Proposal for Task Force Consideration at the 2009 Biennial Meeting Interstate Shellfish Sanitation Conference	☒ **Growing Area** ☐ **Harvesting/Handling/Distribution** ☐ **Administrative**
Name of Submitter:	John Karolus
Affiliation:	Northeast Laboratory Evaluation Officers and Managers (NELEOM)
Address:	Connecticut Department of Agriculture Bureau of Aquaculture PO Box 97 Milford, CT 06460
Phone: **Fax:** **Email:**	203-874-0696 203-783-9976 jjkarolus@snet.net
Proposal Subject:	Method to determine the presence of Male Specific Coliphage in shellfish meats and the Microbiology Checklist for Male-specific Coliphage (MSC)
Specific NSSP Guide Reference:	ISSC Constitution, ByLaws, and Procedures Procedure XVI, Procedure for acceptance and approval of analytical methods for the NSSP. Section IV, Chapter II Growing Areas, .11 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists.
Text of Proposal/ Requested Action	The laboratory procedure is based on the methods described in Burkhardt, W., III, W.D. Watkins, and S.R. Rippey. 1992. Seasonal effects on accumulation of microbial indicator organisms by *Mercenaria mercenaria*. Appl. Environ. Microbiol. 58:826-831; DeBartolomeis, J. and Cabelli, V.J. 1991. Evaluation of an *Escherichia coli* host strain for enumeration of F male specific bacteriophages. Appl. Environ. Microbiol. 57: 1301-1305; Burkhardt, W. III *Enumeration of Male-specific Bacteriophage in water and shellfish tissue.* 2004. Gulf Coast Seafood Laboratory, Office of Seafood, U.S. Food and Drug Administration, Dauphin Island, AL. 31 pg. The laboratory procedure is to be reviewed by the Laboratory Methods Review Committee for consideration as a Type IV Method according to Procedure XVI. The Laboratory Evaluation Checklist - Pages 2, 16, 17, and 18, Microbiology of the Guidance Documents, Chapter II. Growing Areas, .11 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists is attached. It includes a section for the Male-specific Coliphage (MSC). MSC is an important microorganism for monitoring the microbial quality of waters (e.g., sewage treatment, growing area, etc.).
Public Health Significance:	FDA is submitting a proposal to ISSC to allow MSC to be used as a re-opening criterion in cases where unexpected, unusual sewage contamination occurs that may have impacted shellfish harvest areas (not for conditional re-openings). State Laboratory Managers and Laboratory Evaluation Officers need this document to correctly perform the analysis and to evaluate any laboratory performing the Coliphage (Bacteriophage) procedure.
Cost Information (if available):	None
Action by 2005 Laboratory Quality Assurance Committee	Recommended referral of Proposal 05-113 to the appropriate committee as determined by the Conference Chairman.

Action by 2005 Task Force I	Recommended adoption of the Laboratory Quality Assurance Committee recommendation on Proposal 05-113.
Action by 2005 General Assembly	Adopted recommendation of 2005 Task Force I.
Action by USFDA	Concurred with Conference action.
Action by 2007 Laboratory Methods Review Committee	Recommended no action on Proposal 05-113. Rationale - The "no action" on Proposal 05-114 eliminated the need for checklist adoption. The submitter will submit the checklist with the data for method approval to the Executive Office for Conference approval consistent with Procedure XVI.
Action by 2007 Task Force I	Recommended adoption of the Laboratory Methods Review Committee recommendation of no action on Proposal 05-113.
Action by 2007 General Assembly	Adopted recommendation of 2007 Task Force I.
Action by USFDA	December 20, 2007 Concurred with Conference action with the following comments and recommendations for ISSC consideration. The Conference has made considerable progress in its efforts to recognize new and developing analytical methods for the detection of indicators, pathogens, and marine toxins. Much credit goes to the Laboratory Methods Review Committee and its leadership for ensuring a scientifically defensible process for adopting analytical methods under the NSSP. At the 2007 meeting numerous analytical methods were proposed for ISSC adoption. However, many of these methods were lacking the validation and associated data needed by the Laboratory Methods Review Committee to make a final determination regarding their efficacy for use in the NSSP. As a result the General Assembly voted "No Action" on analytical method Proposals 05-107, 05-108, 05-109, 05-111, 05-113, and 05-114. It is FDA's understanding that the intent of the "No Action" vote was not to remove these Proposals from ISSC deliberation as "No Action" normally suggests, but rather to maintain them before the Conference pending submission of additional data for further consideration. The Voting Delegates, by requesting the Proposal submitters provide additional data to the Executive Office for methods approval consistent with Procedure XVI, clearly recognized the importance and utility of these methods and intended to maintain them before the Conference for possible adoption following additional data submission. FDA requests that the ISSC Executive Board confirm FDA's understanding of this outcome. FDA fully supports such a Conference action and encourages the Executive Office to pursue submission of additional data as necessary to move forward with acceptance of these methods.

.11 – Laboratory Evaluation Checklist – Microbiology - 2

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]		
	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 Bacteriophage for Shellfish Meats [PART III]		
PART 1 – QUALITY ASSURANCE			
CODE	**REF**	**ITEM**	
K	8, 11	**Quality Assurance 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	8		2. QA Plan Implemented
K	11		3. 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. Pipet aid used, mouth pipetting not permitted.
CODE	**REF.**	**Bacteriological Examination of Shellfish by Male-specific Bacteriophage Equipment & Supplies**	
			SEE PAGE 3, 4 & 5 FOR RELEVENT EQUIPMENT ITEMS.
K	**31**		**1. Sample containers are sterile, made of glass or some other inert material (i.e., polypropylene), hold 100-125 mL, and treated with sodium thiosulfate.**
C	**27,28,29,30**		**2. The refrigerated centrifuge must have the capacity to accommodate the amount of shellfish samples required for procedure, perform at 9000 x G, and maintain a temperature of 4°C ± 1°C.**
C	**27,28,29,30**		**3. The water bath must be able to maintain 44-46°C and 50-52°C temperature ranges.**
K	**9**		**4. The level of water in the water bath covers the level of liquid and agar in the containers and culture tubes.**
K	**13**		**5. Working thermometers are tagged with identification, date of calibration, calibrated temperature and correction factor.**
K	**4**		**6. All working thermometers are appropriately immersed.**
K	**11**		**7. A standards thermometer has been calibrated by NIST or one of**

			equivalent accuracy at the points -20°, 0°, 35°, 44.5°C, 50° and 121°C. Calibration records maintained.
K	9		8. Standards thermometer is checked annually for accuracy by ice point determination. Results recorded and maintained. Date of most recent determination _____.
K	13		9. Incubator, freezer, refrigerator, autoclave and water bath working thermometers are checked annually against the standards thermometer at the temperatures at which they are used. Records maintained.
C	32		10. Sterile 0.22 or 0.45µm pore size filters are used to prepare the antibiotic solutions using sterile disposable syringes. Check sterility of each lot.
K	27,28,29,30, 31		11. Pre-sterilized plastic or sterile glass syringes are used to filter sterilize the stock antibiotic solution. Check sterility of each lot.
K	31		12. Colonies are counted with the aid of magnification or light box device.
C	32		13. Balance provides a sensitivity of at least 0.01 g.
C	31		14. The temperature of the incubator is maintained at 35-37°C.
K	27,28,29		15. Reusable or disposable pipets-pipettors are used and sterility is checked with each lot.
K	2727,28,29		16. Sterile disposable 15 and 50 mL centrifuge tubes are used and sterility is checked with each lot.
		Media Preparation and Storage	
			SEE PAGES 5 & 6 FOR RELEVENT MEDIA PREPARATION AND STORAGE ITEMS.
K	27,28,29		1. Media is prepared from individual components.
K	27,28,29		2. Media is prepared and sterilized according to the method procedure.
C	27,28,29		3. Streptomycin/ Ampicillin solution is added after the autoclaved bottom agar has tempered to 44 – 46 ° C.
O	27,28,29		4. Storage of MSB bottom agar under refrigeration does not exceed 1 month.
O	27,28,29		5. Unsterilized DS soft agar is stored in a – 20° C freezer for up to 1 month
K	27,28,29		6. The DS soft agar is removed from the freezer and sterilized for 15 minutes at 121° C before use.
O	27,28,29		7. 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.
C	27,28,29		8. Host *stock E. coli* F_{amp} is ATCC 700609.
K	27,28,29		9. The host stock used for growth broth host cells is less than 1 week old.
O	27,28,29		10. Media is warmed to room temperature before use.
		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 12 shellstock are used for analysis.
K	2, 19		11. The sample is weighed to the nearest 0.1 gram
C	9		12. Samples are blended at high speed for 60 seconds.
K	9		13. For other than shellstock, APHA *Recommended Procedures* is followed for the examination of freshly shucked and frozen shellfish meats.
		Sample Analysis	
C	27,28,29		Samples are analyzed according to the approved method.
K	27,28,29		Growth Broth is tempered to 35 – 37° C and vortexed (or shaken) to aerate prior to inoculation
K	27,28,29		Several host cell colonies are transferred to a tube of growth broth to provide log phase growth host cells for sample procedure.
C	27,28,29		Growth broth with host cells is incubated 35 – 37° C for 4 to 6 hours to provide culture in log phase growth.
C	27,28,29		The host cell growth broth is not shaken.
O	27,28,29		At least 30 to 50 grams of blended shellfish meat is weighed into sterile centrifuge tubes; weight is recorded .
C	27,28,29		The blended shellfish meat is centrifuged for 15 minutes at 9000 x g at 4° C.
K	27,28,29		Only supernatant is pipetted off and weight recorded.
K	27,28,29		Supernatant is allowed to warm to room temperature – 20 to 30 minutes.
K	27,28,29		The autoclaved DS soft agar is tempered and held at 50 – 52° C throughout sample procedure.
K	27,28,29		The supernatant is shaken or vortexed before adding to DS soft agar.
K	27,28,29		At least, a total of 7.5 ml of shellfish meat supernatant are plated.
C	27,28,29		2.5 ml of sample are added to 2.5 ml of DS soft agar and 0.2 ml of log phase host cell in growth broth while in the tempering waterbath.
C	27,28,29		DS soft agar/sample/host cell mixture is gently rolled between palms to mix.
C	27,28,29		The soft agar mixture is overlaid bottom agar and swirled gently to distribute.
K	27,28,29		Negative and positive control plates accompany samples.
K	27,28,29		Growth broth is used for negative (blank) control plates.
K	27,28,29		MS2 male specific bacteriophage is used as the positive control.
K	27,28,29		A negative control plate is the first plate and the last plate.
K	27,28,29		The positive control plate is set up after all samples and just before the final negative plate.
C	27,28,29		All plates are incubated at 35 – 37° C for 16 to 20 hours.
		Computation of Results	
C	31		1. Circular zones of clearing (of any diameter) in lawn of host bacteria are plaques.
C	32		2. The desired range of 30 to 300 PFU per plate. If the count exceeds the upper range or if the plaques are not discrete, results should be recorded as *too numerous to count* (TNTC).
K	27		3. The equation used is:

			$\text{PFU/100grams} = \frac{\text{Avg of plate counts}}{\text{ml analyzed/plate}} \times \frac{\text{grams of homogenate}}{\text{grams of supernate}} \times 100$
O	**9**		**2. Round off at the end of your computation using the information in *Recommended Procedures for the Examination For Sea Water and Shellfish.***
K	**27**		**4. Results are reported as PFU/ 100 g for shellfish samples.**

REFERENCES	
1.	*Compendium of Methods for the Microbiological Examination of Foods*, 2nd Edition, APHA. 1984.
2.	Good Laboratory Practice.
3.	"Interim Guides for the Depuration of the Northern Quahog, *Mercenaria mercenaria*, Northeast Marine Health Sciences Laboratory, North Kingstown, RI. 1968.
4.	NBS *Monograph 150*, U.S. Department of Commerce, Washington, D.C. 1976.
5.	*Official Methods of Analyses of the Association of Official Analytical Chemists*, 17th Edition, 2000. Chapter 17.305, page 22.
6.	*Proceedings of the 8th National Shellfish Sanitation Workshop.* 1974.
7.	Public Health Service, *Public Health Report,* Reprint *#1621*. 1947.
8.	*Quality Assurance Principles for Analytical Laboratories*, Association of Official Analytical Chemists. 1991.
9.	*Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition, American Public Health Association. 1970.
10.	Shellfish Sanitation Interpretation #SS-39, Interstate Shellfish Sanitation Conference, 1986.
11.	*Standard Methods for the Examination of Water and Wastewater*, 18th Edition, APHA/WEF/AWWA. 1992.
12.	Title 21, Code of Federal Regulations, Part 58, "Good Laboratory Practice for Nonclinical Laboratory Study", Washington, D.C.
13.	*Standard Methods for the Examination of Dairy Products*, 16th Edition, APHA. 1992.
14.	Fisher, J. 1985. "Measurement of pH". *American Laboratory*. 16:54-60.
15.	Consult pH electrode product literature.
16.	AOAC Methods Validation and Technical Programs – Criteria for Laboratories Performing Food Testing. 1999
17.	*Handbook for Evaluating Water Bacteriological Laboratories*. 1975. US EPA, 670/9-75-006.
18.	Adams, W.N., 1974. NETSU. Personal communication to Dr. Wallace Andrews, FDA.
19.	*Bacteriological Analytical Manual*. 1995. FDA, 8th Edition, AOAC, Arlington,VA.
20.	*NSSP Guide to the Control of Molluscan Shellfish*. 1997. FDA/ISSC.
21.	*Microbiological Methods for Monitoring the Environment, Water and Wastes*. 1978. US EPA, EPA/600/8/78/017.
22.	Furfari, Santo. March 21, 1972. Personal Communication to Dan Hunt, FDA.
27.	Burkhardt, W. III *Enumeration of Male-specific Bacteriophage in water and shellfish tissue*, 2004. Gulf Coast Seafood Laboratory, Office of Seafood, U.S. Food and Drug Administration (or just FDA), Dauphin Island, AL. 31 pg.
28.	Burkhardt, W., III, W.D. Watkins, and S.R. Rippey. 1992. Seasonal effects on accumulation of microbial indicator organisms by Mercenaria mercenaria. Appl. Environ. Microbiol. 58:826-831.
29.	Cabelli, V.J. 1988. Microbial indicator levels in shellfish, water, and sediments from the upper Narragansett Bay conditional shellfish-growing area. *Report to the Narragansett Bay Project*, Providence, RI.
30.	DeBartolomeis, J. and Cabelli, V.J. 1991. Evaluation of an *Escherichia coli* host strain for enumeration of F male-specific bacteriophages. Appl. Environ. Microbiol. 57:1301-1305.
28.	United States Environmental Protection Agency, *Method 1601: Male-specific (F+) and Somatic Coliphage in Water by Two-step Enrichment Procedure,* EPA 821-R-01-030, EPA, Washington, DC, April 2001.
29	United States Environmental Protection Agency, *USEPA Manual of Methods for Virology*, Chapter 16, EPA 600/4-84/013 (N16), Washington DC, June 2001.

NSSP Form LAB-100 rev. 2005-02-18