Section IV. Guidance Documents

Chapter II. Growing Areas

.12 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists

NSSP Guidance Documents provide the public health principles supporting major components of the NSSP and its Model Ordinance, and summaries of the requirements for that component. NSSP Model Ordinance requirements apply only to interstate commerce although most states apply the requirements intrastate. For the most up to date and detailed listing of requirements, the reader should consult the most recent edition of the Model Ordinance.

Laboratory results from the bacteriological and marine toxin testing of shellfish growing waters and meats are widely used in the National Shellfish Sanitation Program (NSSP) to aid in determining the safety of shellfish for human consumption. Experience with the bacteriological and marine Biotoxin analyses of shellfish and shellfish waters have indicated that minor differences in laboratory procedures or techniques might cause wide variations in the results. Improper handling of the sample may also cause variations in results during collection or transportation to the laboratory. To ensure uniformity nationwide in the application of standards for shellfish and shellfish growing waters, a laboratory quality assurance program is necessary to substantiate the validity of analytical results. A laboratory quality assurance program is the systematic application of the practices essential to remove or minimize errors that may occur in any laboratory operation caused by personnel, apparatus, equipment, media, reagents, sampling procedures, and analytical methodology (APHA, 1985). Integral to laboratory quality assurance is a strong program for the external assessment or evaluation of laboratory performance.

Requirements for evaluating laboratories that analyze samples under the NSSP have increased significantly since the 1970's. The number of laboratories participating in the shellfish program has also increased. Several states now have multiple laboratories that provide these analyses. Some states have officially designated city, county or private laboratories to conduct analyses supporting their shellfish sanitation programs. Some states are also authorizing the use of private laboratories to monitor depuration operations. More states are maintaining a marine Biotoxin analytical capability in their laboratories; and more foreign laboratories are involved in the NSSP. Historically, FDA has evaluated all these laboratories. Reduction in FDA staffing has made it difficult to evaluate the many state, county, municipal, and foreign shellfish laboratories operating in support of the NSSP. If states with multiple laboratory support would exercise their option to accept responsibility for evaluating their laboratories by employing a State Shellfish Laboratory Evaluation Officer (State Shellfish LEO), FDA would be able to better meet its NSSP responsibilities.

Selection of State Shellfish Laboratory Evaluation Officers (LEOs) should be based on the following criteria:

(1) The individual must be administratively attached to a State central shellfish sanitation laboratory that has been found by the FDA to be in full conformance with NSSP requirements. To avoid the appearance of impropriety and maintain objectivity in the evaluation process, individuals certified as State Shellfish LEOs will not be allowed to evaluate their own laboratories. FDA will maintain the responsibility for evaluating these laboratories.

(2) The individual must be an experienced analyst and should have laboratory supervision experience. To maintain the integrity of the evaluation process, this individual should not, however, have overall supervisory responsibility for the laboratory or laboratories to be evaluated if deemed necessary by an FDA Laboratory Evaluation Officer, the individual must conduct several laboratory evaluations jointly with the FDA Laboratory Evaluation Officer.

(3) During the joint on-site laboratory evaluations with an FDA Laboratory Evaluation Officer, the individual must demonstrate competence in evaluating the laboratory's capability to support the NSSP. The evaluation will be performed and documented using the most current version of the applicable FDA Shellfish Laboratory Evaluation Checklist.

(4) The individual must submit a written narrative report of the joint on-site evaluation to the FDA co-evaluator for review and comment. The report should consist of the completed FDA Shellfish Laboratory Evaluation Checklist and a narrative discussion that accurately and concisely describes the overall operation of the laboratory. All nonconformities noted should be described in this evaluation write-up; and, where relevant an explanation provided relating the potential impact of the deficiency on the analytical results. Recommendations for corrective action or, if applicable, suggestions to enhance laboratory operations must also be included in this write-up.

The FDA will issue a letter certifying each individual who successfully completes the certification process and will clear the evaluation report(s) for distribution to the laboratories evaluated with copies to the appropriate Shellfish Specialist. Certification is normally effective for a period of three (3) years. Once certified, the individual is then expected to assume the following responsibilities:

* Conduct on-site laboratory evaluations at least every three (3) years. However, more frequent evaluations are strongly encouraged and may be required with marginally performing laboratories, or when major changes in workloads or priorities have occurred or when there has been a substantial turnover of personnel, or, at the specific request of State Shellfish Control Authorities;

* Provide appropriate post-evaluation follow-up for each laboratory evaluated;

* Prepare timely narrative evaluation reports for all laboratories evaluated incorporating the requirements specified in 4 above;

* Distribute completed evaluation reports to the appropriate FDA Laboratory Evaluation Officer and Regional Shellfish Specialist;

* Inform the appropriate FDA Laboratory Evaluation Officer when a laboratory has been found to be nonconforming;

* Develop/coordinate/implement/conduct yearly proficiency testing for all laboratories in the state supporting the NSSP; and,

* Prepare at least annually (in December) a summary list of qualified analysts for each laboratory supporting the NSSP in the state and transmit it to the appropriate FDA Laboratory Evaluation Officer.

Recertification of State Shellfish LEOs will normally occur triennially and will be based on satisfactorily meeting the following criteria:

(1) The individual must continue to be administratively attached to a central state shellfish laboratory which is in full conformance with NSSP requirements;

(2) The individual is not the supervisor of any of the laboratories to be evaluated;

(3) The individual must demonstrate continued competence in evaluating the capability of laboratories to support the NSSP. If considered necessary, the individual will be required to perform one to several joint evaluations with the FDA Laboratory Evaluation Officer;

(4) The individual must submit a written narrative report of the joint evaluation(s) to the FDA co-evaluator for review and comment. The report should consist of the completed FDA Shellfish Laboratory Evaluation Checklist and the narrative portion should be prepared as described above;

(5) The individual must have all state laboratory evaluations, split-sample (proficiency) test examinations, and reports current;

(6) The individual should receive training, as necessary, in laboratory evaluations and analytical procedures to remain proficient.

State Shellfish LEOs who successfully complete this process will be issued a letter of recertification by FDA and be cleared to distribute the evaluation reports to the laboratories evaluated with a copy to the appropriate Regional Shellfish Specialist. Normally recertification is effective for a period of three (3) years. Individuals who fail to meet the requirements for recertification will lose their certification until it is demonstrated that all requirements including adequate training are met.

References

American Public Health Association. 1985. *Standard Methods for the Examination of Water and Wastewater*. 16th Ed. American Public Health Association, American Water Works Association, Water Pollution Control Federation. Washington, D.C.

Food and Drug Administration. 1994. *Standard Procedures for State Shellfish Laboratory Evaluation Officers*. U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Field Programs, Division of Cooperative Programs, Shellfish Safety Branch, Washington, D.C.

National Shellfish Sanitation Program (NSSP) Guide for the Control of Molluscan Shellfish: 2011 Revision

Laboratory Evaluation Checklist - Microbiology

PUBLIC HEALTH SERVICE **U.S. FOOD AND DRUG ADMINISTRATION** **SHELLFISH PROGRAM IMPLEMENTATION BRANCH** **SHELLFISH SAFETY TEAM** **5100 PAINT BRANCH PARKWAY** **COLLEGE PARK, MD 20740-3835** **TEL. 301-436-2151/2147 FAX 301-436-2672**		
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: C- Critical K - Key O - Other NA- Not Applicable Conformity is noted by a "√"		

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 1 - QUALITY ASSURANCE		
CODE	**REF.**	**ITEM**
K	8, 11	**Quality Assurance (QA) Plan**

		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, calibration, maintenance, repair and for performance checks.
		☐ e. Laboratory safety.
		☐ f. Internal performance assessment.
		☐ g. External performance assessment.
C	State's Human Resources Department	☐ 2. In state laboratories, the supervisor meets the state educational and experience requirements for managing a public health laboratory
K	State's Human Resources Department	☐ 3. In state laboratories, the analyst(s) meets the state educational and experience requirements for processing samples in a public health laboratory.
C	USDA Microbiology & EELAP	☐ 4. In private 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	☐ 5. In private laboratories, the analyst(s) must have at least a high school diploma and shall have at least three months of experience in laboratory sciences.
C	8	☐ 6. QA Plan Implemented.
K	11	☐ 7. Participates in a proficiency testing program annually. Specify Program(s)_____
CODE	**REF.**	**Work Area**
O	8,11	☐ 1. Adequate for workload and storage.
K	11	☐ 2. Clean, well lighted.
K	11	☐ 3. Adequate temperature control.
O	11	☐ 4. All work surfaces are nonporous, easily cleaned and disinfected.
K	11	☐ 5. Microbiological quality and density of air is < 15 colonies/plate in a 15 minute exposure determined monthly and results recorded.
O	11	☐ 6. Pipette aid used, mouth pipetting not permitted.
CODE	**REF.**	**Equipment**
O	9	☐ 1. To determine the pH of prepared media, the pH meter has a standard accuracy of 0.1 units.
O	14	☐ 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 effect the accuracy of the pH reading).
K	11	☐ 3. The effect of temperature on the pH is compensated for by an ATC probe or by manual adjustment.
K	8	☐ 4. pH meter is calibrated daily or with each use and records are maintained.
K	11	☐ 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 daily and discarded.

O	8,15	☐	6. Electrode effectiveness is determined daily or with each use. Method of determination_____.
K	9	☐	7. Balance provides a sensitivity of at least 0.1 g at a load of 150 g.
K	11,13	☐	8. Balance checked monthly using NIST Class S or ASTM Class 1 or 2 weights or equivalent and records are maintained.
K	11	☐	9. Refrigerator temperature(s) monitored at least once daily and recorded.
K	1	☐	10. Refrigerator temperature maintained at 0° to 4° C.
C	9	☐	11. The temperature of the incubator is maintained at 35 ± 0.5° C.
C	11	☐	12. Thermometers used in the air incubator(s) are graduated at no greater than 0.5° C increments.
K	9	☐	13 Working thermometer located on top and bottom shelves of use in the air incubator(s).
C	11	☐	14. Temperature of the waterbath is maintained at 44.5 ± 0.2° C under any loading capacity.
C	9	☐	15. The thermometers used in the waterbath are graduated in 0.1° C increments.
O	13	☐	16. The waterbath has adequate capacity for workload.
K	9	☐	17. The level of water in the waterbath covers the level of liquid in the incubating tubes.
K	8, 11	☐	18. Air incubator/waterbath temperatures are taken twice daily and recorded.
K	13	☐	19. Working thermometers are tagged with identification, date of calibration, calibrated temperature and correction factor.
K	4	☐	20. All working thermometers are appropriately immersed.
K	11	☐	21. A standards thermometer has been calibrated by NIST or one of equivalent accuracy at the points 0°, 35° and 44.5° C (45.5° C for ETCP). Calibration records maintained.
K	9	☐	22. Standards thermometer is checked annually for accuracy by ice point determination. Results recorded and maintained. Date of most recent determination_____.
K	13	☐	23. Incubator and waterbath working thermometers are checked annually against the standards thermometer at the temperatures at which they are used. Records maintained.
CODE	**REF.**		**Labware and Glassware Washing**
O	9	☐	1. Utensils and containers are clean borosilicate glass, stainless steel or other noncorroding materials
K	9	☐	2. Culture tubes are of a suitable size to accommodate the volume for nutritive ingredients and samples
K	9	☐	3. Sample containers are made of glass or some other inert material (i.e. polypropylene).
O	9	☐	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	☐	5. Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method is used to ensure

			appropriate volumes.
K	9	☐	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; nor, are pipits larger than 1ml used to deliver 0.1ml.
K	9	☐	7. Reusable sample containers are capable of being properly washed and sterilized.
K	9	☐	8. In washing reusable pipits, 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	9	☐	9. In washing reusable sample containers, glassware and plasticware, the effectiveness of the rinsing procedure is established annually and when detergent (brand or lot) is changed by the Inhibitory Residue Test as described in the current edition of Standard Methods for the Examination of Water and Wastewater. Records are kept. Date of most recent testing_____ Average difference between Groups A and B_____ Average difference between Groups B and D_____ Detergent Brand_____Lot #_____
K	11	☐	10. Once during each day of washing several pieces of glassware (pipettes, sample bottles, etc.) from one batch are tested for residual acid or alkali w/aqueous 0.04% bromthymol blue. Records are maintained.
CODE	**REF.**		**Sterilization and Decontamination**
O	9	☐	1. Autoclave(s) are of sufficient size to accommodate the workload.
O	8	☐	2. Routine autoclave maintenance performed (e.g. pressure relief valves, exhaust trap, chamber drain) and records maintained.
O	8	☐	3. Autoclave(s) and/or steam generators serviced annually or as needed by qualified technician and records maintained.
C	11	☐	4. Autoclave(s) provides a sterilizing temperature of 121° C (tolerance 121 ± 2° C) as determined weekly using a calibrated working maximum registering thermometer or equivalent (thermocouples, platinum resistance thermometers).
K	11	☐	5. An autoclave standards thermometer has been calibrated by the National Institute of Standards and Technology (NIST) or its equivalent at 121° C.
K	16	☐	6. The autoclave standards thermometer is checked every five years for accuracy at either 121° C or at the steam point. Date of most recent determination_____

K	1	☐ 7. Working autoclave thermometers are checked against the autoclave standards thermometer at 121° C yearly. Date of last check _____ Method _____
K	11	☐ 8. Spore suspensions are used monthly to evaluate the effectiveness of the autoclave sterilization process. Results recorded.
O	11	☐ 9. Heat sensitive tape is used with each autoclave batch.
K	11, 13	☐ 10. 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	☐ 11. For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperature in the range of 160° to 180° C.
K	9	☐ 12. 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 when in use.
K	13	☐ 13. Records of temperatures and exposure times are maintained for the operation of the hot-air sterilizing oven during use.
K	11	☐ 14. Spore strips are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. Records are maintained.
K	11	☐ 15. Reusable sample containers are sterilized for 60 minutes at 170° C in a hot-air oven or autoclaved for 15 minutes at 121° C.
O	1	☐ 16. The sterility of reusable/disposable sample containers is determined for each batch/lot.
K	9	☐ 17. Reusable pipettes are stored and sterilized in aluminum or stainless steel canisters or equivalent alternative.
K	9	☐ 18. Reusable pipettes (in canisters) are sterilized in a hot-air oven at 170° C for 2 hours.
O	2	☐ 19. The sterility of reusable/disposable pipettes is determined with each batch/lot. Results are recorded and maintained.
K	18	☐ 20. Hardwood applicators transfer sticks are properly sterilized.
O	13	☐ 21. Spent broth cultures and agar plates are decontaminated by autoclaving for at least 30 minutes before conventional disposal.
CODE	**REF.**	**Media Preparation**
K	3, 5	☐ 1. Media is commercially dehydrated except in the case of medium A-1 which is prepared from the individual components and modified MacConkey agar which may be prepared from its components.
O	11	☐ 2. Dehydrated media and media components properly stored in cool, clean, dry place.
O	11	☐ 3. Dehydrated media are labeled with date of receipt and date opened.
C	12	☐ **4. Caked or expired media are discarded.**
C	11	☐ 5. Make-up water is distilled or deionized *(circle one)* and exceeds 0.5 megohm resistance or is less than 2µ Siemens/cm

			conductivity at 25° C to be tested and recorded monthly for resistance or conductivity *(circle the appropriate)*.
C	11	☐	6. Make-up water is analyzed for residual chlorine monthly and is at a non-detectable level (≤ 0.1 ppm). Records are maintained. Specify method of determination_____.
K	11	☐	7. Make-up water is free from trace (<0.05mg/L) dissolved metals, specifically Cd, Cr, Cu, Ni, Pb, and Zn as determined annually with total heavy metal content < or equal to 1.0mg/L and records are maintained.
K	11	☐	8. Make-up water contains <1000 CFU/ml as determined monthly using the heterotrophic plate count method and records are maintained.
K	11	☐	9. Media are sterilized according to the manufacturer's instructions.
K	9	☐	10. Volume and concentration of media in the tube are suitable for the amount of sample inoculated.
C	11	☐	11. Total time of exposure of sugar broths to autoclave temperatures does not exceed 45 minutes.
C	1	☐	12. Media sterility and positive and negative controls are run with each lot of commercially prepared media or are run with each batch of media prepared from its components as a check of media productivity. Results recorded and records maintained.
O	9	☐	13. Sterile phosphate buffered dilution water is used as the sample diluent.
K	11	☐	14. pH is determined after sterilization to ensure that it is consistent with manufacturer's requirements and records are maintained.
CODE	**REF.**		**Storage of Prepared Culture Media**
O	9	☐	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	☐	2. Brilliant green bile 2% broth and A-1 media are stored in the dark.
K	13	☐	3. Stored media are labeled with expiration date or sterilization date.
O	9	☐	4. Storage of prepared culture media at room temperature does not exceed 7 days.
O	2	☐	5. Storage under refrigeration of prepared media with loose fitting closures shall not exceed 1 month.
O	11	☐	6. Storage under refrigeration of prepared media with screw-cap closures does not exceed 3 months.
K	17	☐	7. All prepared media stored under refrigeration are held at room temperature overnight prior to use. Culture tubes containing any type of precipitate or Durham tubes containing air bubbles are discarded.
PART II - SEAWATER SAMPLES			
CODE	**REF.**		**ITEM**
			Collection and Transportation of Samples
C	11	☐	1. Containers are of suitable size to contain at least 100 ml and to allow headspace for shaking. Seawater samples are collected in clean, sterile, water tight, properly labeled sample containers.

K	1	☐	2. Sample identified with collectors name, harvest area, time and date of collection.
C	9	☐	3. After collection, seawater samples shall be kept at a temperature between 0 and 10° C until examined.
K	1	☐	4. A temperature blank is used to determine the temperature of samples upon receipt at the laboratory. Results are recorded and maintained.
C	9	☐	5. Examination of the sample is initiated as soon as possible after collection. However, seawater samples are not tested if they are held beyond 30 hours of refrigeration.
CODE	**REF.**	**Bacteriological Examination of Seawater by the APHA MPN**	
C	9	☐	1. Lactose broth or lauryl tryptose broth is used as the presumptive medium. *(circle appropriate one)*
C	9	☐	2. Sample and dilutions of sample are mixed vigorously (25 times in a 12" arc in 7 seconds) before inoculation.
C	9	☐	3. In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended).
C	6	☐	4. In a single dilution series not less than 12 tubes are used (for depuration at least 5 tubes are used).
K	6	☐	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_____
K	9	☐	6. Inoculated media are placed in an air incubator at 35 ± 0.5° C for up to 48 ± 3 hours.
K	2	☐	7. Positive and negative control cultures accompany samples throughout the procedure. Records are maintained. Positive Control _____ Negative Control _____
K	9	☐	8. Inoculated media are read after 24 ± 2 hours and 48 ± 3 hours of incubation and transferred at both intervals if positive for gas.
CODE	**REF.**	**Confirmed Test for Seawater by APHA MPN**	
C	9	☐	1. Brilliant green bile 2% broth (BGB) is used as the confirmatory medium for total coliforms.
C	9	☐	2. EC medium is used as the confirmatory medium for fecal coliforms.
K	9, 11	☐	3. Transfers made to BGB/EC by either sterile loop or sterile hardwood applicator stick from positive presumptives incubated for 24 and 48 hours *(Circle the method of transfer)*.
K	2	☐	4. When the inoculation of both EC and BGB broths is performed using the same loop or transfer stick, the order of inoculation is EC first, followed by BGB.
C	9	☐	5. BGB tubes are incubated at 35 ± 0.5° C.
K	9	☐	6. BGB tubes are read after 48 ± 3 hours of incubation.
C	9	☐	7. EC tubes are incubated in a circulating waterbath at 44.5 ± 0.2° C for 24 ± 2 hours.
C	9	☐	8. The presence of any amount of gas or effervescence in the

			culture tube constitutes a positive test.
CODE	**REF.**	**Computation of Results**	
K	9	☐	1. Results of multiple dilution tests are read from tables in *Recommended Procedures*, 4th Edition.
K	7	☐	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".
K	7, 9	☐	3. Results are reported as MPN/100 ml of sample.
CODE	**REF.**	**Bacteriological Examination of Seawater by the MA-1 Method**	
C	5	☐	1. Medium A-1 sterilized for 10 minutes at 121° C.
C	9	☐	2. Sample and dilutions of sample are mixed vigorously (25 times in a 12" arc in 7 seconds) before inoculation.
C	9	☐	3. In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended)
C	6	☐	4. In a single dilution series at least 12 tubes are used.
K	6	☐	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 _____
K	2	☐	6. Positive and negative control cultures accompany samples throughout the procedure. Records are maintained. Positive Control _____ Negative Control _____
C	2,5	☐	7. Inoculated media are placed in an air incubator at 35 ± 0.5° C for 3 ± 0.5 hours of resuscitation.
C	5	☐	8. After 3 ± 0.5 hours resuscitation at 35° C, inoculated media are incubated at 44.5 ± 0.2° C in a circulating waterbath for the remainder of the 24 ± 2 hours.
C	5	☐	9. The presence of any amount of gas or effervescence in the culture tube constitutes a positive test.
CODE	**REF.**	**Computation of Results**	
K	9	☐	1. Results of multiple dilution tests are read from tables in *Recommended Procedures*, 4th Edition.
K	7	☐	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".
K	7, 9	☐	3. Results are reported as MPN/100 ml of sample.
CODE	**REF.**	**Bacteriological Examination of Seawater by Membrane Filtration using mTEC Agar**	
			Equipment
C	23, 24	☐	1. When used for elevated temperature incubation, the temperature of the hot air incubator is maintained at 44.5±0.5° C under any loading capacity.
C	23	☐	2. When using a waterbath for elevated temperature incubation, the level of the water completely covers the plates.

C	23	☐	3. Pre-sterilized plastic or sterile glass culture plates that are clear, flat bottomed, free of bubbles and scratches are used.
K	11	☐	4. Colonies are counted with the aid of magnification.
C	11, 23	☐	5. 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.
O	2	☐	6. Lot number, date of receipt and if provided the expiration date of the membrane filters are recorded.
K	2, 11	☐	7. New lots of membrane filters are checked by comparing recovery of fecal coliform organisms against membrane filters from previously acceptable lots.
C	2	☐	8. The sterility of each lot or autoclave batch of membrane filters are checked before use.
K	2	☐	9. Membrane filters which are beyond their expiration date are not used.
O	11	☐	10. Forceps tips are clean.
O	11	☐	11. Forceps tips are smooth without pitting or corrugations to damage the filters being manipulated.
K	11	☐	12. Forceps are dipped in alcohol and flame sterilized between sample filters.
K	11	☐	13. If indelible graduation marks are used on clear glass or plastic funnels to measure sample volumes, their accuracy is checked 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	☐	14. Membrane filtration units are made of stainless steel, glass or autoclavable plastic free of scratches, corrosion and leaks.
C	11	☐	15. Membrane filter assemblies are autoclave sterilized for 15 minutes at 121°C prior to the start of a filtration series.
O	11, 23, 26	☐	16. A UV sterilization unit is used to disinfect filter assemblies between sample and filtration runs.
K	11	☐	17. If used, the effectiveness of the UV sterilization unit is determined by biological testing monthly. Results are recorded and records maintained.
CODE	**REF.**	**Media Preparation and Storage**	
K	11	☐	1. Phosphate buffered saline is used as the sample diluent.
C	11	☐	2. Phosphate buffered saline is properly sterilized.
K	23	☐	3. A sufficient amount of medium (4-5 ml) is used in each plate.
O	11	☐	4. Refrigerated prepared plates are stored for no more than 2 weeks in sealed plastic bags or containers to minimize evaporation.
CODE	**REF.**	**Sample Analyses**	
C	24	☐	1. mTEC agar is used.
C	23	☐	2. The sample is mixed vigorously (25 times in a 12” arc in 7 seconds) before filtration.
C	23	☐	3. The membrane is placed grid side up within the sterile filter apparatus.
C	23, 25	☐	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	☐	5. Sample volumes are filtered under vacuum
K	26	☐	6. The pressure of the vacuum pump does not exceed 15 psi..
C	23, 26	☐	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	☐	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	☐	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).
K	2, 11	☐	10. Positive and negative control cultures treated like samples accompany test samples throughout the procedure. Positive control _____ Negative control _____ Results are recorded and records maintained.
C	11, 23, 24	☐	11. Inoculated plates are placed inverted either directly in an air incubator or in a watertight, tightly sealed container at 35 + 0.5°C for 2 hours of resuscitation prior to waterbath incubation or in Ethyfoam for incubation in air at 44.5 +0.5°C.
C	11, 23, 24	☐	12. After 2 hours of resuscitation at 35°C watertight sealed containers are transferred to a circulating waterbath at 44.5 + 0.2°C, submerged completely and incubated for 22-24 hours. Individual plates are transferred inverted to a watertight container, tightly sealed and submerged completely in a circulating waterbath at 44.5 + 0.2°C for 22-24 hours of incubation.
CODE	**REF.**	**Computation of Results**	
C	23	☐	1. All yellow, yellow-green or yellow-brown colonies are counted.
C	23	☐	2. Only plates having 80 or fewer colonies are counted. If it is necessary to use plates having more than 80 colonies, counts are given as >80 x 100/the volume filtered.
K	23, 11	☐	3. The number of fecal coliforms is calculated by the following equation: Number of fecal coliforms per 100 ml = [number of colonies counted/volume of sample filtered in ml] x 100.
K	23, 11	☐	4. Results are reported as CFU/100 ml of sample.
PART III - SHELLFISH SAMPLES			
CODE	**REF.**	**ITEM**	
		Collection and Transportation of Samples	
C	9	☐	1. A representative sample of shellstock is collected.
K	9	☐	2. Shellstock is collected in clean, waterproof, puncture resistant containers.
K	9	☐	3. Shellstock labeled with collector's name, type of shellstock, the source, the harvest area, time, date and place (if market sample) of collection.
C	9	☐	4. Shellstock samples are maintained in dry storage between 0 and 10° C until examined.
C	1	☐	5. Examination of the sample is initiated as soon as possible after collection. However, shellfish samples are not examined if the time interval between collection and examination exceeds 24 hours.
CODE	**REF**	**Preparation of Shellstock for Examination**	
K	2,11	☐	1. Shucking knives, scrub brushes and blender jars are (autoclave) sterilized for 15 minutes prior to use.

O	2	☐	2. Blades of shucking knives are not corroded.
O	9	☐	3. Prior to scrubbing and rinsing debris off shellstock, the hands of the analyst are thoroughly washed with soap and water.
O	2	☐	4. The faucet used to provide the potable water for rinsing the shellstock does not contain an aerator.
K	9	☐	5. Shellstock are scrubbed with a stiff, sterile brush and rinsed under water of drinking water quality.
O	9	☐	6. Shellstock are allowed to drain in a clean container or on clean towels prior to opening.
K	9	☐	7. Prior to opening, the hands (or gloved hands) of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol.
K	9	☐	8. Shellstock are not shucked directly through the hinge.
C	9	☐	9. Contents of shellstock (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.
K	9	☐	10. At least 200 grams of shellfish meat is used for analysis.
K	2, 19	☐	11. The sample is weighed to the nearest 0.1 gram and an equal amount by weight of (tempered for ETCP) diluent is added.
O	9	☐	12. Sterile phosphate buffered dilution water is used as the sample diluent.
K	3	☐	13. Sterile phosphate buffered saline is used as a sample diluent for the ETCP procedure.
C	9	☐	14. Samples are blended at high speed for 60 to 120 seconds.
K	9	☐	15. For other shellstock, APHA *Recommended Procedures* are followed for the examination of freshly shucked and frozen shellfish meats.
CODE	**REF.**	**MPN Analysis for Fecal Coliform Organisms, Presumptive Test, APHA**	
C	9	☐	1. Appropriate strength lactose or lauryl tryptose broth is used as presumptive media in the analysis. *(circle appropriate choice)*
K	9	☐	2. Immediately (within 2 minutes) after blending, the ground sample is diluted and inoculated into tubes of presumptive media.
C	9	☐	3. No fewer than 5 tubes per dilution are used in a multiple dilution MPN series.
C	9	☐	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., 22 ml of l:1 diluted sample to 88 ml of diluent or the equivalent for 0.1 g portion.
K	6	☐	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	☐	6. Positive and negative control cultures accompany samples throughout the procedure. Records are maintained. Positive Control _____ Negative Control _____
K	9	☐	7. Inoculated media are incubated at 35 ± 0.5° C.
K	10	☐	8. Presumptive tubes are read at 24 ± 2 hours of incubation and transferred if positive.
CODE	**REF.**	**Confirmed Test for Fecal Coliforms - APHA**	
C	9	☐	1. EC medium is used as the confirmatory medium.
K	9, 11	☐	2. Transfers are made to EC medium by either sterile loop or hardwood sterile applicator sticks from positive presumptives

			incubated for 24 hours *(circle the method of transfer).*
C	9	☐	3. EC tubes are incubated in a circulating waterbath at 44.5 ± 0.2° C for 24 ± 2 hours.
K	9	☐	4. EC tubes are read for gas production after 24 ± 2 hours of incubation.
C	9	☐	5. The presence of any amount of gas or effervescence in the Durham tube constitutes a positive test.
CODE	**REF.**	**Computation of Results for MPN Analyses**	
K	9	☐	1. Results of multiple dilution tests are read from tables in *Recommended Procedures,* 4th Edition and multiplied by the appropriate dilution factor.
K	7	☐	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".
K	9	☐	3. Results are reported as MPN/100 grams of sample.
CODE	**REF.**	**Standard Plate Count Method**	
O	20	☐	1. A standard plate count analysis is performed in conjunction with the analysis for fecal coliform organisms.
K	9	☐	2. In the standard plant count procedure at least four plates, duplicates of two dilutions are used to provide 30 to 300 colonies per plate.
K	2	☐	3. Fifteen to 20 ml of tempered sterile plate count agar is used.
K	9	☐	4. Agar tempering bath maintains the agar at 44 to 46° C.
O	9	☐	5. Temperature control of the plate count agar is used in the tempering bath.
K	9	☐	6. Not more than 1 ml nor less than 0.1 ml of sample or sample dilution is plated.
C	9	☐	7. Samples or sample dilutions to be plated are mixed vigorously (25 times in a 12" arc in 7 seconds) before plating.
K	11	☐	8. Control plates are used to check the sterility of the air, agar and the diluent.
K	9,21	☐	9. Solidified plates are incubated at 35 ± 0.5°C for 48 ± 3 hours inverted and stacked no more than four high.
K	9	☐	10. Quebec Colony Counter or its equivalent is used to provide the necessary magnification and visibility for counting plates.
K	1	☐	11. A hand tally or its equivalent is used for accuracy in counting.
CODE	**REF.**	**Computation of Results**	
K	9	☐	1 Colony counts determined in accordance with Part III, A, Sections 4.31 through 4.33 Recommended *Procedures,* 4th Edition.
O	19	☐	2 Colony counts reported as APC/g of sample.
CODE	**REF.**	**Bacteriological Examination of Shellfish Using the ETCP**	
K	9	☐	1. Sample homogenate is cultured within 2 minutes of blending.
K	3	☐	2. Double strength Modified MacConkey Agar is used.
C	3	☐	3. Hydrated double strength Modified MacConkey Agar is heated to boiling, removed from the heat, and boiled again. This agar is never autoclaved.
K	2, 3	☐	4. Twice boiled, double strength Modified MacConkey Agar and sterile phosphate buffered saline are maintained in a tempering bath at 45 to 50° C until used. Prepared Modified MacConkey Agar is used on the day it is made.
C	2, 3	☐	5. The equivalent of 6 grams of the homogenate is placed into a

			sterile container and the contents brought up to 60 ml with tempered, sterile phosphate buffered saline.
K	3	☐	6. Sixty (60) ml of tempered, twice boiled double strength Modified MacConkey Agar is added.
K	2, 3, 22	☐	7. The container is gently swirled or rotated to mix the contents, which are then, distributed uniformly over 6 to 8 petri plates.
C	1	☐	8. Media and diluent sterility are determined with each use. Results are recorded and records maintained.
C	1	☐	9. To determine media productivity, positive and negative control cultures are pour plated in an appropriate concentration to accompany samples throughout the procedure. Positive control_____ Negative control _____
C	3, 13	☐	10. Plates are incubated inverted within 3 hours of plating in air at 45.5 ± 0.5° C for 18 to 30 hours. Plates are stacked not more than four high.
C	3	☐	11. Incubator temperature is maintained at 45.5 ± 0.5° C.
CODE	**REF.**	**Expression of Results**	
K	11	☐	1. Quebec Colony counter or its equivalent is used to provide the necessary magnification and visibility.
O	1	☐	2. A hand tally or its equivalent is used to aid in counting.
C	3, 6	☐	3. All brick red colonies greater than 0.5mm in diameter are totaled over all the plates and multiplied by a factor of 16.7 to report results as CFU/100 grams of sample.
CODE	**REF.**	**Bacteriological Examination of Soft-shelled Clams and American Oysters for Male Specific Coliphage (MSC)**	
			Equipment and Supplies
K	30	☐	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	☐	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.
C	27, 28	☐	3. The tempering bath(s) must be able to maintain the temperature within 2°C of the set temperature.
K	9	☐	4. The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
C	27, 28	☐	5. 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	☐	6. The sterility of each lot of pre-sterilized syringes and syringe filters is determined.
K	1	☐	7. The sterility of each batch of reusable glass syringes is determined.
C	27, 28	☐	8. The balance used provides a sensitivity of at least 10 mg.
C	27, 28	☐	9. The temperature of the incubator used is maintained between 35 – 37°C.
C	28	☐	**10. Sterile disposable 50 ml centrifuge tubes are used and their sterility is determined with each lot.**
			Media Preparation
K	28	☐	1. Media preparation and sterilization is according to

			thevalidated method.
K	27, 28	☐	2. Bottom agar, double strength soft agar and growth broth are prepared from their individual components.
K	27, 28	☐	3. Soft agar is prepared double strength in volumes of 2.5 ml.
C	27, 28	☐	4. **The streptomycin and ampicillin solutions are added to tempered bottom agar.**
O	27, 28	☐	5. Storage of the bottom agar under refrigeration does not exceed 1 month.
K	27, 28	☐	6. Unsterilized soft agar is stored at -20°C for up to 3 months.
K	27, 28	☐	7. The soft agar is removed from the freezer and sterilized for 15 minutes at 121°C before use.
K	27, 28	☐	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	☐	9. Bottom agar plates are allowed to reach room temperature before use.
			PREPARATION OF THE SOFT-SHELLED CLAMS AND AMERICAN OYSTERS FOR ANALYSIS
K	2, 11	☐	1. Shucking knives, scrub brushes and blender jars areautoclave sterilized for 15 minutes prior to use.
O	2	☐	2. The blades of the shucking knives used are not corroded.
O	9	☐	3. The hands of the analyst are thoroughly washed with soap and water prior to scrubbing and rinsing of debris off the shellfish.
O	2	☐	4. The faucet used to provide the potable water for rinsing the shellfish does not contain an aerator.
K	9	☐	5. The shellfish are scrubbed with a stiff, sterile brush and rinsed under water of drinking water quality.
O	9	☐	6. The shellfish are allowed to drain in a clean container or on clean towels unlayered prior to shucking.
K	9	☐	7. Prior to shucking, the hands (or gloved hands) of the analyst are thoroughly washed with soap and water and rinsed with 70% alcohol.
K	9	☐	8. The shellfish are not directly shucked through the hinge.
C	9	☐	9. **The contents of the shellfish (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	9	☐	10. At least 12 shellfish are used for the analysis.
C	2, 19	☐	11. **The sample is weighed to the nearest 0.1 gram.**
CODE	**REF**	**SAMPLE ANALYSIS**	
C	28	☐	1. *E.coli Famp* ATCC 700891 is the bacterial host strain used in this procedure.
K	27, 28	☐	2. Host cell growth broth is tempered at 35 –37°C and vortexed (or shaken) to aerate prior to inoculation with host cells.
K	27, 28	☐	3. Several host cell colonies are transferred to a tube of tempered, aerated growth broth and incubated at 35 – 37°C to provide host cells in log phase growth for sample analysis.
C	27, 28	☐	4. **Inoculated growth broth is incubated at 35 – 37°C for 4 to 6 hours to provide a host cell culture in log phase growth.**
C	27, 28	☐	**5. After inoculation, the host cell growth broth culture is not shaken.**
C	28	☐	6. **A 2:1 mixture of growth broth to shellfish tissue is used for eluting the MSC.**
C	28	☐	7. **The elution mixture is prepared w/v by weighing the**

			sample and adding two equal portions of growth broth by volume to the shellfish tissue.
C	28	☐	8. **The elution mixture is homogenized at high speed for 180 seconds.**
C	28	☐	9. **Immediately after blending, 33 grams of the homogenized elution mixture are weighed into centrifuge tubes.**
C	28	☐	10. **The homogenized elution mixture is centrifuged for 15 minutes at 9000 x g at 4°C.**
C	27, 28	☐	11. **The supernatant is pipetted off, weighed and the weight recorded.**
C	27, 28	☐	12. **The supernatant is allowed to warm to room temperature about 20 to 30 minutes.**
K	27, 28	☐	13. The autoclaved soft agar is tempered and held at 50 – 52°C throughout the period of sample analysis.
K	27, 28	☐	14. 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	☐	15. The sample supernatant is shaken or vortexed before being added to the tempering soft agar.
C	27, 28	☐	16. **2.5 ml of sample supernatant is added to each tube of tempering soft agar.**
C	27, 28	☐	17. **The soft agar/sample supernatant/host cell mixture is gently rolled between the palms of the hands to mix.**
C	27, 28	☐	18. **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	☐	19. **10 plates are used, 2.5 ml per plate for a total of 25 ml of supernatant analyzed per sample.**
K	27, 28	☐	20. Negative and positive control plates are prepared and accompany each set of samples analyzed.
K	27, 28	☐	21. Growth broth is used as the negative control or blank.
K	27, 28	☐	22. Type strain MS2 (ATCC 15597) male specific bacteriophage is used as the positive control.
K		☐	23. A negative control plate is plated at the beginning and end of each set of samples analyzed.
K	27, 28	☐	24. The positive control is plated after all the samples are analyzed and immediately prior to the final negative control.
C	27, 28	☐	25. **All plates are incubated at 35 – 37°C for 16 to 20 hours.**
			COMPUTATION OF RESULTS
C	27	☐	1. **Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted.**
C	28	☐	2. **The working range of the method is 1 to 100 PFU per plate. When there are no plaques on all ten plates, the count is <6 PFU/100 gm for soft- shelled clams and <7 PFU/ 100 gm for American oysters. If the density exceeds 100 PFU per plate on all plates, the count is given as > 10,000 PFU/100 gm.**
K	28	☐	3. The formula used for determining the density of MSC in PFU/100 gm 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	☐	4. The MSC count is rounded off conventionally to give a whole number.

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National Shellfish Sanitation Program (NSSP) Guide for the Control of Molluscan Shellfish: 2011 Revision

Shellfish Laboratory Evaluation Checklist

Summary of Non Conformitites

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 $\geq$ **4 or**

b. The total # of Key nonconformities is $\geq$ **13 or**

c. The total # of Critical, Key and Other is $\geq$ **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 $\geq$ **1 but** $\leq$ **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. 2005-08-19

Laboratory Evaluation Checklist - PSP

PUBLIC HEALTH SERVICE **U.S. FOOD AND DRUG ADMINISTRATION** **SHELLFISH PROGRAM IMPLEMENTATION BRANCH** **SHELLFISH SAFETY TEAM** **5100 PAINT BRANCH PARKWAY** **COLLEGE PARK, MD 20740-3835** **TEL. 301-436-2151/2147 FAX 301-436-2672**		
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: C- Critical K - Key O - Other NA - Not Applicable Conformity is noted by a "√"		

PART I - QUALITY ASSURANCE		
Code		**Item Description**
		Quality Assurance (QA) Plan
K	☐	1. Written Plan adequately covers all the following: (check √ those that apply) 1. a. Organization of the laboratory. 2. b. Staff training requirements. 3. c. Standard operating procedures. 4. d. Internal quality control measures for equipment, calibration, maintenance, repair and performance. 5. e. Laboratory safety. 6. f. Quality assessment. 7. g. Proper animal care.
C	☐	**2. QA plan implemented.**
		1.2 Work Area
O	☐	1. Adequate for workload and storage.
O	☐	2. Clean and well lighted.
O	☐	3. Adequate temperature control.
O	☐	4. All work surfaces are nonporous and easily cleaned.
C	☐	**5. A separate, quiet area with adequate temperature control for mice acclimation and injection is maintained.**
		1.3 Laboratory Equipment
O	☐	1. The pH meter has a standard accuracy of 0.1 unit.
K	☐	2. pH paper in the appropriate range (i.e. 1-4) is used with minimum accuracy of 0.5 pH units.
K	☐	3. pH electrodes consist of pH half cell and reference half cell or equivalent combination electrode (free from Ag/AgCl or contains an ion exchange barrier to prevent passage of Ag ions into the medium that may result in inaccurate pH readings).
K	☐	4. pH meter is calibrated daily or with each use. Records maintained.
K	☐	5. Effect of temperature has been compensated for by an ATC probe or by manual adjustment.
K	☐	6. A minimum of two standard buffer solutions (2 & 7) is used to calibrate the pH meter. Standard buffer solutions are used once and discarded.
K	☐	7. Electrode efficiency is determined daily or with each use following either slope or millivolt procedure.
K	☐	8. The balance provides a sensitivity of at least 0.1g at a load of 150 grams.
K	☐	9. The balance calibration is checked monthly using NIST Class S or ASTM Class 1or 2 weights or equivalent. Records maintained.
K	☐	10. Refrigerator temperature is maintained between 0 and 4˚C.
O	☐	11. Refrigerator temperature is monitored at least once daily. Record maintained.
K	☐	12. Freezer temperature is maintained at -20°C or below.
O	☐	13. Freezer temperature is monitored at least once daily. Record maintained.
O	☐	14. All glassware is clean.
O	☐	15. Once during each day of washing, several pieces of glassware from each batch washed are tested for residual detergent with aqueous 0.04% bromthymol blue solution. Records are maintained.

		1.4 Reagent and Reference Solution Preparation and Storage
C	☐	**1. Opened PSP reference stand solution (100 µg/ml) is not stored.**
K	☐	2. PSP working standard solution (1 µg/ml) and all dilutions are prepared with dilute HCl, pH 3 water, using 'Class A' volumetric glassware (flasks and pipettes) or prepared gravimetrically.
K	☐	3. Refrigerated storage of PSP working standard solution (1µg/ml) does not exceed 6 months and is checked gravimetrically for evaporation loss.
K	☐	4. PSP working dilutions are discarded after use.
K	☐	5. Make up water is distilled or deionized (*circle one*) and exceeds 0.5 megohm resistance or is less than 2 µ Siemens/cm conductivity at 25°C to be tested and recorded monthly for resistance or conductivity *(circle the appropriate)*.
O	☐	6. Make up water is analyzed for residual chlorine monthly and is at a nondetectable level (≤ 0.1 ppm). Records maintained.
K	☐	7. Make up water is free from trace (< 0.5 mg/l) dissolved metals specifically Cd, Cr, Cu, Ni, Pb, and Zn as determined annually with total heavy metal content ≤1.0 mg/l. Records maintained.
O		8. Makeup water contains < 1000 CFU/ml as determined monthly using the heterotrophic plate count method. Records maintained
		1.5 Collection and Transportation of Samples
O	☐	1. Shellstock are collected in clean, waterproof, puncture resistant containers.
K	☐	2. Samples are appropriately labeled with the collector's name, harvest area and time and date of collection.
K	☐	3. Immediately after collection, shellstock samples are placed in dry storage for transport (e.g. cooler) which is maintained between 0 and 10°C. Upon receipt at the lab, samples are placed under refrigeration.
K	☐	4. The time from collection to completion of the bioassay should not exceed 24 hours. However, if there are significant transportation delays, then shellstock samples are processed immediately as follows (*circle the appropriate choice*): a. Washed, shucked, drained, frozen until extracted; b. Washed, shucked, drained, homogenized and frozen; c. Washed, shucked, drained, extracted, the supernatant decanted and refrigerated (**best choice**); or d. The laboratory has an appropriate contingency plan in place to handle samples which can't be analyzed within 24 hours due to transportation issues.
K	☐	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 PSP TOXIN		
		2.1 Preparation of Sample
C	☐	**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.**
O	☐	2. The outside of the shell is thoroughly cleaned with fresh water.
O	☐	3. Shellstock are opened by cutting adductor muscles.
O	☐	4. The inside of the shell is rinsed with fresh water to remove sand or other foreign material.
O	☐	5. Shellfish meats are removed from the shell by separating adductor muscles and tissue connecting at the hinge.
K	☐	6. Damage to the body of the mollusk is minimized in the process of opening.
O	☐	7. Shucked shellfish are drained on a #10 mesh sieve (or equivalent) without layering for 5 minutes.

K	☐	8. Pieces of shell and drainage are discarded.
C	☐	**9. Drained meats or thawed homogenates are blended at high speed until homogenous** (60 - 120 seconds).
		2.2 Extraction
K	☐	1. 100 grams of homogenized sample is weighed into a beaker.
K	☐	2. An equal amount of 0.1 N/0.18 N HCl is added to the homogenate and thoroughly mixed (*circle the appropriate normality*).
C	☐	**3. pH is checked and, if necessary adjusted to between pH 2.0 and 4.0.**
C	☐	**4. Adjustment of pH is made by the dropwise addition of either the acid (5 N HCl) or base (0.1N NaOH) while constantly stirring the mixture.**
C	☐	**5. The homogenate/acid mixture is promptly brought to a boil, 100 ± 1°C, then gently boiled for 5 minutes**.
O	☐	6. The homogenate/acid mixture is boiled under adequate ventilation (i.e. fume hood).
O	☐	7. The extract is cooled to room temperature.
C	☐	**8. The pH of the extract is determined and adjusted, if necessary to between pH 2 and 4, preferably to pH 3 with the stirred dropwise addition of 5 N HCl to lower the pH or 0.1N NaOH to raise the pH.**
K	☐	9. The extract volume (or mass) is adjusted to 200 mls (or grams) with dilute HCl, pH 3 water.
K	☐	10. The extract is returned to the beaker, stirred to homogeneity and allowed to settle to remove particulates; or, if necessary, an aliquot of the stirred supernatant is centrifuged at 3,000 RPM for 5 minutes before injection.
K	☐	11. If mice cannot be injected immediately then the supernatant should be removed from the centrifuge tubes and refrigerated for up to 24 hours.
K	☐	12. Refrigerated extracts are allowed to reach ambient temperature before being bioassayed.
		2.3 Bioassay
O	☐	1. A 26-gauge hypodermic needle is used for injection.
K	☐	2. Healthy mice in the weight range of 17 -23 grams (19 - 21 grams preferable) from a stock colony are used for routine assays. Mice are not reused for bioassay. Stock strain used _____ Source of mice_____
C	☐	**3. Mice are allowed to acclimate for at least 24 hours prior to injection. In some cases up to 48 hours may be required.**
C	☐	**4. A conversion factor (CF) has been determined as _____. Month and year when current CF determined_____.**
C	☐	**5. CF value is checked weekly if assays are done on several days during the week, or, once each day that assays are performed if they are performed less than once per week.** **Date of most recent CF check _____** **CF verified/CF not verified** *(Circle appropriate choice)*
C	☐	**6. If the CF is not verified, 5 additional mice are injected with the dilution used in the CF check to complete a group of 10 mice. Ten additional mice are also injected with this dilution to produce a second group of 10 mice. The CF is calculated for each group of 10 mice and averaged to give the CF to be used in sample toxicity calculations for the day's or week's work only. All subsequent work must make use of the original laboratory CF value unless this value continues to fail to be verified by routine CF checks.**
C	☐	**7. If the CF fails to be verified, the cause is investigated and the situation corrected. If the cause cannot be determined with reasonable certainty and fails > 3 times per year, the bioassay**

		is restandardized.
O	☐	8. Mice are weighed to the nearest 0.5 gram.
C	☐	**9. Mice are injected intrapertioneally with 1 ml of the acid extract.**
K	☐	10. For the CF check, at least 5 mice are used.
C	☐	**11. At least 3 mice are used per sample in routine assays.**
C	☐	**12. Elapsed time is accurately determined and recorded.**
K	☐	13. If death occurs, the time of death to the nearest second is noted by the last gasping breath.
C	☐	**14. If median death time(2 out of 3 mice injected die) is < 5 minutes, a dilution is made with dilute HCl, pH 3 water, to obtain a median death time in the range of 5 to 7 minutes.**
		2.4 Calculation of Toxicity
C	☐	**1. The death time of each mouse is converted to mouse units (MU) using Sommer's Table (Table 6 *Recommended Procedures*, 4th edition). The death time of mice surviving beyond 60 minutes is considered to be < 0.875 MU.**
K	☐	2. A weight correction in MU is made for each mouse injected using Table 7 in *Recommended Procedures*, 4th edition.
C	☐	**3. The death time of each mouse in MU is multiplied by a weight correction in MU to give the corrected mouse unit (CMU) for each mouse.**
C	☐	**4. The median value of the array of corrected mouse units (CMU) is determined to give the median corrected mouse unit (MCMU).**
C	☐	**5. The concentration of toxin is determined by the formula, MCMU x CF X Dilution Factor X 200.**
C	☐	**6. Any value greater than 80µg/l00 grams of meat is actionable.**

REFERENCES

1. Adams, W.N. and S.A. Furfari. 1984. Evaluation of laboratory performance of the AOAC method for PSP toxin in shellfish. *J. Assoc. Off. Anal. Chem.* Vol 67, 6:1147-1148.
2. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
3. American Public Health Association. 1992. *Standard Method for the Examination of Dairy Products*, 16th Edition. APHA, Washington, D.C.
4. Association of Official Analytical Chemists International. 1990. *Methods of Analysis*, 15th Edition. AOAC, Arlington, VA.
5. APHA/WEF/AWWA. 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA, Washington, D.C.
6. Title 21, Code of Federal Regulations, Part 58, *Good Laboratory Practice for Nonclinical Laboratory Study*. U.S. Government Printing, Washington, D.C.
7. National Research Council. 1996. *Guide for the Care and Use of Laboratory Animals*. National Academy Press, Washington, D.C.
8. Personal communication with USFDA Washington Seafood Laboratory Branch, Office of Seafood, CFSAN, 1998-1999.

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

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
PARALYTIC SHELLFISH POISON 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 PSP Component

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

A. The total # of Critical nonconformities is $\geq$ **3 or**

B. The total # of Key nonconformities is $\geq$ **6 or**

C. The total # of Critical, Key and Other is $\geq$ **10**

2. **Provisionally Conforms Status**: The PSP component of this laboratory is determined to be provisionally conforming to NSSP requirements if the number of critical nonconformities is $\geq$ 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 Rev. 2005-08-19

National Shellfish Sanitation Program (NSSP) Guide for the Control of Molluscan Shellfish: 2011 Revision

Laboratory Evaluation Checklist - Analysis for NSP (Mouse Bioassay)

PUBLIC HEALTH SERVICE **U.S. FOOD AND DRUG ADMINISTRATION** **SHELLFISH PROGRAM IMPLEMENTATION BRANCH** **SHELLFISH SAFETY TEAM** **5100 PAINT BRANCH PARKWAY** **COLLEGE PARK, MD 20740-3835** **TEL. 301-436-2151/2147 FAX 301-436-2672**		
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: C- Critical K - Key O - Other NA- Not Applicable Conformity is noted by a "√"		

Weighted Code		Item Description
		Quality Assurance (QA) Plan
C	☐	**1. Written Plan adequately covers the following (check those that apply):** **1. a. Organization of the laboratory.** **2. b. Staff training requirements.** **3. c. Standard operating procedures.** **4. d. Internal quality control measures for equipment, calibration, maintenance, repair and performance.** **5. e. Laboratory safety.** **6. f. Internal performance assessment.** **7. g. External performance assessment.**
C	☐	2. QA Plan is implemented
		Work Area
O	☐	1. Adequate for workload and storage.
O	☐	2. Clean and well lighted.
O	☐	3. All work surfaces are nonporous and easily cleaned.
C	☐	4. A separate, quiet area with adequate temperature control is maintained for acclimation and injection of mice.
		Laboratory Equipment
K	☐	1. The differing sensitivities in weight measurements required by various steps in the extraction procedure as well as the bioassay are met by the balances being used. 1. a. To determine sample weight, a sensitivity of at least 0.1 g at load of 100 g is required. 2. b. To determine the weight of the lipid extract and its subsequent volume adjustment, a sensitivity of at least 10 mg at loads of 1 and 10 g is required. 3. c. To determine the weight of the mice used in the bioassay, a sensitivity of 0.1 g at a load of 20 g is required.
O	☐	2. The calibrations of the balances are checked monthly using NIST Class S or ASTM Class 1 or 2 weights or equivalent. Records are maintained.
K	☐	3. The temperature maintained by the refrigerator is between 0 and 5°C.
O	☐	4. Refrigerator temperature is monitored at least once daily. Temperatures are recorded and records are maintained.
		Reagents
K	☐	1. Concentrated (12N) HCl is used to acidify the homogenate.
O	☐	2. Reagent grade NaCl is used in the extraction procedure.
C	☐	3. Diethyl ether purified for lipid extraction is used for extracting lipids from the shellfish homogenates.
C	☐	4. Cottonseed oil (0.917 g/ml) or a solvent with a similar density (0.915 to 0.927 g/ml) is used as the toxin delivery system. Name of the solvent if substituted for cottonseed oil. _____ Specify density _____

		Collection and Transportation of Samples
O	☐	1. Shellstock are collected in clean, waterproof, puncture resistant containers.
K	☐	2. Samples are appropriately labeled with the collector's name, the harvest area and the time and date of collection.
K	☐	3. Immediately after collection, shellstock samples are placed in dry storage between 0 and 10°C until analyzed.
K	☐	4. Shellstock samples are analyzed within 24 hours of collection or refrigerated unshucked until analyzed.
K	☐	5. Refrigerated storage of shellstock does not exceed 48 hours.
K	☐	6. If shellstock is refrigerated, only live animals are used in the analysis.
K	☐	7. If shellfish are shucked in a location other than the laboratory, they must be prepared according to steps 1-9 in "Preparation of Sample" section below.
		Preparation of Sample
C	☐	1. At least 12 animals are used per sample.
O	☐	2. The outside of the shell is thoroughly cleaned with fresh water.
K	☐	3. Shellstock are opened by cutting the adductor muscles.
C	☐	4. Shell liquor is discarded.
O	☐	5. The inside of the shells is rinsed with fresh water to remove sand or other foreign material.
K	☐	6. Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
K	☐	7. Damage to the body of the mollusk is minimized in the process of opening.
K	☐	8. 100 - 150 grams of meat are collected or all the available sample if there is less than 100 grams.
O	☐	9. Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for 5 minutes.
K	☐	10. Pieces of shell and drainings are discarded.
C	☐	11. Drained meats are blended at high speed until homogenous (60-120 seconds).
C	☐	12. Shellfish homogenates are digested within 2 hours of blending.
		Digestion of Sample
K	☐	1. All glassware used is clean and properly washed with a succession of at least three fresh water rinses, and a final distilled/deionized rinse to remove residual detergent.
K	☐	2. 100 grams (or entire sample amount if less than 100 grams is available) of homogenized sample is weighted into a beaker.
C	☐	3. 1 ml of concentrated HCl and 5 g NaCl is added to the 100 gram homogenate and thoroughly mixed. (For samples <100 g, add reagents to obtain final concentrations of 0.12N HCl and 5% NaCl.)
C	☐	4. The homogenate is brought to a boil and once 100 ± 1°C (sea level) is reached, gently boil for 5 minutes.
O	☐	5. The beaker is covered with a watch glass or equivalent during boiling to prevent excessive evaporation.
O	☐	6. The homogenate is boiled under adequate ventilation (fume hood).
O	☐	7. The boiled, acidified homogenate is cooled to room temperature or below in a refrigerator or in an ice bath.
		Extraction
C	☐	1. All steps in the extraction procedure which involve any manipulation of diethyl ether are carried out under adequate ventilation.
C	☐	2. 100 ml of diethyl ether is added to the cooled, acidified homogenate in a stoppered centrifuge tube and shaken vigorously for 5 minutes.
O	☐	3. Centrifuge tubes are vented frequently while being shaken and before being centrifuged to avoid accidents.

C	☐	4. The content of the centrifuge tubes are centrifuged at 2000 rpm for 10 to 15 minutes.
C	☐	5. The clear upper ether phase is transferred to a large separatory funnel.
C	☐	6. The contents of the centrifuge tube are extracted three additional times for a total of four times, each time with 100 ml of diethyl ether. The upper phases are combined together in the separatory funnel (as in step 5).
C	☐	7. The ether extract is transferred to a large, clean, dry pre-weighed beaker (discard any emulsion or tissue that may have settled in the funnel.)
C	☐	8. Ether is evaporated to dryness.
C	☐	9. The final lipid residue is weighted and the weight is recorded.
		Bioassay
C	☐	1. The volume of the lipid residue is adjusted by weight to 10 ml (9.17 g) per 100 g shellfish extracted using cottonseed oil. If a solvent with a density similar to cottonseed oil is used, the volume is adjusted to a weight 10 times the density of the solvent. Specify the weight to which the volume is adjusted to _____.
K	☐	2. A 25 gauge hypodermic needle is used for injection.
C	☐	3. Healthy male mice in the weight range of 17 to 23 grams from a stock colony are used for routine assays. Stock strain used _____. Source of the mice _____
C	☐	4. Mice are allowed to acclimate for at least 24 hours prior to injection. In some cases up to 48 hours may be required. Typical length of the period of acclimation is _____.
O	☐	5. Mice are weighed to the nearest 0.1 gram.
C	☐	6. The extract is completely mixed before it is injected.
C	☐	7. Mice are injected intraperitoneally with 1 ml of the lipid extract.
C	☐	8. A total of 5 mice are injected with undiluted or diluted extract as appropriate per sample in routine assays. a. The extract is not diluted when all test/assay mice survive beyond 110 minutes of injection. b. The extract is diluted when 2 of 2 test mice or 3 of 5 assay mice survive for fewer than 110 minutes after injection c. When dilution is required, only dilutions which produce mean/median death times within 110 to 360 minutes of injection are used in the analysis.
C	☐	9. The time of completed injection is recorded.
C	☐	10. Mice are continuously observed for at least 6 hours (360 minutes).
C	☐	11. If death occurs within the period of continuous observation, the time of death to the nearest minute is noted by the last gasping breath.
K	☐	12. If mice survive the test, the time of death is recorded as ">" the period of continuous observation.
		Calculation of Toxicity
C	☐	1. The death time of each mouse is converted to mouse units (MU) using Table 8 in *Recommended Procedures*, 4th Edition.
O	☐	2. Table 8 is interpolated for death times between 110 and 360 minutes that are not listed in the Table.
K	☐	3. A weight correction in MU is made for each mouse injected using Table 8 in *Recommended Procedures*, 4th Edition.
O	☐	4. Table 8 is interpolated to accommodate weights which are not listed.
C	☐	5. The death time for each mouse in MU is multiplied by a weight correction in MU to give the corrected mouse unit (CMU) for each mouse.
C	☐	6. The mean corrected mouse unit of the array of corrected mouse units (CMU) is used when all the mice injected with diluted or undiluted extract die during the period of continuous observation.

C	☐	7. The median corrected mouse unit of the array of corrected mouse units (CMU) is used when at least one mouse either survives the test or dies.
C	☐	8. The concentration of toxin is determined by the formula: Mean or median CMU x Dilution Factor x 10.
C	☐	9. When the time of death is known for certain for all mice injected, toxicity is determinate and the toxin concentration is reported as the number of mouse units per 100 grams of sample.

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

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
NEUROTOXIC SHELLFISH POISON COMPONENT:	
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 NSP Component

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

A. The total # of Critical nonconformities is **≥ 3 or**

B. The total # of Key nonconformities is **≥ 6 or**

C. The total # of Critical, Key and Other is **≥ 10**

2. **Provisionally Conforms Status**: The NSP 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 Analysis for NSP (Mouse Bioassay) 2005-08-19