PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
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SHELLFISH LABORATORY EVALUATION CHECKLIST
Microbiology

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 N/A- 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]
	Membrane Filtration Technique for UV Treated Process Water using mEndo Agar LES [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 Shellfish Meats [PART III]
	Male Specific Coliphage for Wastewater [PART IV]

PART 1 - QUALITY ASSURANCE			
CODE	**REF**		**ITEM**
		1.1 Quality Assurance (QA) Plan	
K	8, 11		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 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 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 electrode isopotential point (pH 7) and 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 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	**9, 36**		**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 Devices (RTDs) and Platinum Resistance Devices (PTDs) with an accuracy and tolerance appropriate for the application.**
C	**11**		**1.4.21 A standards thermometer has been calibrated by NIST or a qualified calibration laboratory using a 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 annually for accuracy by ice point determination. Results recorded and maintained. Date of most recent determination ___________________

C	**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 and tolerance appropriate for the application are used as the laboratory standards thermometer. (*Circle the thermometer type used.*)**
K	13, 32		1.4.24 The accuracy of working thermometers are verified annually against the standards thermometer at the temperatures at which they are used. Discard working temperature-sensing devices that differ by >1°C from the reference/ standards device. 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	**29, 32, 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 using a device traceable to NIST or an equivalent authority at 121°C.
K	16		1.6.5 The autoclave standards thermometer is recalibrated every five (5) years for accuracy at 121°C by a qualified calibration laboratory; or, is verified in-house at the steam point (100°C) if it was 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 against the autoclave standards thermometer at 121°C yearly. Date of last check ___________ Method __________________
K	32		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 ≥160°C.
K	9		1.6.11 A thermometer capable of determining temperatures ≥160°C accurately 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 or reusable loops are properly sterilized. Alternatively, presterilized loops are used for transfers. Method of sterilization ________________________

C	**2**		**1.6.22 The sterility of the hardwood applicator transfer sticks/presterilized loops 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 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 and sterilized 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	9		1.7.9 The volume and concentration of media in the tube are suitable for the amount of sample inoculated.
C	**11**		**1.7.10 Total time of exposure of sugar containing broths to autoclave temperatures does not exceed 45 minutes.**
C	**1**		**1.7.11 Media sterility is determined for each load sterilized. Results are recorded and the records maintained.**
C	**40**		**1.7.12 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		1.7.13 Sterile phosphate buffered dilution water is used as the sample diluent, unless otherwise specified in the method specific sections of this checklist.
K	11		1.7.14 The pH of the prepared media is determined after sterilization to ensure that it is consistent with the manufacturer's requirements, unless otherwise specified in the original method documentation. 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	**9, 34**		**2.2.2 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**		**2.2.3 In a multiple dilution series not less than three (3) tubes per dilution are used (five (5) tubes are recommended).**
C	**6**		**2.2.4 In a single dilution series not less than 12 tubes are used (for depuration at least five (5) tubes are used).**
C	**6**		**2.2.5 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		2.2.6 Inoculated tubes are incubated in air at 35 ± 0.5°C.
C	**2**		**2.2.7 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		2.2.8 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.**
K	9, 11		2.3.3 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**		**2.3.4 BGB tubes are incubated at 35 ± 0.5°C.**
K	9		2.3.5 BGB tubes are read after 48 ± 3 hours of incubation.
C	**9**		**2.3.6 EC tubes are incubated in a circulating waterbath maintained at 44.5 ± 0.2°C.**
C	**9**		**2.3.7 EC tubes are read after 24 ± 2 hours of incubation.**
C	**9**		**2.3.8 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, 30**		**2.5.2 A-1 medium without salicin is used in the analysis. Comparability testing supports use of A-1 medium 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	**9, 34**		**2.5.4 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**		**2.5.5 In a multiple dilution series of not less than three (3) tubes per dilution are used (five (5) tubes are recommended).**
C	**6**		**2.5.6 In a single dilution series at least 12 tubes are used.**
C	**6**		**2.5.7 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**		**2.5.8 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**		**2.5.9 Inoculated tubes are placed in an air incubator at 35 ± 0.5°C for 3 ± 0.5 hours of resuscitation.**
C	**5**		**2.5.10 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**		**2.5.11 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 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	**23, 34**		**2.9.2 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**		**2.9.3 The membrane is placed grid side up within the sterile filter apparatus.**

C	23, 25		2.9.4 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		2.9.5 Sample volumes are filtered under vacuum.
K	26		2.9.6 The pressure of the vacuum pump does not exceed 15 psi.
C	23, 26		2.9.7 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		2.9.8 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		2.9.9 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		2.9.10 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		2.9.11 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		2.9.12 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, 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, 35**		**2.12.1 mEndo Agar LES is used.**
K	11, 21, 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, 35		2.12.3 mEndo Agar LES is prepared using 95% alcohol that is not denatured.
C	**9, 11, 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, 35**		**2.12.5 mEndo Agar LES is never autoclaved.**
K	9, 11, 35		2.12.6 A sufficient amount of medium (4-5 mL) is aseptically aliquoted to each culture plate.

O	9, 11, 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.
K	9, 11, 21, 35		2.12.8 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**		**2.12.9 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, 35**		**2.13.1 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	**11, 21, 35**		**2.13.2 The membrane filter is placed grid side up within the sterile filter apparatus.**
C	**11, 26, 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, 35**		**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, 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, 35		2.13.7 Forceps are dipped in alcohol and flame sterilized between sample filters.
C	**11, 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, 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, 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, 35**		**2.14.3 Results are reported as total coliforms/100mL.**
C	**11, 20, 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	2,32		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.
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.*)**
K	9		3.3.2 Immediately (within two (2) minutes) after blending, the ground sample is diluted and inoculated into tubes of presumptive media.

C	**9**		**3.3.3 No fewer than five (5) tubes per dilution are used in a multiple dilution MPN series.**
C	**9**		**3.3.4 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		3.3.5 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**		**3.3.6 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		3.3.7 Inoculated media are incubated at 35 ± 0.5°C.
K	10		3.3.8 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.**
K	9, 11		3.4.2 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**		**3.4.3 EC tubes are incubated in a circulating waterbath at 44.5 ± 0.2°C**
K	9		3.4.4 EC tubes are read for gas production after 24 ± 2 hours of incubation.
C	**9**		**3.4.5 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 (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 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 Pa rt 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	**3, 13**		**3.8.12 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**		**3.8.13 Plates are stacked no more than three (3) high in the incubator.**

C	**2**		**3.8.14 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 Shellfish Meats for Male Specific Coliphage (MSC)			
		3.10 MSC Equipment and Supplies	
K	2		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 100mL.
K	2		3.10.2 The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
K	1		3.10.3 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.
C	**2**		**3.10.4 The balance used provides a sensitivity of at least mg (0.01g.).**
C	**27, 28, 31**		**3.10.5 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	**1**		**3.10.7 The sterility of disposable 50 mL centrifuge tubes 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 Antibiotic solutions are filter sterilized using sterile 0.22 µm pore size filters.
O	27, 28, 31		3.11.3 Storage of the bottom agar under refrigeration does not exceed six (6) weeks.
K	2		3.11.4 Unsterilized soft agar is stored at ≤-15°C for up to three (3) months.
K	27, 28, 31		3.11.5 The soft agar is sterilized for 15 minutes at 121°C before use.
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		3.11.7 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.**
		3.13 Preparation of the Shellfish for MSC Analysis	
K	2,37		3.13.1 Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2		3.13.2 The blades of shucking knives are not corroded.
O	9		3.13.3 The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2		3.13.4 The faucet used for rinsing the shellfish does not contain an aerator.
K	9		3.13.5 The shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9		3.13.6 The shellfish are allowed to drain in a clean container or on clean towels unlayered prior to shucking.
K	9		3.13.7 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**		**3.13.8 Shellfish are not shucked through the hinge.**
C	**9**		**3.13.9 The contents of shellfish (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	9		3.13.10 A representative sample of at least 12 shellfish is used for the analysis.
K	2, 19		3.13.11 The sample is weighed to the nearest 0.1 gram.
C	**28, 31**		**3.13.12 Two (2) times the weight of the sample sterile growth broth, by volume, is added.**
C	**28, 31**		**3.13.13 Samples are blended at high speed for 180 seconds.**
		3.14 MSC Sample Analysis	
C	**28, 31**		**3.14.1 Immediately after blending, 33 grams of the homogenate is weighed into a centrifuge tube.**
C	**28, 31**		**3.14.2 The homogenized elution mixture is centrifuged for 15 minutes at 9000 x g at 4°C.**
C	**27, 28, 31**		**3.14.3 The supernatant is transferred to a new sterile tube, weighed and the weight recorded.**
C	**27, 28, 31**		**3.14.4 The supernatant is allowed to warm to room temperature prior to analysis.**
K	27, 28, 31		3.14.5 The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28, 31		3.14.6 200 microliters (0.2 mL) of log phase host strain E coli is added to the tempered soft agar immediately prior to adding the sample supernatant.
K	27, 28, 31		3.14.7 The sample supernatant is shaken or vortexed before being added to the tempered soft agar.
C	**27, 28, 31**		**3.14.8 2.5 mL of sample supernatant (avoiding bubbles where necessary) is added to a tube of tempered soft agar.**
C	**27, 28, 31**		**3.14.9 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**		**3.14.10 The tube contents is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**

C	**28, 31**		**3.14.11 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		3.14.12 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		3.14.13 Room temperature growth broth is used as the negative control or blank.
K	27, 28, 31		3.14.14 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		3.14.15 A negative control plate is plated at the end of each set of samples analyzed.
K	27, 28, 31		3.14.16 The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	**27, 28, 31**		**3.14.17 All plates are incubated at 36 ± 1°C for 18 ± 2 hours.**
		3.15 Computation of Results - MSC	
C	**27**		**3.15.1 Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted.**
C	**28, 31, 37**		**3.15.2 The working range of the method is 1 to 200 PFU per plate. When there are no plaques on all ten plates, the 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		3.15.3 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	2		3.15.4 The MSC count is rounded off conventionally to give a whole number.
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	**39**		**4.1.2 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	38		4.2.1 Wastewater samples or appropriately diluted samples are transferred into a sterile vessel.
K	9		4.2.2 The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
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.
C	**27, 28**		**4.2.4 The balance used provides a sensitivity of at least 0.01g.**
C	**27, 28**		**4.2.5 The temperature of the incubator used is maintained at 36 ±1°C.**
K	2		4.2.6 The temperature of the freezer is maintained at ≤ -15°C.
C	**28**		**4.2.7 The sterility of the transfer vessel utilized 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. Antibiotic solutions are filter sterilized using sterile 0.22 µm pore size filters.
O	27, 28		4.3.3 Storage of the bottom agar under refrigeration does not exceed six (6) weeks.
K	27, 28		4.3.4 Unsterilized soft agar is stored at ≤ -15°C for up to three (3) months.
K	27, 28		4.3.5 The soft agar is sterilized for 15 minutes at 119°C to 124°C before use.
K	27, 28		4.3.6 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	27, 28		4.3.7 Bottom agar plates stored under refrigeration are allowed to reach room temperature before use.
		4.4 Preparation of Host Culture for MSC Analysis	
C	**28**		**4.4.1 *E.coli* Famp ATCC 700891 is the bacterial host strain**
K	27, 28		4.4.2 Host cell growth broth is tempered at 36 ± 1°C prior to inoculation with host cells.
K	27, 28		4.4.3 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**		**4.4.4 After inoculation, the host cell growth broth culture is not shaken.**

			4.5 MSC Sample Analysis
C	**38**		**4.5.1 Wastewater 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) 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.**
K	27, 28		4.5.2 The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28		4.5.3 Two hundred microliters (0.2 mL) of log phase host strain E coli is added to the tempered soft agar immediately prior to adding the sample.
C	**27, 28**		**4.5.4 2.5 mL of sample is added to a tube of tempered soft agar.**
C	**27, 28**		**4.5.5 The tube contents is gently rolled between the palms of the hands to mix.**
C	**27, 28**		**4.5.6 The tube contents is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**
C			**4.5.7 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		4.5.8 Negative and positive control plates are prepared and accompany each set of samples analyzed. The results are recorded and records maintained.
K	27, 28		4.5.9 Room Temperature Growth broth is used as the negative control or blank.
K	27, 28		4.5.10 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		4.5.11 A negative control plate is plated at the end of each set of samples analyzed.
K	27, 28		4. 5.12 The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	**27, 28**		**4.5.13 All plates are incubated at 36 ± 1°C for 18 ± 2 hours.**
			4.6 Computation of Results - MSC
C	**38**		**4.6.1 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	**38**		**4.6.2 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		4.6.3 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		4.6.4 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-IV)	
A. Results Total # of Critical (C) Nonconformities in Parts I-IV Total # of Key (K) Nonconformities in Parts I-IV Total # of Critical (C), Key (K), and Other (O) Nonconformities in Parts I-IV	 ____________________ ____________________ ____________________
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. June 2024