control_____** **Negative *Vibrio parahaemolyticus* productivity control_____** **Positive *Vibrio vulnificus* productivity control_____** **Negative *Vibrio vulnificus* productivity control _____**
C	**6, 12**	☐	**1.7.12 The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer requirements and/or method tolerance. Results are recorded, and records are maintained.**
			1.8 Storage of Prepared Culture Media and Reagents
K	5	☐	1.8.1 Prepared culture media are stored in a cool, clean, dry place where excessive evaporation and the danger of contamination is minimized.
K	2	☐	1.8.2 Stored media are labeled with the storage expiration date or sterilization date.
K	2	☐	1.8.3 Storage of prepared culture media at room temperature does not exceed seven (7) days.
K	6	☐	1.8.4 Storage under refrigeration of prepared agar plates in sealed plastic bags shall not exceed two (2) weeks.
K	6	☐	1.8.5 Storage under refrigeration of prepared broth media with loose fitting closures shall not exceed one (1) month.
K	6	☐	1.8.6 Storage under refrigeration of prepared broth media and diluent with screw-cap closures shall not exceed three (3) months.
K	12, 15	☐	1.8.7 Refrigerated prepared plates are dried inverted before use to permit the sample to be completely absorbed into the medium to prevent colony spreading, for direct plating.
K	2, 6	☐	1.8.8 All prepared broth media and diluent stored under refrigeration are warmed to room temperature prior to use, at temperatures that do not exceed the medium's incubation temperature.
K	15	☐	1.8.9 Storage at room temperature of Lysis Solution, Ammonium Acetate Buffer, 20XSSC, 1XSSC/SDS, and 3XSSC/SDS for the hybridization procedure shall not exceed three (3) months.
K	15	☐	1.8.10 Storage under refrigeration of Hybridization Buffer for the hybridization procedure shall not exceed one (1) week.

C	**15**	☐	**1.8.11 NBT/BCIP solution and 1XSSC for the hybridization procedure should be made fresh the day of use.**
PART II – SHELLFISH SAMPLES			
			2.1 Sample Handling and Receipt
C	1, 5, 12, 15	☐	**2.1.1 A representative sample is collected and a chain of custody documenting the history of the sample(s) from collection to final disposal has been established.**
K	5, 15	☐	2.1.2 Shellfish samples are received in clean, waterproof, puncture resistant containers loosely sealed or are rejected for regulatory analysis.
K	1, 5	☐	2.1.3 Samples are received labeled with the collector's (or if PHP, company/processor and collector's) name, the source, the time and date of collection or are rejected for regulatory analysis.
C	**5, 12, 15**	☐	**2.1.4 Immediately after collection, samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10 ºC with ice or cold packs for transport to the laboratory or rejected. Direct contact of the shellfish with ice in the transport container should be avoided. Once received, the samples are placed under refrigeration unless processed immediately.**
K	5, 15	☐	2.1.5 If ice is used in sample transport, samples are rejected if melt water has come in contact with the samples.
C	**15**	☐	**2.1.6 Analysis of the samples is initiated as soon as possible after collection, but not to exceed 36 hours. If processing IQF samples, samples are defrosted under refrigeration for no longer than 36 hours once removed from the freezer.**
			2.2 Preparation of Samples for Analysis
K	2, 11	☐	2.2.1 Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2, 11	☐	2.2.2 Blades of shucking knives are not corroded.
K	5, 11	☐	2.2.3 The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2, 11	☐	2.2.4 The faucet used for rinsing the shellfish does not contain an aerator.
K	5, 11	☐	2.2.5 Shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
K	5, 11	☐	2.2.6 Shellfish are allowed to drain in a clean container or on clean towels prior to opening.
K	2, 5, 11	☐	2.2.7 Immediately prior to shucking, the hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol, or gloves are donned. The gloves, if worn, are latex, nitrile and/or stainless-steel mesh to protect analyst's hands from injury.
C	**5, 11**	☐	**2.2.8 Shellfish are not shucked through the hinge.**
C	**5, 11, 12, 15**	☐	**2.2.9 The contents of the shellfish (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
C	**12, 15**	☐	**2.2.10 A representative sample of 10 to 14 shellfish is used for analysis.**

C	**2, 11**	☐	**2.2.11 The quantity of meat and liquor is sufficient to cover the blender blades or additional shellfish are used in order to ensure sample homogeneity.**
K	5, 12, 13, 15	☐	2.2.12 Either a 1:1 dilution is made, or the sample is homogenized without dilution. If a dilution is made, the sample is weighed to the nearest 0.1 g and an equal amount, by weight, of diluent is added.
K	12, 14, 15	☐	2.2.13 Sterile phosphate buffered saline (pH 7.4) or alkaline peptone water (APW) is used as the sample diluent. If APW is used, sample analysis is conducted immediately.
C	**12, 15**	☐	**2.2.14 Samples are blended ~~at~~ for 90-120 seconds until homogenous.**
PART III – ALKALINE PHOSPHATASE PROBE METHOD FOR *VIBRIO VULNIFICUS* AND *VIBRIO PARAHAEMOLYTICUS* DETECTION IN SHELLFISH			
			3.1 Preparation of Samples for the Alkaline Phosphatase Probe Method: Direct Plating
C	**2, 12, 15**	☐	**3.1.1 For oyster samples, two tenths (0.20) of a gram of the initial 1:1 diluted homogenate (or 0.10 g of undiluted homogenate) and/or appropriate dilutions are used as inoculum. Dilutions are made in sterile PBS or APW. If APW is used, time from initial dilution until plating does not exceed 30 minutes.** **For samples other than oysters, 100 µl of the 1:10 dilution and/or subsequent dilutions should be used as inoculum.**
K	12, 15	☐	3.1.2 For analysis of total *V. parahaemolyticus*, at least one (1) T1N3 plate is inoculated to be probed for the *tlh* gene. For pathogenic *V. parahaemolyticus*, at least two (2) T1N3 plates are inoculated to be probed for the *tdh* gene. For analysis of *V. vulnificus*, at least one (1) VVA plate is inoculated to be probed for the *vvhA* gene.
K	12, 15	☐	3.1.3 Sterile cell spreaders are used to spread each inoculum evenly onto the dry T1N3 and/or VVA agar plates.
C	**2**	☐	**3.1.4 For *V. parahaemolyticus* analysis, a *tdh*+ *V. parahaemolyticus* culture diluted to <10^3 per ml is used as a positive process control. A non-*V. parahaemolyticus* culture is used as a negative process control.** **For *V. vulnificus* analysis, a *V. vulnificus* culture diluted to <10^3 per ml is used as a positive process control. A non-*V. vulnificus* culture is used as a negative process control.**
C	**2**	☐	**3.1.5 The process control cultures accompany the samples throughout incubation and hybridization and color development phases of the method. Results are recorded, and records are maintained.**
C	**12, 15**	☐	**3.1.6 Inoculated plates are incubated 16-24 hours at 35 ± 2 ºC. All plates are used for colony lifts and hybridization, except for those with confluent growth.**

			3.2 Preparation of Samples for the Alkaline Phosphatase Probe Method: APW Enrichment and Colony Isolation
K	11, 12	☐	3.2.1 Sterile phosphate buffered saline (PBS) is used as the sample diluent.
C	**12**	☐	**3.2.2 The 1:10 dilution is prepared gravimetrically with sterile PBS. All successive dilutions are prepared volumetrically.**
C	**12, 16**	☐	**3.2.3 Appropriate sample dilutions are inoculated into sterile APW.** **Specify dilution(s) used:_____** **Specify number of tubes per dilution:_____**
C	**2**	☐	**3.2.4 For *V. parahaemolyticus* analysis, a tdh+ *V. parahaemolyticus* culture diluted to <10^3 per ml is used as a positive process control. A non-*V. parahaemolyticus* culture is used as a negative process control.** **For *V. vulnificus* analysis, a *V. vulnificus* culture diluted to <10^3 per ml is used as a positive process control. A non-*V. vulnificus* culture is used as a negative process control.**
C	**2**	☐	**3.2.5 The process control cultures accompany the samples throughout incubation, isolation and confirmation. Results are recorded, and records are maintained.**
C	**12**	☐	**3.2.6 Inoculated APW enrichment tubes are incubated at 35 ± 2.0 ºC.**
C	**12**	☐	**3.2.7 Tubes are read after 18-24 hours of incubation. Clear tubes are negative. Turbid tubes are positive. Positive tubes are confirmed as *Vibrio parahaemolyticus* or *Vibrio vulnificus* as appropriate.**
K	12	☐	3.2.8 A loopful from the top one (1) cm of APW tubes showing growth is streaked onto TCBS for *V. parahaemolyticus* and mCPC or CC agars for *V. vulnificus* isolation.
C	**12**	☐	**3.2.9 TCBS plates are incubated at 35 ± 2 ºC and mCPC or CC plates are incubated at 35-40 ºC for 18-24 hours.**
C	**12**	☐	**3.2.10 Presumptive colonies are selected meeting these phenotypic characteristics:** **a. *V. parahaemolyticus* appear on TCBS agar as round, opaque, green or bluish colonies, two (2) to three (3) mm in diameter. Interfering large, opaque and yellow colonies are avoided.** **b. *V. vulnificus* appear on mCPC or CC agar as round, flat, opaque, yellow colonies, one (1) to two (2) mm in diameter. Typical positives have "fried egg" appearance. Purple/blue colonies are avoided.**

C	**12**	☐	**3.2.11 A sterile 96-well microtiter plate is filled with 100 µl/well of APW. Presumptive vibrios are picked from a selective agar plate using a sterile toothpick or wood transfer stick to individual wells. The plate is incubated 3-5 hours or overnight at 35 ± 2 °C. A 48-prong replicator is used to replicate/transfer isolates in the wells to an agar plate (T1N3 for *V. parahaemolyticus* and VVA for *V. vulnificus*).**
C	**12**	☐	**3.2.12 Plates are incubated at 35 ± 2 ºC for 18-24 hours.**
			3.3 Alkaline Phosphatase Probe Hybridization: Filter Preparation
C	**12, 15**	☐	**3.3.1 VVA/T1N3 plates are overlaid with labeled (sample number, dilution) #541 Whatman filters for one (1) to 30 minutes.**
K	12, 15	☐	3.3.2 Filters are transferred with colony side up to a plastic or glass Petri dish lid containing one (1) ml of lysis solution to wet the filter.
C	**12, 15**	☐	**3.3.3 Filters are microwaved to dryness, but not brown. Microwave for 15-30 seconds/filter, depending on the wattage of the microwave. Additional heating cycles may be required.**
K	12, 15	☐	3.3.4 Filters are neutralized for five (5) minutes in an appropriate vessel or container with ammonium acetate (4 ml/filter) on a shaker at room temperature.
C	**12, 15**	☐	**3.3.5 #541 Whatman filters are rinsed two (2) times in 1X SSC buffer (10 ml/filter) for 1-2 minutes. Filters may be air dried and stored at this point.**
C	**12, 15**	☐	**3.3.6 Up to 30 filters are incubated in proteinase K solution (10 ml/filter) for 30 minutes at 42 °C with shaking (~50 rpm).**
K	12, 15	☐	3.3.7 Filters are rinsed three (3) times in 1X SSC (10 ml/filter) for 10 minutes at room temperature with shaking at 50-125 rpm. Filters may be air dried and stored at this point.
			3.4 Alkaline Phosphatase Probe Hybridization: Hybridization.
C	**12, 15**	☐	**3.4.1 For total *V. parahaemolyticus* (*tlh*), the 5'AP-labeled probe 5'aa agc gga tta tgc aga agc act g 3' is used. For pathogenic *V. parahaemolyticus* (*tdh*), the 5'AP-labeled probe 5'gg ttc tat tcc aag taa aat gta ttt g 3' is used. For *V. vulnificus* (*vvhA*), the 5'AP-labelled probe 5'ga gct gtc acg gca gtt gga acc a 3' is used.**
C	**12, 15**	☐	**3.4.2 Probes are stored in the refrigerator and are not frozen.**
K	12, 15	☐	3.4.3 A maximum of five (5) filters to be hybridized with the same probe are added to a plastic bag.
C	**12, 15**	☐	**3.4.4 Filters are presoaked in 10-15 ml of hybridization buffer for 30 minutes at 54-± 0.1 ºC for *V. parahaemolyticus* (*tlh* and *tdh*) or 55 ± 0.1 °C for *V. vulnificus* with shaking.**
C	**12, 15**	☐	**3.4.5 Used buffer is discarded and 10 ml of fresh pre-warmed buffer per bag is added. Probe (final concentration of 0.5 pmol/ml) is quickly added to each bag and incubated for 1 hour at 54 ± 0.1 ºC for *Vibrio parahaemolyticus* or 55 ± 0.1 ºC for *Vibrio vulnificus* with shaking.**

K	15	☐	3.4.6 Filters are removed from the bag(s) and transferred to an appropriate vessel or container. Up to 30 filters hybridized with the same probe can be combined.
C	**12, 15**	☐	**3.4.7 Filters are rinsed two (2) times for 10 minutes each in 1X SSC – 1% SDS (for tlh and *Vibrio vulnificus*) or 3X SSC – 1% SDS (for tdh) (10 ml/filter) at 54 ± 0.1 ºC for *Vibrio parahaemolyticus* or 55 ± 0.1 ºC for *Vibrio vulnificus* with shaking.**
K	12, 15	☐	3.4.8 Filters are rinsed five (5) times for five (5) minutes each in 1X SSC (10 ml/filter) at room temperature with shaking.
			3.5 Alkaline Phosphatase Probe Hybridization: Color development.
C	**12, 15**	☐	**3.5.1 In a petri dish containing 20 ml of NBT/BCIP solution, filters (5 or fewer) are added and incubated with gentle shaking at room temperature, or at 35 ºC for faster results. The petri dish is kept covered to omit light.**
K	12, 15	☐	3.5.2 Color development of the positive control is checked every 30 minutes. Reaction time varies.
K	12, 15	☐	3.5.3 Filters are rinsed in tap or deionized/distilled water (10 ml/filter) three (3) times for 10 minutes each to stop color development.
C	**12, 15**	☐	**3.5.4 Reactions of test sample colonies are compared to the positive and negative process control cultures. Positive reactions appear as purple or brown spots, yellow spots are considered negative reactions. Filters are stored in the dark.**
			3.6 Alkaline Phosphatase Probe Hybridization: Computation of Results
C	**12, 15**	☐	**3.6.1 For direct plating, probe-positive colonies are counted and multiplied by the plated dilution factor of the sample to determine the concentration. Note that filter colonies must correspond to colonies visible on the agar plate.**
K	15	☐	3.6.2 For direct plating, results are reported as CFU/g of sample.
C	**12**	☐	**3.6.3 For APW enrichment, upon identification of probe-positive colonies refer to the original positive APW dilutions and record MPN value as derived in Appendix 2 of the FDA Bacteriological Analytical Manual (BAM).**
K	12, 16	☐	3.6.4 For APW enrichments, results are reported as MPN/g of sample or pass/fail in the case of PHP samples.

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LABORATORY:	**DATE OF EVALUATION:**

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

Page ___ of ___

LABORATORY:

Page	Item	Observation

Page ___ of ___

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
MICROBIOLOGICAL COMPONENT: (Part I-III)	
A. Results Total # of Critical (C) Nonconformities in Parts I-III Total # of Key (K) Nonconformities in Parts I-III Total # of Critical, Key and Other (O) Nonconformities in Parts I-III	 _____ _____ _____

B. Criteria for Determining Laboratory Status of the Microbiological Component:

1. **Does Not Conform Status**: The Microbiological component of this laboratory is not in conformity with NSSP requirements if:

   a. The total # of Critical nonconformities is **≥ 4 or**

   b. The total # of Key nonconformities is **≥ 13 or**

   c. The total # of Critical, Key and Other is **≥ 18**

2. **Provisionally Conforms Status**: The microbiological component of this laboratory is determined to be provisionally conforming to NSSP requirements if the number of critical nonconformities is **≥ 1 but ≤ 3**.

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before_____.

Laboratory Signature: _____ Date:_____

LEO Signature: _____ Date:_____

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5100 PAINT BRANCH PARKWAY
COLLEGE PARK, MD 20740-3835
TEL. 240- 402-2151/2055/4960 FAX 301-436-2601

SHELLFISH LABORATORY EVALUATION CHECKLIST

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:** **REGION:**
OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity it noted by a "√"**

C- Critical K - Key O - Other NA- Not Applicable

Check the applicable analytical methods:

☐	Multiple Tube Fermentation Technique for Seawater (APHA)[PART II]
	Multiple Tube Fermentation Technique for Seawater using MA-1 [PART II]
☐	Membrane Filtration Technique for Seawater using mTEC [PART II]
	Multiple Tube Fermentation Technique for Shellfish Meats (APHA)[PART III]
☐	Standard Plate Count for Shellfish Meats [PART III]
☐	Elevated Temperature Coliform Plate Method for Shellfish Meats [PART III]
☐	Male Specific Coliphage for Soft-shelled Clams and American Oysters [PART III]

PART 1 - QUALITY ASSURANCE				
CODE	**REF.**		**ITEM**	
K	8, 11	**1.1 Quality Assurance (QA) Plan**		
		☐	1.1.1	Written Plan (Check those items which apply.)
				a. Organization of the laboratory.
		☐		b. Staff training requirements.
				c. Standard operating procedures.
		☐		d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance, and rejection criteria established.
		☐		e. Laboratory safety.
		☐		f. Internal performance assessment.
				g. External performance assessment.
C	**8**	☐	**1.1.2**	**QA Plan Implemented.**
K	11		1.1.3	The Laboratory participates in a proficiency testing program annually. Specify Program(s)_____
		1.2 Educational/Experience Requirements		
C	**State's Human Resources Department**	☐	**1.2.1**	**In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department	☐	1.2.2	In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**	☐	**1.2.3**	**In commercial laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, or equivalent discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP	☐	1.2.4	In commercial laboratories, the analyst(s) must have at least a high school diploma and shall have at least three months of experience in laboratory sciences.
		1.3 Work Area		
O	8,11	☐	1.3.1	Adequate for workload and storage.
K	11		1.3.2	Clean, well-lighted.
K	11	☐	1.3.3	Adequate temperature control.
O	11	☐	1.3.4	All work surfaces are nonporous, easily cleaned and disinfected.
K	11	☐	1.3.5	Microbiological quality of the air is fewer than 15 colonies for a 15 minute exposure and determined monthly. The results are recorded and records maintained.
		1.4 Laboratory Equipment		
O	9	☐	1.4.1	To determine the pH of prepared media, the pH meter has a standard accuracy of 0.1 units.
O	14	☐	1.4.2	pH electrodes consisting of pH half-cell and reference half-cell or equivalent combination electrode free from (Ag/AgCl) or contains an ion exchange barrier preventing passage of Ag ions into the medium which may affect the accuracy of the pH reading.
K	11	☐	1.4.3	The effect of temperature on the pH is compensated for by an ATC probe or by manual adjustment.
K	8	☐	1.4.4	pH meter is calibrated daily or with each use Results are recorded and records maintained.
K	11	☐	1.4.5	A minimum of two standard buffer solutions is used to calibrate the pH meter. The first must be near the electrode isopotential point (pH 7). The second near the expected sample pH (i.e., pH 4 or pH 10). Standard buffer solutions are used once and discarded.
O	8,15	☐	1.4.6	Electrode acceptability is determined daily or with each use by the millivolt

			procedure or through determination of the slope. (*Circle the method used.*)
K	9	☐	1.4.7 Balance provides a sensitivity of at least 0.1 g at weights of use.
K	11,13	☐	1.4.8 Balance calibrations are checked monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use. Results are recorded and records maintained.
K	11	☐	1.4.9 Refrigerator temperature(s) are monitored at least once daily on workdays Results are recorded and records maintained.
K	1	☐	1.4.10 Refrigerator temperature is maintained at 0 to 4°C.
C	**9**	☐	**1.4.11 The temperature of the incubator is maintained at 35 ± 0.5°C.**
C	**11**	☐	**1.4.12 Thermometers used in the air incubator(s) are graduated in at least 0.1°C increments.**
K	9	☐	1.4.13 Working thermometers are located on top and bottom shelves or appropriately placed based on the results of spatial temperature checks.
C	**11**	☐	**1.4.14 Temperature of the waterbath is maintained at 44.5 ± 0.2°C under all loading conditions.**
C	**9**	☐	**1.4.15 The thermometers used in the waterbath are graduated in at least 0.1°C increments.**
C	**13**	☐	**1.4.16 The waterbath has adequate capacity for workload.**
K	9	☐	1.4.17 The level of water in the waterbath covers the level of liquid in the incubating tubes.
K	8, 11	☐	1.4.18 Air incubator/waterbath temperatures are taken twice daily on workdays. The results are recorded and records maintained.
C	**4**	☐	**1.4.19 All working thermometers are appropriately immersed.**
C	**29, 33**	☐	**1.4.20 Working thermometers are either: calibrated mercury-in-glass thermometers, calibrated non-mercury-in-glass thermometers ~~having the accuracy and tolerance of mercury,~~ or appropriately calibrated ~~low drift~~ electronic devices, including Resistance Temperature Devises (RTDs) and Platinum Resistance Devices (PTDs) ~~with an accursey of less than or equal to ≤ ±0.05°C~~. with an accuracy and tolerance appropriate for the application.**
C	**11**	☐	**1.4.21 A mercury-in-glass standards thermometer has been calibrated by NIST or a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at the points 0, 35 and 44.5°C (45.5°C for ETCP). These calibration records are maintained.**
K	9	☐	1.4.22 Standards thermometers are checked annually for accuracy by ice point determination. Results recorded and maintained. Date of most recent determination_____.
C	**29**	☐	**1.4.23 Either mercury-in-glass thermometers, non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury or low drift electronic resistance thermometers with an accuracy of ≤ ±0.05°C are used as the laboratory standards thermometer. (*Circle the thermometer type used.*)**
K	13		1.4.24 Incubator and waterbath working thermometers are checked annually against the standards thermometer at the temperatures at which they are used. Results are recorded and records maintained.
O	11	☐	1.4.25 Appropriate pipet aids are available and used to inoculate samples. Mouth pipetting is not permitted.
		1.5 Labware and Glassware Washing	
O	9	☐	1.5.1 Utensils and containers are clean borosilicate glass, stainless steel or other noncorroding materials.
K	9	☐	1.5.2 Culture tubes are of a suitable size to accommodate the volume for nutritive ingredients and samples.
K	9	☐	1.5.3 Sample containers are made of glass or some other inert material.

O	9	☐	1.5.4	Dilution bottles and tubes are made of borosilicate glass or plastic and closed with rubber stoppers, caps or screw caps with nontoxic liners.
K	9	☐	1.5.5	Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method is used to ensure appropriate volumes.
C	**9**	☐	**1.5.6**	**Pipettes used to inoculate the sample deliver accurate aliquots, have unbroken tips and are appropriately graduated. Pipettes larger than 10 mL are not used to deliver 1mL aliquots; nor, are pipets larger than 1.1mL used to deliver 0.1 mL aliquots.**
K	9	☐	1.5.7	Reusable sample containers are capable of being properly washed and sterilized.
K	9	☐	1.5.8	In washing reusable pipettes, a succession of at least three fresh water rinses plus a final rinse of distilled/deionized water is used to thoroughly rinse off all the detergent.
C	**2**	☐	**1.5.9**	**An alkaline or acidic detergent is used for washing glassware/labware.**
C	**11**		**1.5.10**	**With each load of labware/glassware washed the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali) with aqueous 0.04% bromothymol blue. Results are recorded and records maintained.**
		1.6 Sterilization and Decontamination		
K	9	☐	1.6.1	Autoclave(s) are of sufficient size to accommodate the workload.
O	8		1.6.2	Routine autoclave maintenance is performed and the records are maintained.
C	**11, 30**	☐	**1.6.3**	**The autoclave provides a sterilizing temperature of 121± 2°C as determined for each load using a calibrated maximum registering thermometer. As an alternative, an appropriate temperature monitoring device is used in place of the maximum registering thermometer when these are unavailable due to the ban on mercury.**
K	11	☐	1.6.4	An autoclave standards thermometer has been calibrated by a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at 121°C. Calibration at 100°C, the steam point, is also recommended but not required.
K	16	☐	1.6.5	The autoclave standards thermometer is checked every five (5) years for accuracy at 121°C by a qualified calibration laboratory; or, is checked in-house at the steam point (100°C) if it has been previously calibrated at both 100°C and 121°C. Any change in temperature at the steam point changes the calibrated temperature at 121°C by the same magnitude. Date of most recent determination_____
K	1	☐	1.6.6	Working autoclave thermometers are checked against the autoclave standards thermometer at 121°C yearly. Date of last check_____Method _____
K	11	☐	1.6.7	Spore strips/suspensions appropriate for use in an autoclave media cycle are used monthly according to manufacturer's instructions to evaluate the effectiveness of the sterilization process. Results are recorded and the records maintained.
O	11		1.6.8	Heat sensitive tape is used with each autoclave batch.
K	11, 13	☐	1.6.9	Autoclave sterilization records including length of sterilization, total heat exposure time and chamber temperature are maintained. Type of record: Autoclave log, computer printout or chart recorder tracings. (*Circle appropriate type or types.)*
K	11		1.6.10	For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperatures in the range of 160 to 180°C.
K	9	☐	1.6.11	A thermometer capable of determining temperatures accurately in the range of 160 to 180°C is used to monitor the operation of the hot-air sterilizing oven
K	13	☐	1.6.12	Records of temperatures and exposure times are maintained for the operation of the hot-air sterilizing oven during use.

K	11	☐	1.6.13 Spore strips/suspensions are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. Records are maintained.
K	11	☐	1.6.14 Reusable sample containers are sterilized for 60 minutes at 170°C in a hot-air oven or autoclaved for 15 minutes at 121°C.
C	**1**	☐	**1.6.15 The sterility of reusable sample containers is determined for each load sterilized. The results are recorded and the records maintained.**
C	**1**	☐	**1.6.16 The sterility of pre-sterilized disposable sample containers is determined for each lot received. Results are recorded and the records maintained.**
K	9	☐	1.6.17 Reusable pipettes are stored and sterilized in aluminum or stainless steel canisters.
K	9	☐	1.6.18 Reusable pipettes (in canisters) are sterilized in a hot-air oven at 170°C for 2 hours.
C	**2**	☐	**1.6.19 The sterility of reusable pipettes is determined with each load sterilized. Results are recorded and records maintained.**
C	**2**	☐	**1.6.20 The sterility of pre-sterilized disposable pipettes is determined with each lot received. Results are recorded and the records maintained.**
K	18	☐	1.6.21 Hardwood applicator transfer sticks are properly sterilized. Method of sterilization _____
C	**2**	☐	**1.6.22 The sterility of the hardwood applicator transfer sticks is checked routinely. Results are recorded and the records maintained.**
O	13	☐	1.6.23 Spent broth cultures and agar plates are decontaminated by autoclaving for at least 30 minutes before conventional disposal.
		1.7 Media Preparation	
K	3, 5	☐	1.7.1 Media is commercially dehydrated except in the case of medium A-1 which must be prepared from the individual components and modified MacConkey agar which may be prepared from its components.
K	11	☐	1.7.2 Media is prepared according to manufacturer's instructions.
O	11		1.7.3 Dehydrated media and media components are properly stored in a cool, clean, dry place.
O	11	☐	1.7.4 Dehydrated media are labeled with date of receipt and date opened.
C	**12**		**1.7.5 Caked or expired media or media components are discarded.**
C	**11**	☐	**1.7.6 Reagent water is distilled or deionized (circle appropriate choice), tested monthly and exceeds 0.5 megohm-cm resistance (2 megohms-cm in-line) or is less than 2.0 µSiemens/cm conductivity at 25°C. (C*ircle the appropriate water quality descriptor determined*.) Results are recorded and the records maintained.**
C	**11**	☐	**1.7.7 Reagent water is analyzed for residual chlorine monthly and is at a non-detectable level (< 0.1 mg/L). Results are recorded and the records maintained.** **Specify method of determination_____.**
K	11	☐	1.7.8 Reagent water contains <100 CFU/mL as determined monthly using the heterotrophic plate count method. Results are recorded and the records maintained.
K	11	☐	1.7.9 Media prepared from commercial dehydrated components are sterilized according to the manufacturer's instructions.
K	9	☐	1.7.10 The volume and concentration of media in the tube are suitable for the amount of sample inoculated.
C	**11**	☐	**1.7.11 Total time of exposure of sugar broths to autoclave temperatures does not exceed 45 minutes.**
C	**1**	☐	**1.7.12 Media sterility is determined for each load sterilized. Results are recorded and the records maintained.**
C	**1**	☐	**1.7.13 Media productivity is determined using media-appropriate, properly diluted positive and negative control cultures for each lot of dehydrated**

				media received or with each batch of media prepared when the medium is made from its individual components.
O	9	☐	1.7.14	Sterile phosphate buffered dilution water is used as the sample diluent.
K	11	☐	1.7.15	The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer's requirements. Results are recorded and records are maintained.
		1.8 Storage of Prepared Culture Media		
K	9	☐	1.8.1	Prepared culture media are stored in a cool, clean, dry space where excessive evaporation and the danger of contamination are minimized.
K	5,11	☐	1.8.2	Brilliant green bile 2% broth and A-1 media are stored in the dark.
K	13	☐	1.8.3	Stored media are labeled with the storage expiration date or the sterilization date.
K	9		1.8.4	Storage of prepared culture media at room temperature does not exceed 7 days.
K	2	☐	1.8.5	Storage under refrigeration of prepared culture media with loose fitting closures shall not exceed 1 month.
K	11	☐	1.8.6	Storage under refrigeration of prepared culture media with screw-cap closures does not exceed 3 months.
K	17	☐	1.8.7	All prepared MPN broth media stored under refrigeration must reach room temperature prior to use. Culture tubes containing any type of precipitate or Durham tubes containing air bubbles are discarded.
		PART II - SEAWATER SAMPLES		
		2.1 Collection and Transportation of Samples		
C	**11**	☐	**2.1.1**	**Sample containers are of a suitable size to contain at least 110 mL of sample and to allow adequate headspace for proper shaking. Seawater samples are collected in clean, sterile, watertight, properly labeled sample containers.**
K	1	☐	2.1.2	Samples are identified with collectors name, harvest area, sampling station, time and date of collection.
C	**9**	☐	**2.1.3**	**Immediately after collection, seawater samples are placed in dry storage (ice chest or equivalent) capable of maintaining a temperature of 0 to 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed in the refrigerator unless processed immediately.**
O	1		2.1.4	A temperature blank is used to represent the temperature of samples upon receipt at the laboratory. Temperature should be equivalent or less than that of the growing area waters. Results are recorded and maintained.
C	**9**	☐	**2.1.5**	**Analysis of the sample is initiated as soon as possible after collection. Seawater samples are not tested if they have been held for more than 30 hours from the time of collection.**
		2.2 Bacteriological Examination of Seawater by the APHA MPN		
C	**9**	☐	**2.2.1**	**Lactose broth or lauryl tryptose broth is used as the presumptive medium.** ***(Circle appropriate one.)***
C	**2**	☐	**2.2.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
C	**9**	☐	**2.2.3**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds) before inoculation.**
C	**9**	☐	**2.2.4**	**In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended).**
C	**6**	☐	**2.2.5**	**In a single dilution series not less than 12 tubes are used (for depuration at least 5 tubes are used).**
C	**6**	☐	**2.2.6**	**In a single dilution series, the volumes analyzed are adequate to meet the needs of routine monitoring.** **Sample volume inoculated _____**

				Range of MPN_____ **Strength of media used_____**
K	9	☐	2.2.7	Inoculated tubes are incubated in air at 35 ± 0.5°C.
C	**2**	☐	**2.2.8**	**Appropriately diluted process control cultures accompany the samples *throughout both the presumptive and confirmed phases of incubation.* Results are recorded and the records maintained.** **Positive process control_____Negative process control _____**
K	9	☐	2.2.9	Inoculated tubes are read after 24 ± 2 hours and 48 ± 3 hours of incubation and transferred at both time interval if positive for growth (the presence of turbidity) and gas or effervescence in the culture tube. These tubes are considered presumptive positive requiring further confirmatory testing.
		2.3 Confirmed Test for Seawater by APHA MPN		
C	**9**	☐	**2.3.1**	**Brilliant green bile 2% broth (BGB) is used as the confirmatory medium for total coliforms.**
C	**9**	☐	**2.3.2**	**EC medium is used as the confirmatory medium for fecal coliforms.**
C	**2**	☐	**2.3.3**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
K	9, 11	☐	2.3.4	Transfers are made to BGB/EC by either sterile loop or sterile hardwood transfer stick from positive presumptive tubes incubated for 24 and 48 hours as appropriate. *(Circle the method of transfer.)*
C	**9**	☐	**2.3.5**	**BGB tubes are incubated at 35 ± 0.5°C.**
K	9		2.3.6	BGB tubes are read after 48 ± 3 hours of incubation.
C	**9**	☐	**2.3.7**	**EC tubes are incubated in a circulating waterbath maintained at 44.5 ± 0.2°C.**
C	**9**	☐	**2.3.8**	**EC tubes are read after 24 ± 2 hours of incubation.**
C	**9**		**2.3.9**	**The presence of turbidity and any amount of gas or effervescence in the culture tube constitutes a positive test.**
		2.4 Computation of Results – APHA MPN		
K	9	☐	2.4.1	Results of multiple dilution tests are read from tables in *Recommended Procedures for the Examination of Sea Water and Shellfish*, Fourth Edition.
K	7	☐	2.4.2	Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**7, 9**	☐	**2.4.3**	**Results are reported as MPN/100 mL of sample.**
		2.5 Bacteriological Examination of Seawater by the MA-1 Method		
C	**5**	☐	**2.5.1**	**A-1 medium complete is used in the analysis.**
C	**2, 31**		**2.5.2**	**A-1 medium without salicin is used in the analysis. Comparability testing supports use of A-1medium without salicin. Study records are available.**
C	**5**	☐	**2.5.3**	**A-1 medium sterilized for 10 minutes at 121°C.**
C	**2**	☐	**2.5.4**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
C	**9**	☐	**2.5.5**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds) before inoculation.**
C	**9**	☐	**2.5.6**	**In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended).**
C	**6**	☐	**2.5.7**	**In a single dilution series at least 12 tubes are used.**

C	**6**	☐	**2.5.8 In a single dilution series, the volumes analyzed are adequate to meet the needs of routine monitoring.** **Sample volume inoculated _____** **Range of MPN _____** **Strength of media used _____**
C	**2**	☐	**2.5.9 Appropriately diluted process control cultures accompany the samples *throughout both resuscitation and waterbath incubation* Results are recorded and the records maintained.** **Positive process control_____Negative process control _____**
C	**2,5**	☐	**2.5.10 Inoculated tubes are placed in an air incubator at 35 ± 0.5°C for 3 ± 0.5 hours of resuscitation.**
C	**5**	☐	**2.5.11 After 3 ± 0.5 hours resuscitation at 35°C, inoculated tubes are incubated at 44.5 ± 0.2°C in a circulating waterbath for the remainder of the 24 ± 2 hours.**
C	**5**	☐	**2.5.12 The presence of turbidity and any amount of gas or effervescence in the culture tube constitutes a positive test.**
		2.6 Computation of Results – APHA MPN	
K	9	☐	2.6.1 Results of multiple dilution tests are read from tables in *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition.
K	7	☐	2.6.2 Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**7, 9**		**2.6.3 Results are reported as MPN/100 mL of sample.**
		2.7 Bacteriological Analysis of Seawater by Membrane Filtration (MF) using mTEC Agar - Materials and Equipment	
C	**23, 24**	☐	**2.7.1 When used for elevated temperature incubation in conjunction with ethafoam resuscitation, the temperature of the hot air incubator is maintained at 44.5 ± 0.5°C under any loading capacity.**
C	**23**	☐	**2.7.2 When using a waterbath for elevated temperature incubation, the level of the water completely covers the plates.**
C	**23**	☐	**2.7.3 Pre-sterilized plastic or sterile glass culture plates that are clear, flat bottomed, free of bubbles and scratches with tight fitting lids are used.**
C	**2**	☐	**2.7.4 The sterility of pre-sterilized culture plates is determined for each lot received. Results are recorded and the records maintained.**
K	11	☐	2.7.5 Colonies are counted with the aid of magnification.
C	**11, 23**		**2.7.6 Membrane filters are made from cellulose ester material, white, grid marked, 47 mm in diameter with a pore size of 0.45 µm and certified by the manufacturer for fecal coliform analyses.**
C	**2**		**2.7.7 Lot number, date of receipt and if provided the expiration date of the membrane filters are recorded and records maintained.**
C	**2**	☐	**2.7.8 When initiating monitoring by mTEC or switching brands or types of membrane filters used and no previous lots of filters are available for comparing acceptable performance, an appropriate method for determining the suitability of the lot is developed and the comparison testing implemented. The results are recorded and this record is maintained.**
K	2, 11	☐	2.7.9 New lots of membrane filters are checked by comparing recovery of fecal coliform organisms against membrane filters from previously acceptable lots.
C	**2**	☐	**2.7.10 The sterility of each lot or autoclave batch of membrane filters are checked before use.**
K	2	☐	2.7.11 Membrane filters which are beyond their expiration date are not used.
O	11		2.7.12 Forceps tips are clean.
O	11	☐	2.7.13 Forceps tips are smooth without pitting or corrugations to damage the filters being manipulated.

K	11	☐	2.7.14 Forceps are dipped in alcohol and flame sterilized between sample filters.
K	11		2.7.15 If indelible graduation marks are used on clear glass or plastic funnels to measure sample volumes, their accuracy is checked gravimetrically or with a Class A graduated cylinder before use and periodically rechecked. Funnels having a tolerance greater than 2.5% are not used. Checks are recorded and records maintained.
K	11	☐	2.7.16 Membrane filtration units are made of stainless steel, glass or autoclavable plastic free of scratches, corrosion and leaks.
C	**11**	☐	**2.7.17 Membrane filter assemblies are autoclave sterilized for 15 minutes at 121°C prior to the start of a filtration series.**
O	11, 23, 26	☐	2.7.18 A UV sterilization unit is used to disinfect filter assemblies between sample and filtration runs.
K	11	☐	2.7.19 The effectiveness of the UV sterilization unit is determined by biological testing monthly. Results are recorded and records maintained.
K	2	☐	2.7.20 Maintenance of the UV sterilization unit is performed as needed. This maintenance is documented and the records maintained.
		2.8 Media Preparation and Storage – MF using mTEC Agar	
K	11	☐	2.8.1 Phosphate buffered saline is used as the sample diluent and filter funnel rinse.
C	**11**		**2.8.2 The phosphate buffered saline is properly sterilized.**
K	23	☐	2.8.3 A sufficient amount of medium (4-5 mL) is used in each plate.
O	11		2.8.4 Refrigerated prepared plates are stored for no more than 2 weeks in sealed plastic bags or containers to minimize evaporation.
		2.9 Sample Analyses - MF using mTEC Agar	
C	**24**	☐	**2.9.1 mTEC agar is used.**
C	**2**	☐	**2.9.2 The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
C	**23**	☐	**2.9.3 The sample is shaken vigorously (25 times in a 12″ arc in 7 seconds) before filtration.**
C	**23**	☐	**2.9.4 The membrane is placed grid side up within the sterile filter apparatus.**
C	**23, 25**		**2.9.5 Sample volumes tested are consistent with the sampling regime employed (i.e., half log or other appropriate dilutions are used with systematic random sampling).**
C	**23**	☐	**2.9.6 Sample volumes are filtered under vacuum.**
K	26		2.9.7 The pressure of the vacuum pump does not exceed 15 psi.
C	**23, 26**	☐	**2.9.8 The sides of the filter funnel are rinsed at least twice with 20-30 mL of sterile phosphate buffered saline after sample filtration.**
C	**23**	☐	**2.9.9 The membrane filter is removed from the filtering apparatus with sterile forceps and rolled onto mTEC agar so that no bubbles form between the filter and the agar.**
C	**11**	☐	**2.9.10 Blanks are run at the beginning of filtration, after every 10th aliquot and at the end of the filtration run to check the sterility of the testing system (phosphate buffered saline, filter funnel, forceps, membrane filter, media and culture plate).**
C	**2, 11**	☐	**2.9.11 Appropriately diluted process control cultures accompany the samples throughout both resuscitation and elevated temperature incubation. Results are recorded and the records maintained.** **Positive process control_____Negative process control _____**
C	**11, 23, 24**	☐	**2.9.12 Inoculated plates are placed inverted into a watertight, tightly sealed container prior to being placed in the air incubator and incubated at 35 + 0.5°C for 2 hours of resuscitation. Alternatively inoculated plates may be placed in ethafoam prior to air incubation at 44.5 ± 0.5°C for 24 ± 2 hours.**

C	**11, 23, 24**	☐	**2.9.13 After 2 hours of resuscitation at 35°C, the watertight, tightly sealed containers are transferred to a circulating waterbath at 44.5 + 0.2°C, submerged completely and incubated for 22-24 hours.**
			2.10 Computation of Results - MF using mTEC Agar
C	**23**	☐	**2.10.1 All yellow, yellow-green or yellow-brown colonies are counted.**
C	**23**	☐	**2.10.2 Only plates having 80 or fewer colonies are counted. If it is unavoidable to use plates having more than 80 colonies, counts are given as >80 x 100/the volume of sample filtered.**
C	**2, 11, 23**	☐	**2.10.3 When multiple dilutions are filtered, the laboratory has developed a procedure for assessing the contribution of all positive dilutions to the final count.**
C	**23, 11**	☐	**2.10.4 The number of fecal coliforms is calculated by the following equation:** **Number of fecal coliforms per 100 mL = [number of colonies counted per plate used in the count / volume (s) of sample filtered in ml] x 100.**
C	**23, 11**	☐	**2.10.5 Results are reported as CFU/100 mL of sample.**
			PART III - SHELLFISH SAMPLES
			3.1 Collection and Transportation of Samples
C	**9**	☐	**3.1.1 A representative sample of shellstock is collected.**
K	9	☐	3.1.2 Shellstock samples are collected in clean, waterproof, puncture resistant containers loosely sealed.
K	9	☐	3.1.3 Shellstock samples are labeled with collector's name, type of shellstock, the source or harvest area, sampling station, time, date and place (if applicable) of collection.
C	**9**	☐	**3.1.4 Immediately after collection, shellfish samples are placed in dry storage(ice chest or equivalent) which is maintained between 0 and 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed under refrigeration unless processed immediately.**
C	**1**	☐	**3.1.5 Analysis of the samples is initiated as soon as possible after collection. Shellfish samples are not tested if the time interval between collection and analysis exceeds 24 hours.**
			3.2 Preparation of Shellfish for Examination
K	2,11	☐	3.2.1 Shucking knives, scrub brushes and blender jars are (autoclave) sterilized for 15 minutes prior to use.
O	2	☐	3.2.2 Blades of shucking knives are not corroded.
O	9	☐	3.2.3 The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2	☐	3.2.4 The faucet used for rinsing the shellstock does not contain an aerator.
K	9		3.2.5 Shellstock are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9	☐	3.2.6 Shellstock are allowed to drain in a clean container or on clean towels prior to opening.
K	9	☐	3.2.7 Immediately prior to shucking, the hands (or gloved hands) of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol.
C	**9**	☐	**3.2.8 Shellstock are not shucked directly through the hinge.**
C	**9**		**3.2.9 Contents of shellstock (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	9	☐	3.2.10 At least 200 grams of shellfish meat or a quantity of meat sufficient to cover the blender blades is used for the analysis.
K	9	☐	3.2.11 A representative sample of at least 12 shellfish is used for the analysis.
K	2	☐	3.2.12 The sample is weighed to the nearest 0.1 gram and an equal amount by weight of diluent is added.
O	9	☐	3.2.13 Sterile phosphate buffered dilution water is used as the sample diluent.

C	**9**	☐	**3.2.14**	**Samples are blended at high speed for 60 to 120 seconds until homogenous.**
K	9		3.2.15	APHA *Recommended Procedures for the Examination of Sea Water And Shellfish*, Fourth Edition is followed for the analysis of previously shucked and frozen shellfish meats.
		3.3 MPN Analysis for Fecal Coliform Organisms, Presumptive Test, APHA		
C	**9**	☐	**3.3.1**	**Appropriate strength lactose or lauryl tryptose broth is used as presumptive media in the analysis. *(Circle the medium used.)***
C	**2**	☐	**3.3.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
K	9	☐	3.3.3	Immediately (within 2 minutes) after blending, the ground sample is diluted and inoculated into tubes of presumptive media.
C	**9**	☐	**3.3.4**	**No fewer than 5 tubes per dilution are used in a multiple dilution MPN series.**
C	**9**	☐	**3.3.5**	**Allowing for the initial 1:1 dilution of the sample, appropriate portions are inoculated (i.e., 2 ml of original 1:1 dilution for the 1 g portion) and diluted for subsequent inoculation (i.e., 20 ml of 1:1 diluted sample to 80 ml of diluent or the equivalent for 0.1 g portion). All successive dilutions are prepared conventionally.**
K	6	☐	3.3.6	In a single dilution series, the volumes examined are adequate to meet the needs of routine monitoring. Sample volume inoculated _____ Range of MPN_____ Strength of media used_____
C	**2**		**3.3.7**	**Appropriately diluted process control cultures accompany the samples throughout both the presumptive and confirmed phases of incubation. Results are recorded and the records maintained.** **Positive Process control_____Negative Process control_____**
K	9	☐	3.3.8	Inoculated media are incubated at 35 ± 0.5°C.
K	10	☐	3.3.9	Tubes are read after 24 ± 2 hours of incubation and transferred if positive for growth (the presence of turbidity and gas or effervescence in the culture tube). These tubes are considered presumptive requiring further confirmatory testing.
		3.4 Confirmed Test for Fecal Coliforms - APHA		
C	**9**	☐	**3.4.1**	**EC medium is used as the confirmatory medium.**
C	**2**	☐	**3.4.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
K	9, 11	☐	3.4.3	Transfers are made to EC medium by either sterile loop or hardwood sterile transfer sticks from positive presumptives. *(Circle the method of transfer.)*
C	**9**	☐	**3.4.4**	**EC tubes are incubated in a circulating waterbath at 44.5 ± 0.2°C**
K	9		3.4.5	EC tubes are read for gas production after 24 ± 2 hours of incubation.
C	**9**	☐	**3.4.6**	**The presence of turbidity and any amount of gas and/or effervescence in the Durham tube constitutes a positive test.**
		3.5 Computation of Results for MPN Analyses		
K	9	☐	3.5.1	Results of multiple dilution tests are read from tables in *Recommended Procedure for the Examination of Sea Water and Shellfish,* 4th Edition and multiplied by the appropriate dilution factor.
K	7	☐	3.5.2	Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**9**	☐	**3.5.3**	**Results are reported as MPN/100 grams of sample.**

		3.6 Standard Plate Count Method		
O	20	☐	3.6.1	A standard plate count (SPC) analysis may be performed in conjunction with the analysis for fecal coliform organisms.
K	9	☐	3.6.2	In the standard plate count procedure at least four plates are used, duplicates of two dilutions. One of the dilutions should produce colonies of 30 to 300 per plate.
K	2	☐	3.6.3	Fifteen to 20 mL of tempered sterile plate count agar is used per plate.
C	**9**		**3.6.4**	**Agar tempering bath maintains the agar at 44-46°C.**
C	**9**	☐	**3.6.5**	**An agar based temperature control having a similar volume and shape as the tempering plate count agar is used in the tempering bath.**
K	9	☐	3.6.6	Samples or sample dilutions to be plated are shaken vigorously (25 times in a 12" arc in 7 seconds) before plating.
C	**9**	☐	**3.6.7**	**Not more than 1 mL nor less than 0.1 mL of sample or sample dilution is plated.**
K	11	☐	3.6.8	Control plates are used to check air quality and the sterility of the agar and the diluent.
K	9,21	☐	3.6.9	Solidified plates are incubated at 35 ± 0.5°C for 48 ± 3 hours inverted and stacked no more than four high.
K	9	☐	3.6.10	Quebec Colony Counter or its equivalent is used to provide the necessary magnification and visibility for counting plates.
K	1	☐	3.6.11	A hand tally or its equivalent is used for accuracy in counting.
		3.7 Computation of Results -SPC		
K	9	☐	3.7.1	Colony counts determined in accordance with Part III, A, Sections 4.31 through 4.33 in *Recommended Procedures for the Examination of Sea Water and Shellfish*, Fourth Edition.
C	**19**		**3.7.2**	**Colony counts are reported as CFU/g of sample.**
		3.8 Bacteriological Analysis of Shellfish Using the ETCP		
C	**2,3**	☐	**3.8.1**	**Prepared modified MacConkey agar is used on the day that it is made.**
K	3		3.8.2	Double strength modified MacConkey agar is used.
C	**3**	☐	**3.8.3**	**Prepared double strength modified MacConkey agar is heated to boiling, removed from the heat, and boiled again. This agar is never autoclaved.**
K	2, 3	☐	3.8.4	Twice boiled, double strength modified MacConkey agar and is maintained in a tempering bath at 45 to 50°C until used.
K	2, 3	☐	3.8.5	Phosphate buffered saline is used as the sample diluent in the ETCP.
C	**2, 3**		**3.8.6**	**The phosphate buffered saline is tempered at 45 - 50°C to prevent premature solidification of the agar.**
C	**9**	☐	**3.8.7**	**The sample homogenate is cultured within 2 minutes of blending.**
C	**2,3**		**3.8.8**	**Six grams of shellfish (12 grams of homogenate if initially diluted 1:1) is placed into a sterile container and the contents brought up to 60 mL with sterile, tempered phosphate buffered saline.**
K	3	☐	3.8.9	Sixty (60) mL of tempered, twice boiled double strength Modified MacConkey Agar is added.
K	2,3, 22	☐	3.8.10	The container is gently swirled or slowly inverted once to mix the contents, which are subsequently distributed uniformly over six plates.
C	**1**	☐	**3.8.11**	**Media and diluent sterility are determined with each use. Results are recorded and the records maintained.**
C	**1**	☐	**3.8.12**	**Media productivity is determined using media appropriate properly diluted pour plated positive and negative control cultures for each batch of Modified MacConkey agar prepared. Positive control culture_____Negative control culture _____**
C	**3, 13**	☐	**3.8.13**	**When solidified, the plates are placed inverted into an air incubator at 45.5 ± 0.5°C for 18 to 30 hours of incubation.**
C	**2**	☐	**3.8.14**	**Plates are stacked no more than three high in the incubator.**

C	**2**	☐	**3.8.15 Appropriately diluted pour plated process control cultures *accompany each set of samples throughout incubation.* The results are recorded and the records maintained.** **Positive process control_____Negative process control_____**
		3.9 Computation of Results - ETCP	
K	11	☐	3.9.1 Quebec Colony counter or its equivalent is used to provide the necessary magnification and visibility for counting.
O	1	☐	3.9.2 A hand tally or its equivalent is used to aid in counting.
C	**3, 6**	☐	**3.9.3 All brick red colonies greater than 0.5 mm in diameter are totaled over all the plates and multiplied by a factor of 16.7.**
C	**3**	☐	**3.9.4 Results are reported as CFU/100 grams of sample.**
		Bacteriological Examination of Soft-shelled Clams and American Oysters for Male Specific Coliphage (MSC)	
		3.10 MSC Equipment and Supplies	
K	30	☐	3.10.1 Sample containers used for the shucked sample are sterile, made of glass or some other inert material (i.e. polypropylene) and hold 100 – 125 mL.
C	**27, 28**	☐	**3.10.2 The refrigerated centrifuge used must have the capacity to accommodate the amount of shellfish sample required for the procedure, perform at 9000 x g and maintain a temperature of 4°C.**
K	9	☐	3.10.3 The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
C	**27, 28**	☐	**3.10.4 Sterile 0.22 µm pore size syringe filters and pre-sterilized plastic or sterile glass syringes are used to sterilize the antibiotic solutions.**
K	1	☐	3.10.5 The sterility of each lot of pre-sterilized syringes and syringe filters is determined. Results are recorded and records maintained.
K	1	☐	3.10.6 The sterility of each batch of reusable glass syringes is determined. Results are recorded and records maintained.
C	**27, 28**	☐	**3.10.7 The balance used provides a sensitivity of at least mg (0.01g.).**
C	**27, 28**		**3.10.8 The temperature of the incubator used is maintained at 36 ± 1°C.**
C	**28**	☐	**3.10.9 Sterile disposable 50 mL centrifuge tubes are used and their sterility is determined with each lot. Results are recorded and records maintained.**
		3.11 MSC Media Preparation	
K	28	☐	3.11.1 Media preparation and sterilization is according to the validated method.
K	27, 28		3.11.2 Bottom agar, double strength soft agar and growth broth are prepared from their individual components.
K	27, 28	☐	3.11.3 Soft agar is prepared double strength in volumes of 2.5 mL.
C	**27, 28**		**3.11.4 The streptomycin and ampicillin solutions are added to tempered bottom agar and vortex for 2 minutes on stir plate.**
O	27, 28	☐	3.11.5 Storage of the bottom agar under refrigeration does not exceed 1 month.
K	27, 28		3.11.6 Unsterilized soft agar is stored at -20 °C -15C for up to 3 months.
K	27, 28	☐	3.11.7 The soft agar is removed from the freezer and sterilized for 15 minutes at 121°C before use.
K	27, 28	☐	3.11.8 Storage of growth broth in the refrigerator in loosely capped tubes/bottles does not exceed 1 month and in screw capped tubes/bottles does not exceed 3 months.
K	27, 28	☐	3.11.9 Bottom agar plates are allowed to reach room temperature before use.
		3.12 Preparation of the Soft-Shelled Clams and American Oysters for MSC Analysis	
K	2,11	☐	3.12.1 Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2	☐	3.12.2 The blades of shucking knives are not corroded.
O	9	☐	3.12.3 The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2	☐	3.12.4 The faucet used for rinsing the shellfish does not contain an aerator.

K	9	☐	3.12.5 The shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9	☐	3.12.6 The shellfish are allowed to drain in a clean container or on clean towels unlayered prior to shucking.
K	9	☐	3.12.7 Immediately prior to shucking, the hands (or gloved hands) of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol.
C	**9**	☐	**3.12.8 Shellfish are not shucked through the hinge.**
C	**9**	☐	**3.12.9 The contents of shellfish (liquor and meat) are shucked into a sterile,tared blender jar or other sterile container.**
K	9	☐	3.12.10 A representative sample of at least 12 shellfish is used for the analysis.
K	2, 19	☐	3.12.11 The sample is weighed to the nearest 0.1 gram.
			3.13 MSC Sample Analysis
C	**28**	☐	**3.13.1 E.coli *Famp* ATCC 700891 is the bacterial host strain used in this procedure.**
K	27, 28	☐	3.13.2 Host cell growth broth is tempered at 36 ± 1°C and vortexed (or shaken) to aerate prior to inoculation with host cells.
K	27, 28	☐	3.13.3 Several host cell colonies are transferred to a tube of tempered, aerated growth broth and incubated at 36 ± 1°C for 4-6 hours to provide host cells in log phase growth for sample analysis.
C	**27, 28**	☐	**3.13.4 After inoculation, the host cell growth broth culture is not shaken.**
C	**28**		**3.13.5 A 2:1 mixture of sterile growth broth to shellfish tissue is used for eluting the MSC.**
C	**28**	☐	**3.13.6 The elution mixture is prepared w/v by weighing the sample and adding two equal portions of sterile growth broth by volume to the shellfish tissue.**
C	**28**	☐	**3.13.7 The elution mixture is homogenized at high speed for 180 seconds.**
C	**28**		**3.13.8 Immediately after blending, 33 grams of the homogenized elution mixture are weighed into centrifuge tubes.**
C	**28**	☐	**3.13.9 The homogenized elution mixture is centrifuged for 15 minutes at 9000 x g at 4°C.**
C	**27, 28**	☐	**3.13.10 The supernatant is pipetted off, weighed and the weight recorded.**
C	**27, 28**		**3.13.11 The supernatant is allowed to warm to room temperature about 20 to 30 minutes.**
K	27, 28	☐	3.13.12 The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28	☐	3.13.13 Two hundred microliters (0.2 mL) of log phase host strain *E coli* is added to the tempering soft agar immediately prior to adding the sample supernatant.
K	27, 28	☐	3.13.14 The sample supernatant is shaken or vortexed before being added to the tempering soft agar.
C	**27, 28**	☐	**3.13.15 2.5 mL of sample supernatant is added to each tube of tempering soft agar.**
C	**27, 28**		**3.13.16 The soft agar/sample supernatant/host cell mixture is gently rolled between the palms of the hands to mix.**
C	**27, 28**	☐	**3.13.17 The soft agar/sample supernatant/host cell mixture is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**
C	**28**	☐	**3.13.18 Ten (10) plates are used, 2.5 mL per plate for a total of 25 mL of supernatant analyzed per sample.**
K	27, 28	☐	3.13.19 Negative and positive control plates are prepared and accompany each set of samples analyzed. The results are recorded and records maintained. Positive control _____
K	27, 28	☐	3.13.20 Growth broth is used as the negative control or blank.
K	27, 28		3.13.21 Type strain MS2 (ATCC 15597) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.
K	2	☐	3.13.22 A negative control plate is plated at the beginning and end of each set of samples analyzed.

K	27, 28	☐	3.13.23 The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	**27, 28**	☐	**3.13.24 All plates are incubated at 36 ± 1°C for 18 ± 2 hours.**
		3.14 Computation of Results - MSC	
C	**27**	☐	**3.14.1 Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted.**
C	**28, 32**	☐	**3.14.2 The working range of the method is 1 to 200 PFU per plate. When there are no plaques on all ten plates, the count is <6 PFU/100 grams for soft-shelled clams, <7 PFU/ 100 grams for American oysters, and <5 PFU/ 100 grams for quahog (hard) clams. If the density exceeds 200 PFU per plate on all plates, the count is given as > 20,000 PFU/100 grams.**
K	28		3.14.3 The formula used for determining the density of MSC in PFU/100 grams is: (0.364) (N) (Ws), where N = total number of plaques counted on all 10 plates and Ws = weight of the supernatant used.
O	9	☐	3.14.4 The MSC count is rounded off conventionally to give a whole number.

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~~32.~~33. NIST Monograph 150 states "the accuracy attainable is principally limited by the characteristics of the thermometer itself."

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS

LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	

MICROBIOLOGICAL COMPONENT: (Part I-III)

A. Results	
Total # of Critical (C) Nonconformities in Parts I-III	_____
Total # of Key (K) Nonconformities in Parts I-III	_____
Total # of Critical, Key and Other (O) Nonconformities in Parts I-III	_____

B. Criteria for Determining Laboratory Status of the Microbiological Component:

1. **Does Not Conform Status**: The Microbiological component of this laboratory is not in conformity with NSSP requirements if:

   a. The total # of Critical nonconformities is **≥ 4 or**

   b. The total # of Key nonconformities is **≥ 13 or**

   c. The total # of Critical, Key and Other is **≥ 18**

2. **Provisionally Conforms Status**: The microbiological component of this laboratory is determined to be provisionally conforming to NSSP requirements if the number of critical nonconformities is **≥ 1 but ≤ 3**.

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before_____.

Laboratory Signature: _____ Date:_____

LEO Signature: _____ Date:_____

NSSP Form LAB-100 Microbiology Rev. October 2015

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240- 402-2151/2055/4960 FAX 301-436-2601
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**
OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: Conformity is noted by a "√"

C- Critical K - Key O - Other NA- Not Applicable

Check the applicable analytical methods:

	MPN Real-time PCR method for *Vibrio vulnificus* detection in Oysters [PART III] SmartCycler II
	MPN Real-time PCR method for *Vibrio parahaemolyticus* detection in Oysters [PART III] SmartCycler II and AB 7500 Fast

PART I – Quality Assurance			
		ITEM	
CODE	REF		
		1.1 Quality Assurance (QA) Plan	
K	4, 6	1.1.1 Written Plan (Check √ those items which apply).	
			a. Organization of the Laboratory.
			b. Staff training requirements.
			c. Standard operating procedures (SOPs).
			d. Internal quality control measures for equipment, their calibration maintenance, repair, performance and rejection criteria established.
			e. Laboratory safety.
			f. Internal performance assessment.
			g. External performance assessment.
C	**4**		**1.1.2 The QA plan is implemented.**
K	6		1.1.3 The Laboratory participates in a proficiency testing program annually. Specify theprogram(s): _____
		1.2 Educational/Experience Requirements	
C	**State's Human Resources Department**		**1.2.1 In state/county laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or equivalent discipline with at least two years of laboratory experience.**
K	State's Human Resources Department		1.2.2 In state/county laboratories, the analysts meet the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3 In commercial laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or equivalent discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4 In commercial laboratories, the analysts must have at least a high school diploma and at least three months of experience in laboratory sciences.
		1.3 Work Area	
O	4, 6		1.3.1 Adequate for workload and storage.
K	6		1.3.2 Clean, well lighted.
K	6		1.3.3 Adequate temperature control.
O	6		1.3.4 All work surfaces are nonporous, easily cleaned and disinfected.
K	6		1.3.5 Microbiological quality of the air contains fewer than 15 colonies/plate for a 15 minute exposure determined monthly. The results are recorded and records maintained.
		1.4 Laboratory Equipment	
K	5		1.4.1 To determine the pH of prepared media and reagents, the pH meter has a standard accuracy of 0.1 pH units.
K	9		1.4.2 pH electrodes consisting of pH half-cell and reference half-cell or equivalent combination electrode free from (Ag/AgCl) or contains an ion exchange barrier preventing passage of Ag ions into the medium which may affect the accuracy of the pH reading.
K	6		1.4.3 The effect of temperature on the pH is compensated for by an internal/external ATC probe or by manual adjustment (*Circle the appropriate type of adjustment*).
K	4		1.4.4 The pH meter is calibrated daily or with each use as per product literature. Results are recorded and records maintained.
K	6		1.4.5 A minimum of two standard buffer solutions are used to calibrate the pH meter. The first is near the electrode isopotential point (pH 7). The second is near the expected sample pH (i.e. pH4 or pH 10). Standard buffer solutions are used once and discarded.
O	4		1.4.6 Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope (*Circle the method used*).
K	5		1.4.7 The balances used provide a sensitivity of at least 0.1 g at the weights of use.

K	6		1.4.8 Balance calibrations are checked monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use. Results are recorded and records maintained.
K	6		1.4.9 Refrigerator temperatures are monitored at least once daily on workdays. Results are recorded and records maintained.
K	1		1.4.10 Refrigerator temperatures are maintained between 0 and 4 °C, except for reagent refrigerators which are maintained between 2 and 8 °C.
C	**7**		**1.4.11 Freezer temperature is maintained at -15 °C or below.**
O	7		1.4.12 Freezer temperature is monitored at least once daily on workdays. Results are recorded and records maintained.
C	**5**		**1.4.13 The temperature of the incubator is maintained at 35 +/- 2.0 °C.**
K	6		1.4.14 Thermometers used in the air incubators are graduated at no greater than 0.5 °C increments.
K	5		1.4.15 Working thermometers are located on top and bottom shelves of use in the air incubator or appropriately placed based on the results of spatial temperature checks.
K	4, 6		1.4.16 Air incubator temperatures are taken twice daily on workdays. Results are recorded and records maintained.
C	**3**		**1.4.17 All working thermometers are appropriately immersed.**
C	**~~2,~~ 20, 23**		**1.4.18 Working thermometers are either: ~~calibrated~~ mercury-in-glass thermometers,~~-calibrated~~ non-mercury-in-glass thermometers having the accuracy and tolerance of mercury, or ~~appropriately calibrated~~low drift electronic devices, including Resistance Temperature Devises (RTDs) and Platinum Resistance Devices (PTDs) with an accuracy of less than or equal to ≤ ±0.05°C.**
C	**6, 20**		**1.4.19 A standards thermometer has been calibrated by NIST or a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at the points 0 and 35. These calibration records are maintained.**
K	3, 5		1.4.20 Standard thermometers are checked annually for accuracy by ice point determination. Results are recorded and maintained. Date of most recent determination:_____
C	**2, 20**		**1.4.21 Either mercury-in-glass thermometers, non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury or low drift electronic resistance thermometers with an accuracy of ≤ 0.05 °C are used as the laboratory standards thermometer (*Circle the thermometer type used*).**
K	3, 8		1.4.22 All working thermometers are checked annually against the standards thermometer at temperature(s) of use. Results are recorded and records maintained.
O	6		1.4.23 Appropriate pipet aids are available and used to inoculate samples.
K	2		1.4.24 Micropipettors are calibrated annually at appropriate volumes used and checked for accuracy quarterly. Results are recorded and records maintained.
		1.5 Labware and Glassware Washing	
K	5		1.5.1 Utensils, containers, glassware and plasticware are clean borosilicate glass, stainless steel or other noncorroding material.
K	5		1.5.2 Culture tubes are new and of a suitable size to accommodate the volume for nutritive ingredients and sample.
K	5		1.5.3 Dilution bottles and tubes are made of borosilicate glass or plastic and closed with secure caps or screw caps with nontoxic liners.
K	5		1.5.4 Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method is used to ensure appropriate volumes.
K	5		1.5.5 In washing reusable pipets, glassware and labware, a succession of at least three fresh water rinses plus a final rinse of deionized water is used to thoroughly rinse off all detergent.
C	**2**		**1.5.6 An alkaline or acidic detergent is used for washing glassware/labware.**
C	**6**		**1.5.7 With each load of labware/glassware washed, the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali as appropriate) with aqueous 0.04% bromothymol blue (BTB) solution. Results are recorded and records maintained.**

		1.6 Sterilization and Decontamination	
K	5		1.6.1 The autoclave is of sufficient size to accommodate the workload.
K	4		1.6.2 Routine autoclave maintenance is performed and the records maintained.
C	**6, 20**		**1.6.3 The autoclave provides a sterilizing temperature of 121 ± 2 °C as determined for each load using a calibrated maximum registering thermometer. As an alternative, an appropriate temperature monitoring device is used in place of the maximum registering thermometer when these are unavailable due to the ban on mercury.**
K	6		1.6.4 An autoclave standards thermometer has been calibrated by a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at 121 °C. Calibration at 100 °C, the steam point is also recommended but not required.
K	10		1.6.5 The autoclave standards thermometer is checked every five years for accuracy at either 121 °C or at 100 °C, the steam point if the thermometer has been previously calibrated at this temperature. Date of most recent determination: _____
K	1		1.6.6 Working autoclave thermometers are checked against the autoclave standards thermometer at 121 °C yearly. Date of last check:_____
K	6		1.6.7 Spore strips/suspensions appropriate for use in an autoclave media cycle are used monthly according to manufacturer's instructions to evaluate the effectiveness of the sterilization process. Results are recorded and the records maintained.
O	6		1.6.8 Heat sensitive tape is used with each autoclave batch.
K	6		1.6.9 Autoclave sterilization records including length of sterilization, total heat exposure time and chamber temperature are maintained. Type of record: Autoclave log, computer printout or chart recorder tracings (*Circle the appropriate type or types*).
K	6		1.6.10 For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperatures in the range of 160 to 180 °C.
K	5		1.6.11 A thermometer capable of determining temperatures accurately in the range of 160 to 180 °C is used to monitor the operation of the hot air sterilizing oven.
K	8		1.6.12 Records of temperature and exposure times are maintained for the operation of the hot-air sterilizing oven.
K	6		1.6.13 Spore strips/suspensions appropriate for use in dry heat are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. Results are recorded and records maintained.
K	5		1.6.14 Reusable pipets are stored and sterilized in aluminum or stainless steel containers.
K	5		1.6.15 Reusable pipets (in canisters) are sterilized in a hot-air oven at 170 °C for 2 hours.
C	**2**		**1.6.16 The sterility of reusable pipets is determined with each load sterilized. Results are recorded and records maintained.**
C	**2**		**1.6.17 The sterility of autoclave sterilized disposable pipet tips and microcentrifuge tubes is determined with each load sterilized. Results are recorded and records maintained.** **If presterilized pipet tips and microcentrifuge tubes are purchased certificate should be maintained and sterility confirmed as in 1.6.18.**
C	**2**		**1.6.18 The sterility of presterilized disposable pipets, pipet tips and microcentrifuge tubes is determined with each lot received. Results are recorded and records maintained.**
K	8		1.6.19 Spent broth cultures and agar plates are properly decontaminated before disposal.
		1.7 Media Preparation	
K	13, 14		1.7.1 Alkaline peptone water (APW) is prepared from the individual components and pH adjusted appropriately.
K	6		1.7.2 Media components are properly stored in a cool dry place.
O	6		1.7.3 Media components are labeled with the analyst's initials, date of receipt and date opened.
O	6		1.7.4 Dehydrated media are labeled with date of receipt and date opened.

C	**6**		**1.7.5 Caked or expired media or media components are discarded.**
C	**6**		**1.7.6 Reagent water for media and diluent preparation is analyzed for residual chlorine monthly and is at a non-detectable level (≤0.1 ppm). Results are recorded and records maintained**
K	6		1.7.7 Reagent water for media and diluent preparation contains <100 CFU/mL as determined monthly using the heterotropic plate count method. Results are recorded and records maintained.
K	5		1.7.8 The volume and concentration of media in the tube is suitable for the amount of sample inoculated.
C	**6**		**1.7.9 Media broths are not in the autoclave for more than 60 minutes.**
C	**1**		**1.7.10 Media and diluent sterility is determined for each load sterilized. Results are recorded and records maintained.**
C	**1**		**1.7.11 Media productivity is determined using media-appropriate positive and negative control cultures for each lot of dehydrated media received or with each batch of media prepared when the medium is made from its individual components.**
C	**6**		**1.7.12 The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer requirements and/or method tolerance. Results are recorded and records are maintained.**
		1.8 Storage of Prepared Culture Media	
K	5		1.8.1 Prepared culture media are stored in a cool, clean, dry place where excessive evaporation and the danger of contamination is minimized.
K	8		1.8.2 Stored media are labeled with the storage expiration date or sterilization date.
K	5		1.8.3 Storage of prepared culture media at room temperature does not exceed 7 days.
K	2		1.8.4 Storage under refrigeration of prepared broth media with loose fitting closures does not exceed 1 month.
K	6		1.8.5 Storage under refrigeration of prepared culture media with screw- cap closures does not exceed 3 months.
K	11		1.8.6 All prepared broth media stored under refrigeration is warmed to room temperature prior to use, without exceeding incubation temperature.
PART II –Samples			
		2.1 Sample Collection, Transportation and Receipt	
C	**2, 6**		**2.1.1 A representative sample is collected and a chain of custody documenting the history of the sample(s) from collection to final disposal has been established.**
K	5		2.1.2 Shellfish samples as received are collected in clean, waterproof, puncture resistant containers loosely sealed or are rejected for regulatory analysis.
K	5		2.1.3 Shellfish samples as received are labeled with the collector's (or if PHP, company/processor and collector's) name, the source, the time and date of collection or are rejected for regulatory analysis.
C	**5**		**2.1.4 Immediately after collection, shellfish samples are placed in dry storage (ice chest or equivalent) which is maintained between 2 and 10 °C with ice or cold packs for transport to the laboratory. Once received, the samples are placed under refrigeration unless processed immediately.**
C	**1**		**2.1.5 Analysis of the samples is initiated as soon as possible after collection, but not to exceed 36 h. If processing IQF samples, samples are defrosted under refrigeration for no longer than 36 h once removed from the freezer.**
		2.2 Preparation of Samples for Analysis	
K	2, 6		2.2.1 Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes.
O	2		2.2.2 Blades of shucking knives are not corroded.
K	5		2.2.3 The hands of the analyst are thoroughly washed with soap and water or new gloves are donned, immediately prior to cleaning the shells of debris.
O	2		2.2.4 The faucet used for rinsing the shellfish does not contain an aerator.
K	5		2.2.5 Shellfish are scrubbed witha stiff, sterile brush and rinsed under tap water of drinking water quality.
K	5		2.2.6 Samples are allowed to drain in a clean container or on clean towels prior to opening
K	5, 15		2.2.7 Immediately prior to shucking, the hands or gloved hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol. The gloves if worn are latex, nitrile and/or stainless steel mesh to protect analyst's hands from injury.

C	**5**		**2.2.8 Shellfish are not shucked through the hinge.**
C	**5**		**2.2.9 The contents of the sample (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
C	**5**		**2.2.10 A representative sample of at least 12 shellfish is used for analysis**
C	**2, 5**		**2.2.11 A quantity of meat and liquor is sufficient to cover the blender blades or additional oysters are used in order to ensure sample homogeneity.**
K	2, 13		2.2.12 The sample can be processed directly or a 1:1 dilution of shellfish:diluent made. If a dilution is made, the sample is weighed to the nearest 0.1 g and an equal amount, by weight, of diluent is added.
K	13		2.2.13 Sterile phosphate buffered saline (pH 7.4) is used as the sample diluent.
C	**5**		**2.2.14 Samples are blended for 60 to 120 seconds until homogenous.**
PART III- PCR method for *Vibrio vulnificus* and *Vibrio parahaemolyticus* detection in Oysters			
		3.1 APW Enrichment	
K	5		3.1.1 Sterile phosphate buffered saline (PBS) is used as the sample diluent.
C	**5, 15**		**3.1.2 The 1:10 dilution is prepared gravimetrically with PBS. All successive dilutions are prepared volumetrically.** **For example, if an initial 1:1 dilution of the sample was used for blending, the 1:10 dilution is prepared by adding 20 g of sample homogenate to 80 ml of PBS. If the homogenate was not diluted, the 1:10 dilution is prepared by adding 10 g of sample homogenate to 90 ml of PBS.**
C	**17**		**3.1.3 Appropriate sample dilutions are inoculated into APW.** **Specify dilution(s) used_____ Specify number of tubes per dilution _____**
C	**2, 15**		**3.1.4 For *V. parahaemolyticus* analysis, a tdh+, trh+ *V. parahaemolyticus* culture diluted to <10^3 per ml is used as a positive process control. A non *V. parahaemolyticus* culture is used as a negative process control.** **For *V. vulnificus* analysis, a *V. vulnificus* culture diluted to <10^3 per ml is used as a positive process control. A non *V. vulnificus* culture is used as a negative process control.** **The process control cultures accompany the samples throughout incubation, isolation, and confirmation. Records are maintained.**
C	**13**		**3.1.5 Inoculated APW enrichment tubes are incubated at 35 +/- 2 °C.**
C	**13**		**3.1.6 Tubes are read after 18 – 24 hours of incubation. Clear tubes are negative. Turbid tubes are positive and shall be further processed.**
		3.2 PCR Reagents	
C	**14, 15**		**3.2.1 Lyophilized primers and probes are stored according to manufacturer's instructions.**
K	14, 15		3.2.2 Fluorescent probes are stored in light occluding tubes or containers.
C	**14, 15, 18, 19**		**3.2.3 The PCR forward and reverse primers and probes are appropriate for the platform.** **<u>For Total and Pathogenic Vp Real-time PCR Method</u>** tdh_269-20: 6FAM-5'-TGACATCCTACATGACTGTG-3'-MGBNFQ trh_133-23:NED/TET-5'-AGAAATACAACAATCAAAACTGA-3'-MGBNFQ tlh_1043: JOE / TEXAS RED-5'- CGCTCGCGTTCACGAAACCGT -3'-BHQ2 IAC_109: CY5-5'- TCTCATGCGTCTCCCTGGTGAATGTG -3'- BHQ2 trh_20F: 5'-TTGCTTTCAGTTTGCTATTGGCT-3' trh_292R: 5'-TGTTTACCGTCATATAGGCGCTT-3' tdh_89F:5'-TCCCTTTTCCTGCCCCC-3' tdh_321R: 5'-CGCTGCCATTGTATAGTCTTTATC-3' tlh_884F: 5'-ACTCAACACAAGAAGAGATCGACAA-3' tlh_1091R: 5'-GATGAGCGGTTGATGTCCAAA-3' IAC_46F: 5'-GACATCGATATGGGTGCCG-3'

			IAC_186R: 5'-CGAGACGATGCAGCCATTC-3' **<u>For Vv Real-time PCR Method</u>** vvhF 5'-TGTTTATGGTGAGAACGGTGACA-3' vvhR 5'-TTCTTTATCTAGGCCCCAAACTTG-3
C	**14, 18**		**3.2.4 Lyophilized forward and reverse primers, and probes, are hydrated with TE buffer to produce a 0.1 mM stock solution.**
C	**14, 18**		**3.2.5 Using molecular grade, nuclease free water, primer and probe stock solutions are diluted to produce a 0.01 mM working solution.**
C	**14, 18**		**3.2.6 Reconstituted primers and probes are stored in a -20 °C manual defrost freezer for up to 5 freeze thaw cycles, not to exceed two years.**
C	**21, 22**		**3.2.7 Platinum *Taq* DNA is stored in -20 °C manual defrost freezer until first use. After first use, can be stored between 2-8 °C.**
C	**21, 22**		**3.2.8 PCR reagents (dNTPs, buffer, MgCl2, fluorescent dyes) are stored in -20 °C manual defrost freezer until first use. After first use, they can be stored between 2-8 °C.**
		3.3 DNA Extraction	
C	**14, 18**		**3.3.1 All microcentrifuge tubes and pipet tips are sterile.**
C	**14, 18**		**3.3.2 Pipet tips have aerosol barriers.**
K	14, 18		3.3.3 Latex or nitrile gloves are worn throughout the extraction and PCR preparation process.
K	14, 18		3.3.4 All work surfaces, centrifuge racks and equipment used in PCR analysis are disinfected immediately prior to DNA extraction, Master Mix preparation and PCR analysis.
C	**14, 18**		**3.3.5 Aseptic technique is observed throughout the extraction and PCR analysis.**
C	**14, 18**		**3.3.6 One thousand (1000) µL aliquots from each positive APW enrichment tube, including the process controls, are extracted.**
C	**14, 18**		**3.3.7 Positive APW aliquots are placed in sterile microcentrifuge tubes and heated at 95-100 °C for 10 minutes.**
K	14, 18		3.3.8 A set of positive and negative process controls are included with each batch of samples in a heating block/boiling bath.
C	**14, 18**		**3.3.9 After boiling, tubes are chilled in ice or immediately frozen in a manual defrost freezer for future analysis. Boil preps may be refrigerated not to exceed 72 hours.**
K	14, 18		3.3.10 Frozen extracts are analyzed within 6 months of frozen storage.
		3.4 Preparation of the Master Mix for PCR	
C	**14, 16, 18**		**3.4.1 Nuclease-free microcentrifuge tubes and pipette tips, with filters, are used in Master Mix preparation.**
C	**14, 16, 18**		**3.4.2 For each reaction, add the specified amount of water, buffer, MgCl2, dNTPs, specific primers, nuclease probes, *Taq*, and internal control DNA is added.**
K	14, 21, 18		3.4.3 The Master Mix is gently vortexed to mix constituents and then briefly spun.
C	**14, 16, 18**		**3.4.4 Twenty-three (23) µL of Master Mix is used for each PCR reaction.**
C	**14, 16, 18**		**3.4.5 Master Mix must be used on the day of preparation or stored at –20 °C until time of use.**
		3.5 PCR	
C	**14, 19**		**3.5.1 If previously frozen, the DNA extracts are completely thawed at temperatures no warmer than room temperature. Immediately prior to use, DNA extracts are centrifuged at >5,000 x g for 2 minutes to remove particulate matter and cell debris.**
C	**14, 19**		**3.5.2 Two (2) µL of DNA template is added to each reaction tube or plate well containing 23 µL of Master Mix for a total PCR reaction volume of 25 µL.**
K	14, 19		3.5.3 Two (2) µL of molecular grade, nuclease free water is added to a reaction tube or plate well containing 23 µL of Master Mix for each batch of Master Mix prepared as a no template control.
C	**14, 19**		**3.5.4 Two (2) µL of DNA template extracted from the negative process control culture is added to a reaction tube or plate well containing 23 µL of Master Mix.**
C	**14, 19**		**3.5.5 Two (2) µL of DNA template extracted from the positive process control culture is added to a reaction tube or plate well containing 23 µL of Master Mix**.

O	14, 19		3.5.6 Two (2) µL of DNA template extracted from the positive control culture (prepared separately from the positive process control) is added to a reaction tube or plate well containing 23 µL of Master Mix as the positive PCR control.
K	14, 19		3.5.7 Immediately prior to loading the reaction tubes or plates into the instrument they are centrifuged for 3-5 seconds to ensure that all reagents and the DNA template are in the bottom of the tube to optimize the PCR amplification process.
C	16		**3.5.8 After centrifugation, tubes or plates are inserted into the instrument.**
		3.6 PCR Amplification	
C	**14, 19**		**3.6.1 The appropriate instrument platform is used for the protocol.**
K	16		3.6.2 Manufacturer's instructions are followed in operating the instrument.
C	**14, 19**		**3.6.3 The PCR cycle parameters used are appropriate for the protocol.**
K	14, 19		3.6.4 Optical calibrations for the dyes being used are current, per the instrument manufacturer's recommendations.
C	**14, 19**		**3.6.5 The analysis settings are adjusted as specified in the protocol.**
		3.7 Computation of Results	
K	14, 19		3.7.1 All runs in which the positive control generates a Ct value for the target(s) of interest and the negative control reaction generates no Ct value for the target(s), but a Ct value for the internal control are considered valid.
C	**2**		**3.7.2 Data is quality checked by the analyst.**
C	**14, 19**		**3.7.3 All reactions in a valid run which generate a Ct value for the target(s) of interest with a sigmoidal amplification curve are considered to be positive.**
C	**16**		**3.7.4 Any sample which does not demonstrate a sigmoidal amplification curve may have a reported positive/negative determination that is discrepant from the instrument if appropriately justified using the raw fluorescent data.**
K	16		3.7.5 All reactions in a valid run which do not generate a Ct value for the target(s) of interest, but do generate a Ct value for the internal control are considered negative.
C	**16**		**3.7.6 Any reaction in which no Ct value is generated for the target(s) of interest or the internal control is considered invalid and should be re-tested.**
C	**13**		**3.7.7 Upon determination of positive reactions, refer to the original positive dilutions of APW and record MPN values as derived from the calculator in Appendix 2 of the FDA Bacteriological Analytical Manual (BAM).**
K	13		3.7.8 For APW enrichment, results are reported as MPN/g of sample.

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LABORATORY:	DATE of EVALUATION:

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY of NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORYSTATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
MICROBIOLOGICAL COMPONENT: (Part I-III)	
A. Results Total # of Critical (C) Nonconformities in Parts I-III Total # of Key (K) Nonconformities in Parts I-III Total # of Critical, Key and Other (O) Nonconformities in Parts I-III	

B. Criteria for Determining Laboratory Status of the Microbiological Component:

1. **Does Not Conform Status**: The Microbiological component of this laboratory is not in conformity with NSSP requirements if:

   a. The total # of Critical nonconformities is **≥ 4 or**_____

   b. The total # of Key nonconformities is **≥ 13 or**_____

   c. The total # of Critical, Key and Other is **≥18** _____

2. **Provisionally Conforms Status**: The microbiological component of this laboratory is determined to be provisionally conforming to NSSP requirements if the number of critical nonconformities is **≥ 1**

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory
Evaluation Officer on or before_____.

Laboratory Signature: _____ Date:_____

LABORATORY:

Page	Item	Observation

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240-402-2151/2055/4960 FAX 301-436-2601

SHELLFISH LABORATORY EVALUATION CHECKLIST
Diarrhetic Shellfish Poisoning Toxins (DSP) LC-MS/MS

LABORATORY:		
ADDRESS:		
TELEPHONE:	FAX:	EMAIL:
DATE OF EVALUATION:	DATE OF REPORT:	LAST EVALUATION:

LABORATORY REPRESENTED BY:	TITLE:
LABORATORY EVALUATION OFFICER:	SHELLFISH SPECIALIST:
OTHER OFFICIALS PRESENT:	TITLE:

Items which do not conform are noted by:

C – Critical K - Key O - Other NA - Not Applicable Conformity is noted by a "'1"

PART I – QUALITY ASSURANCE			
Code	REF		Item Description
			1.1 Quality Assurance (QA) Plan
K	1, 7, 8		1.1.1 Written Plan adequately covers all the following: (check those that apply) a. Organization of the laboratory b. Staff training requirements c. Standard operating procedures d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance and rejection criteria established e. Laboratory safety f. Internal performance assessment g. External performance assessment
C	5		**1.1.2 QA Plan is implemented.**
			1.2 Educational/Experience Requirements
C	State's Human Resources Department		**1.2.1 In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2 In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	USDA Microbiology & EELAP		**1.2.3 In commercial/private laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, chemistry, or another appropriate discipline with at least two (2) years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4 In commercial/private laboratories, the analyst must have at least a high school diploma and shall have at least three (3) months of experience in laboratory sciences.
C	3		**1.2.5 LC-MS Operator must be trained in the operation and maintenance of the specific liquid chromatography-mass spectrometry system used.**
			1.3 Work Area
O	1		1.3.1 Adequate for workload and storage.
O	1		1.3.2 Clean and well lighted.
O	1		1.3.3 Adequate temperature control.
O	8		1.3.4 All work surfaces are nonporous and easily cleaned.

			1.4 Laboratory Equipment
C	**3**		**1.4.1 A heat block or water bath capable of heating samples to 76 ± 2 °C.**
K	2		1.4.2 Balances provide an appropriate sensitivity at the weights of use, at least 0.1 g for laboratory precision balances and 0.1 mg for analytical balances.
K	7, 8		1.4.3 The balance calibration is checked monthly using NIST class S, ASTM class 1 or 2 weights or equivalent. Results are recorded and records are maintained.
K	1		1.4.4 Refrigerator temperature is maintained between 0 and 4 ºC.
K	7		1.4.5 Refrigerator temperature is monitored at least once daily. Results are recorded and records maintained.
K	2		1.4.6 Freezer temperature is maintained at -10 ºC or below.
K	7		1.4.7 Freezer temperature is monitored at least once daily. Results are recorded and records maintained.
C	**10**		**1.4.8 All in-service thermometers are properly calibrated and immersed.**
K	4		1.4.9 All glassware is clean.
K	3, 12		1.4.10 An ultra-performance liquid chromatography system (UPLC) equipped with the following is used: a. mobile phase system capable of delivering a pulse-free flow of 0.12 mL/min b. solvent degasser (optional) c. autosampler (refrigerated preferred) with loop suitable for five (5) µL injections d. column compartment capable of controlling temperature at 40 ºC e. a data collection system (e.g., computer, integrator)
C	**3**		**1.4.11 A mass spectrometer equipped with the following is used:** **a. an electrospray ionization source operating in negative ion mode and** **b. multiple reaction monitoring scan mode capability.** **c. if a divert valve is used to divert LC flow at the beginning and end of each chromatographic run, the switching time should be at least one minute before the first peak elution and at least one minute after the last peak elution.**
K	2		1.4.12 Autopipettors are calibrated for the appropriate volumes used and checked annually for accuracy. Results are recorded and records are maintained.
K	3		1.4.13 A centrifuge capable of generating 2000 x g and holding 15 mL and 50 mL polypropylene tubes is used.

			1.5 Reagents and Reference Solution Preparation and Storage
C	**3**		**1.5.1 All solvents and reagents used are analytical or LC grade materials.**
O	7		1.5.2 Water contains < 100 CFU/ml determined monthly using the heterotrophic plate count method. Results are recorded and records are maintained. (Not required for bottled reagent grade or HPLC grade water when used immediately upon opening. If the bottle of water is not used entirely immediately, the water must be tested as above prior to continued use.)
K	7		1.5.3 Reagents are properly stored and labeled with the date of receipt, date opened or date prepared and expiration date.
C	**3**		**1.5.4 The mobile phase system used to analyze DSP toxins consists of:** **A: 2 mM ammonium formate and 50 mM formic acid in water** **B: 2 mM ammonium formate and 50 mM formic acid in 95% acetonitrile/5% water**
O	2		1.5.5 Mobile phase is ~~filtered~~ degassed manually before use if the UPLC does not have a degasser or if the degasser is not in use.
C	**3**		**1.5.6 Only certified reference materials are used for standard solutions. Source of the reference standard: _____**
C	**6**		**1.5.7 All primary standards are stored appropriately as per supplier recommendations.**
C	**6**		**1.5.8 All standards used are within their expiration date.**
C	**2, 3**		**1.5.9 All standards are prepared either gravimetrically or using appropriate positive displacement pipettes or syringes.**
C	**3**		**1.5.10 Working standards are made up from primary standard by dilution with the toxin-free, extraction solvent (i.e., 100% methanol).**
			1.6 Collection and Transportation of Samples
O	5, 1		1.6.1 Shellstock are collected in clean, waterproof, puncture resistant containers.
K	5, 1		1.6.2 Samples are appropriately labeled with the collector's name, type of shellstock, the harvest area, and time and date of collection.
C	5, 1		**1.6.3 Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10°C with ice or cold packs for transport to the laboratory.**

K	2		1.6.4 Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur, the laboratory has an appropriate contingency plan in place to handle the samples. For samples shipped live in accordance with 1.6.3, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt. The contingency plan also addresses field and/or laboratory processing that ensures the integrity of the sample or extract until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.
C	2		**1.6.5 Frozen shucked product or homogenates are allowed to thaw completely and all liquid is included as part of the sample before being processed further.**
PART II – EXAMINATION OF SHELLFISH FOR DSP TOXINS			
			2.1 Preparation of Sample
C	2		**2.1.1 At least 12 animals are used per sample or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish (e.g., three (3) geoduck gut balls).**
O	5		2.1.2 The outside of the shell is thoroughly cleaned with fresh water.
O	5		2.1.3 Shellstock are opened by cutting the adductor muscles.
O	5		2.1.4 The inside surfaces of the shells are rinsed with fresh water to remove sand and other foreign materials.
O	5		2.1.5 Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	5		**2.1.6 Damage to the body of the mollusk is minimized in the process of opening.**
O	5		2.1.7 Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for five (5) minutes.
K	5		2.1.8 Pieces of shell and drainage are discarded.
C	2, 5		**2.1.9 Drained meats or previously cooled/refrigerated shucked meats and their drip loss liquid or thawed homogenates with their freeze-thaw liquid are blended at high speed until homogenous (60-120 seconds).**

			2.2 Sample Extraction
K	2		2.2.1 Sample homogenates are extracted as soon as possible (preferably the same day) or stored in the freezer at -10 ºC or below.
C	**3**		**2.2.2 Two (2.00) ± 0.05 g of homogenized sample is weighed into a 50 mL polypropylene centrifuge tube and subsequently extracted.**
C	**3**		**2.2.3 The sample homogenate is extracted with 9 mL of 100% methanol and vortexed to mix.**
K	3		2.2.4 The sample homogenate/extract mixture is centrifuged for 10 minutes at 2000 x g and the supernatant decanted into a clean container (e.g. new polypropylene tube or glass vial).
C	**3**		**2.2.5 The tissue pellet is reextracted with nine (9) mL of 100% methanol and homogenized to mix.**
K	3		2.2.6 The sample homogenate/extract mixture is centrifuged for 10 minutes at 2000 x g and the supernatant combined with the supernatant in 2.2.4.
K	3		2.2.7 The total extract volume ~~in the polypropylene tube~~ is adjusted to 20 mL with 100% methanol.
K	3		2.2.8 The crude extract is hydrolyzed or stored in the freezer at < -~~20~~ 10 °C.
			2.3 Sample Hydrolysis and Cleanup
K	3		2.3.1 A two (2) mL aliquot of the sample extract is transferred to an appropriately sized* ~~16 × 100 mm~~ glass tube with a phenolic PTFE lined screw cap using a positive displacement pipette or syringe. *Note: A 16 x 100 mm tube will have sufficient volume to perform the hydrolysis and hexane wash steps and fit in a standard 15 mL centrifuge tube adaptor.
K	3		2.3.2 The sample extract is hydrolyzed by adding 250 µL of 2.5 M NaOH and the sample is homogenized with a vortex mixer for 30 seconds.
C	**3**		**2.3.3 Sample tube caps are securely fastened to prevent extract loss, and the weight of the sample tube is recorded. The sample tube is heated at 76 °C for 40 minutes, then allowed to cool to room temperature, dried, and re-weighed. If the weight has dropped by more than 0.1 g, lost volume is replaced using 100% MeOH.**
K	3		2.3.4 Samples are neutralized with 250 µL of 2.5 M HCL and vortexed to mix.
~~K~~O	3		2.3.5 Nonpolar lipids are removed by adding seven (7) mL of hexanes to the hydrolyzed sample extract and vortexing to mix (2.3.5 – 2.3.7 ~~Optional~~Preferred).
K	3		2.3.6 The sample extract/hexane mixture is partitioned by centrifuging for 10 minutes at 2000 x g (2.3.5 – 2.3.7 ~~Optional~~Preferred).

K	3		2.3.7 The hexane layer is removed with a glass pipette and one (1) mL of the hydrolyzed methanolic extract is removed and filtered into an LC-MS ~~certified~~ glass autosampler vial using a 0.2 µm PTFE syringe tip filter (2.3.5 – 2.3.7 ~~Optional~~Preferred).
K	2, 3		2.3.8 The cleaned-up extract is loaded into the autosampler immediately for analysis.
			2.4 Analysis
C	**3**		**2.4.1 Analytes are detected in standards and samples using the mass transitions in the table (negative ion mode).**

Compound	Q1 (m/z)	Q3 (m/z)
OA	-803.5	-255.2
OA	-803.5	-113.1
OA	-803.5	-151.1
DTX2	-803.5	-255.2
DTX2	-803.5	-113.1
DTX2	-803.5	-151.1
DTX1	-817.5	-255.2
DTX1	-817.5	-113.1
DTX1	-817.5	-151.1

K	3		2.4.2 Other system parameters such as collision energy are optimized for the specific system using standards before analysis.
C	3		**2.4.3 A standard calibration curve of at least six (6) concentrations is performed ~~before and after each set of samples~~at the beginning of each run. An additional curve is required if a run lasts longer than 24 hours.**
K	3		2.4.4 Five (5) µL of extract is injected for analysis.
K	2, 12		2.4.5 Samples are stored in the sample compartment of the autosampler at ≤ 10°C during analysis~~.~~. Otherwise the samples must be analyzed within 24 hours if the autosampler is held at room temperature.
K	3		2.4.6 A column heater is used and the temperature is maintained at 40 °C, with a tolerance as specified by the manufacturer, during the analysis.
C	3		**2.4.7 An Acquity UPLC BEH C18 1.0 × 150 mm, 1.7 µm particle size (or equivalent) analytical column is used for analyte separation**
C	**3**		**2.4.8 Analytes are separated on the LC column using gradient elution.**

K	2		2.4.9 The column is stored following the manufacturer's instructions when not in use.
K	**2**		2.4.10 Dead volume in the system is minimized by the use of short lengths of connecting tubing of small internal diameter between the sample injector and the column and between the column and detector.
C	**3**		**2.4.11 ~~Procedural~~ A matrix or procedural b~~B~~lank~~s~~ (i.e. NRC CRM Zero-Mus or equivalent negative matrix, or methanol carried through sample preparation process at the same time as the samples) should be ~~analyzed before and after extracted samples~~included in each analytical batch.**

			2.5 System Suitability
~~C~~	~~3~~		~~2.5.1 Each calibration curve should be derived from at least six (6) calibration points and the linear regression of the combined curves should yield a correlation coefficient (R^2) ≥ 0.98. Results are recorded and records are maintained.~~
~~C~~	~~3~~		~~2.5.2 If a calibration curve yields a correlation coefficient ≤ 0.98, or if non-linearity is visually observed, or if the variation in the slopes between the first and second calibration curves exceeds 25%, a new calibration curve is prepared and samples are reanalyzed.~~
C	3		**2.5.~~3~~1 The retention time of analytes in ~~all matrix solution should be within 3% that of the toxin standards.~~all samples are within 3% that of one of the intermediate toxin standards, measured from the apex of the peak.**
C	3		**2.5.~~4~~2 Chromatographic separation must be sufficient for resolving OA and DTX2. Peak resolution (Rs) of OA/DTX2 should ≥ 1 when calculated using the equation below (RT is retention time and W is peak width at baseline; Peak 1 is OA and Peak 2 is DTX2).** $$Rs = 2 \times (RT2 - RT1)/(W1 \mp W2)$$
K	2, 3		2.5.~~5~~3 Each chromatographic peak must be defined by at least 10 data points.
C	3		**2.5.4 Reagent blanks (methanol) are analyzed after the high calibration standard, periodically (as determined by the laboratory's internal verification), and after fortified samples to ensure that analyte carryover is not occurring. Analyte carryover is defined as a confirmed peak > LOD.**
C	3		**2.5.5 To confirm the presence of each DST, two (2) mass transitions must be observed above the limit of detection (LOD).** **The transition yielding the highest signal-to-noise ratio (S/N) is used for quantitation (i.e. 817.5 → 255.2 for DTX-1, 803.5 → 255.2 for OA and DTX-2). The transition yielding the second highest S/N is used for confirmation. The S/N of the peak used for confirmation is ≥ 3.**
C	3		**2.5.6 The ratio of the abundance of the confirmation ion transition to the quantitation ion transition is calculated for each toxin. These ion ratios must be within ± 20% of that of the toxin standards in order to confirm toxin identity.**
C	3		**2.5.7 When ≥ 10 samples are analyzed, analysts must show that the calibration has not significantly drifted using an option provided below:**

			OPTION 1 – USE OF BRACKETING CALIBRATION CURVES
C	3		2.5.7.1 A second standard curve is analyzed at the end of the analytical sequence and the averaged peak areas are used for the linear regression.
C	3		2.5.7.2 The linear regression of the averaged calibration curves must yield an $R^2 \geq 0.98$. Results are recorded and records are maintained.
C	3		2.5.7.3 A new calibration curve is prepared, and samples are reanalyzed, if any of the following are observed: a) The average of bracketing calibration curves yields an $R^2 < 0.98$. b) The difference in the slope between bracketing calibration curves exceeds 25%. c) The difference in retention times of the standards in the bracketing standard curves exceeds 3%.
			OPTION 2 – USE OF A CONTINUING CALIBRATION VERIFICATION (CCV) STANDARD
C	3		2.5.7.4 The linear regression of the single calibration curve must yield an $R^2 \geq 0.99$.
C	3		2.5.7.5 A continuing calibration verification (CCV) standard, matching on of the intermediate standards from the calibration curve, is analyzed after every 10 samples and at the end of a run.
C	3		2.5.7.6 A new calibration curve is prepared, and samples are reanalyzed, if any of the following are observed: a) The calibration curve yields an $R^2 < 0.99$. b) The retention time of a CCV sample exceeds 3% of the corresponding standard.* c) The peak area of a CCV sample exceeds ± 15% compared to the corresponding standard in the calibration curve.* * Samples immediately preceding and post the failed CCV shall be reanalyzed with a new standard curve.
C	3		2.5.8 Repeated injections of calibration or control samples at a concentration near the action level agree within ± 10% (as determined through the use of the coefficient of variation).
~~C~~	~~3~~		~~2.5.6 A new calibration curve is performed, or one mid-point calibration standard is analyzed, at least every 10 samples to ensure that no retention time shifts or loss in signal intensity has occurred.~~
~~K~~	~~2~~		~~2.5.7 Peak asymmetry must be <0.9 or >1.3.~~

~~C~~	~~3~~		~~2.5.8 Reagent blanks (methanol) are analyzed after the high calibration standard and periodically after fortified samples to insure that analyte carryover is not occurring.~~
~~C~~	~~2~~		~~2.5.9 Repeated injections of calibrated standards/samples agree within ± 5% (as determined through the use of the coefficient of variation).~~
~~C~~	~~3~~		~~2.5.10 To confirm the presence of each DST, two (2) mass transitions must be observed above the limit of detection (LOD). The transition yielding the highest signal-to-noise ratio (S/N) is used for quantitation (i.e., 817.5 → 151.1 for DTX-1, 803.5 → 151.1 for OA and DTX-2). The transition yielding the second highest S/N is used for confirmation. The S/N of the peak used for confirmation is ≥ 3.~~
~~C~~	~~3~~		~~2.5.11 The ratio of the abundance of the quantitative ion transition to the confirmation ion transition is calculated for each toxin. These ion ratios must be within ± 20% of that of the toxin standards in order to confirm toxin identity.~~
			2.6 Calculation of Sample Toxicity

C	~~4, 15~~3, 11		**2.6.1 The toxicity of the individual toxins is calculated as follows:** $\frac{ug}{g} toxin = C \times \frac{V}{W}$ X Hyd X ReTx~~X Hyd X ReTx~~ **where:** **C = the concentration in µg/ml of the extract injected, determined using the standard curve** **V = total volume of homogenate and extraction solvent mL)** **W = weight (g) of tissue homogenate extracted** **Hyd = dilution factor for hydrolysis (1.25) ~~Hyd = dilution factor for hydrolysis (1.25)~~** **ReTx = relative toxicity of toxin vs. Okadaic Acid ~~ReTx = relative toxicity of toxin vs. Okadaic Acid~~**

Relative Toxicity Values ~~Relative Toxicity Values~~

Toxin~~Toxin~~	ReTx~~ReTx~~
OA~~OA~~	1~~1~~
~~DTX1~~DTX1	~~1~~1
~~DTX2~~DTX2	~~0.6~~0.6

The individual toxicities for each toxin are summed to obtain the overall sample toxicity in ppm OA equivalents. ~~The individual toxicities for each toxin are summed to obtain the overall sample toxicity in ppm OA equivalents~~

C	~~12~~9		**2.6.2 Any value at or above ~~0.~~0.16 ppm ~~OA equivalents~~OA equivalents (mg/kg or µg/g) of the sum of any analytes present is actionable. Shellfish Program Management is made aware of positive result. Laboratory action ~~to identify positive result~~ is_____ .**

REFERENCES

1. American Public Health Association. 1984. *Compendium for the Microbiological Examination of foods,* 2nd Edition. APHA. Washington D.C.
2. Good Laboratory Practice. 21 CFR 58.
3. Interstate Shellfish Sanitation Conference (ISSC), Proposal 17-103 Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Method for the Determination of Diarrhetic Shellfish Poisoning (DSP) Toxins in Shellfish
4. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
5. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.

6. Consult reference standard product literature.

7. APHA/WEF/AWWA. 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA, Washington, D.C.

8. American Public Health Association. 1992. *Standard Methods for the Examination of Dairy Products*, 16th Edition. APHA, Washington, D.C.

9. U.S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 2015. *NSSP Guide for the Control of Molluscan Shellfish.* FDA/ISSC, Washington, D.C. and Columbia, S.C.

10. U.S. Department of Commerce. 1976. NBS Monograph 150. U.S. Department of Commerce, Washington, D.C.

11. The EFSA Journal. 2009. Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on Marine Biotoxins in Shellfish – Summary on regulated marine biotoxins. 1306, 1-23.

12. Deeds, J.R. and M.D. Celiz, Personal Communication, Addendum to proposal 19-136 DSP LC MS/MS Additional Ruggedness Testing; effect of the sample storage temperature during analysis (24 hrs), Email Received by ISSC Checklist Subcommittee 2/18/2021.

LABORATORY:			DATE OF EVALUATION:
SHELLFISH LABORATORY EVALUATION CHECKLIST **SUMMARY OF NONCONFORMITIES**			
Page	**Item**	**Observation**	**Documentation Required**

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	

DIARRHETIC SHELLFISH POISON (DSP) COMPONENT: PARTS I AND II

A. Results

Total # of **Critical (C)** Nonconformities	_____
Total # of Key (K) Nonconformities	_____
Total # of Critical, Key, and Other (O) Nonconformities	_____

B. Criteria for Determining Laboratory Status of the DSP Component

1. **Conforms Status: The DSP component of this Laboratory is in conformity with NSSP requirements if all of the following apply.**
   - **a. No Critical nonconformities.**
   - **b. and <6 Key nonconformities.**
   - **c. and <12 Total nonconformities.**

2. **Provisionally Conforms Status:** The DSP component of this laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply.
   - a. the number of critical nonconformities is ≥ 1 but **< 4**.
   - b. and **< 6** Key nonconformities.
   - c. and **< 12** Total nonconformities.

3. **Does Not Conform Status**: The DSP component of this laboratory is not in conformity with NSSP requirements when any of the following apply.
   - a. The total # of Critical nonconformities is **≥4**.
   - b. or the total # of Key nonconformities is **≥6.**
   - c. or the total # of Critical, Key, or Other is **≥12.**

C. Laboratory Status (*circle appropriate)*

Does Not Conform – Provisionally Conforms – Conforms

Acknowledgement by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before_____.

Laboratory Signature:_____Date:_____

LEO Signature: Date:

Addendum to Proposal 19-136:

Single Laboratory Validation (SLV) Protocol for Submission to the Interstate Shellfish Sanitation Conference (ISSC) For Method Approval

Submitter: Jonathan Deeds, Ph.D.

FDA Center for Food Safety and Applied Nutrition

5001 Campus Drive

College Park, MD 20740

Phone: 240-402-1474

jonathan.deeds@fda.hhs.gov

Additional Ruggedness Testing: Effect of the sample storage temperature during analysis (24 hrs)

To assess the effect of sample storage temperature during analysis on accuracy/trueness, two sub-samples from each of 10 extracts from previously spiked samples, representing two different matrix sources (5 samples each from matrix sources A and B), each spiked at 5 different concentrations bracketing the regulatory guidance level (8, 12, 16, 24, and 32 µg/ 100 g), were tested at 10°C and also at room temperature (approx. 24°C) after storage at room temperature for 24 hrs. After hydrolysis and hexane washing, each sample was filtered, as described previously, into two LC-vials. One set was analyzed using a refrigerated sample injector set to 10°C, while the second set was stored at room temperature (approx. 24 °), protected from light, for 24 hrs, then injected using the same sample injector with the refrigeration turned off. This entire procedure was repeated on separate days so that in total 20 samples were tested at 10°C and at 24°C after storage for 24 hrs. The data handling procedures outlined in the Marine Biotoxin and Non-MPN Based Microbiological Methods SOP for Ruggedness were used for reporting these results. Data for the ruggedness testing of the sample storage temperature during analysis are presented in tables 25, 26, and 27. The variance ratio showed no significant differences between the two treatments for any of the three toxins, and no significant differences were found in the measured concentrations if injected at 10°C or at room temperature after storage for 24 hrs. We conclude from this data that during analysis, finished samples can be stored at room temperature for up to 24 hrs. without significantly affecting the analytical results.

Table 25. Ruggedness Testing for Sample Storage Temperature During Analysis for DTX1 in Clam

DTX1		10°C	Room Temp (ca. 24°C)
Sample	**Spiked Concentration (µg/100g)**	**Determined Concentration (µg/100g)**	**Determined Concentration (µg/100g)**
1	8	6.984	7.413
2	12	10.835	10.784
3	16	14.455	14.093
4	24	21.329	20.961
5	32	27.770	28.106
6	8	7.144	6.889
7	12	11.219	10.575
8	16	14.870	14.364
9	24	22.573	21.815
10	32	30.958	30.344
11	8	7.215	7.073
12	12	10.571	10.225
13	16	14.086	13.856
14	24	20.865	22.201
15	32	27.650	28.030
16	8	6.933	6.834
17	12	10.295	10.516
18	16	14.266	14.350
19	24	21.179	21.666
20	32	29.179	38.408
Skewness		**0.47**	**0.82**
Variance		**64.7**	**82.4**
Variance Ratio =1.3, no significant difference			
Paired t-test (two-tailed): P=0.405, no significant difference			
Mean of differences: 0.84; 95% confidence interval: -0.09-1.8			

Table 26. Ruggedness Testing for Sample Storage Temperature During Analysis for DTX2 in Clam

DTX2		10°	Room Temp (ca. 24°C)
Sample	**Spiked Concentration (µg/100g)**	**Calculated Concentration (µg/100g)**	**Calculated Concentration (µg/100g)**
1	8	7.215	7.006
2	12	10.268	10.253
3	16	13.664	13.326
4	24	20.378	19.954
5	32	27.649	26.761
6	8	7.236	6.590
7	12	10.281	9.823
8	16	13.925	13.553
9	24	21.863	20.364
10	32	30.780	28.513
11	8	6.430	6.489
12	12	9.428	9.308
13	16	12.624	12.949
14	24	18.985	20.738
15	32	24.391	26.468
16	8	6.395	6.396
17	12	9.325	9.644
18	16	12.226	13.653
19	24	19.168	20.313
20	32	26.528	34.255
Skewness		**0.592**	**0.714**
Variance		**59.4**	**69.8**
Variance Ratio =1.2, no significant difference			
Paired t-test (two-tailed): P=0.410, no significant difference			
Mean of differences: 1.1; 95% confidence interval: 0.30-1.9			

Table 27. Ruggedness Testing for Sample Storage Temperature During Analysis for OA in Clam

OA		10°	Room Temp (ca. 24°C)
Sample	**Spiked Concentration (µg/100g)**	**Calculated Concentration (µg/100g)**	**Calculated Concentration (µg/100g)**
1	8	7.236	7.018
2	12	10.856	10.399
3	16	14.356	13.736
4	24	22.198	20.748
5	32	29.823	27.436
6	8	6.954	6.853
7	12	11.179	10.421
8	16	15.896	14.149
9	24	23.409	21.384
10	32	33.059	29.430
11	8	6.578	6.945
12	12	9.645	10.685
13	16	13.321	14.136
14	24	20.296	22.180
15	32	25.723	27.905
16	8	6.521	6.621
17	12	9.813	10.376
18	16	13.580	14.253
19	24	20.433	21.889
20	32	28.225	37.651
Skewness		**0.542**	**0.784**
Variance		**70.2**	**79.4**
Variance Ratio =1.1, no significant difference/variance homogeneous			
Paired t-test (two-tailed): P=0.667, no significant difference			
Mean of differences: 1.6; 95% confidence interval: 0.63-2.6			

PUBLIC HEALTH SERVICE **U.S. FOOD AND DRUG ADMINISTRATION** **OFFICE OF FOOD SAFETY** **SHELLFISH AND AQUACULTURE POLICY BRANCH** **~~5100 PAINT BRANCH PARKWAY~~5001 CAMPUS DRIVE** **COLLEGE PARK, MD 20740-3835** **TEL. 240- 402-~~2151~~4960/9258/2055~~/4960~~ FAX 301-436-2601**

SHELLFISH LABORATORY EVALUATION CHECKLIST		
LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:** **~~REGION:~~**
OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity it noted by a "√"**

C- Critical K - Key O - Other NA- Not Applicable

Check the applicable analytical methods:	
	Multiple Tube Fermentation Technique for Seawater (APHA)[PART II]
	Multiple Tube Fermentation Technique for Seawater using MA-1 [PART II]
	Membrane Filtration Technique for Seawater using mTEC [PART II]
	Multiple Tube Fermentation Technique for Shellfish Meats (APHA)[PART III]
	Standard Plate Count for Shellfish Meats [PART III]
	Elevated Temperature Coliform Plate Method for Shellfish Meats [PART III]
	Male Specific Coliphage for ~~Soft-shelled Clams and American Oysters~~Shellfish Meats [PART III]
	Membrane Filtration Technique for Seawater using mTEC [Part II]
	Membrane Filtration Technique for UV Treated Process Water using mEndo Agar LES [Part II]
	Multiple Tube Fermentation Technique for Shellfish Meats (APHA) [Part III]

PART 1 - QUALITY ASSURANCE				
CODE	**REF.**			**ITEM**
K	8, 11	**1.1 Quality Assurance (QA) Plan**		
			1.1.1	Written Plan (Check those items which apply.)
				a. Organization of the laboratory.
				b. Staff training requirements.
				c. Standard operating procedures.
				d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance, and rejection criteria established.
				e. Laboratory safety.
				f. Internal performance assessment.
				g. External performance assessment.
C	**8**		**1.1.2**	**QA Plan Implemented.**
K	11		1.1.3	The Laboratory participates in a proficiency testing program annually. Specify Program(s)_____
		1.2 Educational/Experience Requirements		
C	**State's Human Resources Department**		**1.2.1**	**In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2	In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3**	**In commercial laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, or equivalent discipline with at least two (2) years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4	In commercial laboratories, the analyst(s) must have at least a high school diploma and shall have at least three (3) months of experience in laboratory sciences.
		1.3 Work Area		
O	8,11		1.3.1	Adequate for workload and storage.
K	11		1.3.2	Clean, well-lighted.
K	11		1.3.3	Adequate temperature control.
O	11		1.3.4	All work surfaces are nonporous, easily cleaned and disinfected.
K	11		1.3.5	Microbiological quality of the air is fewer than 15 colonies for a 15 minute exposure and determined monthly. The results are recorded and records maintained.
		1.4 Laboratory Equipment		
O	9		1.4.1	To determine the pH of prepared media, the pH meter has a standard accuracy of 0.1 units.
O	14		1.4.2	pH electrodes consisting of pH half-cell and reference half-cell or equivalent combination electrode free from (Ag/AgCl) or contains an ion exchange barrier preventing passage of Ag ions into the medium which may affect the accuracy of the pH reading.
K	11		1.4.3	The effect of temperature on the pH is compensated for by an ATC probe or by manual adjustment.
K	8		1.4.4	pH meter is calibrated daily or with each use Results are recorded and records maintained.
K	11		1.4.5	A minimum of two (2) standard buffer solutions is used to calibrate the pH meter. The first must be near the electrode isopotential point (pH 7). The second near the expected sample pH (i.e., pH 4 or pH 10). Standard buffer solutions are used once and discarded.
O	8,15		1.4.6	Electrode acceptability is determined daily or with each use by the millivolt

				procedure or through determination of the slope. (*Circle the method used.*)
K	9		1.4.7	Balance provides a sensitivity of at least 0.1 g at weights of use.
K	11,13		1.4.8	Balance calibrations are checked monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use. Results are recorded and records maintained.
K	11		1.4.9	Refrigerator temperature(s) are monitored at least once daily on workdays Results are recorded and records maintained.
K	1		1.4.10	Refrigerator temperature is maintained at 0 to 4°C.
C	**9**		**1.4.11**	**The temperature of the incubator is maintained at 35 ± 0.5°C.**
C	**11**		**1.4.12**	**Thermometers used in the air incubator(s) are graduated in at least 0.1°C increments.**
K	9		1.4.13	Working thermometers are located on top and bottom shelves or appropriately placed based on the results of spatial temperature checks.
C	**11**		**1.4.14**	**Temperature of the waterbath is maintained at 44.5 ± 0.2°C under all loading conditions.**
C	**9**		**1.4.15**	**The thermometers used in the waterbath are graduated in at least 0.1°C increments.**
C	**13**		**1.4.16**	**The waterbath has adequate capacity for workload.**
K	9		1.4.17	The level of water in the waterbath covers the level of liquid in the incubating tubes.
K	8, 11		1.4.18	Air incubator/waterbath temperatures are taken twice daily on workdays. The results are recorded and records maintained.
C	**4**		**1.4.19**	**All working thermometers are appropriately immersed.**
C	~~**2**~~**9**		**1.4.20**	**Working thermometers are either: calibrated mercury-in-glass thermometers, calibrated non-mercury-in-glass thermometers, or appropriately calibrated electronic devices, including Resistance Temperature Devises (RTDs) and Platinum Resistance Devices (PTDs).**
C	**11**		**1.4.21**	**A standards thermometer has been calibrated by NIST or a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at the points 0, 35 and 44.5°C (45.5°C for ETCP). These calibration records are maintained.**
K	9		1.4.22	Standards thermometers are checked annually for accuracy by ice point determination. Results recorded and maintained. Date of most recent determination_____.
C	~~**2**~~**9**		**1.4.23**	**Either mercury-in-glass thermometers, non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury or low drift electronic resistance thermometers with an accuracy of ≤at least ±0.05°C are used as the laboratory standards thermometer. (*Circle the thermometer type used.*)**
K	13		1.4.24	The accuracy of working thermometers is checked annually against the standards thermometer either at the temperatures at which they are used or by ice point determination. Results are recorded and records maintained.
O	11		1.4.25	Appropriate pipet aids are available and used to inoculate samples. Mouth pipetting is not permitted.
		1.5 Labware and Glassware Washing		
O	9		1.5.1	Utensils and containers are clean borosilicate glass, stainless steel or other noncorroding materials.
K	9		1.5.2	Culture tubes are of a suitable size to accommodate the volume for nutritive ingredients and samples.
K	9		1.5.3	Sample containers are made of glass or some other inert material.
O	9		1.5.4	Dilution bottles and tubes are made of borosilicate glass or plastic and closed

				with rubber stoppers, caps, or screw caps with nontoxic liners.
K	9		1.5.5	Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method is used to ensure appropriate volumes.
C	**9**		**1.5.6**	**Pipettes used to inoculate the sample deliver accurate aliquots, have unbroken tips and are appropriately graduated. Pipettes larger than 10 mL are not used to deliver 1mL aliquots; nor, are pipets larger than 1.1mL used to deliver 0.1 mL aliquots.**
K	9		1.5.7	Reusable sample containers are capable of being properly washed and sterilized.
K	9		1.5.8	In washing reusable pipettes, a succession of at least three (3) fresh water rinses plus a final rinse of distilled/deionized water is used to thoroughly rinse off all the detergent.
C	**2**		**1.5.9**	**An alkaline or acidic detergent is used for washing glassware/labware.**
C	**11**		**1.5.10**	**With each load of labware/glassware washed the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali) with aqueous 0.04% bromothymol blue. Results are recorded and records maintained.**
		1.6 Sterilization and Decontamination		
K	9		1.6.1	Autoclave(s) are of sufficient size to accommodate the workload.
O	8		1.6.2	Routine autoclave maintenance is performed and the records are maintained.
C	**~~30~~29, ~~33~~32, ~~34~~33**		**1.6.3**	**The autoclave provides sterilization conditions suitable to the load contents. Sterilization temperature range may be 119ºC - 124ºC as determined by the lab's equipment Quality Assurance Verification Testing and recommended practices from the media manufacturer. Sterilization is determined for each load using a working -maximum registering thermometer or an appropriate working temperature monitoring device.**
K	11		1.6.4	An autoclave standards thermometer has been calibrated by a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at 121°C. Calibration at 100°C, the steam point, is also recommended but not required.
K	16		1.6.5	The autoclave standards thermometer is checked every five (5) years for accuracy at 121°C by a qualified calibration laboratory; or, is checked in-house at the steam point (100°C) if it has been previously calibrated at both 100°C and 121°C. Any change in temperature at the steam point changes the calibrated temperature at 121°C by the same magnitude. Date of most recent determination_____
K	1		1.6.6	Working autoclave thermometers are checked against the autoclave standards thermometer at 121°C yearly. Date of last check _____ Method _____
K	11		1.6.7	Spore strips/suspensions appropriate for use in an autoclave media cycle are used monthly according to manufacturer's instructions to evaluate the effectiveness of the sterilization process. Results are recorded and the records maintained.
O	11		1.6.8	Heat sensitive tape is used with each autoclave batch.
K	11, 13		1.6.9	Autoclave sterilization records including length of sterilization, total heat exposure time and chamber temperature are maintained. Type of record: Autoclave log, computer printout or chart recorder tracings. (*Circle appropriate type or types.)*
K	11		1.6.10	For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperatures in the range of 160 to 180°C.
K	9		1.6.11	A thermometer capable of determining temperatures accurately in the range of 160 to 180°C is used to monitor the operation of the hot-air sterilizing oven
K	13		1.6.12	Records of temperatures and exposure times are maintained for the operation of

				the hot-air sterilizing oven during use.
K	11		1.6.13	Spore strips/suspensions are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. Records are maintained.
K	11		1.6.14	Reusable sample containers are sterilized for 60 minutes at 170°C in a hot-air oven or autoclaved for 15 minutes at 121°C.
C	**1**		**1.6.15**	**The sterility of reusable sample containers is determined for each load sterilized. The results are recorded and the records maintained.**
C	**1**		**1.6.16**	**The sterility of pre-sterilized disposable sample containers is determined for each lot received. Results are recorded and the records maintained.**
K	9		1.6.17	Reusable pipettes are stored and sterilized in aluminum or stainless steel canisters.
K	9		1.6.18	Reusable pipettes (in canisters) are sterilized in a hot-air oven at 170°C for two (2) hours.
C	**2**		**1.6.19**	**The sterility of reusable pipettes is determined with each load sterilized. Results are recorded and records maintained.**
C	**2**		**1.6.20**	**The sterility of pre-sterilized disposable pipettes is determined with each lot received. Results are recorded and the records maintained.**
K	18		1.6.21	Hardwood applicator transfer sticks are properly sterilized. Method of sterilization _____
C	**2**		**1.6.22**	**The sterility of the hardwood applicator transfer sticks is checked routinely. Results are recorded and the records maintained.**
O	13		1.6.23	Spent broth cultures and agar plates are decontaminated by autoclaving for at least 30 minutes before conventional disposal.
		1.7 Media Preparation		
K	3, 5		1.7.1	Media is commercially dehydrated except in the case of ~~medium~~ A-1 medium, which must be prepared from the individual components and modified MacConkey agar which may be prepared from its components.
K	11		1.7.2	Media is prepared according to manufacturer's instructions.
O	11		1.7.3	Dehydrated media and media components are properly stored in a cool, clean, dry place.
O	11		1.7.4	Dehydrated media are labeled with date of receipt and date opened.
C	**12**		**1.7.5**	**Caked or expired media or media components are discarded.**
C	**11**		**1.7.6**	**Reagent water is distilled or deionized (circle appropriate choice), tested monthly, and exceeds 0.5 megohm-cm resistance (2 megohms-cm in-line) or is less than 2.0 µSiemens/cm conductivity at 25°C. (*Circle the appropriate water quality descriptor determined.*) Results are recorded and the records maintained.**
C	**11**		**1.7.7**	**Reagent water is analyzed for residual chlorine monthly and is at a non-detectable level (< 0.1 mg/L). Results are recorded and the records maintained.** **Specify method of determination_____.**
K	11		1.7.8	Reagent water contains <100 CFU/mL as determined monthly using the heterotrophic plate count method. Results are recorded and the records maintained.
K	11		1.7.9	Media prepared from commercially dehydrated components are sterilized according to the manufacturer's instructions.
K	9		1.7.10	The volume and concentration of media in the tube are suitable for the amount of sample inoculated.
C	**11**		**1.7.11**	**Total time of exposure of sugar containing broths to autoclave temperatures does not exceed 45 minutes.**
C	**1**		**1.7.12**	**Media sterility is determined for each load sterilized. Results are recorded**

				and the records maintained.
C	**1**		**1.7.13**	**Media productivity is determined using media-appropriate, properly diluted positive and negative control cultures for each lot of dehydrated media received or with each batch of media prepared when the medium is made from its individual components.**
O	9		1.7.14	Sterile phosphate buffered dilution water is used as the sample diluent.
K	11		1.7.15	The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer's requirements. Results are recorded and records are maintained.
		1.8 Storage of Prepared Culture Media		
K	9		1.8.1	Prepared culture media are stored in a cool, clean, dry space where excessive evaporation and the danger of contamination are minimized.
K	5,11		1.8.2	Brilliant green bile 2% broth and A-1 media are stored in the dark.
K	13		1.8.3	Stored media are labeled with the storage expiration date or the sterilization date.
K	9		1.8.4	Storage of prepared culture media at room temperature does not exceed seven (7) days.
K	2		1.8.5	Storage under refrigeration of prepared culture media with loose fitting closures shall not exceed one (1) month.
K	11		1.8.6	Storage under refrigeration of prepared culture media with screw-cap closures does not exceed three (3) months.
K	17		1.8.7	All prepared MPN broth media stored under refrigeration must reach room temperature prior to use. Culture tubes containing any type of precipitate or Durham tubes containing air bubbles are discarded.
PART II - SEAWATER SAMPLES				
		2.1 Collection and Transportation of Samples		
C	**11**		**2.1.1**	**Sample containers are of a suitable size to contain at least 110 mL of sample and to allow adequate headspace for proper shaking. Seawater samples are collected in clean, sterile, watertight, properly labeled sample containers.**
K	1		2.1.2	Samples are identified with collector's name, harvest area, sampling station, time and date of collection.
C	**9**		**2.1.3**	**Immediately after collection, seawater samples are placed in dry storage (ice chest or equivalent) capable of maintaining a temperature of 0 to 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed in the refrigerator unless processed immediately.**
O	1		2.1.4	A temperature blank is used to represent the temperature of samples upon receipt at the laboratory. Temperature should be equivalent or less than that of the growing area waters. Results are recorded and maintained.
C	**9**		**2.1.5**	**Analysis of the sample is initiated as soon as possible after collection. Seawater samples are not tested if they have been held for more than 30 hours from the time of collection.**
		2.2 Bacteriological Examination of Seawater by the APHA MPN		
C	**9**		**2.2.1**	**Lactose broth or lauryl tryptose broth is used as the presumptive medium.** ***(Circle appropriate one.)***
C	**2**		**2.2.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
C	**9, ~~35~~34**		**2.2.3**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) before inoculation.**
C	**9**		**2.2.4**	**In a multiple dilution series not less than three (3) tubes per dilution are used (Five (5) tubes are recommended).**

C	**6**		**2.2.5**	**In a single dilution series not less than 12 tubes are used (for depuration at least five (5) tubes are used).**
C	**6**		**2.2.6**	**In a single dilution series, the volumes analyzed are adequate to meet the needs of routine monitoring.** **Sample volume inoculated _____** **Range of MPN_____** **Strength of media used_____**
K	9		2.2.7	Inoculated tubes are incubated in air at 35 ± 0.5°C.
C	**2**		**2.2.8**	**Appropriately diluted process control cultures accompany the samples *throughout both the presumptive and confirmed phases of incubation.* Results are recorded and the records maintained.** **Positive process control _____ Negative process control _____**
K	9		2.2.9	Inoculated tubes are read after 24 ± 2 hours and 48 ± 3 hours of incubation and transferred at both time interval if positive for growth (the presence of turbidity) and gas or effervescence in the culture tube. These tubes are considered presumptive positive requiring further confirmatory testing.
		2.3 Confirmed Test for Seawater by APHA MPN		
C	**9**		**2.3.1**	**Brilliant green bile 2% broth (BGB) is used as the confirmatory medium for total coliforms.**
C	**9**		**2.3.2**	**EC medium is used as the confirmatory medium for fecal coliforms.**
C	**2**		**2.3.3**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
K	9, 11		2.3.4	Transfers are made to BGB/EC by either sterile loop or sterile hardwood transfer stick from positive presumptive tubes incubated for 24 and 48 hours as appropriate. *(Circle the method of transfer.)*
C	**9**		**2.3.5**	**BGB tubes are incubated at 35 ± 0.5°C.**
K	9		2.3.6	BGB tubes are read after 48 ± 3 hours of incubation.
C	**9**		**2.3.7**	**EC tubes are incubated in a circulating waterbath maintained at 44.5 ± 0.2°C.**
C	**9**		**2.3.8**	**EC tubes are read after 24 ± 2 hours of incubation.**
C	**9**		**2.3.9**	**The presence of turbidity and any amount of gas or effervescence in the culture tube constitutes a positive test.**
		2.4 Computation of Results – APHA MPN		
K	9		2.4.1	Results of multiple dilution tests are read from tables in *Recommended Procedures for the Examination of Sea Water and Shellfish*, Fourth Edition.
K	7		2.4.2	Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**7, 9**		**2.4.3**	**Results are reported as MPN/100 mL of sample.**
		2.5 Bacteriological Examination of Seawater by the MA-1 Method		
C	**5**		**2.5.1**	**A-1 medium complete is used in the analysis.**
C	**2, ~~31~~30**		**2.5.2**	**A-1 medium without salicin is used in the analysis. Comparability testing supports use of A-1medium without salicin. Study records are maintained and are available upon request.**
C	**5**		**2.5.3**	**A-1 medium sterilized for 10 minutes at 121°C.**
C	**2**		**2.5.4**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records**

				maintained. **Positive productivity control _____Negative productivity control _____**
C	**9, 3534**		**2.5.5**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in seven (7) seconds by hand or for at least 15 seconds when using a mechanical shaker) before inoculation.**
C	**9**		**2.5.6**	**In a multiple dilution seriesof not less than three (3) tubes per dilution are used (five (5) tubes are recommended).**
C	**6**		**2.5.7**	**In a single dilution series at least 12 tubes are used.**
C	**6**		**2.5.8**	**In a single dilution series, the volumes analyzed are adequate to meet the needs of routine monitoring.** **Sample volume inoculated _____** **Range of MPN _____** **Strength of media used _____**
C	**2**		**2.5.9**	**Appropriately diluted process control cultures accompany the samples *throughout both resuscitation and waterbath incubation* Results are recorded and the records maintained.** **Positive process control _____ Negative process control _____**
C	**2,5**		**2.5.10**	**Inoculated tubes are placed in an air incubator at 35 ± 0.5°C for 3 ± 0.5 hours of resuscitation.**
C	**5**		**2.5.11**	**After 3 ± 0.5 hours resuscitation at 35°C, inoculated tubes are incubated at 44.5 ± 0.2°C in a circulating waterbath for the remainder of the 24 ± 2 hours.**
C	**5**		**2.5.12**	**The presence of turbidity and any amount of gas or effervescence in the culture tube constitutes a positive test.**
		2.6 Computation of Results – APHA MPN		
K	9		2.6.1	Results of multiple dilution tests are read from tables in *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition.
K	7		2.6.2	Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**7, 9**		**2.6.3**	**Results are reported as MPN/100 mL of sample.**
		2.7 Bacteriological Analysis of Seawater by Membrane Filtration (MF) using mTEC Agar - Materials and Equipment		
C	**23, 24**		**2.7.1**	**When used for elevated temperature incubation in conjunction with ethafoam resuscitation, the temperature of the hot air incubator is maintained at 44.5 ± 0.5°C under any loading capacity.**
C	**23**		**2.7.2**	**When using a waterbath for elevated temperature incubation, the level of the water completely covers the plates.**
C	**23**		**2.7.3**	**Pre-sterilized plastic or sterile glass culture plates that are clear, flat bottomed, free of bubbles and scratches with tight fitting lids are used.**
C	**2**		**2.7.4**	**The sterility of pre-sterilized culture plates is determined for each lot received. Results are recorded and the records maintained.**
K	11		2.7.5	Colonies are counted with the aid of magnification.
C	**11, 23**		**2.7.6**	**Membrane filters are made from cellulose ester material, white, grid marked, 47 mm in diameter with a pore size of 0.45 µm and certified by the manufacturer for fecal coliform analyses.**
C	**2**		**2.7.7**	**Lot number, date of receipt and if provided the expiration date of the membrane filters are recorded and records maintained.**
C	**2**		**2.7.8**	**When initiating monitoring by mTEC or switching brands or types of membrane filters used and no previous lots of filters are available for comparing acceptable performance, an appropriate method for determining the suitability of the lot is developed and the comparison**

				testing implemented. The results are recorded and ~~this~~ the record is maintained.
K	2, 11		2.7.9	New lots of membrane filters are checked by comparing recovery of fecal coliform organisms against membrane filters from previously acceptable lots.
C	**2**		**2.7.10**	**The sterility of each lot or autoclave batch of membrane filters are checked before use.**
K	2		2.7.11	Membrane filters which are beyond their expiration date are not used.
O	11		2.7.12	Forceps tips are clean.
O	11		2.7.13	Forceps tips are smooth without pitting or corrugations to damage the filters being manipulated.
K	11		2.7.14	Forceps are dipped in alcohol and flame sterilized between sample filters.
K	11		2.7.15	If indelible graduation marks are used on clear glass or plastic funnels to measure sample volumes, their accuracy is checked gravimetrically or -with a Class A graduated cylinder before use and periodically rechecked. Funnels having a tolerance greater than 2.5% are not used. Checks are recorded and records maintained.
K	11		2.7.16	Membrane filtration units are made of stainless steel, glass or autoclavable plastic free of scratches, corrosion and leaks.
C	**11**		**2.7.17**	**Membrane filter assemblies are autoclave sterilized for 15 minutes at 121°C prior to the start of a filtration series.**
O	11, 23, 26		2.7.18	A UV sterilization unit is used to disinfect filter assemblies between sample and filtration runs.
K	11		2.7.19	The effectiveness of the UV sterilization unit is determined by biological testing monthly. Results are recorded and records maintained.
K	2		2.7.20	Maintenance of the UV sterilization unit is performed as needed. This maintenance is documented and the records maintained.
		2.8 Media Preparation and Storage – MF using mTEC Agar		
K	11		2.8.1	Phosphate buffered saline is used as the sample diluent and filter funnel rinse.
C	**11**		**2.8.2**	**The phosphate buffered saline is properly sterilized.**
K	23		2.8.3	A sufficient amount of medium (4-5 mL) is used in each plate.
O	11		2.8.4	Refrigerated prepared plates are stored for no more than two (2) weeks in sealed plastic bags or containers to minimize evaporation.
		2.9 Sample Analyses - MF using mTEC Agar		
C	**24**		**2.9.1**	**mTEC agar is used.**
C	**2**		**2.9.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
C	**23, ~~35~~34**		**2.9.3**	**The sample is shaken vigorously (25 times in a 12″ arc in seven (7) seconds by hand or for at least 15 seconds when using a mechanical shaker) before filtration.**
C	**23**		**2.9.4**	**The membrane is placed grid side up within the sterile filter apparatus.**
C	**23, 25**		**2.9.5**	**Sample volumes tested are consistent with the sampling regime employed (i.e., half log or other appropriate dilutions are used with systematic random sampling).**
C	**23**		**2.9.6**	**Sample volumes are filtered under vacuum.**
K	26		2.9.7	The pressure of the vacuum pump does not exceed 15 psi.
C	**23, 26**		**2.9.8**	**The sides of the filter funnel are rinsed at least twice with 20-30 mL of sterile phosphate buffered saline after sample filtration.**
C	**23**		**2.9.9**	**The membrane filter is removed from the filtering apparatus with sterile forceps and rolled onto mTEC agar so that no bubbles form between the filter and the agar.**
C	**11**		**2.9.10**	**Blanks are run at the beginning of filtration, after every 10th aliquot and at**

				the end of the filtration run to check the sterility of the testing system (phosphate buffered saline, filter funnel, forceps, membrane filter, media and culture plate).
C	**2, 11**		**2.9.11**	**Appropriately diluted process control cultures accompany the samples throughout both resuscitation and elevated temperature incubation. Results are recorded and the records maintained.** **Positive process control _____ Negative process control _____**
C	**11, 23, 24**		**2.9.12**	**Inoculated plates are placed inverted into a watertight, tightly sealed container prior to being placed in the air incubator and incubated at 35 ±~~+~~ 0.5°C for two (2) hours of resuscitation. Alternatively, inoculated plates may be placed in ethafoam prior to air incubation at 44.5 ± 0.5°C for 24 ± 2 hours.**
C	**11, 23, 24**		**2.9.13**	**After 2 hours of resuscitation at 35°C, the watertight, tightly sealed containers are transferred to a circulating waterbath at 44.5 ±~~+~~ 0.2°C, submerged completely and incubated for 22-24 hours.**
		2.10 Computation of Results - MF using mTEC Agar		
C	**23**		**2.10.1**	**All yellow, yellow-green or yellow-brown colonies are counted.**
C	**23**		**2.10.2**	**Only plates having 80 or fewer colonies are counted. If it is unavoidable to use plates having more than 80 colonies, counts are given as >80 x 100/the volume of sample filtered.**
C	**2, 11, 23**		**2.10.3**	**When multiple dilutions are filtered, the laboratory has developed a procedure for assessing the contribution of all positive dilutions to the final count.**
C	**23, 11**		**2.10.4**	**The number of fecal coliforms is calculated by the following equation:** **Number of fecal coliforms per 100 mL = [number of colonies counted per plate used in the count / volume (s) of sample filtered in ml] x 100.**
C	**23, 11**		**2.10.5**	**Results are reported as CFU/100 mL of sample.**
		2.11 Bacteriological Analysis of UV Treated Process Water Samples by Membrane Filtration (MF) using mEndo Agar LES – Materials and Equipment		
C	**9, 11, 21**		**2.11.1**	**Pre-sterilized plastic or sterile glass culture plates that are clear, flat bottomed, free of bubbles and scratches are used.**
C	**2**		**2.11.2**	**The sterility of pre-sterilized culture plates is determined for each lot received. Results are recorded and the records maintained.**
C	**11, 19, 21**		**2.11.3**	**Membrane filters are made from cellulose ester material, white, grid marked, 47 mm diameter with a pore size of 0.45µm and certified by the manufacturer for total coliform analysis.**
C	**2**		**2.11.4**	**Membrane filter lot numbers, dates of receipt and expiration dates are recorded and the records maintained.**
C	**2**		**2.11.5**	**If previous lots of agar or membrane filters are not available for comparability testing, an appropriate method for determining lot suitability has been developed and comparison testing implemented when the following has occurred:** **a. initiating monitoring by mEndo Agar LES;** **b. changing agar manufacturers; or** **c. changing brands of membrane filters used.** **The results are recorded and the records are maintained.**
K	2, 11		2.11.6	Recovery of total coliforms from new lots of membrane filters and mEndo Agar LES is compared against the recovery from the previously acceptable lot.
C	**2**		**2.11.7**	**The sterility of each lot and autoclave batch of membrane filters is verified before use.**
K	2		2.11.8	Expired membrane filters are not used.

K	9, 11, 19, 21		2.11.9 Membrane filtration units are made of stainless steel, glass or autoclavable plastic free of scratches, corrosion and leaks.
K	11		2.11.10 If indelible graduation marks are used on clear glass or plastic funnels to measure sample volumes, their accuracy is checked gravimetrically with a Class A graduated cylinder before use and periodically rechecked. Funnels having a tolerance greater than 2.5% are not used. Checks are recorded and records maintained.
C	**9, 11**		**2.11.11 Membrane filter assemblies are autoclave sterilized for 15 minutes at 121+/- 2°C prior to the start of a filtration run. A new run occurs when there is a break of 30 minutes or more between the previous filtration run.**
O	11, 19, 26, ~~36~~35		2.11.12 A UV sterilization unit is used to disinfect filter assemblies between sample and filtration runs.
K	11		2.11.13 The effectiveness of the UV sterilization unit is determined by biological testing monthly. Results are recorded and the records are maintained.
K	2		2.11.14 Maintenance of the UV sterilization unit is performed as needed. Maintenance is documented and the records maintained.
O	9, 11		2.11.15 Forceps tips are clean and smooth without pitting or corrugations.
		2.12 Media Preparation and Storage	
C	**9, 11, 19, 21, ~~36~~35**		**2.12.1 mEndo Agar LES is used.**
K	11, 21, ~~36~~35		2.12.2 mEndo Agar LES is prepared aseptically using pre-sterilized glassware, sterile reagent water and pre-sterilized stir bar.
K	9, 11, ~~36~~35		2.12.3 mEndo Agar LES is prepared using 95% alcohol that is not denatured.
C	**9, 11, ~~36~~35**		**2.12.4 mEndo Agar LES is brought to near boiling; removed from the heat and tempered at 44-50°C before dispensing.**
C	**9, 11, ~~36~~35**		**2.12.5 mEndo Agar LES is never autoclaved.**
K	9, 11, ~~36~~35		2.12.6 A sufficient amount of medium (4-5 mL) is aseptically aliquoted to each culture plate.
O	9, 11, ~~36~~35		2.12.7 Prepared plates of mEndo Agar LES are stored at 4°C in the dark for no more than two (2) weeks in sealed plastic bags or containers to minimize evaporation.
C	**2**		**2.12.8 Appropriate, properly diluted positive and negative productivity controls for mEndo Agar LES medium are used. Results are recorded and the records maintained.** **Positive productivity control _____** **Negative productivity control _____**
K	9, 11, 21, ~~36~~35		2.12.9 Sterile phosphate buffered water or sterile phosphate buffered saline is used as a sample blank, filter funnel rinse and process and productivity control diluent for UV treated process water samples.
C	**11**		**2.12.10 The phosphate buffered water/saline is properly sterilized and the sterility is tested before being placed in service. Results are recorded and records maintained**
		2.13 Sample Analysis	
C	**9, 11, ~~36~~35**		**2.13.1 The sample is shaken vigorously (25 times in a 12-inch arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) before filtration.**
C	**11, 21, ~~36~~35**		**2.13.2 The membrane filter is placed grid side up within the sterile filter apparatus**.
C	**11, 26, ~~36~~ 35**		**2.13.3 A 100 mL quantity of sample is filtered under vacuum.**
K	26		2.13.4 The pressure of the vacuum pump does not exceed 15 psi.
C	**9, 11, 26,**		**2.13.5 The sides of the filter funnel are rinsed at least twice with 20-30 mL of**

	~~36~~35			**sterile phosphate buffered water/saline as appropriate after filtration.**
C	**9, 11, ~~36~~35**		**2.13.6**	**The membrane filter is removed from the filtering apparatus with sterile forceps and rolled onto mEndo Agar LES so that no bubbles form between the filter and the agar.**
K	9, 11, ~~36~~35		2.13.7	Forceps are dipped in alcohol and flame sterilized between sample filters.
C	**11, ~~36~~35**		**2.13.8**	**Blanks are run at the beginning and at the end of the filtration run to check the sterility of the testing system (phosphate buffered water/saline, filter funnels, forceps, membrane filters, media and culture plates).**
C	**2, ~~36~~35**		**2.13.9**	**An appropriate properly diluted positive process control culture accompanies the sample throughout incubation. Results are recorded and the records are maintained.** **Positive process control _____**
C	**9, 11, ~~36~~35**		**2.13.10**	**Inoculated plates are incubated inverted at 35+/- 0.5°C for 22 to 24 hours.**
K	2, 9, 11		2.13.11	An appropriate level of humidity is maintained in the incubator to prevent the plates from drying out.
		2.14 Computation of Results		
K	9, 11		2.14.1	Colonies are counted with the aid of magnification.
C	**9, 11, 19, 21, 23**		**2.14.2**	**All metallic sheen colonies are counted as total coliforms.**
C	**9, 11, 21, ~~36~~35**		**2.14.3**	**Results are reported as total coliforms/100mL.**
C	**11, 20, ~~36~~35**		**2.14.4**	**When no colonies are observed, results are reported as <1.0 coliform/100mL (nondetectable)**
PART III - SHELLFISH SAMPLES				
		3.1 Collection and Transportation of Samples		
C	**9**		**3.1.1**	**A representative sample of shellstock is collected.**
K	9		3.1.2	Shellstock samples are collected in clean, waterproof, puncture resistant containers loosely sealed.
K	9		3.1.3	Shellstock samples are labeled with collector's name, type of shellstock, the source or harvest area, sampling station, time, date and place (if applicable) of collection.
C	**9**		**3.1.4**	**Immediately after collection, shellfish samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed under refrigeration unless processed immediately.**
C	**1**		**3.1.5**	**Analysis of the samples is initiated as soon as possible after collection. Shellfish samples are not tested if the time interval between collection and analysis exceeds 24 hours.**
		3.2 Preparation of Shellfish for Examination		
K	2,~~11~~32		3.2.1	Shucking knives, scrub brushes and blender jars are (autoclave) sterilized for 15 minutes prior to use.
O	2, 32		3.2.2	Blades of shucking knives are not corroded.
O	9, 32		3.2.3	The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2, 32		3.2.4	The faucet used for rinsing the shellstock does not contain an aerator.
K	9, 32		3.2.5	Shellstock are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9, 32		3.2.6	Shellstock are allowed to drain in a clean container or on clean towels prior to opening.
K	1, 9, 32		3.2.7	Immediately prior to shucking, the hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol or clean gloves are donned.
C	**9, 32**		**3.2.8**	**Shellstock are not shucked directly through the hinge.**

C	**9, 32**		**3.2.9**	**Contents of shellstock (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	2, 9		3.2.10	At least 200 grams of shellfish meat or a quantity of meat sufficient to cover the blender blades is used for the analysis.
K	9		3.2.11	A representative sample of at least 12 shellfish is used for the analysis.
K	2		3.2.12	The sample is weighed to the nearest 0.1 gram and an equal amount by weight of diluent is added.
O	9		3.2.13	Sterile phosphate buffered dilution water is used as the sample diluent.
C	**9**		**3.2.14**	**Samples are blended at high speed for 60 to 120 seconds until homogenous.**
K	9		3.2.15	APHA *Recommended Procedures for the Examination of Sea Water And Shellfish*, Fourth Edition is followed for the analysis of previously shucked and frozen shellfish meats.
		3.3 MPN Analysis for Fecal Coliform Organisms, Presumptive Test, APHA		
C	**9**		**3.3.1**	**Appropriate strength lactose or lauryl tryptose broth is used as presumptive media in the analysis. *(Circle the medium used.)***
C	**2**		**3.3.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
K	9		3.3.3	Immediately (within two (2) minutes) after blending, the ground sample is diluted and inoculated into tubes of presumptive media.
C	**9**		**3.3.4**	**No fewer than five (5) tubes per dilution are used in a multiple dilution MPN series.**
C	**9**		**3.3.5**	**Allowing for the initial 1:1 dilution of the sample, appropriate portions are inoculated (i.e., 2 ml of original 1:1 dilution for the 1 g portion) and diluted for subsequent inoculation (i.e., 20 ml of 1:1 diluted sample to 80 ml of diluent or the equivalent for 0.1 g portion). All successive dilutions are prepared conventionally.**
K	6		3.3.6	In a single dilution series, the volumes examined are adequate to meet the needs of routine monitoring. Sample volume inoculated _____ Range of MPN_____ Strength of media used_____
C	**2**		**3.3.7**	**Appropriately diluted process control cultures accompany the samples throughout both the presumptive and confirmed phases of incubation. Results are recorded and the records maintained.** **Positive Process control _____ Negative Process control_____**
K	9		3.3.8	Inoculated media are incubated at 35 ± 0.5°C.
K	10		3.3.9	Tubes are read after 24 ± 2 hours of incubation and transferred if positive for growth (the presence of turbidity and gas or effervescence in the culture tube). These tubes are considered presumptive requiring further confirmatory testing.
		3.4 Confirmed Test for Fecal Coliforms - APHA		
C	**9**		**3.4.1**	**EC medium is used as the confirmatory medium.**
C	**2**		**3.4.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
K	9, 11		3.4.3	Transfers are made to EC medium by either sterile loop or hardwood sterile transfer sticks from positive presumptives. *(Circle the method of transfer.)*
C	**9**		**3.4.4**	**EC tubes are incubated in a circulating waterbath at 44.5 ± 0.2°C**
K	9		3.4.5	EC tubes are read for gas production after 24 ± 2 hours of incubation.
C	**9**		**3.4.6**	**The presence of turbidity and any amount of gas and/or effervescence in the Durham tube constitutes a positive test.**

		3.5 Computation of Results for MPN Analyses		
K	9		3.5.1	Results of multiple dilution tests are read from tables in *Recommended Procedure for the Examination of Sea Water and Shellfish,* 4th Edition and multiplied by the appropriate dilution factor.
K	7		3.5.2	Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**9**		**3.5.3**	**Results are reported as MPN/100 grams of sample.**
		3.6 Standard Plate Count Method		
O	20		3.6.1	A standard plate count (SPC) analysis may be performed in conjunction with the analysis for fecal coliform organisms.
K	9		3.6.2	In the standard plate count procedure at least four (4) plates are used, duplicates of two (2) dilutions. One (1) of the dilutions should produce colonies of 30 to 300 per plate.
K	2		3.6.3	~~Fifteen~~ 15 to 20 mL of tempered sterile plate count agar is used per plate.
C	**9**		**3.6.4**	**Agar tempering bath maintains the agar at 44-46°C.**
C	**9**		**3.6.5**	**An agar based temperature control having a similar volume and shape as the tempering plate count agar is used in the tempering bath.**
K	9		3.6.6	Samples or sample dilutions to be plated are shaken vigorously (25 times in a 12" arc in seven (7) seconds) before plating.
C	**9**		**3.6.7**	**Not more than 1 mL nor less than 0.1 mL of sample or sample dilution is plated.**
K	11		3.6.8	Control plates are used to check air quality and the sterility of the agar and the diluent.
K	9, 21		3.6.9	Solidified plates are incubated at 35 ± 0.5°C for 48 ± 3 hours inverted and stacked no more than four (4) high.
K	9		3.6.10	Quebec Colony Counter or its equivalent is used to provide the necessary magnification and visibility for counting plates.
K	1		3.6.11	A hand tally or its equivalent is used for accuracy in counting.
		3.7 Computation of Results -SPC		
K	9		3.7.1	Colony counts determined in accordance with Part III, A, Sections 4.31 through 4.33 in *Recommended Procedures for the Examination of Sea Water and Shellfish*, Fourth Edition.
C	**19**		**3.7.2**	**Colony counts are reported as CFU/grams of sample.**
		3.8 Bacteriological Analysis of Shellfish Using the ETCP		
C	**2, 3**		**3.8.1**	**Prepared modified MacConkey agar is used on the day that it is made.**
K	3		3.8.2	Double strength modified MacConkey agar is used.
C	**3**		**3.8.3**	**Prepared double strength modified MacConkey agar is heated to boiling, removed from the heat, and boiled again. This agar is never autoclaved.**
K	2, 3		3.8.4	Twice boiled, double strength modified MacConkey agar and is maintained in a tempering bath at 45 to 50°C until used.
K	2, 3		3.8.5	Phosphate buffered saline is used as the sample diluent in the ETCP.
C	**2, 3**		**3.8.6**	**The phosphate buffered saline is tempered at 45 - 50°C to prevent premature solidification of the agar.**
C	**9**		**3.8.7**	**The sample homogenate is cultured within two (2) minutes of blending.**
C	**2, 3**		**3.8.8**	**Six (6) grams of shellfish (12 grams of homogenate if initially diluted 1:1) is placed into a sterile container and the contents brought up to 60 mL with sterile, tempered phosphate buffered saline.**
K	3		3.8.9	Sixty (60) mL of tempered, twice boiled double strength Modified MacConkey Agar is added.
K	2, 3, 22		3.8.10	The container is gently swirled or slowly inverted once to mix the contents,

				which are subsequently distributed uniformly over six (6) plates.
C	**1**		**3.8.11**	**Media and diluent sterility are determined with each use. Results are recorded and the records maintained.**
C	**1**		**3.8.12**	**Media productivity is determined using media appropriate properly diluted pour plated positive and negative control cultures for each batch of Modified MacConkey agar prepared.** **Positive control culture _____ Negative control culture _____**
C	**3, 13**		**3.8.13**	**When solidified, the plates are placed inverted into an air incubator at ~~45~~ 45.5 ± 0.5°C for 18 to 30 hours of incubation.**
C	**2**		**3.8.14**	**Plates are stacked no more than three (3) high in the incubator.**
C	**2**		**3.8.15**	**Appropriately diluted pour plated process control cultures *accompany each set of samples throughout incubation.* The results are recorded and the records maintained.** **Positive process control_____ Negative process control_____**
		3.9 Computation of Results - ETCP		
K	11		3.9.1	Quebec Colony counter or its equivalent is used to provide the necessary magnification and visibility for counting.
O	1		3.9.2	A hand tally or its equivalent is used to aid in counting.
C	**3, 6**		**3.9.3**	**All brick red colonies greater than 0.5 mm in diameter are totaled over all the plates and multiplied by a factor of 16.7.**
C	**3**		**3.9.4**	**Results are reported as CFU/100 grams of sample.**
		Bacteriological Examination of ~~Soft-shelled Clams and American Oysters~~Shellfish Meats for Male Specific Coliphage (MSC)		
		3.10 MSC Equipment and Supplies		
K	~~3~~02		3.10.1	Sample containers used for the shucked sample are sterile, made of glass or some other inert material (i.e. polypropylene) and hold at least 100~~—125~~ mL.
~~**C**~~	~~**27, 28**~~		~~**3.10.2**~~	~~**The refrigerated centrifuge used must have the capacity to accommodate the amount of shellfish sample required for the procedure, perform at 9000 x g and maintain a temperature of 4°C.**~~
K	~~9~~2		3.10.~~3~~2	The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
~~**C**~~	~~**27, 28**~~		~~**3.10.4**~~	~~**Sterile 0.22 µm pore size syringe filters and pre-sterilized plastic or sterile glass syringes are used to sterilize the antibiotic solutions.**~~
K	1		3.10.~~5~~3	The sterility of each batch/lot of pre-sterilized or reusable syringes, ~~and syringe~~ filters, and/or filter units is determined. Results are recorded and records maintained.
~~**K**~~	~~**1**~~		~~3.10.6~~	~~The sterility of each batch of reusable glass syringes is determined. Results are recorded and records maintained.~~
C	~~**27, 28**~~2		**3.10.~~7~~4**	**The balance used provides a sensitivity of at least mg (0.01g.).**
C	**27, 28, 31**		**3.10.~~8~~5**	**The temperature of the incubator used is maintained at 36 ± 1°C.**
K	2		3.10.6	The temperature of the freezer is maintained at ≤ -15° C.
C	~~**2**~~1		**3.10.~~9~~7**	**~~Sterile~~ The sterility of disposable 50 mL centrifuge tubes ~~are used and their sterility~~ is determined with each lot. Results are recorded and records maintained.**
		3.11 MSC Media Preparation		
K	28, 31		3.11.1	Media preparation and sterilization is according to the validated method.
K	27, 28, 31		3.11.2	~~Bottom agar, double strength soft agar and growth broth are prepared from their individual components~~Antibiotic solutions are filter sterilized using sterile 0.22 µm pore size filters..
~~**K**~~	~~**27, 28**~~		~~3.11.3~~	~~Soft agar is prepared double strength in volumes of 2.5 mL.~~
~~**C**~~	~~**27, 28**~~		~~**3.11.4**~~	~~**The streptomycin and ampicillin solutions are added to tempered bottom agar and vortex for 2 minutes on stir plate.**~~

O	27, 28, 31		3.11.5 3 Storage of the bottom agar under refrigeration does not exceed 1 month six (6) weeks.
K	27, 282		3.11.6 4 Unsterilized soft agar is stored at ≤ 20 °C -15° C for up to three (3) months.
K	27, 28, 31		3.11.7 5 The soft agar is removed from the freezer and sterilized for 15 minutes at 121°C before use.
K	27, 28		3.11.8 Storage of growth broth in the refrigerator in loosely capped tubes/bottles does not exceed 1 month and in screw capped tubes/bottles does not exceed 3 months.
K	28, 31		3.11.6 Storage under refrigeration of prepared growth broth with screw-cap closures shall not exceed three (3) months and with loose fitting closures shall not exceed one (1) month.
K	2, 27, 28, 31		3.11.9 7 Bottom agar plates stored under refrigeration are allowed to reach room temperature before use.
		3.12 Preparation of Host Culture for MSC Analysis	
C	**28, 31**		**3.12.1 E. coli *Famp* ATCC 700891 is the bacterial host strain.**
K	27, 28, 31		3.12.2 Host cell growth broth is tempered at 36 ± 1° C prior to inoculation with host cells.
K	27, 28, 31		3.12.3 Several host cell colonies are transferred to a tube of tempered growth broth and incubated at 36 ± 1° C for 4-6 hours to provide host cells in log phase growth for sample analysis.
C	**27, 28, 31**		**3.12.4 After inoculation, the host cell growth broth culture is not shaken.**
		3.12 13 Preparation of the Soft-Shelled Clams and American OystersShellfish for MSC Analysis	
K	2, 11 36		3.1213.1 Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2		3.1213.2 The blades of shucking knives are not corroded.
O	9		3.1213.3 The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2		3.1213.4 The faucet used for rinsing the shellfish does not contain an aerator.
K	9		3.1213.5 The shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9		3.1213.6 The shellfish are allowed to drain in a clean container or on clean towels unlayered prior to shucking.
K	9		3.132.7 Immediately prior to shucking, the hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol or clean gloves are donned.
C	**9**		**3.1213.8 Shellfish are not shucked through the hinge.**
C	**9**		**3.1213.9 The contents of shellfish (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	9		3.1213.10 A representative sample of at least 12 shellfish is used for the analysis.
K	2, 19		3.1213.11 The sample is weighed to the nearest 0.1 gram.
C	**28, 31**		**3.13.2 Two (2) times the weight of the sample of sterile growth broth, by volume, is added.**
C	**28, 31**		**3.13.13 Samples are blended at high speed for 180 seconds.**
		3.13 14 MSC Sample Analysis	
C	**28**		**3.13.1 E. coli *Famp* ATCC 700891 is the bacterial host strain used in this procedure.**
K	27, 28		3.13.2 Host cell growth broth is tempered at 36 ± 1°C and vortexed (or shaken) to aerate prior to inoculation with host cells.
K	27, 28		3.13.3 Several host cell colonies are transferred to a tube of tempered, aerated growth broth and incubated at 36 ± 1°C for 4-6 hours to provide host cells in log phase growth for sample analysis.
C	**27, 28**		**3.13.4 After inoculation, the host cell growth broth culture is not shaken.**
C	**28**		**3.13.5 A 2:1 mixture of sterile growth broth to shellfish tissue is used for eluting**

			~~the MSC.~~
~~C~~	~~28~~		~~3.13.6 The elution mixture is prepared w/v by weighing the sample and adding two equal portions of sterile growth broth by volume to the shellfish tissue.~~
~~C~~	~~28~~		~~3.13.7 The elution mixture is homogenized at high speed for 180 seconds.~~
C	28, 31		**3.~~13.8~~14.1 Immediately after blending, 33 grams of the ~~homogenized~~ homogenate ~~elution mixture are~~ is weighed into a centrifuge tube~~s~~.**
C	28, 31		**3.~~13.9~~14.2 The homogenized elution mixture is centrifuged for 15 minutes at 9000 x g at 4°C.**
C	27, 28, 31		**3.~~13.10~~14.3 The supernatant is ~~pipetted off~~transferred to a new stertile tube, weighed, and the weight recorded.**
C	27, 28, 31		**3.~~13.11~~14.4 The supernatant is allowed to warm to room temperature ~~about 20 to 30 minutes~~prior to analysis.**
K	27, 28, 31		3.~~13.12~~14.5 The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28, 31		3.~~13.13~~14.6 ~~Two hundred~~200 microliters (0.2 mL) of log phase host strain *E coli* is added to the ~~tempering~~ tempered soft agar immediately prior to adding the sample supernatant.
K	27, 28, 31		3.~~13.14~~14.7 The sample supernatant is shaken or vortexed before being added to the ~~tempering~~ tempered soft agar.
C	27, 28, 31		**3.~~13.15~~14.8 2.5 mL of sample supernatant (avoiding bubbles where necessary) is added to ~~each~~ a tube of ~~tempering~~ tempered soft agar.**
C	27, 28, 31		**3.~~13.16~~14.9 The tube of soft agar/sample supernatant/host cell mixture is gently rolled between the palms of the hands to mix.**
C	27, 28, 31		**3.~~13.17~~14.10 The ~~soft agar/sample supernatant/host cell mixture~~tube contents is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**
C	28, 31		**3.~~13.18~~14.11 ~~Ten (~~10~~)~~ plates are used for analysis of each sample with~~,~~ 2.5 mL of sample supernatant per plate for a total of 25 mL of supernatant analyzed per sample, unless fewer than 25 mL of supernatant is obtained from the sample in which all supernatant is plated.~~.~~**
K	27, 28, 31		3.~~13.19~~14.12 Negative and positive control plates are prepared and accompany each set of samples analyzed. The results are recorded and records maintained. Positive control _____
K	27, 28, 31		3.~~13.20~~14.13 Room temperature g~~G~~rowth broth is used as the negative control or blank.
K	27, 28, 31		3.~~13.21~~14.14 Type strain MS2 (ATCC 15597-B1) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.
K	2		3.~~13.22~~14.15 A negative control plate is plated at the ~~beginning and~~ end of each set of samples analyzed.
K	27, 28, 31		3.~~13.23~~14.16 The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	27, 28, 31		**3.~~13.24~~14.17 All plates are incubated at 36 ± 1° C for 18 ± 2 hours.**
		3.~~14~~ 15 Computation of Results - MSC	
C	27		**3.~~14~~15.1 Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted.**
C	28, 31, ~~32~~36		**3.~~14~~15.2 The working range of the method is 1 to 200 PFU per plate. When there are no plaques on all ten plates, the ~~count~~ reported value is <6 PFU/100 grams for soft- shelled clams, <7 PFU/ 100 grams for American oysters, and <5 PFU/ 100 grams for quahog (hard) clams. If the density exceeds 200 PFU per plate on all plates, the count is given as > 20,000 PFU/100 grams.**
K	28, 31		3.~~14~~15.3 The formula used for determining the density of MSC in PFU/100 grams is: (0.364) (N) (Ws), where N = total number of plaques counted on all 10 plates and Ws = weight of the supernatant used.
O	~~9~~2		3.~~14~~15.4 The MSC count is rounded off conventionally to give a whole number.

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SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
MICROBIOLOGICAL COMPONENT: (Part I-III)	
A. Results Total # of Critical (C) Nonconformities in Parts I-III Total # of Key (K) Nonconformities in Parts I-III Total # of Critical, Key and Other (O) Nonconformities in Parts I-III	 _____ _____ _____

B. Criteria for Determining Laboratory Status of the Microbiological Component:

1. **Does Not Conform Status**: The Microbiological component of this laboratory is not in conformity with NSSP requirements if:

   a. The total # of Critical nonconformities is **≥ 4 or**

   b. The total # of Key nonconformities is **≥ 13 or**

   c. The total # of Critical, Key and Other is **≥ 18**

2. **Provisionally Conforms Status**: The microbiological component of this laboratory is determined to be provisionally conforming to NSSP requirements if the number of critical nonconformities is **≥ 1 but ≤ 3**.

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature: _____ Date:_____

LEO Signature: _____ Date:_____

NSSP Form LAB-100 Microbiology Rev. October 2015

Receptor Binding Assay for Paralytic Shellfish Poisoning (PSP)

PART I – Quality Assurance

ITEM			
CODE	**REF**		
		1.1 Quality Assurance (QA) Plan	
K	1, 2, 3	1.1.1 Written Plan (Check √ those items which apply).	
			a. Organization of the Laboratory.
			b. Staff training requirements. Training must include radiation lab safety.
			c. Standard operating procedures (SOPs).
			d. Internal quality control measures for equipment, their calibration maintenance, repair, performance and rejection criteria established.
			e. Laboratory safety. Radiation safety practices (e.g., handling and disposal) must be included.
			f. Internal performance assessment.
			g. External performance assessment.
C	**2**		**1.1.2 The QA plan is implemented.**
		1.2 Educational/Experience Requirements	
C	**State's Human Resources Department**		**1.2.1 In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2 In state/county laboratories, the analysts meet the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3 In commercial laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or other appropriate discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4 In commercial laboratories, the analysts must have at least a high school diploma and at least three months of experience in laboratory sciences.
C	**6**		**1.2.5 Training regarding radiation laboratory safety, handling and disposal practices ~~and verification of licensing must be provided~~is documented and records are maintained.**
C	**15**		**1.2.6 Laboratory has a Nuclear Regulatory Commission (NRC) or equivalent state license for the use of tritiated saxitioxin in this assay. Alternatively, the laboratory uses less than 50 µCi per year and adheres to the American Radiolabeled Chemical (ARC) exemption status.**
		1.3 Work Area	
O	2		1.3.1 The work area is adequate for the workload and storage.
K	2		1.3.2 The work area is clean and well lighted.
K	2		1.3.3 The work area has adequate temperature control.
O	3		1.3.4 All work surfaces are nonporous, easily cleaned and disinfected.
C	**3,4**		**1.3.5 The work area is located in an appropriate space designated for low-level radiation work. Radioactive materials are only handled and manipulated in designated areas which are clearly identified and labeled accordingly.**
		1.4 Laboratory Equipment	
C	**4**		**1.4.1 Any lab equipment that may come into contact with [^{3}H]-STX at any point in the preparation or assay procedures must be specially labelled and must**

			remain in the work area designated for low-level radiation work.
O	5		1.4.2 The pH meter has a standard accuracy of 0.1 pH units.
K	7		1.4.3 The pH electrodes being used consist of a pH half cell and reference half cell or equivalent combination electrode/triode free from silver/silver chloride (Ag/AgCl) or contains an ion exchange barrier to prevent the passage of silver (Ag) ions into the substance being measured.
K	3, 8		1.4.4 The pH meter is calibrated daily when in use. Results are recorded and records maintained.
K	1		1.4.5 The effect of temperature on the pH has been compensated for by an ATC probe, use of a triode, or by manual adjustment.
K	1		1.4.6 The pH meter manufacturer instructions are followed for calibration, or a minimum of two (2) standard buffer solutions is used to calibrate the pH meter. If the calibration sequence of standard buffer solutions is not stipulated by the manufacturer, the first must be near the isopotential point (pH 7) and the second near the expected sample (i.e., pH 4 or pH 10). Standard buffer solutions are used once and discarded.
O	9		1.4.7 Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope.
K	6		1.4.8 pH paper in the appropriate pH range (i.e., 1-5), if used, measures accurately to a minimum of 0.5 pH units over the covered pH range.
K	6		1.4.9 The differing sensitivities in weight measurements required by the various steps in the assay are met by the balance(s) being used. a. To prepare Phenyl methylsulfonyl fluoride solution (PMSF), the balance used must have a sensitivity of at least 0.001 gram at a load of 1 gram. b. For sample extraction, the balance used must have a sensitivity of at least 0.1 gram at a load of 100 grams. c. For MOPS buffer preparation, the balance used must have a sensitivity of at least 0.01 gram at a load of 100 grams.
K	1, 3		1.4.10 Balance calibrations are checked monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use.
			1.4.11 Balances must be calibrated by an external service at least once per year. Results are recorded and records maintained.
K	2		1.4.12 Refrigerator temperatures are maintained between 0 and 4 °C. Freezer security for ^{3}HSTX and cold STX must meet state and federal requirements for these materials.
K	1		1.4.13 Refrigerator temperatures are monitored at least once daily on workdays. Results are recorded and records maintained.
C	**4, 6, 10**		**1.4.14 Freezer temperature used to store [^{3}H] STX standard, rat brain membrane tissue preparation, interassay calibration standard (QC check) and archived shellfish tissue homogenate is maintained at -80 °C or below. Freezer security for ^{3}HSTX and cold STX must meet state and federal requirements for these materials.**
K	6, 10		1.4.15 Freezer temperature used for all other purposes is maintained at -20 °C or below.
O	1		1.4.16 Freezer temperature is monitored at least once daily on workdays. Results are recorded and records maintained.
O	8		1.4.17 All glassware is clean.
C	**3**		**1.4.18 An alkaline or acid-based detergent is used for washing glassware/labware.**
C	**1**		**1.4.19 With each load of labware/glassware washed, the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali as appropriate) with aqueous 0.04% bromothymol blue (BTB) solution. Results are recorded and records maintained.**
C	**6**		**1.4.20 Micropipettors are calibrated for the appropriate volumes used and checked**

			annually for accuracy. Results are recorded and records are maintained.
C	**11**		**1.4.21 Scintillation counter is serviced according to manufacturer specifications and calibrated annually. Results are recorded and records maintained.**
C	**4**		**1.4.22 Minimum radiation safety equipment and protocols include the following: A wipe-test is conducted in the radiation work area as described in the QA plan. Results are recorded and records maintained.**
		1.5 Reference Solution Reagent Storage, Preparation and Security	
C	**12**		**1.5.1 [^{3}H] STX standard is stored in a freezer at -80 °C or below.**
C	**10**		**1.5.2 Concentration of [^{3}H] STX standard is calculated from the lot information provided by the supplier with each batch.**
K	6		1.5.3 Unopened diHCl STX standard may be stored at room temperature or refrigerated.
C	**10**		**1.5.4 Preparation of MOPS assay buffer includes the following:** **a. 100 mM MOPS/L.** **b. 100 mM choline chloride/L.** **c. pH adjustment to 7.4 with NaOH.** **e. refrigerated storage at 4 °C.** **d. Maintained ice cold while in use.**
C	**10**		**1.5.6 Bulk standard curve dilutions are stored at 4 °C for up to one (1) month.**
K	1		1.5.7 Reagent water is distilled or deionized *(circle appropriate choice)* and is analyzed monthly for the following criteria, with all results recorded and records maintained: a. Exceeds 0.5 megohm-cm resistivity (2 megohm-cm in-line) or less than 2.0 µSiemens/cm conductivity at 25 °C *(circle appropriate choice).* b. Residual chlorine is at a non-detectable level (<0.1 ppm). Specify method of determination_____. c. Water contains <100 CFU/mL using the heterotrophic plate count method.
		1.6 Rat Brain Membrane Tissue Preparation and Storage	
C	**10**		**1.6.1 MOPS/choline chloride/phenyl methylsulfonyl floride (PMSF), pH 7.4 is used in preparing rat brain membrane tissue. PMSF is added to MOPS/choline chloride fresh on the day of use.**
C	**10**		**1.6.2 The cerebral cortex of 6-week old Sprague-Dawley rats is used in membrane tissue preparations, placed in iced MOPS/choline chloride/PMSF buffer (pH 7.4; 1 brain/12.5 mL) and homogenized with no visible chunks remaining in the homogenate. This procedure is repeated until twenty (20) rat brains have been processed.**
C	**10**		**1.6.3 The homogenized cerebral cortex tissue from the twenty (20) rat brain cortices is pooled and centrifuged at 20000 x g for 15 minutes at 4 °C.**
K	10		1.6.4 The pellet of the centrifuged rat brain tissue preparation is fully resuspended in ice cold MOPS/choline chloride/PMSF buffer (up to 10 mL/brain).
K	10		1.6.5 The resuspended rat brain tissue preparations are pooled and the centrifuge tubes used for these preparations are rinsed with a small amount of MOPS/choline chloride/PMSF buffer to recover all the rat brain tissue**.**
K	10		1.6.6 The total volume of the pooled rat brain tissue is adjusted to 200 mL with MOPS/choline chloride/PMSF buffer while iced.
K	10		1.6.7 The iced contents of the pooled rat brain tissue are blended using a Polytron at 70% power or a small hand- held blender at low speed for 20 seconds to obtain a homogeneous membrane tissue preparation.
C	**10**		**1.6.8 Two (2) mL/tube of the pooled, homogeneous rat brain membrane tissue preparation is aliquoted into cryovials, frozen and stored at -80 °C for up to six (6) months.**
		1.7 Rat Brain Membrane Tissue Protein Receptor Determination	
C	**10**		**1.7.1 The protein/receptor concentration of the rat brain membrane tissue preparation is determined for each new batch using a Pierce Micro BCA Protein Assay Reagent Kit No. 23235 (micro plate method) or No. 23225 (tube**

			method) or equivalent.
C	**10**		**1.7.2 The dilution of the protein/receptor concentration of the rat brain membrane tissue preparation needed to obtain a working stock of 1 mg/mL is determined.**
K	10		1.7.3 Dilutions of the protein/receptor concentration of the rat brain membrane tissue preparation of less than 1:4 are not used as they may be too viscous.
PART II – Analysis of Shellfish Samples for PSP Toxins – RBA			
		2.1 Collection and Transportation of Samples	
C	**5**		**2.1.1 A representative sample of shellfish is collected.**
K	5		2.1.2 Shellfish samples are collected in clean, waterproof, puncture resistant containers loosely sealed.
K	5		2.1.3 Shellfish samples are labeled with the collector's name, type of shellstock, the source or harvest area, sampling station, time, date and place (if applicable) of collection.
C	**5**		**2.1.4 Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10 °C with ice or cold packs for transport to the laboratory.**
K	6, 13		2.1.5 Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur, the laboratory has an appropriate contingency plan in place to handle these samples. For samples shipped live in accordance with 2.1.4, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt. The contingency plan also addresses field and/or laboratory processing that ensures the integrity of the sample or extract until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.
			2.2 Preparation of Samples for Analysis – Homogenization
C	**5, 6**		**2.2.1 At least 12 animals are used per sample, or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish or collection conditions.**
O	5		2.2.2 The outside of the shell is thoroughly cleaned with fresh water.
O	5		2.2.3 Shellstock are opened by cutting the adductor muscles.
O	5		2.2.4 The inside surfaces of the shells and meats are rinsed with fresh water to remove sand or other foreign material.
O	5		2.2.5 Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	**5**		**2.2.6 Damage to the body of the mollusk is minimized in the process of opening.**
O	5		2.2.7 Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for 5 minutes.
K	5		2.2.8 Pieces of shell and drainage are discarded.

C	**5, 6**		**2.2.4 Meats are blended at high speed until homogenous (60 – 120 seconds), using the following criteria:** **a. Freshly drained/air dried meats are placed into the blender for homogenization.** **b. Previously frozen shucked, rinsed, and drained meats are completely thawed, then placed in the blender with all freeze-thaw liquid for homogenization.** **c. Previously frozen homogenates are completely thawed then placed in the blender with all freeze-thaw liquid for homogenization.**
K	6, 13		2.2.5 Homogenates should be extracted immediately. If homogenates must be stored, they should be frozen.
			2.3 Preparation of Samples for Analysis – Extraction
K	5, 10		2.3.1 0.1 M HCl is used for extractions.
K	5, 10		2.3.2 At least f~~F~~ive (5) grams of tissue +/- 0.1g is extracted using ~~an equal amount~~a 1:1 mass to volume ratio of 0.1 M HCl.
C	**10**		**2.3.3 The pH of the sample is checked and adjusted as necessary to between 3.0–4.0.**
C	**10**		**2.3.4 Adjustment of the pH is accomplished by dropwise addition of either 5 N HCl or 0.1 N NaOH, as appropriate, while constantly stirring the sample.**
C	**6**		**2.3.5 The sample is promptly brought to a boil at 99.0 +/- 1.0 °C and gently boiled for 5 minutes.**
O	6		2.3.6 The sample is boiled under adequate ventilation (e.g., fume hood).
O	10		2.3.7 The sample is allowed to cool to room temperature.
C	**10**		**2.3.8 The pH of the cooled mixture after boiling is between 3.0 - 4.0, adjusted if necessary, with the dropwise addition of 5 M HCl to lower the pH or 0.1 M NaOH to raise the pH, as appropriate, while constantly stirring the mixture.**
K	5, 10		2.3.9 The volume of the sample is adjusted to the original (pre-boiling) volume, by adding 0.001N HCl (pH 3 water).
K	10		2.3.10 The sample is stirred gently to homogeneity, then treated as follows: a. The sample is allowed to settle to remove particulates, then the supernatant is carefully decanted into a clean container; then b. an aliquot of the sample is centrifuged at 3000 x g for 10 minutes, then the supernatant is carefully decanted into a clean container.
K	6, 10		2.3.11 The sample extract is analyzed immediately, refrigerated at 4 °C in a sealed container for up to 24 hours, or frozen at -20 °C.
			2.4 Sample Assay
K	6		2.4.1 One analyst performs the entire plate set-up for the assay.
K	6		2.4.2 Microtubes containing dilutions and samples are vortexed immediately before dispensing.
K	10		2.4.3 The standard curve consists of at least 7 concentrations (minimum 6 x 10^{-10} M and maximum 6 x 10^{-6} M).
C	**10**		**2.4.4 The rat brain membrane tissue preparation is kept on ice and mixed often during addition to the plate to maintain a homogenous suspension.**
K	10		2.4.5 Each day an assay is conducted, a standard curve~~, reference blank, and an inter-assay QC calibration standard~~ is required. However, filter plates of the same lot must be used if the assay requires multiple plates to accommodate all samples. If the filter plate lot changes over the course of a day, a new standard curve must be performed for the new lot of filter plates. An inter-assay QC calibration and reference blank are required for each plate analyzed.
C	**10**		**2.4.6 The standard curve, reference blank, interassay QC calibration standard, and test samples are all run in triplicate.**
K	10		2.4.7 Assay buffer is added to the plate before any other components of the assay, in order to properly wet the filter membrane.

K	10		2.4.8 All wells of the plate (including any unused wells) are filled with MOPS/choline chloride buffer during vacuum filtration, in order to ensure even pressure and filtration across the plate.
C	**10**		**2.4.9 Appropriate scintillation cocktail is used, depending on the type of scintillation counter (traditional or microplate).**
K	10		2.4.10 ~~If~~ [^{3}H] STX working solution is checked for counts per minute (CPM) ~~it should be~~ and is consistent ~~and~~ within 15% of the expected value.
C	10		**2.4.11 An appropriate dark adaptation interval is employed, based on type of scintillation counter (traditional or microplate).**
K	10		2.4.12 Standard curve fitting is calculated using appropriate software program.
C	**10**		**2.4.13 Slope of standard curve is between -0.8 and -1.2 (the theoretical slope is -1.0). If the slope falls outside these criteria, the assay results are rejected and the assay must be repeated.**
C	**10**		**2.4.14 The relative standard deviation of triplicate CPM for standards and samples must be less than 30%. If greater than 30%, the assay results are rejected and the assay must be repeated.**
C	**10**		**2.4.15 The IC_{50} is in acceptable range (2.0 nM +/- 30%). If the IC_{50} is outside this range, the assay results are rejected and the assay must be repeated**
C	**10**		**2.4.16 The inter-assay QC calibration standard (QC check) sample is in the acceptable range (3 nM +/- 30%). If the QC check sample is outside this range, the assay results are rejected and the assay must be repeated.**
C	**10**		**2.4.17 Sample dilutions are quantified only if B/B_0 is between 0.2 – 0.7. If B/B_0 is greater than 0.7, then the sample is reported as below the limit of detection. If B/B_0 is less than 0.2, then the sample should be further diluted and repeated if a quantification is needed.**
K	4		2.4.18 Assay materials are cleaned and disposed of in accordance with federal, state, and local requirements.
		2.5 Calculation of Sample Toxicity	
C	**10**		**2.5.1 When more than one dilution falls within B/B_0 of 0.2 – 0.7, all wells corresponding to these dilutions are used to calculate sample toxicity.**
C	**10**		**2.5.2 Sample toxicity is calculated as follows:** **(nM STX equiv.) x (sample dilution) x (210 µL total volume/35 µL sample = mM STX equivalent in extract** **(nM STX diHCl equiv. in extract) x 1L/1000 mL x 372 ng/nmol x1 µg/1000 ng =µg STX diHCl equiv./mL** **µg STX diHCl equiv./mL x mL extract/g shellfish x 1000 g/kg =µg STX diHCl equiv./kg**
C	**14**		**2.5.3 Any value equal to or greater than 80 µg STX diHCl equiv./100 g) of sample is actionable.**
C			**Shellfish Program Management is made aware of positive result. Laboratory action to identify positive result is:_____.**

References:

1. American Public Health Association (APHA). 1992. *Standard Methods for Examination of Water and Wastewater*, 18th Edition. APHA/AWWA/WEF, Washington, D.C.

2. American Public Health Association (APHA). 1984. *Compendium of Methods for the Microbiological Examination of Foods*, 2nd Edition. APHA, Washington, D.C.

3. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Diary Products*, 16th Edition. APHA, Washington, D.C.

4. Appendix C: Radiation Safety Requirements, ISSC Proposal 13-114 Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination.

5. American Public Health Association (APHA). 1970. Recommended Procedures for the Examination of Sea Water and Shellfish, Fourth Edition. APHA, Washington, D.C.

6. Good Laboratory Practice.

7. Fisher J. 1985. Measurement of pH. *American Laboratory* 16:54-60.

8. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories*. AOAC, Arlington, VA.

9. Consult pH electrode product literature.

10. Association of Official Analytical Chemists (AOAC). 2016. Official Method 2011.27 Paralytic Shellfish Toxins (PSTs) in Shellfish Receptor Binding Assay.

11. Consult instrument manufacturer instructions.

12. Technical Data Sheet, American Radiolabeled Chemicals, Inc. 101 Arc Drive, St. Louis, MO 63146.

13. Wilt, d. s. (ed). 1974. Proceedings of the 8th National Shellfish Sanitation Workshop. U. S. Food and Drug Administration, Washington, D.C.

14. U. S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 2017. NSSP *Guide for the Control of Molluscan Shellfish*. FDA/ISSC, Washington D.C. and Columbia, S.C.

15. U. S. Nuclear Regulatory Commission Materials, Section 30.18, 10 CFR Part 30, and American Radiolabeled Chemicals Licenses.

Forr

PART 1 - QUALITY ASSURANCE			
CODE	**REF.**		**ITEM**
K	8, 11	**1.1 Quality Assurance (QA) Plan**	
			1.1.1 Written Plan (Check those items which apply.)
			a. Organization of the laboratory.
			b. Staff training requirements.
			c. Standard operating procedures.
			d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance, and rejection criteria established.
			e. Laboratory safety.
			f. Internal performance assessment.
			g. External performance assessment.
C	**8**		**1.1.2 QA Plan Implemented.**
K	11		1.1.3 The Laboratory participates in a proficiency testing program annually. Specify Program(s)_____
		1.2 Educational/Experience Requirements	
C	**State's Human Resources Department**		**1.2.1 In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2 In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3 In commercial laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, or equivalent discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4 In commercial laboratories, the analyst(s) must have at least a high school diploma and shall have at least three months of experience in laboratory science
		1.3 Work Area	
O	8,11		1.3.1 Adequate for workload and storage.
K	11		1.3.2 Clean, well-lighted.
K	11		1.3.3 Adequate temperature control.
O	11		1.3.4 All work surfaces are nonporous, easily cleaned and disinfected.
K	11		1.3.5 Microbiological quality of the air is fewer than 15 colonies for a 15 minute exposure and determined monthly. The results are recorded and records maintained.
		1.4 Laboratory Equipment	
O	9		1.4.1 To determine the pH of prepared media, the pH meter has a standard accuracy of 0.1 units.
O	14		1.4.2 pH electrodes consisting of pH half-cell and reference half-cell or equivalent combination electrode free from (Ag/AgCl) or contains an ion exchange barrier preventing passage of Ag ions into the medium which may affect the accuracy of the pH reading.
K	11		1.4.3 The effect of temperature on the pH is compensated for by an ATC probe or by manual adjustment.
K	8		1.4.4 pH meter is ca libra ted daily or with each use Results are recorded and records maintained.
K	11		1.4.5 A minimum of two standard buffer solutions is used to calibrate the pH meter. The first must be near the electrode isopotential point (pH 7). The second near the expected sample pH (i.e., pH 4 or pH 10). Standard buffer solutions are used once and discarded.
O	8,15		1.4.6 Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope. (*Circle the method used.*)

K	9		1.4.7	Balance provides a sensitivity of at least 0.1 g at weights of use.
K	11,13		1.4.8	Balance calibrations are checked monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use. Results are recorded and records maintained.
K	11		1.4.9	Refrigerator temperature(s) are monitored at least once daily on workdays Results are recorded and records maintained.
K	1		1.4.10	Refrigerator temperature is maintained at 0 to 4°C.
C	**9**		**1.4.11**	**The temperature of the incubator is maintained at 35 ± 0.5°C.**
C	**11**		**1.4.12**	**Thermometers used in the air incubator(s) are graduated in at least 0.1°C increments.**
K	9		1.4.13	Working thermometers are located on top and bottom shelves or appropriately placed based on the results of spatial temperature checks.
C	**11**		**1.4.14**	**Temperature of the waterbath is maintained at 44.5 ± 0.2°C under all loading conditions.**
C	**9**		**1.4.15**	**The thermometers used in the waterbath are graduated in at least 0.1°C increments.**
C	**13**		**1.4.16**	**The waterbath has adequate capacity for workload.**
K	9		1.4.17	The level of water in the waterbath covers the level of liquid in the incubating tubes.
K	8, 11		1.4.18	Air incubator/waterbath temperatures are taken twice daily on workdays. The results are recorded and records maintained.
C	**4**		**1.4.19**	**All working thermometers are appropriately immersed.**
C	**29**		**1.4.20**	**Working thermometers are either: calibrated mercury-in-glass thermometers, calibrated non-mercury-in-glass thermometers, or appropriately calibrated electronic devices, including Resistance Temperature Devises (RTDs) and Platinum Resistance Devices (PTDs).**
C	**11**		**1.4.21**	**A standards thermometer has been calibrated by NIST or a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at the points 0, 35 and 44.5°C (45.5°C for ETCP). These calibration records are maintained.**
K	9		1.4.22	Standards thermometers are checked annually for accuracy by ice point determination. Results recorded and maintained. Date of most recent determination_____.
C	**29**		**1.4.23**	**Either mercury-in-glass thermometers, non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury or low drift electronic resistance thermometers with an accuracy of ≤ ±0.05°C are used as the laboratory standards thermometer. (*Circle the thermometer type used.*)**
K	13		1.4.24	The accuracy of working thermometers is checked annually against the standards thermometer either at the temperatures at which they are used or by ice point determination. Results are recorded and records maintained.
O	11		1.4.25	Appropriate pipet aids are available and used to inoculate samples. Mouth pipetting is not permitted.
		1.5 Labware and Glassware Washing		
O	9		1.5.1	Utensils and containers are clean borosilicate glass, stainless steel or other noncorroding materials.
K	9		1.5.2	Culture tubes are of a suitable size to accommodate the volume for nutritive ingredients and samples.
K	9		1.5.3	Sample containers are made of glass or some other inert material.
O	9		1.5.4	Dilution bottles and tubes are made of borosilicate glass or plastic and closed with rubber stoppers, caps or screw caps with nontoxic liners.

K	9		1.5.5	Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method is used to ensure appropriate volumes.
C	**9**		**1.5.6**	**Pipettes used to inoculate the sample deliver accurate aliquots, have unbroken tips and are appropriately graduated. Pipettes larger than 10 mL are not used to deliver 1mL aliquots; nor, are pipets larger than 1.1mL used to deliver 0.1 mL aliquots.**
K	9		1.5.7	Reusable sample containers are capable of being properly washed and sterilized.
K	9		1.5.8	In washing reusable pipettes, a succession of at least three fresh water rinses plus a final rinse of distilled/deionized water is used to thoroughly rinse off all the detergent.
C	**2**		**1.5.9**	**An alkaline or acidic detergent is used for washing glassware/labware.**
C	**11**		**1.5.10**	**With each load of labware/glassware washed the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali) with aqueous 0.04% bromothymol blue. Results are recorded and records maintained.**
		1.6 Sterilization and Decontamination		
K	9		1.6.1	Autoclave(s) are of sufficient size to accommodate the workload.
O	8		1.6.2	Routine autoclave maintenance is performed and the records are maintained.
C	**30, 33, 34**		**1.6.3**	**The autoclave provides sterilization conditions suitable to the load contents. Sterilization temperature range may be 119ºC - 124ºC as determined by the lab's equipment Quality Assurance Verification Testing and recommended practices from the media manufacturer. Sterilization is determined for each load using a working maximum registering thermometer or an appropriate working temperature monitoring device.**
K	11		1.6.4	An autoclave standards thermometer has been calibrated by a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at 121°C. Calibration at 100°C, the steam point, is also recommended but not required.
K	16		1.6.5	The autoclave standards thermometer is checked every five (5) years for accuracy at 121°C by a qualified calibration laboratory; or, is checked in-house at the steam point (100°C) if it has been previously calibrated at both 100°C and 121°C. Any change in temperature at the steam point changes the calibrated temperature at 121°C by the same magnitude. Date of most recent determination_____
K	1		1.6.6	Working autoclave thermometers are checked against the autoclave standards thermometer at 121°C yearly. Date of last check _____ Method _____
K	11		1.6.7	Spore strips/suspensions appropriate for use in an autoclave media cycle are used monthly according to manufacturer's instructions to evaluate the effectiveness of the sterilization process. Results are recorded and the records maintained.
O	11		1.6.8	Heat sensitive tape is used with each autoclave batch.
K	11, 13		1.6.9	Autoclave sterilization records including length of sterilization, total heat exposure time and chamber temperature are maintained. Type of record: Autoclave log, computer printout or chart recorder tracings. (*Circle appropriate type or types.)*
K	11		1.6.10	For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperatures in the range of 160 to 180°C.
K	9		1.6.11	A thermometer capable of determining temperatures accurately in the range of 160 to 180°C is used to monitor the operation of the hot-air sterilizing oven
K	13		1.6.12	Records of temperatures and exposure times are maintained for the operation of the hot-air sterilizing oven during use.

K	11		1.6.13 Spore strips/suspensions are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. Records are maintained.
K	11		1.6.14 Reusable sample containers are sterilized for 60 minutes at 170°C in a hot-air oven or autoclaved for 15 minutes at 121°C.
C	**1**		**1.6.15 The sterility of reusable sample containers is determined for each load sterilized. The results are recorded and the records maintained.**
C	**1**		**1.6.16 The sterility of pre-sterilized disposable sample containers is determined for each lot received. Results are recorded and the records maintained.**
K	9		1.6.17 Reusable pipettes are stored and sterilized in aluminum or stainless steel canisters.
K	9		1.6.18 Reusable pipettes (in canisters) are sterilized in a hot-air oven at 170°C for 2 hours.
C	**2**		**1.6.19 The sterility of reusable pipettes is determined with each load sterilized. Results are recorded and records maintained.**
C	**2**		**1.6.20 The sterility of pre-sterilized disposable pipettes is determined with each lot received. Results are recorded and the records maintained.**
K	18		1.6.21 Hardwood applicator transfer sticks are properly sterilized. Method of sterilization _____
C	**2**		**1.6.22 The sterility of the hardwood applicator transfer sticks is checked routinely. Results are recorded and the records maintained.**
O	13		1.6.23 Spent broth cultures and agar plates are decontaminated by autoclaving for at least 30 minutes before conventional disposal.
		1.7 Media Preparation	
K	3, 5		1.7.1 Media is commercially dehydrated except in the case of medium A-1 which must be prepared from the individual components and modified MacConkey agar which may be prepared from its components.
K	11		1.7.2 Media is prepared according to manufacturer's instructions.
O	11		1.7.3 Dehydrated media and media components are properly stored in a cool, clean, dry place.
O	11		1.7.4 Dehydrated media are labeled with date of receipt and date opened.
C	**12**		**1.7.5 Caked or expired media or media components are discarded.**
C	**11**		**1.7.6 Reagent water is distilled or deionized (circle appropriate choice), tested monthly and exceeds 0.5 megohm-cm resistance (2 megohms-cm in-line) or is less than 2.0 µSiemens/cm conductivity at 25°C. (C*ircle the appropriate water quality descriptor determined*.) Results are recorded and the records maintained.**
C	**11**		**1.7.7 Reagent water is analyzed for residual chlorine monthly and is at a non-detectable level (< 0.1 mg/L). Results are recorded and the records maintained.** **Specify method of determination_____.**
K	11		1.7.8 Reagent water contains<100 CFU/mL as determined monthly using the heterotrophic plate count method. Results are recorded and the records maintained.
K	11		1.7.9 Media prepared from commercial dehydrated components are sterilized according to the manufacturer's instructions.
K	9		1.7.10 The volume and concentration of media in the tube are suitable for the amount of sample inoculated.
C	**11**		**1.7.11 Total time of exposure of sugar broths to autoclave temperatures does not exceed 45 minutes.**
C	**1**		**1.7.12 Media sterility is determined for each load sterilized. Results are recorded and the records maintained.**

C	**1**		**1.7.13 Media productivity is determined using media-appropriate, properly diluted positive and negative control cultures for each lot of dehydrated media received or with each batch of media prepared when the medium is made from its individual components.**
O	9		1.7.14 Sterile phosphate buffered dilution water is used as the sample diluent, unless otherwise specified in the method specific sections of Parts II and III of this checklist.
K	11		1.7.15 The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer's requirements. Results are recorded and records are maintained.
		1.8 Storage of Prepared Culture Media	
K	9		1.8.1 Prepared culture media are stored in a cool, clean, dry space where excessive evaporation and the danger of contamination are minimized.
K	5,11		1.8.2 Brilliant green bile 2% broth and A-1 media are stored in the dark.
K	13		1.8.3 Stored media are labeled with the storage expiration date or the sterilization date.
K	9		1.8.4 Storage of prepared culture media at room temperature does not exceed 7 days.
K	2		1.8.5 Storage under refrigeration of prepared culture media with loose fitting closures shall not exceed 1 month.
K	11		1.8.6 Storage under refrigeration of prepared culture media with screw-cap closures does not exceed 3 months.
K	17		1.8.7 All prepared MPN broth media stored under refrigeration must reach room temperature prior to use. Culture tubes containing any type of precipitate or Durham tubes containing air bubbles are discarded.
		PART II - SEAWATER SAMPLES	
		2.1 Collection and Transportation of Samples	
C	**11**		**2.1.1 Sample containers are of a suitable size to contain at least 110 mL of sample and to allow adequate headspace for proper shaking. Seawater samples are collected in clean, sterile, watertight, properly labeled sample containers.**
K	1		2.1.2 Samples are identified with collector's name, harvest area, sampling station, time and date of collection.
C	**9**		**2.1.3 Immediately after collection, seawater samples are placed in dry storage (ice chest or equivalent) capable of maintaining a temperature of 0 to 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed in the refrigerator unless processed immediately.**
O	1		2.1.4 A temperature blank is used to represent the temperature of samples upon receipt at the laboratory. Temperature should be equivalent or less than that of the growing area waters. Results are recorded and maintained.
C	**9**		**2.1.5 Analysis of the sample is initiated as soon as possible after collection. Seawater samples are not tested if they have been held for more than 30 hours from the time of collection.**
		2.2 Bacteriological Examination of Seawater by the APHA MPN	
C	**9**		**2.2.1 Lactose broth or lauryl tryptose broth is used as the presumptive medium.** ***(Circle appropriate one.)***
C	**2**		**2.2.2 The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control**
C	**9, 35**		**2.2.3 Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) before inoculation.**
C	**9**		**2.2.4 In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended).**
C	**6**		**2.2.5 In a single dilution series not less than 12 tubes are used (for depuration at least 5 tubes are used).**

C	**6**		**2.2.6**	**In a single dilution series, the volumes analyzed are adequate to meet the needs of routine monitoring.** **Sample volume inoculated _____** **Range of MPN_____** **Strength of media used_____**
K	9		2.2.7	Inoculated tubes are incubated in air at 35 ± 0.5°C.
C	**2**		**2.2.8**	**Appropriately diluted process control cultures accompany the samples *throughout both the presumptive and confirmed phases of incubation.* Results are recorded and the records maintained.** **Positive process control _____ Negative process control _____**
K	9		2.2.9	Inoculated tubes are read after 24 ± 2 hours and 48 ± 3 hours of incubation and transferred at both time interval if positive for growth (the presence of turbidity) and gas or effervescence in the culture tube. These tubes are considered presumptive positive requiring further confirmatory testing.
		2.3 Confirmed Test for Seawater by APHA MPN		
C	**9**		**2.3.1**	**Brilliant green bile 2% broth (BGB) is used as the confirmatory medium for total coliforms.**
C	**9**		**2.3.2**	**EC medium is used as the confirmatory medium for fecal coliforms.**
C	**2**		**2.3.3**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
K	9, 11		2.3.4	Transfers are made to BGB/EC by either sterile loop or sterile hardwood transfer stick from positive presumptive tubes incubated for 24 and 48 hours as appropriate. *(Circle the method of transfer.)*
C	**9**		**2.3.5**	**BGB tubes are incubated at 35 ± 0.5°C.**
K	9		2.3.6	BGB tubes are read after 48 ± 3 hours of incubation.
C	**9**		**2.3.7**	**EC tubes are incubated in a circulating waterbath maintained at 44.5 ± 0.2°C.**
C	**9**		**2.3.8**	**EC tubes are read after 24 ± 2 hours of incubation.**
C	**9**		**2.3.9**	**The presence of turbidity and any amount of gas or effervescence in the culture tube constitutes a positive test.**
		2.4 Computation of Results – APHA MPN		
K	9		2.4.1	Results of multiple dilution tests are read from tables in *Recommended Procedures for the Examination of Sea Water and Shellfish*, Fourth Edition.
K	7		2.4.2	Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**7, 9**		**2.4.3**	**Results are reported as MPN/100 mL of sample.**
		2.5 Bacteriological Examination of Seawater by the MA-1 Method		
C	**5**		**2.5.1**	**A-1 medium complete is used in the analysis.**
C	**2, 31**		**2.5.2**	**A-1 medium without salicin is used in the analysis. Comparability testing supports use of A-1medium without salicin. Study records are available.**
C	**5**		**2.5.3**	**A-1 medium sterilized for 10 minutes at 121°C.**
C	**2**		**2.5.4**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control**

C	**9, 35**		**2.5.5 Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) before inoculation.**
C	**9**		**2.5.6 In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended).**
C	**6**		**2.5.7 In a single dilution series at least 12 tubes are used.**
C	**6**		**2.5.8 In a single dilution series, the volumes analyzed are adequate to meet the needs of routine monitoring.** **Sample volume inoculated _____** **Range of MPN_____** **Strength of media used _____**
C	**2**		**2.5.9 Appropriately diluted process control cultures accompany the samples *throughout both resuscitation and waterbath incubation* Results are recorded and the records maintained.** **Positive process control _____ Negative process control _____**
C	**2,5**		**2.5.10 Inoculated tubes are placed in an air incubator at 35 ± 0.5°C for 3 ± 0.5 hours of resuscitation.**
C	**5**		**2.5.11 After 3 ± 0.5 hours resuscitation at 35°C, inoculated tubes are incubated at 44.5 ± 0.2°C in a circulating waterbath for the remainder of the 24 ± 2 hours.**
C	**5**		**2.5.12 The presence of turbidity and any amount of gas or effervescence in the culture tube constitutes a positive test.**
		2.6 Computation of Results – APHA MPN	
K	9		2.6.1 Results of multiple dilution tests are read from tables in *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition.
K	7		2.6.2 Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**7, 9**		**2.6.3 Results are reported as MPN/100 mL of sample.**
		2.7 Bacteriological Analysis of Seawater by Membrane Filtration (MF) using mTEC Agar - Materials and Equipment	
C	**23, 24**		**2.7.1 When used for elevated temperature incubation in conjunction with ethafoam resuscitation, the temperature of the hot air incubator is maintained at 44.5 ± 0.5°C under any loading capacity.**
C	**23**		**2.7.2 When using a waterbath for elevated temperature incubation, the level of the water completely covers the plates.**
C	**23**		**2.7.3 Pre-sterilized plastic or sterile glass culture plates that are clear, flat bottomed, free of bubbles and scratches with tight fitting lids are used.**
C	**2**		**2.7.4 The sterility of pre-sterilized culture plates is determined for each lot received. Results are recorded and the records maintained.**
K	11		2.7.5 Colonies are counted with the aid of magnification.
C	**11, 23**		**2.7.6 Membrane filters are made from cellulose ester material, white, grid marked, 47 mm in diameter with a pore size of 0.45 µmand certified by the manufacturer for fecal coliform analyses.**
C	**2**		**2.7.7 Lot number, date of receipt and if provided the expiration date of the membrane filters are recorded and records maintained.**
C	**2**		**2.7.8 When initiating monitoring by mTEC or switching brands or types of membrane filters used and no previous lots of filters are available for comparing acceptable performance, an appropriate method for determining the suitability of the lot is developed and the comparison testing implemented. The results are recorded and this record is maintained.**

K	2, 11		2.7.9	New lots of membrane filters are checked by comparing recovery of fecal coliform organisms against membrane filters from previously acceptable lots.
C	**2**		**2.7.10**	**The sterility of each lot or autoclave batch of membrane filters are checked before use.**
K	2		2.7.11	Membrane filters which are beyond their expiration date are not used.
O	11		2.7.12	Forceps tips are clean.
O	11		2.7.13	Forceps tips are smooth without pitting or corrugations to damage the filters being manipulated.
K	11		2.7.14	Forceps are dipped in alcohol and flame sterilized between sample filters.
K	11		2.7.15	If indelible graduation marks are used on clear glass or plastic funnels to measure sample volumes, their accuracy is checked gravimetrically or with a Class A graduated cylinder before use and periodically rechecked. Funnels having a tolerance greater than 2.5% are not used. Checks are recorded and records maintained.
K	11		2.7.16	Membrane filtration units are made of stainless steel, glass or autoclavable plastic free of scratches, corrosion and leaks.
C	**11**		**2.7.17**	**Membrane filter assemblies are autoclave sterilized for 15 minutes at 121°C prior to the start of a filtration series.**
O	11, 23, 26		2.7.18	A UV sterilization unit is used to disinfect filter assemblies between sample and filtration runs.
K	11		2.7.19	The effectiveness of the UV sterilization unit is determined by biological testing monthly. Results are recorded and records maintained.
K	2		2.7.20	Maintenance of the UV sterilization unit is performed as needed. This maintenance is documented and the records maintained.
		2.8 Media Preparation and Storage – MF using mTEC Agar		
K	11		2.8.1	Phosphate buffered saline is used as the sample diluent and filter funnel rinse.
C	**11**		**2.8.2**	**The phosphate buffered saline is properly sterilized.**
K	23		2.8.3	A sufficient amount of medium (4-5 mL) is used in each plate.
O	11		2.8.4	Refrigerated prepared plates are stored for no more than 2 weeks in sealed plastic bags or containers to minimize evaporation.
		2.9 Sample Analyses - MF using mTEC Agar		
C	**24**		**2.9.1**	**mTEC agar is used.**
C	2		**2.9.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control**
C	**23, 35**		**2.9.3**	**The sample is shaken vigorously (25 times in a 12″ arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) before filtration**
C	**23**		**2.9.4**	**The membrane is placed grid side up within the sterile filter apparatus.**
C	**23, 25**		**2.9.5**	**Sample volumes tested are consistent with the sampling regime employed (i.e., half log or other appropriate dilutions are used with systematic random sampling).**
C	**23**		**2.9.6**	**Sample volumes are filtered under vacuum.**
K	26		2.9.7	The pressure of the vacuum pump does not exceed 15 psi.
C	**23, 26**		**2.9.8**	**The sides of the filter funnel are rinsed at least twice with 20-30 mL of sterile phosphate buffered saline after sample filtration.**
C	**23**		**2.9.9**	**The membrane filter is removed from the filtering apparatus with sterile forceps and rolled onto mTEC agar so that no bubbles form between the filter and the agar.**
C	**11**		**2.9.10**	**Blanks are run at the beginning of filtration, after every 10th aliquot and at the end of the filtration run to check the sterility of the testing system (phosphate buffered saline, filter funnel, forceps, membrane filter, media and culture plate).**

C	**2, 11**		**2.9.11 Appropriately diluted process control cultures accompany the samples throughout both resuscitation and elevated temperature incubation. Results are recorded and the records maintained.** **Positive process control _____ Negative process control _____**
C	**11, 23, 24**		**2.9.12 Inoculated plates are placed inverted into a watertight, tightly sealed container prior to being placed in the air incubator and incubated at 35 + 0.5°C for 2 hours of resuscitation. Alternatively inoculated plates may be placed in ethafoam prior to air incubation at 44.5 ± 0.5°C for 24 ± 2 hours.**
C	**11, 23, 24**		**2.9.13 After 2 hours of resuscitation at 35°C, the watertight, tightly sealed containers are transferred to a circulating waterbath at 44.5 + 0.2°C, submerged completely and incubated for 22-24 hours.**
		2.10 Computation of Results - MF using mTEC Agar	
C	**23**		**2.10.1 All yellow, yellow-green or yellow-brown colonies are counted.**
C	**23**		**2.10.2 Only plates having 80 or fewer colonies are counted. If it is unavoidable to use plates having more than 80 colonies, counts are given as >80 x 100/the volume of sample filtered.**
C	**2, 11, 23**		**2.10.3 When multiple dilutions are filtered, the laboratory has developed a procedure for assessing the contribution of all positive dilutions to the final count.**
C	**23, 11**		**2.10.4 The number of fecal coliforms is calculated by the following equation:** **Number of fecal coliforms per 100 mL = [number of colonies counted per plate used in the count / volume (s) of sample filtered in ml] x 100.**
C	**23, 11**		**2.10.5 Results are reported as CFU/100 mL of sample.**
		2.11 Bacteriological Analysis of UV Treated Process Water Samples by Membrane Filtration (MF) using mEndo Agar LES – Materials and Equipment	
C	**9, 11, 21**		**2.11.1 Pre-sterilized plastic or sterile glass culture plates that are clear, flat bottomed, free of bubbles and scratches are used.**
C	**2**		**2.11.2 The sterility of pre-sterilized culture plates is determined for each lot received. Results are recorded and the records maintained.**
C	**11, 19, 21**		**2.11.3 Membrane filters are made from cellulose ester material, white, grid marked, 47 mm diameter with a pore size of 0.45µm and certified by the manufacturer for total coliform analysis.**
C	**2**		**2.11.4 Membrane filter lot numbers, dates of receipt and expiration dates are recorded and the records maintained.**
C	**2**		**2.11.5 If previous lots of agar or membrane filters are not available for comparability testing, an appropriate method for determining lot suitability has been developed and comparison testing implemented when the following has occurred:** **a. initiating monitoring by mEndo Agar LES;** **b. changing agar manufacturers; or** **c. changing brands of membrane filters used.** **The results are recorded and the records are maintained.**
K	2, 11		2.11.6 Recovery of total coliforms from new lots of membrane filters and mEndo Agar LES is compared against the recovery from the previously acceptable lot.
C	**2**		**2.11.7 The sterility of each lot and autoclave batch of membrane filters is verified before use.**
K	2		2.11.8 Expired membrane filters are not used.
K	9, 11, 19, 21		2.11.9 Membrane filtration units are made of stainless steel, glass or autoclavable plastic free of scratches, corrosion and leaks.

K	11		2.11.10 If indelible graduation marks are used on clear glass or plastic funnels to measure sample volumes, their accuracy is checked gravimetrically with a Class A graduated cylinder before use and periodically rechecked. Funnels having a tolerance greater than 2.5% are not used. Checks are recorded and records maintained.
C	**9, 11**		**2.11.11 Membrane filter assemblies are autoclave sterilized for 15 minutes at 121+/- 2°C prior to the start of a filtration run. A new run occurs when there is a break of 30 minutes or more between the previous filtration run.**
O	11, 19, 26, 36		2.11.12 A UV sterilization unit is used to disinfect filter assemblies between sample and filtration runs.
K	11		2.11.13 The effectiveness of the UV sterilization unit is determined by biological testing monthly. Results are recorded and the records are maintained.
K	2		2.11.14 Maintenance of the UV sterilization unit is performed as needed. Maintenance is documented and the records maintained.
O	9, 11		2.11.15 Forceps tips are clean and smooth without pitting or corrugations.
		2.12 Media Preparation and Storage	
C	**9, 11, 19, 21, 36**		**2.12.1 mEndo Agar LES is used.**
K	11, 21, 36		2.12.2 mEndo Agar LES is prepared aseptically using pre-sterilized glassware, sterile reagent water and pre-sterilized stir bar.
K	9, 11, 36		2.12.3 mEndo Agar LES is prepared using 95% alcohol that is not denatured.
C	**9, 11, 36**		**2.12.4 mEndo Agar LES is brought to near boiling; removed from the heat and tempered at 44-50°C before dispensing.**
C	**9, 11, 36**		**2.12.5 mEndo Agar LES is never autoclaved.**
K	9, 11, 36		2.12.6 A sufficient amount of medium (4-5 mL) is aseptically aliquoted to each culture plate.
O	9, 11, 36		2.12.7 Prepared plates of mEndo Agar LES are stored at 4°C in the dark for no more than two (2) weeks in sealed plastic bags or containers to minimize evaporation.
C	**2**		**2.12.8 Appropriate, properly diluted positive and negative productivity controls fo mEndo Agar LES medium are used. Results are recorded and the record maintained.** **Positive productivity control _____** **Negative productivity control _____**
K	9, 11, 21, 36		2.12.9 Sterile phosphate buffered water or sterile phosphate buffered saline is used as a sample blank, filter funnel rinse and process and productivity control diluent for UV treated process water samples.
C	**11**		**2.12.10 The phosphate buffered water/saline is properly sterilized and the sterility is tested before being placed in service. Results are recorded and records maintained**
		2.13 Sample Analysis	
C	**9, 11, 36**		**2.13.1 The sample is shaken vigorously (25 times in a 12-inch arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) before filtration.**
C	**11, 21, 36**		**2.13.2 The membrane filter is placed grid side up within the sterile filter apparatus**.
C	**11, 26, 36**		**2.13.3 A 100 mL quantity of sample is filtered under vacuum.**
K	26		2.13.4 The pressure of the vacuum pump does not exceed 15 psi.
C	**9, 11, 26, 36**		**2.13.5 The sides of the filter funnel are rinsed at least twice with 20-30 mL of sterile phosphate buffered water/saline as appropriate after filtration.**
C	**9, 11, 36**		**2.13.6 The membrane filter is removed from the filtering apparatus with sterile forceps and rolled onto mEndo Agar LES so that no bubbles form between the filter and the agar.**

K	9, 11, 36		2.13.7 Forceps are dipped in alcohol and flame sterilized between sample filters.
C	**11, 36**		**2.13.8 Blanks are run at the beginning and at the end of the filtration run to check the sterility of the testing system (phosphate buffered water/saline, filter funnels, forceps, membrane filters, media and culture plates).**
C	**2, 36**		**2.13.9 An appropriate properly diluted positive process control culture accompanies the sample throughout incubation. Results are recorded and the records are maintained.** **Positive process control _____**
C	**9, 11, 36**		**2.13.10 Inoculated plates are incubated inverted at 35+/- 0.5°C for 22 to 24 hours.**
K	2, 9, 11		2.13.11 An appropriate level of humidity is maintained in the incubator to prevent the plates from drying out.
		2.14 Computation of Results	
K	9, 11		2.14.1 Colonies are counted with the aid of magnification.
C	**9, 11, 19, 21, 23**		**2.14.2 All metallic sheen colonies are counted as total coliforms.**
C	**9, 11, 21, 36**		**2.14.3 Results are reported as total coliforms/100mL.**
C	**11, 20, 36**		**2.14.4 When no colonies are observed, results are reported as <1.0 coliform/100mL (nondetectable)**
		PART III - SHELLFISH SAMPLES	
		3.1 Collection and Transportation of Samples	
C	**9**		**3.1.1 A representative sample of shellstock is collected.**
K	9		3.1.2 Shellstock samples are collected in clean, waterproof, puncture resistant containers loosely sealed.
K	9		3.1.3 Shellstock samples are labeled with collector's name, type of shellstock, the source or harvest area, sampling station, time, date and place (if applicable) of collection.
C	**9**		**3.1.4 Immediately after collection, shellfish samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed under refrigeration unless processed immediately.**
C	**1**		**3.1.5 Analysis of the samples is initiated as soon as possible after collection. Shellfish samples are not tested if the time interval between collection and analysis exceeds 24 hours.**
		3.2 Preparation of Shellfish for Examination	
K	2,11		3.2.1 Shucking knives, scrub brushes and blender jars are (autoclave) sterilized for 15 minutes prior to use.
O	2		3.2.2 Blades of shucking knives are not corroded.
O	9		3.2.3 The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2		3.2.4 The faucet used for rinsing the shellstock does not contain an aerator.
K	9		3.2.5 Shellstock are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9		3.2.6 Shellstock are allowed to drain in a clean container or on clean towels prior to opening.
K	1, 9		3.2.7 Immediately prior to shucking, the hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol or clean gloves are donned.
C	**9**		**3.2.8 Shellstock are not shucked directly through the hinge.**
C	**9**		**3.2.9 Contents of shellstock (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	9		3.2.10 At least 200 grams of shellfish meat or a quantity of meat sufficient to cover the blender blades is used for the analysis.

K	9		3.2.11 A representative sample of at least 12 shellfish is used for the analysis.
K	2		3.2.12 The sample is weighed to the nearest 0.1 gram and an equal amount by weight of diluent is added.
~~O~~	~~9~~		~~3.2.13 Sterile phosphate buffered dilution water is used as the sample diluent.~~
C	**9**		**3.2.13 Samples are blended at high speed for 60 to 120 seconds until homogenous.**
K	9		3.2.14 APHA *Recommended Procedures for the Examination of Sea Water And Shellfish*, Fourth Edition is followed for the analysis of previously shucked and frozen shellfish meats.
		3.3 MPN Analysis for Fecal Coliform Organisms, Presumptive Test, APHA	
C	**9**		**3.3.1 Appropriate strength lactose or lauryl tryptose broth is used as presumptive media in the analysis. *(Circle the medium used.)***
C	**2**		**3.3.2 The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____ Negative productivity control _____**
K	9		3.3.3 Immediately (within 2 minutes) after blending, the ground sample is diluted and inoculated into tubes of presumptive media.
C	**9**		**3.3.4 No fewer than 5 tubes per dilution are used in a multiple dilution MPN series.**
C	**9**		**3.3.5 Allowing for the initial 1:1 dilution of the sample, appropriate portions are inoculated (i.e., 2 ml of original 1:1 dilution for the 1 g portion) and diluted for subsequent inoculation (i.e., 20 ml of 1:1 diluted sample to 80 ml of diluent or the equivalent for 0.1 g portion). All successive dilutions are prepared conventionally.**
K	6		3.3.6 In a single dilution series, the volumes examined are adequate to meet the needs of routine monitoring. Sample volume inoculated _____ Range of MPN _____ Strength of media used _____
C	**2**		**3.3.7 Appropriately diluted process control cultures accompany the samples throughout both the presumptive and confirmed phases of incubation. Results are recorded and the records maintained.** **Positive Process control _____ Negative Process control _____**
K	9		3.3.8 Inoculated media are incubated at 35 ± 0.5°C.
K	10		3.3.9 Tubes are read after 24 ± 2 hours of incubation and transferred if positive for growth (the presence of turbidity and gas or effervescence in the culture tube). These tubes are considered presumptive requiring further confirmatory testing.
		3.4 Confirmed Test for Fecal Coliforms - APHA	
C	**9**		**3.4.1 EC medium is used as the confirmatory medium.**
C	**2**		**3.4.2 The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____ Negative productivity control**
K	9, 11		3.4.3 Transfers are made to EC medium by either sterile loop or hardwood sterile transfer sticks from positive presumptives. *(Circle the method of transfer.)*
C	**9**		**3.4.4 EC tubes are incubated in a circulating waterbath at 44.5 ± 0.2°C**
K	9		3.4.5 EC tubes are read for gas production after 24 ± 2 hours of incubation.
C	**9**		**3.4.6 The presence of turbidity and any amount of gas and/or effervescence in the Durham tube constitutes a positive test.**
		3.5 Computation of Results for MPN Analyses	
K	9		3.5.1 Results of multiple dilution tests are read from tables in *Recommended Procedure for the Examination of Sea Water and Shellfish,* 4th Edition and multiplied by the appropriate dilution factor.

K	7		3.5.2	Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**9**		**3.5.3**	**Results are reported as MPN/100 grams of sample.**
		3.6 Standard Plate Count Method		
O	20		3.6.1	A standard plate count (SPC) analysis maybe performed in conjunction with the analysis for fecal coliform organisms.
K	9		3.6.2	In the standard plate count procedure at least four plates are used, duplicates of two dilutions. One of the dilutions should produce colonies of 30 to 300 per plate.
K	2		3.6.3	Fifteen to 20 mL of tempered sterile plate count agar is used per plate.
C	**9**		**3.6.4**	**Agar tempering bath maintains the agar at 44-46°C.**
C	**9**		**3.6.5**	**An agar based temperature control having a similar volume and shape as the tempering plate count agar is used in the tempering bath.**
K	9		3.6.6	Samples or sample dilutions to be plated are shaken vigorously (25 times in a 12" arc in 7 seconds) before plating.
C	**9**		**3.6.7**	**Not more than 1 mL nor less than 0.1 mL of sample or sample dilution is plated.**
K	11		3.6.8	Control plates are used to check air quality and the sterility of the agar and the diluent.
K	9,21		3.6.9	Solidified plates are incubated at 35 ± 0.5°C for 48 ± 3 hours inverted and stacked no more than four high.
K	9		3.6.10	Quebec Colony Counter or its equivalent is used to provide the necessary magnification and visibility for counting plates.
K	1		3.6.11	A hand tally or its equivalent is used for accuracy in counting.
		3.7 Computation of Results -SPC		
K	9		3.7.1	Colony counts determined in accordance with Part III, A, Sections 4.31 through 4.33 in *Recommended Procedures for the Examination of Sea Water and Shellfish*, Fourth Edition.
C	**19**		**3.7.2**	**Colony counts are reported as CFU/g of sample.**
		3.8 Bacteriological Analysis of Shellfish Using the ETCP		
C	**2,3**		**3.8.1**	**Prepared modified MacConkey agar is used on the day that it is made.**
K	3		3.8.2	Double strength modified MacConkey agar is used.
C	**3**		**3.8.3**	**Prepared double strength modified MacConkey agar is heated to boiling, removed from the heat, and boiled again. This agar is never autoclaved.**
K	2, 3		3.8.4	Twice boiled, double strength modified MacConkey agar and is maintained in a tempering bath at 45 to 50°C until used.
K	2, 3		3.8.5	Phosphate buffered saline is used as the sample diluent in the ETCP.
C	**2, 3**		**3.8.6**	**The phosphate buffered saline is tempered at 45 - 50°C to prevent premature solidification of the agar.**
C	**9**		**3.8.7**	**The sample homogenate is cultured within 2 minutes of blending.**
C	**2,3**		**3.8.8**	**Six grams of shellfish (12 grams of homogenate if initially diluted 1:1) is placed into a sterile container and the contents brought up to 60 mL with sterile, tempered phosphate buffered saline.**
K	3		3.8.9	Sixty (60) mL of tempered, twice boiled double strength Modified MacConkey Agar is added.
K	2,3, 22		3.8.10	The container is gently swirled or slowly inverted once to mix the contents, which are subsequently distributed uniformly over six plates.
C	**1**		**3.8.11**	**Media and diluent sterility are determined with each use. Results are recorded and the records maintained.**

C	**1**		**3.8.12 Media productivity is determined using media appropriate properly diluted pour plated positive and negative control cultures for each batch of Modified MacConkey agar prepared. Positive control culture _____ Negative control culture _____**
C	**3, 13**		**3.8.13 When solidified, the plates are placed inverted into an air incubator at 45.5 ± 0.5°C for 18 to 30 hours of incubation.**
C	**2**		**3.8.14 Plates are stacked no more than three high in the incubator.**
C	**2**		**3.8.15 Appropriately diluted pour plated process control cultures *accompany each set of samples throughout incubation.* The results are recorded and the records maintained. Positive process control_____Negative process control_____**
		3.9 Computation of Results - ETCP	
K	11		3.9.1 Quebec Colony counter or its equivalent is used to provide the necessary magnification and visibility for counting.
O	1		3.9.2 A hand tally or its equivalent is used to aid in counting.
C	**3, 6**		**3.9.3 All brick red colonies greater than 0.5 mm in diameter are totaled over all the plates and multiplied by a factor of 16.7.**
C	**3**		**3.9.4 Results are reported as CFU/100 grams of sample.**
		Bacteriological Examination of Soft-shelled Clams and American Oysters for Male Specific Coliphage (MSC)	
		3.10 MSC Equipment and Supplies	
K	30		3.10.1 Sample containers used for the shucked sample are sterile, made of glass or some other inert material (i.e. polypropylene) and hold 100 – 125 mL.
C	**27, 28**		**3.10.2 The refrigerated centrifuge used must have the capacity to accommodate the amount of shellfish sample required for the procedure, perform at 9000 x g and maintain a temperature of 4°C.**
K	9		3.10.3 The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
C	**27, 28**		**3.10.4 Sterile 0.22µm pore size syringe filters and pre-sterilized plastic or sterile glass syringes are used to sterilize the antibiotic solutions.**
K	1		3.10.5 The sterility of each lot of pre-sterilized syringes and syringe filters is determined. Results are recorded and records maintained.
K	1		3.10.6 The sterility of each batch of reusable glass syringes is determined. Results are recorded and records maintained.
C	**27, 28**		**3.10.7 The balance used provides a sensitivity of at least mg (0.01g.).**
C	**27, 28**		**3.10.8 The temperature of the incubator used is maintained at 36 ± 1°C.**
C	**28**		**3.10.9 Sterile disposable 50 mL centrifuge tubes are used and their sterility is determined with each lot. Results are recorded and records maintained.**
		3.11 MSC Media Preparation	
K	28		3.11.1 Media preparation and sterilization is according to the validated method.
K	27, 28		3.11.2 Bottom agar, double strength soft agar and growth broth are prepared from their individual components.
K	27, 28		3.11.3 Soft agar is prepared double strength in volumes of 2.5 mL.
C	**27, 28**		**3.11.4 The streptomycin and ampicillin solutions are added to tempered bottom agar and vortex for 2 minutes on stir plate.**
O	27, 28		3.11.5 Storage of the bottom agar under refrigeration does not exceed 1 month.
K	27, 28		3.11.6 Unsterilized soft agar is stored at -20 °C -15C for up to 3 months.
K	27, 28		3.11.7 The soft agar is removed from the freezer and sterilized for 15 minutes at 121°C before use.
K	27, 28		3.11.8 Storage of growth broth in the refrigerator in loosely capped tubes/bottles does not exceed 1 month and in screw capped tubes/bottles does not exceed 3 months.
K	27, 28		3.11.9 Bottom agar plates are allowed to reach room temperature before use.

		3.12 Preparation of the Soft-Shelled Clams and American Oysters for MSC Analysis	
K	2,11		3.12.1 Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2		3.12.2 The blades of shucking knives are not corroded.
O	9		3.12.3 The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2		3.12.4 The faucet used for rinsing the shellfish does not contain an aerator.
K	9		3.12.5 The shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9		3.12.6 The shellfish are allowed to drain in a clean container or on clean towels unlayered prior to shucking.
K	9		3.12.7 Immediately prior to shucking, the hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol or clean gloves are donned.
C	**9**		**3.12.8 Shellfish are not shucked through the hinge.**
C	**9**		**3.12.9 The contents of shellfish (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	9		3.12.10 A representative sample of at least 12 shellfish is used for the analysis.
K	2, 19		3.12.11 The sample is weighed to the nearest 0.1 gram.
		3.13 MSC Sample Analysis	
C	**28**		**3.13.1 E. coli *Famp* ATCC 700891 is the bacterial host strain used in this procedure.**
K	27, 28		3.13.2 Host cell growth broth is tempered at 36 ± 1°C and vortexed (or shaken) to aerate prior to inoculation with host cells.
K	27, 28		3.13.3 Several host cell colonies are transferred to a tube of tempered, aerated growth broth and incubated at 36 ± 1°C for 4-6 hours to provide host cells in log phase growth for sample analysis.
C	**27, 28**		**3.13.4 After inoculation, the host cell growth broth culture is not shaken.**
C	**28**		**3.13.5 A 2:1 mixture of sterile growth broth to shellfish tissue is used for eluting the MSC.**
C	**28**		**3.13.6 The elution mixture is prepared w/v by weighing the sample and adding two equal portions of sterile growth broth by volume to the shellfish tissue.**
C	**28**		**3.13.7 The elution mixture is homogenized at high speed for 180 seconds.**
C	**28**		**3.13.8 Immediately after blending, 33 grams of the homogenized elution mixture are weighed into centrifuge tubes.**
C	**28**		**3.13.9 The homogenized elution mixture is centrifuged for 15 minutes at 9000 x g at 4°C.**
C	**27, 28**		**3.13.10 The supernatant is pipetted off, weighed and the weight recorded.**
C	**27, 28**		**3.13.11 The supernatant is allowed to warm to room temperature about 20 to 30 minutes.**
K	27, 28		3.13.12 The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28		3.13.13 Two hundred microliters (0.2 mL) of log phase host strain *E coli* is added to the tempering soft agar immediately prior to adding the sample supernatant.
K	27, 28		3.13.14 The sample supernatant is shaken or vortexed before being added to the tempering soft agar.
C	**27, 28**		**3.13.15 2.5 mL of sample supernatant is added to each tube of tempering soft agar.**
C	**27, 28**		**3.13.16 The soft agar/sample supernatant/host cell mixture is gently rolled between the palms of the hands to mix.**
C	**27, 28**		**3.13.17 The soft agar/sample supernatant/host cell mixture is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**
C	**28**		**3.13.18 Ten (10) plates are used, 2.5 mL per plate for a total of 25 mL of supernatant analyzed per sample.**

K	27, 28		3.13.19 Negative and positive control plates are prepared and accompany each set of samples analyzed. The results are recorded and records maintained. Positive control _____
K	27, 28		3.13.20 Growth broth is used as the negative control or blank.
K	27, 28		3.13.21 Type strain MS2 (ATCC 15597) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.
K	2		3.13.22 A negative control plate is plated at the beginning and end of each set of samples analyzed.
K	27, 28		3.13.23 The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	**27, 28**		**3.13.24 All plates are incubated at 36 ± 1°C for 18 ± 2 hours.**
		3.14 Computation of Results - MSC	
C	**27**		**3.14.1 Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted.**
C	**28, 32**		**3.14.2 The working range of the method is 1 to 200 PFU per plate. When there are no plaques on all ten plates, the count is <6 PFU/100 grams for soft-shelled clams, <7 PFU/ 100 grams for American oysters, and <5 PFU/ 100 grams for quahog (hard) clams. If the density exceeds 200 PFU per plate on all plates, the count is given as > 20,000 PFU/100 grams.**
K	28		3.14.3 The formula used for determining the density of MSC in PFU/100 grams is: (0.364) (N) (Ws), where N = total number of plaques counted on all 10 plates and Ws = weight of the supernatant used.
O	9		3.14.4 The MSC count is rounded off conventionally to give a whole number.

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PART 1 - QUALITY ASSURANCE				
CODE	**REF.**			**ITEM**
K	8, 11	**1.1 Quality Assurance (QA) Plan**		
			1.1.1	Written Plan (Check those items which apply.)
				a. Organization of the laboratory.
				b. Staff training requirements.
				c. Standard operating procedures.
				d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance, and rejection criteria established.
				e. Laboratory safety.
				f. Internal performance assessment.
				g. External performance assessment.
C	**8**		**1.1.2**	**QA Plan Implemented.**
K	11		1.1.3	The Laboratory participates in a proficiency testing program annually. Specify Program(s)_____
		1.2 Educational/Experience Requirements		
C	**State's Human Resources Department**		**1.2.1**	**In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2	In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3**	**In commercial laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, or equivalent discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4	In commercial laboratories, the analyst(s) must have at least a high school diploma and shall have at least three months of experience in laboratory science
		1.3 Work Area		
O	8,11		1.3.1	Adequate for workload and storage.
K	11		1.3.2	Clean, well-lighted.
K	11		1.3.3	Adequate temperature control.
O	11		1.3.4	All work surfaces are nonporous, easily cleaned and disinfected.
K	11		1.3.5	Microbiological quality of the air is fewer than 15 colonies for a 15 minute exposure and determined monthly. The results are recorded and records maintained.
		1.4 Laboratory Equipment		
O	9		1.4.1	To determine the pH of prepared media, the pH meter has a standard accuracy of 0.1 units.
O	14		1.4.2	pH electrodes consisting of pH half-cell and reference half-cell or equivalent combination electrode free from (Ag/AgCl) or contains an ion exchange barrier preventing passage of Ag ions into the medium which may affect the accuracy of the pH reading.
K	11		1.4.3	The effect of temperature on the pH is compensated for by an ATC probe or by manual adjustment.
K	8		1.4.4	pH meter is calibrated daily or with each use. Results are recorded and records maintained.
K	11		1.4.5	A minimum of two standard buffer solutions is used to calibrate the pH meter. The first must be near the electrode isopotential point (pH 7). The second near the expected sample pH (i.e., pH 4 or pH 10). Standard buffer solutions are used once and discarded.

O	8,15		1.4.6 Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope. (*Circle the method used.*)

K	9		1.4.7 Balance provides a sensitivity of at least 0.1 g at weights of use.
K	11,13		1.4.8 Balance calibrations are verified ~~checked~~ monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use. Results are recorded and records maintained.
K	11		1.4.9 Refrigerator temperature(s) are monitored at least once daily on workdays Results are recorded and records maintained.
K	1		1.4.10 Refrigerator temperature is maintained at 0 to 4°C.
C	**9**		**1.4.11 The temperature of the incubator is maintained at 35 ± 0.5°C.**
C	**11**		**1.4.12 Thermometers used in the air incubator(s) are graduated in at least 0.1°C increments.**
K	9		1.4.13 Working thermometers are located on top and bottom shelves or appropriately placed based on the results of spatial temperature checks.
C	**11**		**1.4.14 Temperature of the waterbath is maintained at 44.5 ± 0.2°C under all loading conditions.**
C	**9**		**1.4.15 The thermometers used in the waterbath are graduated in at least 0.1°C increments.**
C	**13**		**1.4.16 The waterbath has adequate capacity for workload.**
K	9		1.4.17 The level of water in the waterbath covers the level of liquid in the incubating tubes.
K	8, 11		1.4.18 Air incubator/waterbath temperatures are taken twice daily on workdays. The results are recorded and records maintained.
C	**4**		**1.4.19 All working thermometers are appropriately immersed.**
C	**29**		**1.4.20 Working thermometers are either: calibrated mercury-in-glass thermometers, calibrated non-mercury-in-glass thermometers, or appropriately calibrated electronic devices, including Resistance Temperature Devises (RTDs) and Platinum Resistance Devices (PTDs).**
C	**11**		**1.4.21 A standards thermometer has been calibrated by NIST or a qualified calibration laboratory using a ~~primary~~ standard traceable to NIST or an equivalent authority at the points 0, 35 and 44.5°C (45.5°C for ETCP). These calibration records are maintained.**
K	9		1.4.22 Standards thermometers are verified ~~checked~~ annually for accuracy by ice point determination. Results recorded and maintained. Date of most recent determination_____
C	**29**		**1.4.23 Either mercury-in-glass thermometers, non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury or low drift electronic resistance thermometers with an accuracy and tolerance appropriate for the application ~~with an accuracy of ≤±0.05°C~~ are used as the laboratory standards thermometer. (*Circle the thermometer type used.*)**
K	13		1.4.24 The accuracy of working thermometers is checked annually against the standards thermometer either at the temperatures at which they are used or by ice point determination. Results are recorded and records maintained.
O	11		1.4.25 Appropriate pipet aids are available and used to inoculate samples. Mouth pipetting is not permitted.
		1.5 Labware and Glassware Washing	
O	9		1.5.1 Utensils and containers are clean borosilicate glass, stainless steel or other noncorroding materials.
K	9		1.5.2 Culture tubes are of a suitable size to accommodate the volume for nutritive ingredients and samples.
K	9		1.5.3 Sample containers are made of glass or some other inert material.
O	9		1.5.4 Dilution bottles and tubes are made of borosilicate glass or plastic and closed with rubber stoppers, caps or screw caps with nontoxic liners.

K	9		1.5.5	Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method is used to ensure appropriate volumes.
C	**9**		**1.5.6**	**Pipettes used to inoculate the sample deliver accurate aliquots, have unbroken tips and are appropriately graduated. Pipettes larger than 10 mL are not used to deliver 1mL aliquots; nor, are pipets larger than 1.1mL used to deliver 0.1 mL aliquots.**
K	9		1.5.7	Reusable sample containers are capable of being properly washed and sterilized.
K	9		1.5.8	In washing reusable pipettes, a succession of at least three fresh water rinses plus a final rinse of distilled/deionized water is used to thoroughly rinse off all the detergent.
C	**2**		**1.5.9**	**An alkaline or acidic detergent is used for washing glassware/labware.**
C	**11**		**1.5.10**	**With each load of labware/glassware washed the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali) with aqueous 0.04% bromothymol blue. Results are recorded and records maintained.**
		1.6 Sterilization and Decontamination		
K	9		1.6.1	Autoclave(s) are of sufficient size to accommodate the workload.
O	8		1.6.2	Routine autoclave maintenance is performed and the records are maintained.
C	**30, 33, 34**		**1.6.3**	**The autoclave provides sterilization conditions suitable to the load contents. Sterilization temperature range may be 119ºC - 124ºC as determined by the lab's equipment Quality Assurance Verification Testing and recommended practices from the media manufacturer. Sterilization is determined for each load using a working maximum registering thermometer or an appropriate working temperature monitoring device.**
K	11		1.6.4	An autoclave standards thermometer has been calibrated ~~by a qualified calibration laboratory~~ using a device traceable ~~primary standard traceable~~ to NIST or an equivalent authority at 121°C. ~~Calibration at 100°C, the steam point, is also recommended but not required.~~
K	16		1.6.5	The autoclave standards thermometer is recalibrated ~~checked~~ every five (5) years for accuracy at 121°C by a qualified calibration laboratory; or, is verified ~~checked~~ in-house at the steam point (100°C) if it was ~~has been~~ previously calibrated at both 100°C and 121°C. Any change in temperature at the steam point changes the calibrated temperature at 121°C by the same magnitude. Date of most recent determination_____
K	1		1.6.6	Working autoclave thermometers are verified ~~checked~~ against the autoclave standards thermometer at 121°C yearly. Date of last check _____ Method _____
K	33~~11~~		1.6.7	Spore strips/suspensions with a kill time appropriate for use in an autoclave liquid media cycle are used monthly according to manufacturer's instructions to evaluate the effectiveness of the sterilization process. Results are recorded and the records maintained.
O	11		1.6.8	Heat sensitive tape is used with each autoclave batch.
K	11, 13		1.6.9	Autoclave sterilization records including length of sterilization, total heat exposure time and chamber temperature are maintained. Type of record: Autoclave log, computer printout or chart recorder tracings. (*Circle appropriate type or types.)*
K	11		1.6.10	For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperatures in the range of 160 to 180°C.
K	9		1.6.11	A thermometer capable of determining temperatures accurately in the range of 160 to 180°C is used to monitor the operation of the hot-air sterilizing oven
K	13		1.6.12	Records of temperatures and exposure times are maintained for the operation of the hot-air sterilizing oven during use.

K	11		1.6.13 Spore strips/suspensions are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. Records are maintained.
K	11		1.6.14 Reusable sample containers are sterilized for 60 minutes at 170°C in a hot-air oven or autoclaved for 15 minutes at 121°C.
C	**1**		**1.6.15 The sterility of reusable sample containers is determined for each load sterilized. The results are recorded and the records maintained.**
C	**1**		**1.6.16 The sterility of pre-sterilized disposable sample containers is determined for each lot received. Results are recorded and the records maintained.**
K	9		1.6.17 Reusable pipettes are stored and sterilized in aluminum or stainless steel canisters.
K	9		1.6.18 Reusable pipettes (in canisters) are sterilized in a hot-air oven at 170°C for 2 hours.
C	**2**		**1.6.19 The sterility of reusable pipettes is determined with each load sterilized. Results are recorded and records maintained.**
C	**2**		**1.6.20 The sterility of pre-sterilized disposable pipettes is determined with each lot received. Results are recorded and the records maintained.**
K	18		1.6.21 Hardwood applicator transfer sticks or <u>reusable loops</u> are properly sterilized. <u>Alternatively, presterilized loops are used for transfers</u>. Method of sterilization _____
C	**2**		**1.6.22 The sterility of the hardwood applicator transfer sticks/<u>presterilized loops</u> is checked routinely. Results are recorded and the records maintained.**
O	13		1.6.23 Spent broth cultures and agar plates are decontaminated by autoclaving for at least 30 minutes before conventional disposal.
		1.7 Media Preparation	
K	3, 5		1.7.1 Media is commercially dehydrated except in the case of medium A-1 which must be prepared from the individual components and modified MacConkey agar which may be prepared from its components.
K	11		1.7.2 Media is prepared <u>and sterilized</u> according to manufacturer's instructions.
O	11		1.7.3 Dehydrated media and media components are properly stored in a cool, clean, dry place.
O	11		1.7.4 Dehydrated media are labeled with date of receipt and date opened.
C	**12**		**1.7.5 Caked or expired media or media components are discarded.**
C	**11**		**1.7.6 Reagent water is distilled or deionized (circle appropriate choice), tested monthly and exceeds 0.5 megohm-cm resistance (2 megohms-cm in-line) or is less than 2.0 µSiemens/cm conductivity at 25°C. (C*ircle the appropriate water quality descriptor determined*.) Results are recorded and the records maintained.**
C	**11**		**1.7.7 Reagent water is analyzed for residual chlorine monthly and is at a non-detectable level (< 0.1 mg/L). Results are recorded and the records maintained.** **Specify method of determination _____.**
K	11		1.7.8 Reagent water contains<100 CFU/mL as determined monthly using the heterotrophic plate count method. Results are recorded and the records maintained.
~~K~~	~~11~~		~~1.7.9 Media prepared from commercial dehydrated components are sterilized according to the manufacturer's instructions.~~
K	9		1.7.9~~10~~ The volume and concentration of media in the tube are suitable for the amount of sample inoculated.
C	**11**		**1.7.10~~1~~ Total time of exposure of sugar broths to autoclave temperatures does not exceed 45 minutes.**

C	**1**		**1.7.11~~2~~ Media sterility is determined for each load sterilized. Results are recorded and the records maintained.**

C	**1**		**1.7.12~~3~~**	**Media productivity is determined using media-appropriate, properly diluted positive and negative control cultures for each lot of dehydrated media received or with each batch of media prepared when the medium is made from its individual components.**
O	9		1.7.13~~4~~	Sterile phosphate buffered dilution water is used as the sample diluent.
K	11		1.7.14~~5~~	The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer's requirements. Results are recorded and records are maintained.
		1.8 Storage of Prepared Culture Media		
K	9		1.8.1	Prepared culture media are stored in a cool, clean, dry space where excessive evaporation and the danger of contamination are minimized.
K	5,11		1.8.2	Brilliant green bile 2% broth and A-1 media are stored in the dark.
K	13		1.8.3	Stored media are labeled with the storage expiration date or the sterilization date.
K	9		1.8.4	Storage of prepared culture media at room temperature does not exceed 7 days.
K	2		1.8.5	Storage under refrigeration of prepared culture media with loose fitting closures shall not exceed 1 month.
K	11		1.8.6	Storage under refrigeration of prepared culture media with screw-cap closures does not exceed 3 months.
K	17		1.8.7	All prepared MPN broth media stored under refrigeration must reach room temperature prior to use. Culture tubes containing any type of precipitate or Durham tubes containing air bubbles are discarded.
		PART II - SEAWATER SAMPLES		
		2.1 Collection and Transportation of Samples		
C	**11**		**2.1.1**	**Sample containers are of a suitable size to contain at least 110 mL of sample and to allow adequate headspace for proper shaking. Seawater samples are collected in clean, sterile, watertight, properly labeled sample containers.**
K	1		2.1.2	Samples are identified with collector's name, harvest area, sampling station, time and date of collection.
C	**9**		**2.1.3**	**Immediately after collection, seawater samples are placed in dry storage (ice chest or equivalent) capable of maintaining a temperature of 0 to 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed in the refrigerator unless processed immediately.**
O	1		2.1.4	A temperature blank is used to represent the temperature of samples upon receipt at the laboratory. Temperature should be equivalent or less than that of the growing area waters. Results are recorded and maintained.
C	**9**		**2.1.5**	**Analysis of the sample is initiated as soon as possible after collection. Seawater samples are not tested if they have been held for more than 30 hours from the time of collection.**
		2.2 Bacteriological Examination of Seawater by the APHA MPN		
C	**9**		**2.2.1**	**Lactose broth or lauryl tryptose broth is used as the presumptive medium.** ***(Circle appropriate one.)***
C	**2**		**2.2.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _**
C	**9, 35**		**2.2.3**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) before inoculation.**
C	**9**		**2.2.4**	**In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended).**
C	**6**		**2.2.5**	**In a single dilution series not less than 12 tubes are used (for depuration at least 5 tubes are used).**

C	**6**		**2.2.6**	**In a single dilution series, the volumes analyzed are adequate to meet the needs of routine monitoring.** **Sample volume inoculated _____** **Range of MPN_____** **Strength of media used_____**
K	9		2.2.7	Inoculated tubes are incubated in air at 35 ± 0.5°C.
C	**2**		**2.2.8**	**Appropriately diluted process control cultures accompany the samples *throughout both the presumptive and confirmed phases of incubation.* Results are recorded and the records maintained.** **Positive process control _____ Negative process control _____**
K	9		2.2.9	Inoculated tubes are read after 24 ± 2 hours and 48 ± 3 hours of incubation and transferred at both time interval if positive for growth (the presence of turbidity) and gas or effervescence in the culture tube. These tubes are considered presumptive positive requiring further confirmatory testing.
		2.3 Confirmed Test for Seawater by APHA MPN		
C	**9**		**2.3.1**	**Brilliant green bile 2% broth (BGB) is used as the confirmatory medium for total coliforms.**
C	**9**		**2.3.2**	**EC medium is used as the confirmatory medium for fecal coliforms.**
C	**2**		**2.3.3**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
K	9, 11		2.3.4	Transfers are made to BGB/EC by either sterile loop or sterile hardwood transfer stick from positive presumptive tubes incubated for 24 and 48 hours as appropriate. *(Circle the method of transfer.)*
C	**9**		**2.3.5**	**BGB tubes are incubated at 35 ± 0.5°C.**
K	9		2.3.6	BGB tubes are read after 48 ± 3 hours of incubation.
C	**9**		**2.3.7**	**EC tubes are incubated in a circulating waterbath maintained at 44.5 ± 0.2°C.**
C	**9**		**2.3.8**	**EC tubes are read after 24 ± 2 hours of incubation.**
C	**9**		**2.3.9**	**The presence of turbidity and any amount of gas or effervescence in the culture tube constitutes a positive test.**
		2.4 Computation of Results – APHA MPN		
K	9		2.4.1	Results of multiple dilution tests are read from tables in *Recommended Procedures for the Examination of Sea Water and Shellfish*, Fourth Edition.
K	7		2.4.2	Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**7, 9**		**2.4.3**	**Results are reported as MPN/100 mL of sample.**
		2.5 Bacteriological Examination of Seawater by the MA-1 Method		
C	**5**		**2.5.1**	**A-1 medium complete is used in the analysis.**
C	**2, 31**		**2.5.2**	**A-1 medium without salicin is used in the analysis. Comparability testing supports use of A-1medium without salicin. Study records are available.**
C	**5**		**2.5.3**	**A-1 medium sterilized for 10 minutes at 121°C.**
C	**2**		**2.5.4**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _**

C	**9, 35**		**2.5.5 Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) before inoculation.**
C	**9**		**2.5.6 In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended).**
C	**6**		**2.5.7 In a single dilution series at least 12 tubes are used.**
C	**6**		**2.5.8 In a single dilution series, the volumes analyzed are adequate to meet the needs of routine monitoring.** **Sample volume inoculated _____** **Range of MPN_____** **Strength of media used _____**
C	**2**		**2.5.9 Appropriately diluted process control cultures accompany the samples *throughout both resuscitation and waterbath incubation* Results are recorded and the records maintained.** **Positive process control _____Negative process control _____**
C	**2,5**		**2.5.10 Inoculated tubes are placed in an air incubator at 35 ± 0.5°C for 3 ± 0.5 hours of resuscitation.**
C	**5**		**2.5.11 After 3 ± 0.5 hours resuscitation at 35°C, inoculated tubes are incubated at 44.5 ± 0.2°C in a circulating waterbath for the remainder of the 24 ± 2 hours.**
C	**5**		**2.5.12 The presence of turbidity and any amount of gas or effervescence in the culture tube constitutes a positive test.**
		2.6 Computation of Results – APHA MPN	
K	9		2.6.1 Results of multiple dilution tests are read from tables in *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition.
K	7		2.6.2 Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**7, 9**		**2.6.3 Results are reported as MPN/100 mL of sample.**
		2.7 Bacteriological Analysis of Seawater by Membrane Filtration (MF) using mTEC Agar - Materials and Equipment	
C	**23, 24**		**2.7.1 When used for elevated temperature incubation in conjunction with ethafoam resuscitation, the temperature of the hot air incubator is maintained at 44.5 ± 0.5°C under any loading capacity.**
C	**23**		**2.7.2 When using a waterbath for elevated temperature incubation, the level of the water completely covers the plates.**
C	**23**		**2.7.3 Pre-sterilized plastic or sterile glass culture plates that are clear, flat bottomed, free of bubbles and scratches with tight fitting lids are used.**
C	**2**		**2.7.4 The sterility of pre-sterilized culture plates is determined for each lot received. Results are recorded and the records maintained.**
K	11		2.7.5 Colonies are counted with the aid of magnification.
C	**11, 23**		**2.7.6 Membrane filters are made from cellulose ester material, white, grid marked, 47 mm in diameter with a pore size of 0.45 µmand certified by the manufacturer for fecal coliform analyses.**
C	**2**		**2.7.7 Lot number, date of receipt and if provided the expiration date of the membrane filters are recorded and records maintained.**
C	**2**		**2.7.8 When initiating monitoring by mTEC or switching brands or types of membrane filters used and no previous lots of filters are available for comparing acceptable performance, an appropriate method for determining the suitability of the lot is developed and the comparison testing implemented. The results are recorded and this record is maintained.**

K	2, 11		2.7.9 New lots of membrane filters are checked by comparing recovery of fecal coliform organisms against membrane filters from previously acceptable lots.
C	**2**		**2.7.10 The sterility of each lot or autoclave batch of membrane filters are checked before use.**
K	2		2.7.11 Membrane filters which are beyond their expiration date are not used.
O	11		2.7.12 Forceps tips are clean.
O	11		2.7.13 Forceps tips are smooth without pitting or corrugations to damage the filters being manipulated.
K	11		2.7.14 Forceps are dipped in alcohol and flame sterilized between sample filters.
K	11		2.7.15 If indelible graduation marks are used on clear glass or plastic funnels to measure sample volumes, their accuracy is checked gravimetrically or with a Class A graduated cylinder before use and periodically rechecked. Funnels having a tolerance greater than 2.5% are not used. Checks are recorded and records maintained.
K	11		2.7.16 Membrane filtration units are made of stainless steel, glass or autoclavable plastic free of scratches, corrosion and leaks.
C	**11**		**2.7.17 Membrane filter assemblies are autoclave sterilized for 15 minutes at 121°C prior to the start of a filtration series.**
O	11, 23, 26		2.7.18 A UV sterilization unit is used to disinfect filter assemblies between sample and filtration runs.
K	11		2.7.19 The effectiveness of the UV sterilization unit is determined by biological testing monthly. Results are recorded and records maintained.
K	2		2.7.20 Maintenance of the UV sterilization unit is performed as needed. This maintenance is documented and the records maintained.
		2.8 Media Preparation and Storage – MF using mTEC Agar	
K	11		2.8.1 Phosphate buffered saline is used as the sample diluent and filter funnel rinse.
C	**11**		**2.8.2 The phosphate buffered saline is properly sterilized.**
K	23		2.8.3 A sufficient amount of medium (4-5 mL) is used in each plate.
O	11		2.8.4 Refrigerated prepared plates are stored for no more than 2 weeks in sealed plastic bags or containers to minimize evaporation.
		2.9 Sample Analyses - MF using mTEC Agar	
C	**24**		**2.9.1 mTEC agar is used.**
C	**2**		**2.9.2 The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _**
C	**23, 35**		**2.9.3 The sample is shaken vigorously (25 times in a 12″ arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) before filtration.**
C	**23**		**2.9.4 The membrane is placed grid side up within the sterile filter apparatus.**
C	**23, 25**		**2.9.5 Sample volumes tested are consistent with the sampling regime employed (i.e., half log or other appropriate dilutions are used with systematic random sampling).**
C	**23**		**2.9.6 Sample volumes are filtered under vacuum.**
K	26		2.9.7 The pressure of the vacuum pump does not exceed 15 psi.
C	**23, 26**		**2.9.8 The sides of the filter funnel are rinsed at least twice with 20-30 mL of sterile phosphate buffered saline after sample filtration.**
C	**23**		**2.9.9 The membrane filter is removed from the filtering apparatus with sterile forceps and rolled onto mTEC agar so that no bubbles form between the filter and the agar.**
C	**11**		**2.9.10 Blanks are run at the beginning of filtration, after every 10th aliquot and at the end of the filtration run to check the sterility of the testing system (phosphate buffered saline, filter funnel, forceps, membrane filter, media and culture plate).**

C	**2, 11**		**2.9.11 Appropriately diluted process control cultures accompany the samples throughout both resuscitation and elevated temperature incubation. Results are recorded and the records maintained.** **Positive process control _____Negative process control _____**
C	**11, 23, 24**		**2.9.12 Inoculated plates are placed inverted into a watertight, tightly sealed container prior to being placed in the air incubator and incubated at 35 +0.5°C for 2 hours of resuscitation. Alternatively inoculated plates may be placed in ethafoam prior to air incubation at 44.5 ± 0.5°C for 24 ± 2 hours.**
C	**11, 23, 24**		**2.9.13 After 2 hours of resuscitation at 35°C, the watertight, tightly sealed containers are transferred to a circulating waterbath at 44.5 + 0.2°C, submerged completely and incubated for 22-24 hours.**
		2.10 Computation of Results - MF using mTEC Agar	
C	**23**		**2.10.1 All yellow, yellow-green or yellow-brown colonies are counted.**
C	**23**		**2.10.2 Only plates having 80 or fewer colonies are counted. If it is unavoidable to use plates having more than 80 colonies, counts are given as >80 x 100/the volume of sample filtered.**
C	**2, 11, 23**		**2.10.3 When multiple dilutions are filtered, the laboratory has developed a procedure for assessing the contribution of all positive dilutions to the final count.**
C	**23, 11**		**2.10.4 The number of fecal coliforms is calculated by the following equation:** **Number of fecal coliforms per 100 mL = [number of colonies counted per plate used in the count / volume (s) of sample filtered in ml] x 100.**
C	**23, 11**		**2.10.5 Results are reported as CFU/100 mL of sample.**
		2.11 Bacteriological Analysis of UV Treated Process Water Samples by Membrane Filtration (MF) using mEndo Agar LES – Materials and Equipment	
C	**9, 11, 21**		**2.11.1 Pre-sterilized plastic or sterile glass culture plates that are clear, flat bottomed, free of bubbles and scratches are used.**
C	**2**		**2.11.2 The sterility of pre-sterilized culture plates is determined for each lot received. Results are recorded and the records maintained.**
C	**11, 19, 21**		**2.11.3 Membrane filters are made from cellulose ester material, white, grid marked, 47 mm diameter with a pore size of 0.45µm and certified by the manufacturer for total coliform analysis.**
C	**2**		**2.11.4 Membrane filter lot numbers, dates of receipt and expiration dates are recorded and the records maintained.**
C	**2**		**2.11.5 If previous lots of agar or membrane filters are not available for comparability testing, an appropriate method for determining lot suitability has been developed and comparison testing implemented when the following has occurred:** **a. initiating monitoring by mEndo Agar LES;** **b. changing agar manufacturers; or** **c. changing brands of membrane filters used.** **The results are recorded and the records are maintained.**
K	2, 11		2.11.6 Recovery of total coliforms from new lots of membrane filters and mEndo Agar LES is compared against the recovery from the previously acceptable lot.
C	**2**		**2.11.7 The sterility of each lot and autoclave batch of membrane filters is verified before use.**
K	2		2.11.8 Expired membrane filters are not used.
K	9, 11, 19, 21		2.11.9 Membrane filtration units are made of stainless steel, glass or autoclavable plastic free of scratches, corrosion and leaks.

K	11		2.11.10 If indelible graduation marks are used on clear glass or plastic funnels to measure sample volumes, their accuracy is checked gravimetrically with a Class A graduated cylinder before use and periodically rechecked. Funnels having a tolerance greater than 2.5% are not used. Checks are recorded and records maintained.
C	**9, 11**		**2.11.11 Membrane filter assemblies are autoclave sterilized for 15 minutes at 121+/- 2°C prior to the start of a filtration run. A new run occurs when there is a break of 30 minutes or more between the previous filtration run.**
O	11, 19, 26, 36		2.11.12 A UV sterilization unit is used to disinfect filter assemblies between sample and filtration runs.
K	11		2.11.13 The effectiveness of the UV sterilization unit is determined by biological testing monthly. Results are recorded and the records are maintained.
K	2		2.11.14 Maintenance of the UV sterilization unit is performed as needed. Maintenance is documented and the records maintained.
O	9, 11		2.11.15 Forceps tips are clean and smooth without pitting or corrugations.
		2.12 Media Preparation and Storage	
C	**9, 11, 19, 21, 36**		**2.12.1 mEndo Agar LES is used.**
K	11, 21, 36		2.12.2 mEndo Agar LES is prepared aseptically using pre-sterilized glassware, sterile reagent water and pre-sterilized stir bar.
K	9, 11, 36		2.12.3 mEndo Agar LES is prepared using 95% alcohol that is not denatured.
C	**9, 11, 36**		**2.12.4 mEndo Agar LES is brought to near boiling; removed from the heat and tempered at 44-50°C before dispensing.**
C	**9, 11, 36**		**2.12.5 mEndo Agar LES is never autoclaved.**
K	9, 11, 36		2.12.6 A sufficient amount of medium (4-5 mL) is aseptically aliquoted to each culture plate.
O	9, 11, 36		2.12.7 Prepared plates of mEndo Agar LES are stored at 4°C in the dark for no more than two (2) weeks in sealed plastic bags or containers to minimize evaporation.
C	**2**		**2.12.8 Appropriate, properly diluted positive and negative productivity controls for mEndo Agar LES medium are used. Results are recorded and the record maintained.** **Positive productivity control _____.** **Negative productivity control _____**
K	9, 11, 21, 36		2.12.9 Sterile phosphate buffered water or sterile phosphate buffered saline is used as a sample blank, filter funnel rinse and process and productivity control diluent for UV treated process water samples.
C	**11**		**2.12.10 The phosphate buffered water/saline is properly sterilized and the sterility is tested before being placed in service. Results are recorded and records maintained**
		2.13 Sample Analysis	
C	**9, 11, 36**		**2.13.1 The sample is shaken vigorously (25 times in a 12-inch arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) before filtration.**
C	**11, 21, 36**		**2.13.2 The membrane filter is placed grid side up within the sterile filter apparatus.**
C	**11, 26, 36**		**2.13.3 A 100 mL quantity of sample is filtered under vacuum.**
K	26		2.13.4 The pressure of the vacuum pump does not exceed 15 psi.
C	**9, 11, 26, 36**		**2.13.5 The sides of the filter funnel are rinsed at least twice with 20-30 mL of sterile phosphate buffered water/saline as appropriate after filtration.**
C	**9, 11, 36**		**2.13.6 The membrane filter is removed from the filtering apparatus with sterile forceps and rolled onto mEndo Agar LES so that no bubbles form between the filter and the agar.**

K	9, 11, 36		2.13.7 Forceps are dipped in alcohol and flame sterilized between sample filters.
C	**11, 36**		**2.13.8 Blanks are run at the beginning and at the end of the filtration run to check the sterility of the testing system (phosphate buffered water/saline, filter funnels, forceps, membrane filters, media and culture plates).**
C	**2, 36**		**2.13.9 An appropriate properly diluted positive process control culture accompanies the sample throughout incubation. Results are recorded and the records are maintained.** **Positive process control _____**
C	**9, 11, 36**		**2.13.10 Inoculated plates are incubated inverted at 35+/- 0.5°C for 22 to 24 hours.**
K	2, 9, 11		2.13.11 An appropriate level of humidity is maintained in the incubator to prevent the plates from drying out.
		2.14 Computation of Results	
K	9, 11		2.14.1 Colonies are counted with the aid of magnification.
C	**9, 11, 19, 21, 23**		**2.14.2 All metallic sheen colonies are counted as total coliforms.**
C	**9, 11, 21, 36**		**2.14.3 Results are reported as total coliforms/100mL.**
C	**11, 20, 36**		**2.14.4 When no colonies are observed, results are reported as <1.0 coliform/100mL (nondetectable)**
		PART III - SHELLFISH SAMPLES	
		3.1 Collection and Transportation of Samples	
C	**9**		**3.1.1 A representative sample of shellstock is collected.**
K	9		3.1.2 Shellstock samples are collected in clean, waterproof, puncture resistant containers loosely sealed.
K	9		3.1.3 Shellstock samples are labeled with collector's name, type of shellstock, the source or harvest area, sampling station, time, date and place (if applicable) of collection.
C	**9**		**3.1.4 Immediately after collection, shellfish samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed under refrigeration unless processed immediately.**
C	**1**		**3.1.5 Analysis of the samples is initiated as soon as possible after collection. Shellfish samples are not tested if the time interval between collection and analysis exceeds 24 hours.**
		3.2 Preparation of Shellfish for Examination	
K	2,11		3.2.1 Shucking knives, scrub brushes and blender jars are (autoclave) sterilized for 15 minutes prior to use.
O	2		3.2.2 Blades of shucking knives are not corroded.
O	9		3.2.3 The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2		3.2.4 The faucet used for rinsing the shellstock does not contain an aerator.
K	9		3.2.5 Shellstock are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9		3.2.6 Shellstock are allowed to drain in a clean container or on clean towels prior to opening.
K	1, 9		3.2.7 Immediately prior to shucking, the hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol or clean gloves are donned.
C	**9**		**3.2.8 Shellstock are not shucked directly through the hinge.**
C	**9**		**3.2.9 Contents of shellstock (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	9		3.2.10 At least 200 grams of shellfish meat or a quantity of meat sufficient to cover the blender blades is used for the analysis.

K	9		3.2.11 A representative sample of at least 12 shellfish is used for the analysis.
K	2		3.2.12 The sample is weighed to the nearest 0.1 gram and an equal amount by weight of diluent is added.
O	9		3.2.13 Sterile phosphate buffered dilution water is used as the sample diluent.
C	**9**		**3.2.14 Samples are blended at high speed for 60 to 120 seconds until homogenous.**
K	9		3.2.15 APHA *Recommended Procedures for the Examination of Sea Water And Shellfish*, Fourth Edition is followed for the analysis of previously shucked and frozen shellfish meats.
		3.3 MPN Analysis for Fecal Coliform Organisms, Presumptive Test, APHA	
C	**9**		**3.3.1 Appropriate strength lactose or lauryl tryptose broth is used as presumptive media in the analysis. *(Circle the medium used.)***
C	**2**		**3.3.2 The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
K	9		3.3.3 Immediately (within 2 minutes) after blending, the ground sample is diluted and inoculated into tubes of presumptive media.
C	**9**		**3.3.4 No fewer than 5 tubes per dilution are used in a multiple dilution MPN series.**
C	**9**		**3.3.5 Allowing for the initial 1:1 dilution of the sample, appropriate portions are inoculated (i.e., 2 ml of original 1:1 dilution for the 1 g portion) and diluted for subsequent inoculation (i.e., 20 ml of 1:1 diluted sample to 80 ml of diluent or the equivalent for 0.1 g portion). All successive dilutions are prepared conventionally.**
K	6		3.3.6 In a single dilution series, the volumes examined are adequate to meet the needs of routine monitoring. Sample volume inoculated _____ Range of MPN_____ Strength of media used_____
C	**2**		**3.3.7 Appropriately diluted process control cultures accompany the samples throughout both the presumptive and confirmed phases of incubation. Results are recorded and the records maintained.** **Positive Process control _____ Negative Process control_____**
K	9		3.3.8 Inoculated media are incubated at 35 ± 0.5°C.
K	10		3.3.9 Tubes are read after 24 ± 2 hours of incubation and transferred if positive for growth (the presence of turbidity and gas or effervescence in the culture tube). These tubes are considered presumptive requiring further confirmatory testing.
		3.4 Confirmed Test for Fecal Coliforms - APHA	
C	**9**		**3.4.1 EC medium is used as the confirmatory medium.**
C	**2**		**3.4.2 The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _**
K	9, 11		3.4.3 Transfers are made to EC medium by either sterile loop or hardwood sterile transfer sticks from positive presumptives. *(Circle the method of transfer.)*
C	**9**		**3.4.4 EC tubes are incubated in a circulating waterbath at 44.5 ± 0.2°C**
K	9		3.4.5 EC tubes are read for gas production after 24 ± 2 hours of incubation.
C	**9**		**3.4.6 The presence of turbidity and any amount of gas and/or effervescence in the Durham tube constitutes a positive test.**
		3.5 Computation of Results for MPN Analyses	
K	9		3.5.1 Results of multiple dilution tests are read from tables in *Recommended Procedure for the Examination of Sea Water and Shellfish,* 4th Edition and multiplied by the appropriate dilution factor.

K	7		3.5.2	Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**9**		**3.5.3**	**Results are reported as MPN/100 grams of sample.**
		3.6 Standard Plate Count Method		
O	20		3.6.1	A standard plate count (SPC) analysis maybe performed in conjunction with the analysis for fecal coliform organisms.
K	9		3.6.2	In the standard plate count procedure at least four plates are used, duplicates of two dilutions. One of the dilutions should produce colonies of 30 to 300 per plate.
K	2		3.6.3	Fifteen to 20 mL of tempered sterile plate count agar is used per plate.
C	**9**		**3.6.4**	**Agar tempering bath maintains the agar at 44-46°C.**
C	**9**		**3.6.5**	**An agar based temperature control having a similar volume and shape as the tempering plate count agar is used in the tempering bath.**
K	9		3.6.6	Samples or sample dilutions to be plated are shaken vigorously (25 times in a 12" arc in 7 seconds) before plating.
C	**9**		**3.6.7**	**Not more than 1 mL nor less than 0.1 mL of sample or sample dilution is plated.**
K	11		3.6.8	Control plates are used to check air quality and the sterility of the agar and the diluent.
K	9,21		3.6.9	Solidified plates are incubated at 35 ± 0.5°C for 48 ± 3 hours inverted and stacked no more than four high.
K	9		3.6.10	Quebec Colony Counter or its equivalent is used to provide the necessary magnification and visibility for counting plates.
K	1		3.6.11	A hand tally or its equivalent is used for accuracy in counting.
		3.7 Computation of Results -SPC		
K	9		3.7.1	Colony counts determined in accordance with Part III, A, Sections 4.31 through 4.33 in *Recommended Procedures for the Examination of Sea Water and Shellfish*, Fourth Edition.
C	**19**		**3.7.2**	**Colony counts are reported as CFU/g of sample.**
		3.8 Bacteriological Analysis of Shellfish Using the ETCP		
C	**2,3**		**3.8.1**	**Prepared modified MacConkey agar is used on the day that it is made.**
K	3		3.8.2	Double strength modified MacConkey agar is used.
C	**3**		**3.8.3**	**Prepared double strength modified MacConkey agar is heated to boiling, removed from the heat, and boiled again. This agar is never autoclaved.**
K	2, 3		3.8.4	Twice boiled, double strength modified MacConkey agar and is maintained in a tempering bath at 45 to 50°C until used.
K	2, 3		3.8.5	Phosphate buffered saline is used as the sample diluent in the ETCP.
C	**2, 3**		**3.8.6**	**The phosphate buffered saline is tempered at 45 - 50°C to prevent premature solidification of the agar.**
C	**9**		**3.8.7**	**The sample homogenate is cultured within 2 minutes of blending.**
C	**2,3**		**3.8.8**	**Six grams of shellfish (12 grams of homogenate if initially diluted 1:1) is placed into a sterile container and the contents brought up to 60 mL with sterile, tempered phosphate buffered saline.**
K	3		3.8.9	Sixty (60) mL of tempered, twice boiled double strength Modified MacConkey Agar is added.
K	2,3, 22		3.8.10	The container is gently swirled or slowly inverted once to mix the contents, which are subsequently distributed uniformly over six plates.
C	**1**		**3.8.11**	**Media and diluent sterility are determined with each use. Results are recorded and the records maintained.**

C	**1**		**3.8.12 Media productivity is determined using media appropriate properly diluted pour plated positive and negative control cultures for each batch of Modified MacConkey agar prepared. Positive control culture _____ Negative control culture _____**
C	**3, 13**		**3.8.13 When solidified, the plates are placed inverted into an air incubator at 45.5 ± 0.5°C for 18 to 30 hours of incubation.**
C	**2**		**3.8.14 Plates are stacked no more than three high in the incubator.**
C	**2**		**3.8.15 Appropriately diluted pour plated process control cultures *accompany each set of samples throughout incubation.* The results are recorded and the records maintained. Positive process control_____ Negative process control_____**
		3.9 Computation of Results - ETCP	
K	11		3.9.1 Quebec Colony counter or its equivalent is used to provide the necessary magnification and visibility for counting.
O	1		3.9.2 A hand tally or its equivalent is used to aid in counting.
C	**3, 6**		**3.9.3 All brick red colonies greater than 0.5 mm in diameter are totaled over all the plates and multiplied by a factor of 16.7.**
C	**3**		**3.9.4 Results are reported as CFU/100 grams of sample.**
		Bacteriological Examination of Soft-shelled Clams and American Oysters for Male Specific Coliphage (MSC)	
		3.10 MSC Equipment and Supplies	
K	30		3.10.1 Sample containers used for the shucked sample are sterile, made of glass or some other inert material (i.e. polypropylene) and hold 100 – 125 mL.
C	**27, 28**		**3.10.2 The refrigerated centrifuge used must have the capacity to accommodate the amount of shellfish sample required for the procedure, perform at 9000 x g and maintain a temperature of 4°C.**
K	9		3.10.3 The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
C	**27, 28**		**3.10.4 Sterile 0.22 µm pore size syringe filters and pre-sterilized plastic or sterile glass syringes are used to sterilize the antibiotic solutions.**
K	1		3.10.5 The sterility of each lot of pre-sterilized syringes and syringe filters is determined. Results are recorded and records maintained.
K	1		3.10.6 The sterility of each batch of reusable glass syringes is determined. Results are recorded and records maintained.
C	**27, 28**		**3.10.7 The balance used provides a sensitivity of at least mg (0.01g.).**
C	**27, 28**		**3.10.8 The temperature of the incubator used is maintained at 36 ± 1°C.**
C	**28**		**3.10.9 Sterile disposable 50 mL centrifuge tubes are used and their sterility is determined with each lot. Results are recorded and records maintained.**
		3.11 MSC Media Preparation	
K	28		3.11.1 Media preparation and sterilization is according to the validated method.
K	27, 28		3.11.2 Bottom agar, double strength soft agar and growth broth are prepared from their individual components.
K	27, 28		3.11.3 Soft agar is prepared double strength in volumes of 2.5 mL.
C	**27, 28**		**3.11.4 The streptomycin and ampicillin solutions are added to tempered bottom agar and vortex for 2 minutes on stir plate.**
O	27, 28		3.11.5 Storage of the bottom agar under refrigeration does not exceed 1 month.
K	27, 28		3.11.6 Unsterilized soft agar is stored at -20 °C -15C for up to 3 months.
K	27, 28		3.11.7 The soft agar is removed from the freezer and sterilized for 15 minutes at 121°C before use.
K	27, 28		3.11.8 Storage of growth broth in the refrigerator in loosely capped tubes/bottles does not exceed 1 month and in screw capped tubes/bottles does not exceed 3 months.
K	27, 28		3.11.9 Bottom agar plates are allowed to reach room temperature before use.

		3.12 Preparation of the Soft-Shelled Clams and American Oysters for MSC Analysis	
K	2,11		3.12.1 Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2		3.12.2 The blades of shucking knives are not corroded.
O	9		3.12.3 The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2		3.12.4 The faucet used for rinsing the shellfish does not contain an aerator.
K	9		3.12.5 The shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9		3.12.6 The shellfish are allowed to drain in a clean container or on clean towels unlayered prior to shucking.
K	9		3.12.7 Immediately prior to shucking, the hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol or clean gloves are donned.
C	**9**		**3.12.8 Shellfish are not shucked through the hinge.**
C	**9**		**3.12.9 The contents of shellfish (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	9		3.12.10 A representative sample of at least 12 shellfish is used for the analysis.
K	2, 19		3.12.11 The sample is weighed to the nearest 0.1 gram.
		3.13 MSC Sample Analysis	
C	**28**		**3.13.1 E. coli *Famp* ATCC 700891 is the bacterial host strain used in this procedure.**
K	27, 28		3.13.2 Host cell growth broth is tempered at 36 ± 1°C and vortexed (or shaken) to aerate prior to inoculation with host cells.
K	27, 28		3.13.3 Several host cell colonies are transferred to a tube of tempered, aerated growth broth and incubated at 36 ± 1°C for 4-6 hours to provide host cells in log phase growth for sample analysis.
C	**27, 28**		**3.13.4 After inoculation, the host cell growth broth culture is not shaken.**
C	**28**		**3.13.5 A 2:1 mixture of sterile growth broth to shellfish tissue is used for eluting the MSC.**
C	**28**		**3.13.6 The elution mixture is prepared w/v by weighing the sample and adding two equal portions of sterile growth broth by volume to the shellfish tissue.**
C	**28**		**3.13.7 The elution mixture is homogenized at high speed for 180 seconds.**
C	**28**		**3.13.8 Immediately after blending, 33 grams of the homogenized elution mixture are weighed into centrifuge tubes.**
C	**28**		**3.13.9 The homogenized elution mixture is centrifuged for 15 minutes at 9000 x g at 4°C.**
C	**27, 28**		**3.13.10 The supernatant is pipetted off, weighed and the weight recorded.**
C	**27, 28**		**3.13.11 The supernatant is allowed to warm to room temperature about 20 to 30 minutes.**
K	27, 28		3.13.12 The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28		3.13.13 Two hundred microliters (0.2 mL) of log phase host strain *E coli* is added to the tempering soft agar immediately prior to adding the sample supernatant.
K	27, 28		3.13.14 The sample supernatant is shaken or vortexed before being added to the tempering soft agar.
C	**27, 28**		**3.13.15 2.5 mL of sample supernatant is added to each tube of tempering soft agar.**
C	**27, 28**		**3.13.16 The soft agar/sample supernatant/host cell mixture is gently rolled between the palms of the hands to mix.**
C	**27, 28**		**3.13.17 The soft agar/sample supernatant/host cell mixture is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**
C	**28**		**3.13.18 Ten (10) plates are used, 2.5 mL per plate for a total of 25 mL of supernatant analyzed per sample.**

K	27, 28		3.13.19 Negative and positive control plates are prepared and accompany each set of samples analyzed. The results are recorded and records maintained. Positive control ___ ____
K	27, 28		3.13.20 Growth broth is used as the negative control or blank.
K	27, 28		3.13.21 Type strain MS2 (ATCC 15597) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.
K	2		3.13.22 A negative control plate is plated at the beginning and end of each set of samples analyzed.
K	27, 28		3.13.23 The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	**27, 28**		**3.13.24 All plates are incubated at 36 ± 1°C for 18 ± 2 hours.**
		3.14 Computation of Results - MSC	
C	**27**		**3.14.1 Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted.**
C	**28, 32**		**3.14.2 The working range of the method is 1 to 200 PFU per plate. When there are no plaques on all ten plates, the count is <6 PFU/100 grams for soft-shelled clams, <7 PFU/ 100 grams for American oysters, and <5 PFU/ 100 grams for quahog (hard) clams. If the density exceeds 200 PFU per plate on all plates, the count is given as > 20,000 PFU/100 grams.**
K	28		3.14.3 The formula used for determining the density of MSC in PFU/100 grams is: (0.364) (N) (Ws), where N = total number of plaques counted on all 10 plates and Ws = weight of the supernatant used.
O	9		3.14.4 The MSC count is rounded off conventionally to give a whole number.

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SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	

MICROBIOLOGICAL COMPONENT: (Part I-III)

A. Results	
Total # of Critical (C) Nonconformities in Parts I-III	_____
Total # of Key (K) Nonconformities in Parts I-III	_____
Total # of Critical, Key and Other (O) Nonconformities in Parts I-III	_____

B. Criteria for Determining Laboratory Status of the Microbiological Component:

1. **Does Not Conform Status**: The Microbiological component of this laboratory is not in conformity with NSSP requirements if:

   a. The total # of Critical nonconformities is **≥ 4 or**

   b. The total # of Key nonconformities is **≥ 13 or**

   c. The total # of Critical, Key and Other is **≥ 18**

2. **Provisionally Conforms Status**: The microbiological component of this laboratory is determined to be provisionally conforming to NSSP requirements if the number of critical nonconformities is **≥ 1 but ≤ 3**.

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature: _____ Date: _____

LEO Signature: _____ Date: _____

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PART 1 - QUALITY ASSURANCE			
CODE	**REF.**		**ITEM**
K	8, 11	**1.1 Quality Assurance (QA) Plan**	
		1.1.1	Written Plan (Check those items which apply.)
			a. Organization of the laboratory.
			b. Staff training requirements.
			c. Standard operating procedures.
			d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance, and rejection criteria established.
			e. Laboratory safety.
			f. Internal performance assessment.
			g. External performance assessment.
C	**8**	**1.1.2**	**QA Plan Implemented.**
K	11	1.1.3	The Laboratory participates in a proficiency testing program annually. Specify Program(s)_____
		1.2 Educational/Experience Requirements	
C	**State's Human Resources Department**	**1.2.1**	**In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department	1.2.2	In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**	**1.2.3**	**In commercial laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, or equivalent discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP	1.2.4	In commercial laboratories, the analyst(s) must have at least a high school diploma and shall have at least three months of experience in laboratory science
		1.3 Work Area	
O	8,11	1.3.1	Adequate for workload and storage.
K	11	1.3.2	Clean, well-lighted.
K	11	1.3.3	Adequate temperature control.
O	11	1.3.4	All work surfaces are nonporous, easily cleaned and disinfected.
K	11	1.3.5	Microbiological quality of the air is fewer than 15 colonies for a 15 minute exposure and determined monthly. The results are recorded and records maintained.
		1.4 Laboratory Equipment	
O	9	1.4.1	To determine the pH of prepared media, the pH meter has a standard accuracy of 0.1 units.
O	14	1.4.2	pH electrodes consisting of pH half-cell and reference half-cell or equivalent combination electrode free from (Ag/AgCl) or contains an ion exchange barrier preventing passage of Ag ions into the medium which may affect the accuracy of the pH reading.
K	11	1.4.3	The effect of temperature on the pH is compensated for by an ATC probe or by manual adjustment.
K	8	1.4.4	pH meter is calibrated daily or with each use Results are recorded and records maintained.
K	11	1.4.5	A minimum of two standard buffer solutions is used to calibrate the pH meter. The first must be near the electrode isopotential point (pH 7). The second near the expected sample pH (i.e., pH 4 or pH 10). Standard buffer solutions are used once and discarded.
O	8,15	1.4.6	Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope. (*Circle the method used.*)

K	9		1.4.7 Balance provides a sensitivity of at least 0.1 g at weights of use.
K	11,13		1.4.8 Balance calibrations are checked monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use. Results are recorded and records maintained.
K	11		1.4.9 Refrigerator temperature(s) are monitored at least once daily on workdays Results are recorded and records maintained.
K	1		1.4.10 Refrigerator temperature is maintained at 0 to 4°C.
C	**9**		**1.4.11 The temperature of the incubator is maintained at 35 ± 0.5°C.**
C	**11**		**1.4.12 Thermometers used in the air incubator(s) are graduated in at least 0.1°C increments.**
K	9		1.4.13 Working thermometers are located on top and bottom shelves or appropriately placed based on the results of spatial temperature checks.
C	**11**		**1.4.14 Temperature of the waterbath is maintained at 44.5 ± 0.2°C under all loading conditions.**
C	**9**		**1.4.15 The thermometers used in the waterbath are graduated in at least 0.1°C increments.**
C	**13**		**1.4.16 The waterbath has adequate capacity for workload.**
K	9		1.4.17 The level of water in the waterbath covers the level of liquid in the incubating tubes.
K	8, 11		1.4.18 Air incubator/waterbath temperatures are taken twice daily on workdays. The results are recorded and records maintained.
C	**4**		**1.4.19 All working thermometers are appropriately immersed.**
C	**29**		**1.4.20 Working thermometers are either: calibrated mercury-in-glass thermometers, calibrated non-mercury-in-glass thermometers, or appropriately calibrated electronic devices, including Resistance Temperature Devises (RTDs) and Platinum Resistance Devices (PTDs).**
C	**11**		**1.4.21 A standards thermometer has been calibrated by NIST or a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at the points 0, 35 and 44.5°C (45.5°C for ETCP). These calibration records are maintained.**
K	9		1.4.22 Standards thermometers are checked annually for accuracy by ice point determination. Results recorded and maintained. Date of most recent determination_____.
C	**29**		**1.4.23 Either mercury-in-glass thermometers, non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury or low drift electronic resistance thermometers with an accuracy of ≤ ±0.05°C are used as the laboratory standards thermometer. (*Circle the thermometer type used.*)**
K	13		1.4.24 The accuracy of working thermometers is checked annually against the standards thermometer either at the temperatures at which they are used or by ice point determination. Results are recorded and records maintained.
O	11		1.4.25 Appropriate pipet aids are available and used to inoculate samples. Mouth pipetting is not permitted.
		1.5 Labware and Glassware Washing	
O	9		1.5.1 Utensils and containers are clean borosilicate glass, stainless steel or other noncorroding materials.
K	9		1.5.2 Culture tubes are of a suitable size to accommodate the volume for nutritive ingredients and samples.
K	9		1.5.3 Sample containers are made of glass or some other inert material.
O	9		1.5.4 Dilution bottles and tubes are made of borosilicate glass or plastic and closed with rubber stoppers, caps or screw caps with nontoxic liners.

K	9		1.5.5 Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method is used to ensure appropriate volumes.
C	**9**		**1.5.6 Pipettes used to inoculate the sample deliver accurate aliquots, have unbroken tips and are appropriately graduated. Pipettes larger than 10 mL are not used to deliver 1mL aliquots; nor, are pipets larger than 1.1mL used to deliver 0.1 mL aliquots.**
K	9		1.5.7 Reusable sample containers are capable of being properly washed and sterilized.
K	9		1.5.8 In washing reusable pipettes, a succession of at least three fresh water rinses plus a final rinse of distilled/deionized water is used to thoroughly rinse off all the detergent.
C	**2**		**1.5.9 An alkaline or acidic detergent is used for washing glassware/labware.**
C	**11**		**1.5.10 With each load of labware/glassware washed the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali) with aqueous 0.04% bromothymol blue. Results are recorded and records maintained.**
		1.6 Sterilization and Decontamination	
K	9		1.6.1 Autoclave(s) are of sufficient size to accommodate the workload.
O	8		1.6.2 Routine autoclave maintenance is performed and the records are maintained.
C	**30, 33, 34**		**1.6.3 The autoclave provides sterilization conditions suitable to the load contents. Sterilization temperature range may be 119ºC - 124ºC as determined by the lab's equipment Quality Assurance Verification Testing and recommended practices from the media manufacturer. Sterilization is determined for each load using a working maximum registering thermometer or an appropriate working temperature monitoring device.**
K	11		1.6.4 An autoclave standards thermometer has been calibrated by a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at 121°C. Calibration at 100°C, the steam point, is also recommended but not required.
K	16		1.6.5 The autoclave standards thermometer is checked every five (5) years for accuracy at 121°C by a qualified calibration laboratory; or, is checked in-house at the steam point (100°C) if it has been previously calibrated at both 100°C and 121°C. Any change in temperature at the steam point changes the calibrated temperature at 121°C by the same magnitude. Date of most recent determination_____
K	1		1.6.6 Working autoclave thermometers are checked against the autoclave standards thermometer at 121°C yearly. Date of last check _____ Method _____
K	11		1.6.7 Spore strips/suspensions appropriate for use in an autoclave media cycle are used monthly according to manufacturer's instructions to evaluate the effectiveness of the sterilization process. Results are recorded and the records maintained.
O	11		1.6.8 Heat sensitive tape is used with each autoclave batch.
K	11, 13		1.6.9 Autoclave sterilization records including length of sterilization, total heat exposure time and chamber temperature are maintained. Type of record: Autoclave log, computer printout or chart recorder tracings. (*Circle appropriate type or types.)*
K	11		1.6.10 For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperatures in the range of 160 to 180°C.
K	9		1.6.11 A thermometer capable of determining temperatures accurately in the range of 160 to 180°C is used to monitor the operation of the hot-air sterilizing oven
K	13		1.6.12 Records of temperatures and exposure times are maintained for the operation of the hot-air sterilizing oven during use.

K	11		1.6.13 Spore strips/suspensions are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. Records are maintained.
K	11		1.6.14 Reusable sample containers are sterilized for 60 minutes at 170°C in a hot-air oven or autoclaved for 15 minutes at 121°C.
C	**1**		**1.6.15 The sterility of reusable sample containers is determined for each load sterilized. The results are recorded and the records maintained.**
C	**1**		**1.6.16 The sterility of pre-sterilized disposable sample containers is determined for each lot received. Results are recorded and the records maintained.**
K	9		1.6.17 Reusable pipettes are stored and sterilized in aluminum or stainless steel canisters.
K	9		1.6.18 Reusable pipettes (in canisters) are sterilized in a hot-air oven at 170°C for 2 hours.
C	**2**		**1.6.19 The sterility of reusable pipettes is determined with each load sterilized. Results are recorded and records maintained.**
C	**2**		**1.6.20 The sterility of pre-sterilized disposable pipettes is determined with each lot received. Results are recorded and the records maintained.**
K	18		1.6.21 Hardwood applicator transfer sticks are properly sterilized. Method of sterilization _____
C	**2**		**1.6.22 The sterility of the hardwood applicator transfer sticks is checked routinely. Results are recorded and the records maintained.**
O	13		1.6.23 Spent broth cultures and agar plates are decontaminated by autoclaving for at least 30 minutes before conventional disposal.
		1.7 Media Preparation	
K	3, 5		1.7.1 Media is commercially dehydrated except in the case of medium A-1 which must be prepared from the individual components and modified MacConkey agar which may be prepared from its components.
K	11		1.7.2 Media is prepared according to manufacturer's instructions.
O	11		1.7.3 Dehydrated media and media components are properly stored in a cool, clean, dry place.
O	11		1.7.4 Dehydrated media are labeled with date of receipt and date opened.
C	**12**		**1.7.5 Caked or expired media or media components are discarded.**
C	**11**		**1.7.6 Reagent water is distilled or deionized (circle appropriate choice), tested monthly and exceeds 0.5 megohm-cm resistance (2 megohms-cm in-line) or is less than 2.0 µSiemens/cm conductivity at 25°C. (C*ircle the appropriate water quality descriptor determined*.) Results are recorded and the records maintained.**
C	**11**		**1.7.7 Reagent water is analyzed for residual chlorine monthly and is at a non-detectable level (< 0.1 mg/L). Results are recorded and the records maintained.** **Specify method of determination_____.**
K	11		1.7.8 Reagent water contains<100 CFU/mL as determined monthly using the heterotrophic plate count method. Results are recorded and the records maintained.
K	11		1.7.9 Media prepared from commercial dehydrated components are sterilized according to the manufacturer's instructions.
K	9		1.7.10 The volume and concentration of media in the tube are suitable for the amount of sample inoculated.
C	**11**		**1.7.11 Total time of exposure of sugar broths to autoclave temperatures does not exceed 45 minutes.**
C	**1**		**1.7.12 Media sterility is determined for each load sterilized. Results are recorded and the records maintained.**

C	**37~~1~~**		**1.7.13 Media productivity is determined using media-appropriate, properly diluted positive and negative control cultures for each lot of dehydrated media received or with each batch of media prepared when the medium is made from its individual components.** **Media: _____ Positive control: _____ Negative control: _____** **Media: _____ Positive control: _____ Negative control: _____** **Media: _____ Positive control: _____ Negative control: _____** **Media: _____ Positive control: _____ Negative control: _____** **Media: _____ Positive control: _____ Negative control: _____** **Media: _____ Positive control: _____ Negative control: _____** **Media: _____ Positive control: _____ Negative control: _____**
O	9		1.7.14 Sterile phosphate buffered dilution water is used as the sample diluent.
K	11		1.7.15 The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer's requirements. Results are recorded and records are maintained.
		1.8 Storage of Prepared Culture Media	
K	9		1.8.1 Prepared culture media are stored in a cool, clean, dry space where excessive evaporation and the danger of contamination are minimized.
K	5,11		1.8.2 Brilliant green bile 2% broth and A-1 media are stored in the dark.
K	13		1.8.3 Stored media are labeled with the storage expiration date or the sterilization date.
K	9		1.8.4 Storage of prepared culture media at room temperature does not exceed 7 days.
K	2		1.8.5 Storage under refrigeration of prepared culture media with loose fitting closures shall not exceed 1 month.
K	11		1.8.6 Storage under refrigeration of prepared culture media with screw-cap closures does not exceed 3 months.
K	17		1.8.7 All prepared MPN broth media stored under refrigeration must reach room temperature prior to use. Culture tubes containing any type of precipitate or Durham tubes containing air bubbles are discarded.
		PART II - SEAWATER SAMPLES	
		2.1 Collection and Transportation of Samples	
C	**11**		**2.1.1 Sample containers are of a suitable size to contain at least 110 mL of sample and to allow adequate headspace for proper shaking. Seawater samples are collected in clean, sterile, watertight, properly labeled sample containers.**
K	1		2.1.2 Samples are identified with collector's name, harvest area, sampling station, time and date of collection.
C	**9**		**2.1.3 Immediately after collection, seawater samples are placed in dry storage (ice chest or equivalent) capable of maintaining a temperature of 0 to 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed in the refrigerator unless processed immediately.**
O	1		2.1.4 A temperature blank is used to represent the temperature of samples upon receipt at the laboratory. Temperature should be equivalent or less than that of the growing area waters. Results are recorded and maintained.
C	**9**		**2.1.5 Analysis of the sample is initiated as soon as possible after collection. Seawater samples are not tested if they have been held for more than 30 hours from the time of collection.**

		2.2 Bacteriological Examination of Seawater by the APHA MPN	
C	**9**		**2.2.1 Lactose broth or lauryl tryptose broth is used as the presumptive medium. *(Circle appropriate one.)***
~~**C**~~	~~**2**~~		~~**2.2.2The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.**~~ ~~**Positive productivity control Negative productivity control**~~
C	**9, 35**		**2.2.3 Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) before inoculation.**
C	**9**		**2.2.4 In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended).**
C	**6**		**2.2.5 In a single dilution series not less than 12 tubes are used (for depuration at least 5 tubes are used).**

C	**6**		**2.2.6 In a single dilution series, the volumes analyzed are adequate to meet the needs of routine monitoring.** **Sample volume inoculated _____** **Range of MPN_____** **Strength of media used_____**
K	9		2.2.7 Inoculated tubes are incubated in air at 35 ± 0.5°C.
C	**2**		**2.2.8 Appropriately diluted process control cultures accompany the samples *throughout both the presumptive and confirmed phases of incubation.* Results are recorded and the records maintained.** **Positive process control _____ Negative process control _____**
K	9		2.2.9 Inoculated tubes are read after 24 ± 2 hours and 48 ± 3 hours of incubation and transferred at both time interval if positive for growth (the presence of turbidity) and gas or effervescence in the culture tube. These tubes are considered presumptive positive requiring further confirmatory testing.
		2.3 Confirmed Test for Seawater by APHA MPN	
C	**9**		**2.3.1 Brilliant green bile 2% broth (BGB) is used as the confirmatory medium for total coliforms.**
C	**9**		**2.3.2 EC medium is used as the confirmatory medium for fecal coliforms.**
~~**C**~~	~~**2**~~		~~**2.3.3The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.**~~ ~~**Positive productivity control Negative productivity control**~~
K	9, 11		2.3.4 Transfers are made to BGB/EC by either sterile loop or sterile hardwood transfer stick from positive presumptive tubes incubated for 24 and 48 hours as appropriate. *(Circle the method of transfer.)*
C	**9**		**2.3.5 BGB tubes are incubated at 35 ± 0.5°C.**
K	9		2.3.6 BGB tubes are read after 48 ± 3 hours of incubation.
C	**9**		**2.3.7 EC tubes are incubated in a circulating waterbath maintained at 44.5 ± 0.2°C.**
C	**9**		**2.3.8 EC tubes are read after 24 ± 2 hours of incubation.**
C	**9**		**2.3.9 The presence of turbidity and any amount of gas or effervescence in the culture tube constitutes a positive test.**
		2.4 Computation of Results – APHA MPN	
K	9		2.4.1 Results of multiple dilution tests are read from tables in *Recommended Procedures for the Examination of Sea Water and Shellfish*, Fourth Edition.
K	7		2.4.2 Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**7, 9**		**2.4.3 Results are reported as MPN/100 mL of sample.**
		2.5 Bacteriological Examination of Seawater by the MA-1 Method	
C	**5**		**2.5.1 A-1 medium complete is used in the analysis.**
C	**2, 31**		**2.5.2 A-1 medium without salicin is used in the analysis. Comparability testing supports use of A-1medium without salicin. Study records are available.**
C	**5**		**2.5.3 A-1 medium sterilized for 10 minutes at 121°C.**
~~**C**~~	~~**2**~~		~~**2.5.4The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.**~~ ~~**Positive productivity control Negative productivity control**~~

C	**9, 35**		**2.5.5 Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) before inoculation.**
C	**9**		**2.5.6 In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended).**
C	**6**		**2.5.7 In a single dilution series at least 12 tubes are used.**
C	**6**		**2.5.8 In a single dilution series, the volumes analyzed are adequate to meet the needs of routine monitoring.** **Sample volume inoculated _____** **Range of MPN_____** **Strength of media used _____**
C	**2**		**2.5.9 Appropriately diluted process control cultures accompany the samples *throughout both resuscitation and waterbath incubation* Results are recorded and the records maintained.** **Positive process control _____ Negative process control _____**
C	**2,5**		**2.5.10 Inoculated tubes are placed in an air incubator at 35 ± 0.5°C for 3 ± 0.5 hours of resuscitation.**
C	**5**		**2.5.11 After 3 ± 0.5 hours resuscitation at 35°C, inoculated tubes are incubated at 44.5 ± 0.2°C in a circulating waterbath for the remainder of the 24 ± 2 hours.**
C	**5**		**2.5.12 The presence of turbidity and any amount of gas or effervescence in the culture tube constitutes a positive test.**
		2.6 Computation of Results – APHA MPN	
K	9		2.6.1 Results of multiple dilution tests are read from tables in *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition.
K	7		2.6.2 Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**7, 9**		**2.6.3 Results are reported as MPN/100 mL of sample.**
		2.7 Bacteriological Analysis of Seawater by Membrane Filtration (MF) using mTEC Agar - Materials and Equipment	
C	**23, 24**		**2.7.1 When used for elevated temperature incubation in conjunction with ethafoam resuscitation, the temperature of the hot air incubator is maintained at 44.5 ± 0.5°C under any loading capacity.**
C	**23**		**2.7.2 When using a waterbath for elevated temperature incubation, the level of the water completely covers the plates.**
C	**23**		**2.7.3 Pre-sterilized plastic or sterile glass culture plates that are clear, flat bottomed, free of bubbles and scratches with tight fitting lids are used.**
C	**2**		**2.7.4 The sterility of pre-sterilized culture plates is determined for each lot received. Results are recorded and the records maintained.**
K	11		2.7.5 Colonies are counted with the aid of magnification.
C	**11, 23**		**2.7.6 Membrane filters are made from cellulose ester material, white, grid marked, 47 mm in diameter with a pore size of 0.45 µm and certified by the manufacturer for fecal coliform analyses.**
C	**2**		**2.7.7 Lot number, date of receipt and if provided the expiration date of the membrane filters are recorded and records maintained.**
C	**2**		**2.7.8 When initiating monitoring by mTEC or switching brands or types of membrane filters used and no previous lots of filters are available for comparing acceptable performance, an appropriate method for determining the suitability of the lot is developed and the comparison testing implemented. The results are recorded and this record is maintained.**

K	2, 11		2.7.9 New lots of membrane filters are checked by comparing recovery of fecal coliform organisms against membrane filters from previously acceptable lots.
C	**2**		**2.7.10 The sterility of each lot or autoclave batch of membrane filters are checked before use.**
K	2		2.7.11 Membrane filters which are beyond their expiration date are not used.
O	11		2.7.12 Forceps tips are clean.
O	11		2.7.13 Forceps tips are smooth without pitting or corrugations to damage the filters being manipulated.
K	11		2.7.14 Forceps are dipped in alcohol and flame sterilized between sample filters.
K	11		2.7.15 If indelible graduation marks are used on clear glass or plastic funnels to measure sample volumes, their accuracy is checked gravimetrically or with a Class A graduated cylinder before use and periodically rechecked. Funnels having a tolerance greater than 2.5% are not used. Checks are recorded and records maintained.
K	11		2.7.16 Membrane filtration units are made of stainless steel, glass or autoclavable plastic free of scratches, corrosion and leaks.
C	**11**		**2.7.17 Membrane filter assemblies are autoclave sterilized for 15 minutes at 121°C prior to the start of a filtration series.**
O	11, 23, 26		2.7.18 A UV sterilization unit is used to disinfect filter assemblies between sample and filtration runs.
K	11		2.7.19 The effectiveness of the UV sterilization unit is determined by biological testing monthly. Results are recorded and records maintained.
K	2		2.7.20 Maintenance of the UV sterilization unit is performed as needed. This maintenance is documented and the records maintained.
		2.8 Media Preparation and Storage – MF using mTEC Agar	
K	11		2.8.1 Phosphate buffered saline is used as the sample diluent and filter funnel rinse.
C	**11**		**2.8.2 The phosphate buffered saline is properly sterilized.**
K	23		2.8.3 A sufficient amount of medium (4-5 mL) is used in each plate.
O	11		2.8.4 Refrigerated prepared plates are stored for no more than 2 weeks in sealed plastic bags or containers to minimize evaporation.
		2.9 Sample Analyses - MF using mTEC Agar	
C	**24**		**2.9.1 mTEC agar is used.**
~~C~~	~~2~~		~~2.9.2The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.~~ ~~Positive productivity control Negative productivity control~~
C	**23, 35**		**2.9.3 The sample is shaken vigorously (25 times in a 12″ arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) before filtration.**
C	**23**		**2.9.4 The membrane is placed grid side up within the sterile filter apparatus.**
C	**23, 25**		**2.9.5 Sample volumes tested are consistent with the sampling regime employed (i.e., half log or other appropriate dilutions are used with systematic random sampling).**
C	**23**		**2.9.6 Sample volumes are filtered under vacuum.**
K	26		2.9.7 The pressure of the vacuum pump does not exceed 15 psi.
C	**23, 26**		**2.9.8 The sides of the filter funnel are rinsed at least twice with 20-30 mL of sterile phosphate buffered saline after sample filtration.**
C	**23**		**2.9.9 The membrane filter is removed from the filtering apparatus with sterile forceps and rolled onto mTEC agar so that no bubbles form between the filter and the agar.**
C	**11**		**2.9.10 Blanks are run at the beginning of filtration, after every 10th aliquot and at the end of the filtration run to check the sterility of the testing system (phosphate buffered saline, filter funnel, forceps, membrane filter, media and culture plate).**

C	**2, 11**		**2.9.11 Appropriately diluted process control cultures accompany the samples throughout both resuscitation and elevated temperature incubation. Results are recorded and the records maintained.** **Positive process control _____ Negative process control _____**
C	**11, 23, 24**		**2.9.12 Inoculated plates are placed inverted into a watertight, tightly sealed container prior to being placed in the air incubator and incubated at 35 + 0.5°C for 2 hours of resuscitation. Alternatively inoculated plates may be placed in ethafoam prior to air incubation at 44.5 ± 0.5°C for 24 ± 2 hours.**
C	**11, 23, 24**		**2.9.13 After 2 hours of resuscitation at 35°C, the watertight, tightly sealed containers are transferred to a circulating waterbath at 44.5 + 0.2°C, submerged completely and incubated for 22-24 hours.**
		2.10 Computation of Results - MF using mTEC Agar	
C	**23**		**2.10.1 All yellow, yellow-green or yellow-brown colonies are counted.**
C	**23**		**2.10.2 Only plates having 80 or fewer colonies are counted. If it is unavoidable to use plates having more than 80 colonies, counts are given as >80 x 100/the volume of sample filtered.**
C	**2, 11, 23**		**2.10.3 When multiple dilutions are filtered, the laboratory has developed a procedure for assessing the contribution of all positive dilutions to the final count.**
C	**23, 11**		**2.10.4 The number of fecal coliforms is calculated by the following equation:** **Number of fecal coliforms per 100 mL = [number of colonies counted per plate used in the count / volume (s) of sample filtered in ml] x 100.**
C	**23, 11**		**2.10.5 Results are reported as CFU/100 mL of sample.**
		2.11 Bacteriological Analysis of UV Treated Process Water Samples by Membrane Filtration (MF) using mEndo Agar LES – Materials and Equipment	
C	**9, 11, 21**		**2.11.1 Pre-sterilized plastic or sterile glass culture plates that are clear, flat bottomed, free of bubbles and scratches are used.**
C	**2**		**2.11.2 The sterility of pre-sterilized culture plates is determined for each lot received. Results are recorded and the records maintained.**
C	**11, 19, 21**		**2.11.3 Membrane filters are made from cellulose ester material, white, grid marked, 47 mm diameter with a pore size of 0.45µm and certified by the manufacturer for total coliform analysis.**
C	**2**		**2.11.4 Membrane filter lot numbers, dates of receipt and expiration dates are recorded and the records maintained.**
C	**2**		**2.11.5 If previous lots of agar or membrane filters are not available for comparability testing, an appropriate method for determining lot suitability has been developed and comparison testing implemented when the following has occurred:** **a. initiating monitoring by mEndo Agar LES;** **b. changing agar manufacturers; or** **c. changing brands of membrane filters used.** **The results are recorded and the records are maintained.**
K	2, 11		2.11.6 Recovery of total coliforms from new lots of membrane filters and mEndo Agar LES is compared against the recovery from the previously acceptable lot.
C	**2**		**2.11.7 The sterility of each lot and autoclave batch of membrane filters is verified before use.**
K	2		2.11.8 Expired membrane filters are not used.
K	9, 11, 19, 21		2.11.9 Membrane filtration units are made of stainless steel, glass or autoclavable plastic free of scratches, corrosion and leaks.

K	11		2.11.10 If indelible graduation marks are used on clear glass or plastic funnels to measure sample volumes, their accuracy is checked gravimetrically with a Class A graduated cylinder before use and periodically rechecked. Funnels having a tolerance greater than 2.5% are not used. Checks are recorded and records maintained.
C	**9, 11**		**2.11.11 Membrane filter assemblies are autoclave sterilized for 15 minutes at 121+/-2°C prior to the start of a filtration run. A new run occurs when there is a break of 30 minutes or more between the previous filtration run.**
O	11, 19, 26, 36		2.11.12 A UV sterilization unit is used to disinfect filter assemblies between sample and filtration runs.
K	11		2.11.13 The effectiveness of the UV sterilization unit is determined by biological testing monthly. Results are recorded and the records are maintained.
K	2		2.11.14 Maintenance of the UV sterilization unit is performed as needed. Maintenance is documented and the records maintained.
O	9, 11		2.11.15 Forceps tips are clean and smooth without pitting or corrugations.
		2.12 Media Preparation and Storage	
C	**9, 11, 19, 21, 36**		**2.12.1 mEndo Agar LES is used.**
K	11, 21, 36		2.12.2 mEndo Agar LES is prepared aseptically using pre-sterilized glassware, sterile reagent water and pre-sterilized stir bar.
K	9, 11, 36		2.12.3 mEndo Agar LES is prepared using 95% alcohol that is not denatured.
C	**9, 11, 36**		**2.12.4 mEndo Agar LES is brought to near boiling; removed from the heat and tempered at 44-50°C before dispensing.**
C	**9, 11, 36**		**2.12.5 mEndo Agar LES is never autoclaved.**
K	9, 11, 36		2.12.6 A sufficient amount of medium (4-5 mL) is aseptically aliquoted to each culture plate.
O	9, 11, 36		2.12.7 Prepared plates of mEndo Agar LES are stored at 4°C in the dark for no more than two (2) weeks in sealed plastic bags or containers to minimize evaporation.
~~**C**~~	~~**2**~~		~~**2.12.8 Appropriate, properly diluted positive and negative productivity controls for mEndo Agar LES medium are used. Results are recorded and the record maintained.**~~ ~~**Positive productivity control Negative**~~ ~~**productivity control**~~
K	9, 11, 21, 36		2.12.9 Sterile phosphate buffered water or sterile phosphate buffered saline is used as a sample blank, filter funnel rinse and process and productivity control diluent for UV treated process water samples.
C	**11**		**2.12.10 The phosphate buffered water/saline is properly sterilized and the sterility is tested before being placed in service. Results are recorded and records maintained**
		2.13 Sample Analysis	
C	**9, 11, 36**		**2.13.1 The sample is shaken vigorously (25 times in a 12-inch arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) before filtration.**
C	**11, 21, 36**		**2.13.2 The membrane filter is placed grid side up within the sterile filter apparatus**.
C	**11, 26, 36**		**2.13.3 A 100 mL quantity of sample is filtered under vacuum.**
K	26		2.13.4 The pressure of the vacuum pump does not exceed 15 psi.
C	**9, 11, 26, 36**		**2.13.5 The sides of the filter funnel are rinsed at least twice with 20-30 mL of sterile phosphate buffered water/saline as appropriate after filtration.**

C	9, 11, 36		2.13.6	The membrane filter is removed from the filtering apparatus with sterile forceps and rolled onto mEndo Agar LES so that no bubbles form between the filter and the agar.

K	9, 11, 36		2.13.7 Forceps are dipped in alcohol and flame sterilized between sample filters.
C	**11, 36**		**2.13.8 Blanks are run at the beginning and at the end of the filtration run to check the sterility of the testing system (phosphate buffered water/saline, filter funnels, forceps, membrane filters, media and culture plates).**
C	**2, 36**		**2.13.9 An appropriate properly diluted positive process control culture accompanies the sample throughout incubation. Results are recorded and the records are maintained.** **Positive process control _____**
C	**9, 11, 36**		**2.13.10 Inoculated plates are incubated inverted at 35+/- 0.5°C for 22 to 24 hours.**
K	2, 9, 11		2.13.11 An appropriate level of humidity is maintained in the incubator to prevent the plates from drying out.
		2.14 Computation of Results	
K	9, 11		2.14.1 Colonies are counted with the aid of magnification.
C	**9, 11, 19, 21, 23**		**2.14.2 All metallic sheen colonies are counted as total coliforms.**
C	**9, 11, 21, 36**		**2.14.3 Results are reported as total coliforms/100mL.**
C	**11, 20, 36**		**2.14.4 When no colonies are observed, results are reported as <1.0 coliform/100mL (nondetectable)**
PART III - SHELLFISH SAMPLES			
		3.1 Collection and Transportation of Samples	
C	**9**		**3.1.1 A representative sample of shellstock is collected.**
K	9		3.1.2 Shellstock samples are collected in clean, waterproof, puncture resistant containers loosely sealed.
K	9		3.1.3 Shellstock samples are labeled with collector's name, type of shellstock, the source or harvest area, sampling station, time, date and place (if applicable) of collection.
C	**9**		**3.1.4 Immediately after collection, shellfish samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed under refrigeration unless processed immediately.**
C	**1**		**3.1.5 Analysis of the samples is initiated as soon as possible after collection. Shellfish samples are not tested if the time interval between collection and analysis exceeds 24 hours.**
		3.2 Preparation of Shellfish for Examination	
K	2,11		3.2.1 Shucking knives, scrub brushes and blender jars are (autoclave) sterilized for 15 minutes prior to use.
O	2		3.2.2 Blades of shucking knives are not corroded.
O	9		3.2.3 The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2		3.2.4 The faucet used for rinsing the shellstock does not contain an aerator.
K	9		3.2.5 Shellstock are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9		3.2.6 Shellstock are allowed to drain in a clean container or on clean towels prior to opening.
K	1, 9		3.2.7 Immediately prior to shucking, the hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol or clean gloves are donned.
C	**9**		**3.2.8 Shellstock are not shucked directly through the hinge.**
C	**9**		**3.2.9 Contents of shellstock (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	9		3.2.10 At least 200 grams of shellfish meat or a quantity of meat sufficient to cover the blender blades is used for the analysis.

K	9		3.2.11 A representative sample of at least 12 shellfish is used for the analysis.
K	2		3.2.12 The sample is weighed to the nearest 0.1 gram and an equal amount by weight of diluent is added.
O	9		3.2.13 Sterile phosphate buffered dilution water is used as the sample diluent.
C	**9**		**3.2.14 Samples are blended at high speed for 60 to 120 seconds until homogenous.**
K	9		3.2.15 APHA *Recommended Procedures for the Examination of Sea Water And Shellfish*, Fourth Edition is followed for the analysis of previously shucked and frozen shellfish meats.
		3.3 MPN Analysis for Fecal Coliform Organisms, Presumptive Test, APHA	
C	**9**		**3.3.1 Appropriate strength lactose or lauryl tryptose broth is used as presumptive media in the analysis.** ***(Circle the medium used.)***
~~**C**~~	~~**2**~~		~~**3.3.2 The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.**~~ ~~**Positive productivity control Negative productivity control**~~
K	9		3.3.3 Immediately (within 2 minutes) after blending, the ground sample is diluted and inoculated into tubes of presumptive media.
C	**9**		**3.3.4 No fewer than 5 tubes per dilution are used in a multiple dilution MPN series.**
C	**9**		**3.3.5 Allowing for the initial 1:1 dilution of the sample, appropriate portions are inoculated (i.e., 2 ml of original 1:1 dilution for the 1 g portion) and diluted for subsequent inoculation (i.e., 20 ml of 1:1 diluted sample to 80 ml of diluent or the equivalent for 0.1 g portion). All successive dilutions are prepared conventionally.**
K	6		3.3.6 In a single dilution series, the volumes examined are adequate to meet the needs of routine monitoring. Sample volume inoculated _____ Range of MPN_____ Strength of media used_____
C	**2**		**3.3.7 Appropriately diluted process control cultures accompany the samples throughout both the presumptive and confirmed phases of incubation. Results are recorded and the records maintained.** **Positive Process control _____ Negative Process control_____**
K	9		3.3.8 Inoculated media are incubated at 35 ± 0.5°C.
K	10		3.3.9 Tubes are read after 24 ± 2 hours of incubation and transferred if positive for growth (the presence of turbidity and gas or effervescence in the culture tube). These tubes are considered presumptive requiring further confirmatory testing.
		3.4 Confirmed Test for Fecal Coliforms - APHA	
C	**9**		**3.4.1 EC medium is used as the confirmatory medium.**
~~**C**~~	~~**2**~~		~~**3.4.2 The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.**~~ ~~**Positive productivity control Negative productivity control**~~
K	9, 11		3.4.3 Transfers are made to EC medium by either sterile loop or hardwood sterile transfer sticks from positive presumptives. *(Circle the method of transfer.)*
C	**9**		**3.4.4 EC tubes are incubated in a circulating waterbath at 44.5 ± 0.2°C**
K	9		3.4.5 EC tubes are read for gas production after 24 ± 2 hours of incubation.
C	**9**		**3.4.6 The presence of turbidity and any amount of gas and/or effervescence in the Durham tube constitutes a positive test.**
		3.5 Computation of Results for MPN Analyses	
K	9		3.5.1 Results of multiple dilution tests are read from tables in *Recommended Procedure for the Examination of Sea Water and Shellfish,* 4th Edition and multiplied by the appropriate dilution factor.

K	7		3.5.2 Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**9**		**3.5.3 Results are reported as MPN/100 grams of sample.**
		3.6 Standard Plate Count Method	
O	20		3.6.1 A standard plate count (SPC) analysis maybe performed in conjunction with the analysis for fecal coliform organisms.
K	9		3.6.2 In the standard plate count procedure at least four plates are used, duplicates of two dilutions. One of the dilutions should produce colonies of 30 to 300 per plate.
K	2		3.6.3 Fifteen to 20 mL of tempered sterile plate count agar is used per plate.
C	**9**		**3.6.4 Agar tempering bath maintains the agar at 44-46°C.**
C	**9**		**3.6.5 An agar based temperature control having a similar volume and shape as the tempering plate count agar is used in the tempering bath.**
K	9		3.6.6 Samples or sample dilutions to be plated are shaken vigorously (25 times in a 12" arc in 7 seconds) before plating.
C	**9**		**3.6.7 Not more than 1 mL nor less than 0.1 mL of sample or sample dilution is plated.**
K	11		3.6.8 Control plates are used to check air quality and the sterility of the agar and the diluent.
K	9,21		3.6.9 Solidified plates are incubated at 35 ± 0.5°C for 48 ± 3 hours inverted and stacked no more than four high.
K	9		3.6.10 Quebec Colony Counter or its equivalent is used to provide the necessary magnification and visibility for counting plates.
K	1		3.6.11 A hand tally or its equivalent is used for accuracy in counting.
		3.7 Computation of Results -SPC	
K	9		3.7.1 Colony counts determined in accordance with Part III, A, Sections 4.31 through 4.33 in *Recommended Procedures for the Examination of Sea Water and Shellfish*, Fourth Edition.
C	**19**		**3.7.2 Colony counts are reported as CFU/g of sample.**
		3.8 Bacteriological Analysis of Shellfish Using the ETCP	
C	**2,3**		**3.8.1 Prepared modified MacConkey agar is used on the day that it is made.**
K	3		3.8.2 Double strength modified MacConkey agar is used.
C	**3**		**3.8.3 Prepared double strength modified MacConkey agar is heated to boiling, removed from the heat, and boiled again. This agar is never autoclaved.**
K	2, 3		3.8.4 Twice boiled, double strength modified MacConkey agar and is maintained in a tempering bath at 45 to 50°C until used.
K	2, 3		3.8.5 Phosphate buffered saline is used as the sample diluent in the ETCP.
C	**2, 3**		**3.8.6 The phosphate buffered saline is tempered at 45 - 50°C to prevent premature solidification of the agar.**
C	**9**		**3.8.7 The sample homogenate is cultured within 2 minutes of blending.**
C	**2,3**		**3.8.8 Six grams of shellfish (12 grams of homogenate if initially diluted 1:1) is placed into a sterile container and the contents brought up to 60 mL with sterile, tempered phosphate buffered saline.**
K	3		3.8.9 Sixty (60) mL of tempered, twice boiled double strength Modified MacConkey Agar is added.
K	2,3, 22		3.8.10 The container is gently swirled or slowly inverted once to mix the contents, which are subsequently distributed uniformly over six plates.
C	**1**		**3.8.11 Media and diluent sterility are determined with each use. Results are recorded and the records maintained.**

~~**C**~~	~~**1**~~		~~**3.8.12 Media productivity is determined using media appropriate properly diluted pour plated positive and negative control cultures for each batch of Modified MacConkey agar prepared.**~~ ~~**Positive control culture Negative control culture**~~
C	**3, 13**		**3.8.13 When solidified, the plates are placed inverted into an air incubator at 45.5 ± 0.5°C for 18 to 30 hours of incubation.**
C	**2**		**3.8.14 Plates are stacked no more than three high in the incubator.**
C	**2**		**3.8.15 Appropriately diluted pour plated process control cultures *accompany each set of samples throughout incubation.* The results are recorded and the records maintained.** **Positive process control_____Negative process control_____**
		3.9 Computation of Results - ETCP	
K	11		3.9.1 Quebec Colony counter or its equivalent is used to provide the necessary magnification and visibility for counting.
O	1		3.9.2 A hand tally or its equivalent is used to aid in counting.
C	**3, 6**		**3.9.3 All brick red colonies greater than 0.5 mm in diameter are totaled over all the plates and multiplied by a factor of 16.7.**
C	**3**		**3.9.4 Results are reported as CFU/100 grams of sample.**
		Bacteriological Examination of Soft-shelled Clams and American Oysters for Male Specific Coliphage (MSC)	
		3.10 MSC Equipment and Supplies	
K	30		3.10.1 Sample containers used for the shucked sample are sterile, made of glass or some other inert material (i.e. polypropylene) and hold 100 – 125 mL.
C	**27, 28**		**3.10.2 The refrigerated centrifuge used must have the capacity to accommodate the amount of shellfish sample required for the procedure, perform at 9000 x g and maintain a temperature of 4°C.**
K	9		3.10.3 The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
C	**27, 28**		**3.10.4 Sterile 0.22µm pore size syringe filters and pre-sterilized plastic or sterile glass syringes are used to sterilize the antibiotic solutions.**
K	1		3.10.5 The sterility of each lot of pre-sterilized syringes and syringe filters is determined. Results are recorded and records maintained.
K	1		3.10.6 The sterility of each batch of reusable glass syringes is determined. Results are recorded and records maintained.
C	**27, 28**		**3.10.7 The balance used provides a sensitivity of at least mg (0.01g.).**
C	**27, 28**		**3.10.8 The temperature of the incubator used is maintained at 36 ± 1°C.**
C	**28**		**3.10.9 Sterile disposable 50 mL centrifuge tubes are used and their sterility is determined with each lot. Results are recorded and records maintained.**
		3.11 MSC Media Preparation	
K	28		3.11.1 Media preparation and sterilization is according to the validated method.
K	27, 28		3.11.2 Bottom agar, double strength soft agar and growth broth are prepared from their individual components.
K	27, 28		3.11.3 Soft agar is prepared double strength in volumes of 2.5 mL.
C	**27, 28**		**3.11.4 The streptomycin and ampicillin solutions are added to tempered bottom agar and vortex for 2 minutes on stir plate.**
O	27, 28		3.11.5 Storage of the bottom agar under refrigeration does not exceed 1 month.
K	27, 28		3.11.6 Unsterilized soft agar is stored at -20 °C -15C for up to 3 months.
K	27, 28		3.11.7 The soft agar is removed from the freezer and sterilized for 15 minutes at 121°C before use.
K	27, 28		3.11.8 Storage of growth broth in the refrigerator in loosely capped tubes/bottles does not exceed 1 month and in screw capped tubes/bottles does not exceed 3 months.
K	27, 28		3.11.9 Bottom agar plates are allowed to reach room temperature before use.

		3.12 Preparation of the Soft-Shelled Clams and American Oysters for MSC Analysis	
K	2,11		3.12.1 Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2		3.12.2 The blades of shucking knives are not corroded.
O	9		3.12.3 The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2		3.12.4 The faucet used for rinsing the shellfish does not contain an aerator.
K	9		3.12.5 The shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9		3.12.6 The shellfish are allowed to drain in a clean container or on clean towels unlayered prior to shucking.
K	9		3.12.7 Immediately prior to shucking, the hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol or clean gloves are donned.
C	**9**		**3.12.8 Shellfish are not shucked through the hinge.**
C	**9**		**3.12.9 The contents of shellfish (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	9		3.12.10 A representative sample of at least 12 shellfish is used for the analysis.
K	2, 19		3.12.11 The sample is weighed to the nearest 0.1 gram.
		3.13 MSC Sample Analysis	
C	**28**		**3.13.1 E. coli *Famp* ATCC 700891 is the bacterial host strain used in this procedure.**
K	27, 28		3.13.2 Host cell growth broth is tempered at 36 ± 1°C and vortexed (or shaken) to aerate prior to inoculation with host cells.
K	27, 28		3.13.3 Several host cell colonies are transferred to a tube of tempered, aerated growth broth and incubated at 36 ± 1°C for 4-6 hours to provide host cells in log phase growth for sample analysis.
C	**27, 28**		**3.13.4 After inoculation, the host cell growth broth culture is not shaken.**
C	**28**		**3.13.5 A 2:1 mixture of sterile growth broth to shellfish tissue is used for eluting the MSC.**
C	**28**		**3.13.6 The elution mixture is prepared w/v by weighing the sample and adding two equal portions of sterile growth broth by volume to the shellfish tissue.**
C	**28**		**3.13.7 The elution mixture is homogenized at high speed for 180 seconds.**
C	**28**		**3.13.8 Immediately after blending, 33 grams of the homogenized elution mixture are weighed into centrifuge tubes.**
C	**28**		**3.13.9 The homogenized elution mixture is centrifuged for 15 minutes at 9000 x g at 4°C.**
C	**27, 28**		**3.13.10 The supernatant is pipetted off, weighed and the weight recorded.**
C	**27, 28**		**3.13.11 The supernatant is allowed to warm to room temperature about 20 to 30 minutes.**
K	27, 28		3.13.12 The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28		3.13.13 Two hundred microliters (0.2 mL) of log phase host strain *E coli* is added to the tempering soft agar immediately prior to adding the sample supernatant.
K	27, 28		3.13.14 The sample supernatant is shaken or vortexed before being added to the tempering soft agar.
C	**27, 28**		**3.13.15 2.5 mL of sample supernatant is added to each tube of tempering soft agar.**
C	**27, 28**		**3.13.16 The soft agar/sample supernatant/host cell mixture is gently rolled between the palms of the hands to mix.**
C	**27, 28**		**3.13.17 The soft agar/sample supernatant/host cell mixture is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**
C	**28**		**3.13.18 Ten (10) plates are used, 2.5 mL per plate for a total of 25 mL of supernatant analyzed per sample.**

K	27, 28		3.13.19 Negative and positive control plates are prepared and accompany each set of samples analyzed. The results are recorded and records maintained. Positive control__
K	27, 28		3.13.20 Growth broth is used as the negative control or blank.
K	27, 28		3.13.21 Type strain MS2 (ATCC 15597) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.
K	2		3.13.22 A negative control plate is plated at the beginning and end of each set of samples analyzed.
K	27, 28		3.13.23 The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	**27, 28**		**3.13.24 All plates are incubated at 36 ± 1°C for 18 ± 2 hours.**
		3.14 Computation of Results - MSC	
C	**27**		**3.14.1 Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted.**
C	**28, 32**		**3.14.2 The working range of the method is 1 to 200 PFU per plate. When there are no plaques on all ten plates, the count is <6 PFU/100 grams for soft-shelled clams, <7 PFU/ 100 grams for American oysters, and <5 PFU/ 100 grams for quahog (hard) clams. If the density exceeds 200 PFU per plate on all plates, the count is given as > 20,000 PFU/100 grams.**
K	28		3.14.3 The formula used for determining the density of MSC in PFU/100 grams is: (0.364) (N) (Ws), where N = total number of plaques counted on all 10 plates and Ws = weight of the supernatant used.
O	9		3.14.4 The MSC count is rounded off conventionally to give a whole number.

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Proposal No. _____
Date Received: _____

Proposal for Task Force Consideration at the ISSC 2022 Biennial Meeting *(Tab to go to next field)*	1. a. ☐ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	ISSC Laboratory Committee
3. Affiliation	
4. Address Line 1	
5. Address Line 2	
6. City, State, Zip	
7. Phone	
8. Fax	
9. Email	
10. Proposal Subject	Guidance for Laboratory Method Matrix Extensions
11. Specific NSSP Guide Reference	PROCEDURE XV. PROCEDURE FOR THE APPROVAL OF ANALYTICAL METHODS FOR THE NSSP and Section IV Guidance Documents – Chapter II. Growing Areas
12. Text of Proposal/ Requested Action	PROCEDURE XV. PROCEDURE FOR THE APPROVAL OF ANALYTICAL METHODS FOR THE NSSP 10. ~~For methods already adopted into the NSSP, consideration of expanding a method to a new molluscan shellfish species is accomplished using the "ISSC Method Application Format for Biotoxin Methods Matrix Extension" and the "ISSC Method Application Format for Microbiology Methods Matrix Extension." The simplified, reduced approach to method validation for expanding an NSSP method to new molluscan shellfish species is visually represented in the "Matrix Extension Guidelines" schematic.~~ For methods already adopted into the NSSP, additional work must be done in order to expand the use of that method to a new molluscan shellfish matrix. To determine if a Matrix Extension is needed, please refer to the guidance provided in the NSSP Guide for the Control of Molluscan Shellfish, Section IV. Guidance Documents, Chapter II. Growing Areas .21 - Guidance for Laboratory Method Matrix Extensions. If a matrix extension is needed, the necessary information, studies, and data to be provided to the Laboratory Committee for consideration are summarized on the "ISSC Method Application Format for Biotoxin Methods Matrix Extension" and the "ISSC Method Application Format for Microbiology Methods Matrix Extension" documents available on the Laboratory tab of the ISSC website. This simplified, reduced approach to method validation for expanding an NSSP method to a new molluscan shellfish matrix is visually represented in the "Matrix Extension Guidelines" schematic, also available on the ISSC website. Section IV Guidance Documents – Chapter II. Growing Areas .20 Quantitative Analytical Method Verification

	This guidance is provided to aid laboratories verifying the performance of an NSSP Approved Method or Approved Limited Use Method of analysis being transferred from the originating laboratory/submitter to the implementing laboratory before being placed in service by the implementing laboratory. When a laboratory implements an NSSP method for the first time, the method performance must be verified in that laboratory. In addition, when a laboratory expands an existing method to a new shellfish matrix, method performance may need to be verified. Guidance outlined in .21 should be followed to determine if the new shellfish matrix is in the same matrix category as matrices previously implemented in the laboratory. If so, the method does not need to be verified. However, if the new shellfish matrix is in a different matrix category, then the method performance must be verified. The following performance criteria are to be verified: recovery, measurement uncertainty, precision (repeatability and intermediate precision), linear range, limit of detection (LOD), limit of quantitation (LOQ), and comparability. Section IV Guidance Documents – Chapter II. Growing Areas (new section .21) .21 Laboratory Method Matrix Extensions Validating Use of an Analytical Method With A New Shellfish Matrix Analytical methods employed in the National Shellfish Sanitation Program (NSSP) are validated for their intended use before being adopted. Since differing characteristics of various molluscan shellfish matrices may impact the performance of certain methods, each validation is specific only to the shellfish species or matrices that were included in the validation studies. In order to expand the use of any method already adopted into the NSSP for use with other molluscan shellfish matrices, additional validation studies need to be done. Based on proximate composition data (i.e. the amount of protein, fat, and carbohydrates in each species), as well as a review of existing empirical data where methods have been tested using multiple species, the Matrix Category Table below was developed to help determine if a Matrix Extension study is needed. If a new shellfish species of interest is in the same matrix category (i.e. vertical column of the table) as an already validated species, then the method should not require further validation. For example, if a method has already been validated for use with the Eastern Oyster (*Crassostrea virginica*), and the new species of interest is the Pacific Oyster (*Crassostrea gigas*), then a matrix extension study is not necessary. If a new species of interest is in a different matrix category from all previously validated species, then a Matrix Extension validation study should be conducted and data submitted to the ISSC for review following the process outlined in the ISSC Constitution, Bylaws, and Procedures, Procedure XV (10.). For example, if a method has already been validated for use with the Eastern Oyster (*Crassostrea virginica*) and the Soft Shell Clam (*Mya*

arenaria), and the new species of interest is the Atlantic Surf Clam (*Spisula solidissima*), then a matrix extension study is needed.

If the new species of interest is not found in the Matrix Category Table, a request to add the new species should be submitted to the ISSC Executive Office. The following information should be included in the request: common and scientific name of species, rationale for inclusion, and any available data for categorization (e.g, proximate composition, empirical data on use).

Regardless of the categorization of the species of interest, certain analytical methods require more species-specific data. The results of these studies will supersede the groupings described in the table below if significant matrix effects are identified.

1. For methods utilizing liquid chromatography, analyses shall be conducted to ensure sufficient separation of target analyte from sample matrix peaks through analysis of peak resolution utilizing retention times (e.g., AOAC[1]). Chromatograms supporting the analyses with labels noting peaks of interest as well as matrix peaks shall accompany the data package.

2. For methods utilizing mass spectrometry, comparison of neat and matrix-fortified standards shall be conducted to assess matrix effects on ionization.

1	2	3	4	5	6	7	8
Oysters	***Hard Clams***	***Non-US Hard Clams***	***Geoducks****	***Soft Clams***	***Mussels***	***Estuarine Mussels (non-***	***Scallops*** **
Eastern Oyster (*Crassostrea virginica*)	Atlantic Surfclam (*Spisula solidissima*)	Wedge Shell Clam (*Donax cuneatus*)	Pacific Geoduck Clam (*Panopea generosa*; formerly *P. abrupta*)	Softshell Clam (*Mya arenaria*)	Blue Mussel (*Mytilus edulis*)	Asian Green Mussel (*Perna viridis*)	Sea Scallop (*Placopecten magellanicus*)
Edible Oyster (*Ostrea edulis*)	Ocean Quahog (*Arctica islandica*)	Asiatic Hard Clam (*Meretrix meretrix*)	Atlantic Geoduck Clam (*Panopea bitruncata*)		Mediterranean Mussel (*Mytilus galloprovincialis*)		Rock Scallop (*Crassodoma gigantea*)
Olympia Oyster (*Ostrea lurida*)	Northern Quahog (*Mercenaria mercenaria*)				California Mussel (*Mytilus californianus*)		Bay Scallop (*Argopecten irradians*)
Pacific Oyster (*Crassostrea gigas*)	Southern Quahog (*Mercenaria campechiensis*)				Chilean Mussel (*Mytilus chelensis*)		Peruvian Scallop (*Argopecten purpuratus*)
	Northern Razor Clam (*Siliqua patula*)				Korean Mussel (*Mytilus coruscus*)		
	Pacific Littleneck Clam (*Protothaca staminea*)						
	Butter Clam (*Saxidomus gigantea*)						
	Sunray Venus Clam (*Macrocallista nimbosa*)						
	Japanese Littleneck Clam (*Venerupis philippinarum*)						

*Geoducks are generally analyzed as whole animals for microbiological methods and gutballs only for biotoxin methods. If a different form of the animal is to be processed (i.e., gutball for micro method or whole animal for biotoxin method), it should be considered a separate matrix.

**Scallops can be analyzed as whole animal or muscle excluded. These different forms of the animal should be considered a separate matrix. Methods for muscle only will not be considered as the product is not within the NSSP.

1 Association of Official Analytical Chemists. "AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals". Arlington, VA. 2002.

13. Public Health Significance	To ensure accurate reporting of analytical results within the NSSP, methods must be demonstrated to be fit-for-purpose. The program has recognized the potential interference from different shellfish types. This proposal is intended to provide additional detail on the conditions under which a matrix extension validation study is needed compared to when a method verification study is required.
14. Cost Information	Dependent upon the level of validation/verification needed.
15. Research Needs Information (Optional)	
a. Proposed specific	

research need/ problem to be addressed	
b. Explain the relationship between proposed research need and program change recommended in the proposal	
c. Estimated cost	
d. Proposed sources of funding	
e. Time frame anticipated	
For Research Guidance Committee Use Only	Relative priority rank in terms of resolving research need ☐ Immediate ☐ Required ☐ Valuable ☐ Important ☐ Other

1	2	3	4	5	6	7	8
Oysters	***Hard Clams***	***Non-US Hard Clams***	***Geoducks****	***Soft Clams***	***Mussels***	***Estuarine Mussels (non-Mytilus)***	***Scallops*** **
Eastern Oyster (*Crassostrea virginica*) Edible Oyster (*Ostrea edulis*) Olympia Oyster (*Ostrea lurida*) Pacific Oyster (*Crassostrea gigas*)	Atlantic Surfclam (*Spisula solidissima*) Ocean Quahog (*Arctica islandica*) Northern Quahog (*Mercenaria mercenaria*) Southern Quahog (*Mercenaria campechiensis*) Northern Razor Clam (*Siliqua patula*) Pacific Littleneck Clam (*Protothaca staminea*) Butter Clam (*Saxidomus gigantea*) Sunray Venus Clam (*Macrocallista nimbosa*) Japanese Littleneck Clam (*Venerupis philippinarum*)	Wedge Shell Clam (*Donax cuneatus*) Asiatic Hard Clam (*Meretrix meretrix*)	Pacific Geoduck Clam (*Panopea generosa* ; formerly *P. abrupta*) Atlantic Geoduck Clam (*Panopea bitruncata*)	Softshell Clam (*Mya arenaria*)	Blue Mussel (*Mytilus edulis*) Mediterranean Mussel (*Mytilus galloprovincialis*) California Mussel (*Mytilus californianus*) Chilean Mussel (*Mytilus chelensis*) Korean Mussel (*Mytilus coruscus*)	Asian Green Mussel (*Perna viridis*)	Sea Scallop (*Placopecten magellanicus*) Rock Scallop (*Crassodoma gigantea*) Bay Scallop (*Argopecten irradians*) Peruvian Scallop (*Argopecten purpuratus*)

*Geoducks are generally analyzed as whole animals for microbiological methods and gutballs only for biotoxin methods. If a different form of the animal is to be processed (i.e., gutball for micro method or whole animal for biotoxin method), it should be considered a separate matrix.

**Scallops can be analyzed as whole animal or muscle excluded. These different forms of the animal should be considered a separate matrix. Methods for muscle only will not be considered as the product is not within the NSSP.

	Source*	Type of seafood	Species (if specified)	Nutritional Composition per 100 g			Comment
				Protein (g)	Total Lipid (g)	Carbohydrate (g)	
Oysters	FDC	Pacific oyster	*Crassostrea gigas*	9.5	2.3	5.0	
	FDC	Eastern oyster	*Crassostrea virginica*	5.7	1.7	2.7	
	V&G 2017	Eastern oyster		14.0	4.9		
	V&G 2017	Oyster, mixed		18.8	3.6		
	FAO	Cupped oysters, flesh, raw		8.9	1.8	5.3	
	FAO	Pacific cupped oyster, flesh, raw (n.s.)		9.7	1.8	5.8	
	FAO	Pacific cupped oyster, farmed, flesh, raw		9.6	1.6	7.3	
	FAO	Mangrove cupped oyster, flesh, raw		8.4	1.7	3.3	
	FAO	American cupped oyster, flesh, raw (n.s.)		5.6	1.4	3.8	
	FAO	American cupped oyster, flesh, farmed, raw (USA)		5.1	1.4	4.7	
	FAO	American cupped oyster, flesh, wild, raw (USA)		6.0	1.2	3.4	
	FAO	Flat oysters, flesh, raw		9.4	2.1	2.6	
	FAO	European flat oyster, flesh, raw		10.8	1.6	0.9	
Clams	FDC	Hard clam/southern quahog	*Mercenaria campechiensis*	5.7		2.9	
	V&G 2017	Clam, mixed		25.5	1.9		
	FAO	Venus clams, flesh, raw		9.2	1.2	3.5	
	FAO	Striped venus, flesh, raw		9.2	1.6	3.8	
Geoduck	Oliveira et al. 2011	Pacific geoduck - mantle	*Panopea abrupta*	14.3	2.6	6.9	%wt per wt
	Oliveira et al. 2011	Pacific geoduck - siphon	*Panopea abrupta*	15.3	0.7	2.2	%wt per wt
Scallops	FDC	Sea scallop (Chilean scallop)	*Argopecten purpuratus*	15.0	0.9	0.9	
	FDC	**MAGDALENA BAY SCALLOPS**	*Argopecten circularis*	15.0	0.9	6.2	
	FDC	North Atlantic Sea Scallop	*Placopecten magellanicus?*	12.4	0.4	3.5	
	FDC	Giant Sea Scallop	*Placopecten magellanicus*	15.0	0.9	0.9	
	FDC	Bay scallops		15.0	0.9	2.7	
	FDC	Sea scallop	*Placopecten magellanicus*	13.3	0.9	0.9	
	V&G 2017	Scallop, mixed		17.0	0.8		
	V&G 2017	Scallop, bay and sea		23.2	1.4		
	FAO	Scallops, flesh, raw		14.9	0.7	2.2	
	FAO	Great Atlantic scallop, flesh, raw		14.8	0.8	0.9	
Mussels	FDC	farmed Chilean mussel	*Mytilus chelensis*	13.9	2.5	4.1	
	FDC	Blue mussel	*Mytllus edulis*	11.9	2.2	3.7	
	G&V 2009	estuarine mussels	*Perna viridis*	28.4	1.7	6.5	*glycogen*
	G&V 2009	estuarine mussels	*Meretrix meretrix*	24.5	1.5	3.9	*glycogen*
	FAO	Mytilus mussels, flesh, raw		11.4	2.0	5.0	
	FAO	Korean mussel, flesh, raw	*Mytilus coruscus*	12.4	2.6	8.8	
	FAO	Blue mussel, flesh, raw	*Mytllus edulis*	11.8	1.9	3.9	
	FAO	Mediterranean mussel, flesh, raw (n.s.)		9.6	1.8	3.4	
	FAO	Mediterranean mussel, wild, flesh, raw		10.2	1.8	3.5	
	FAO	Mediterranean mussel, farmed, flesh, raw		8.3	1.9	3.8	
	FAO	Perna mussel, flesh, raw		13.5	2.0	2.5	
	FAO	New Zealand mussel, flesh, raw (New Zealand)		10.7	1.8	3.9	
	FAO	Green mussel, flesh, raw		9.3	0.9	2.0	
Cockle	FDC	cockle, Alaskan Native		13.5	0.7	4.7	

	Nutritional Composition per 100 g		
	Protein (g)	Total Lipid (g)	Carbohydrate (g)
All Oysters			
Avgerage	9.3	2.1	4.1
SD	3.6	1.0	1.7
RSD	38.9	48.1	41.6
Oysters excluding "mixed"			
Avg	8.6	1.7	4.1
SD	2.5	0.3	1.7
RSD	29.1	16.4	41.0
All Clams (incl. Geoduck)			
Avgerage	13.2	1.6	3.9
SD	6.4	0.6	1.6
RSD	48.4	40.0	42.3
Clams excluding "mixed" and Geoduck			
Avg	8.0	1.4	3.4
SD	1.7	0.2	0.4
RSD	20.5	13.8	11.8
Geoduck			
Avgerage	14.8	1.6	4.6
SD	0.5	0.9	2.4
RSD	3.4	57.2	51.8
All Scallops			
Avgerage	15.6	0.9	2.3
SD	2.8	0.2	1.8
RSD	17.9	25.9	78.1
All Mussels			
Avgerage	13.5	1.9	4.2
SD	5.8	0.4	1.7
RSD	42.8	21.6	39.8
Mussels excluding estuarine			
Avg	11.2	1.9	4.1
SD	1.7	0.4	1.7
RSD	15.1	21.4	41.5

***Sources:**

FDC	USDA FoodDataCentral
FAO	FAO/INFOODS Global Food Composition Database For Fish and Shellfish, version 1.0 (uFiSh1.0)
V&G 2017	V. Venugopal, K. Gopakumar 2017
G&V 2009	S. GOPALAKRISHNAN1 & K. VIJAYAVEL 2009
Oliveira et al. 2011	Oliveira et al. 2011