ISSC

Interstate Shellfish Sanitation Conference

SUMMARY OF ACTIONS

2009 Biennial Meeting

Manchester, New Hampshire
October 17 – 23, 2009
Submitted to FDA November 20, 2009

ISSC 2009 SUMMARY OF ACTIONS

Proposal Subject: *Vibrio vulnificus* Risk Management Plan for Oysters exemption for licensed shellfish harvesters and certified dealers who produce fewer than 1.5 million raw oysters per year and/or sell all of their oysters directly to retailers.

Specific NSSP Guide Reference: Section II, Chapter II Risk Assessment and Risk Management @.04 *Vibrio vulnificus* Risk Management for Oysters, New B.

Text of Proposal/ Requested Action Add a new section; Section II, Chapter II Risk Assessment and Risk Management @.04 B. *Vibrio vulnificus* Risk Management for Oysters.

A. For states having 2 or more etiologically confirmed shellfish-borne *Vibrio vulnificus* illnesses since 1995 traced to the consumption of commercially harvested raw or undercooked oysters that originated from the waters of that state (Source State), the Authority shall develop and implement *a Vibrio vulnificus* Management Plan.

B. **Exemptions. This section does not apply to licensed shellfish harvesters and certified shellfish dealers who produce fewer than 1.5 million raw oysters per year and/or sell all of their oysters directly to retailers.**

~~B.~~ **C.** The Source State's *Vibrio vulnificus* Management Plan shall define the administrative procedures and resources necessary to accomplish (i.e. establish and maintain) involvement by the state in a collective illness reduction program. The goal of the *Vibrio vulnificus* Management Plan will be to reduce the rate of etiologically confirmed shellfish-borne *Vibrio vulnificus* septicemia illnesses reported collectively by California, Florida, Louisiana, and Texas, from the consumption of commercially harvested raw or undercooked oysters by 40 percent for years 2005 and 2006 (average) and by 60 percent for years 2007 and 2008 (average) from the average illness rate for the years 1995 -1999 of 0.303/million. The list of states (California, Florida, Louisiana, Texas) used to calculate rate reduction may be adjusted if after a thorough review, epidemiological and statistical data demonstrates that it would be appropriate. The illness rate shall be calculated as the number of illnesses per unit of population. The goal may be reevaluated prior to the year 2006 and adjusted in the event that new science, data, or information becomes available. State's compliance with the Plan will require States to maintain a minimum of 60% reduction in years subsequent to 2008. Determination and compliance after 2008 will be based on two-year averages beginning in 2009.

Public Health Significance: The *Vibrio vulnificus* Risk Management Plan for Oysters was introduced to the ISSC as being modeled after the U.S. Egg Safety Action Plan. The NSSP which has been in existence since 1925 is far more restrictive than FDA's October 2004 proposed rule for Egg Safety and the Prevention of Salmonella Enteritidis in Shell Eggs During Production and certain egg producers.

The most recent data from the Centers for Disease Control and Prevention (CDC) show that SE illnesses have essentially remained steady for the past several years. CDC estimated that 118,000 illnesses were caused by consumption of SE-contaminated eggs in 2001. Accordingly, FDA believes that further actions to improve egg safety–building upon the safe consumer handling labeling and egg refrigeration at retail rule of 2000–are the most effective way to achieve our public health goals of a 50% reduction in overall salmonellosis and a 50% reduction in SE outbreaks by 2010.

In comparison to an annual average of less than 40 *V.v.* infections to high-risk consumers that are attributed to shellfish, approximately half of those persons infected die, there are approximately 40,000 cases of salmonellosis reported in the United States annually. Because many milder S.E.cases are not diagnosed or reported, the actual number of S.E. infections may be thirty or more times greater. It is estimated that approximately 600 persons die each year with acute salmonellosis. Just as with *V.v.* infections, Salmonellosis infections are more common in the summer than winter. Young children, the elderly, and the immunocompromised are the most likely to have severe S.E. infections.

Since the FDA has proposed a rule that exempts certain egg producers from the rule and t rule is far less burdensome to the egg industry than the *Vibrio vulnificus* Risk Manageme Plan for Oysters is on the Gulf oyster industry, an exemption should be given to oyst producers as suggested.

Cost Information (if available): None

Action by 2005 Task Force I Recommended referral of Proposal 05-100 to the appropriate committee as determined by the Conference Chairperson.

Action by 2005 General Assembly Adopted recommendation of 2005 Task Force I.

Action by USFDA Concurred with Conference action.

Action by 2007 Vibrio Management Committee Recommended adoption of Proposal 05-100 as a research need. More data is needed on the number of small harvesters and the number of small dealers; the percentage of all harvesters and dealers in the affected states that are in this category; the number of illnesses attributable to these small harvesters or dealers; other food commodities that allow exemptions from public health requirements based on the small size of the harvester/producer/processor; and the pathogens of concern with these other foods.

Action by 2007 Task Force I Recommended adoption of the Vibrio Management Committee recommendation on Proposal 05-100.

Action by 2007 General Assembly Adopted recommendation of 2007 Task Force I.

Action by USFDA December 20, 2007
Concurred with Conference action.

Action by 2009 Research Guidance Committee Recommended no action. Rationale: No data presented.

Action by 2009 Task Force I Recommended referral of Proposal 05-100 to the Executive Board. The Task Force stongly urges the Executive Board to identify approaches to gather the information necessary for further deliberation of the issue.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force I on Proposal 05-100.

Action by Executive Board 10/23/2009	Approved referral of Proposal 05-100 to the *Vibrio* Management Committee. The *Vibrio* Management Committee will be asked to hold a conference call within the next 30 days to identify the types of information needed and who best can acquire that data.

Proposal Subject: Re-Opening Criteria Based on New Indicator of Sewage-Borne Viral Pathogens

Specific NSSP Guide Reference: NSSP Guide Model Ordinance Section II, Chapter IV. Shellstock Growing Areas @ .03 Growing Area Classification A. General (5) Status of Growing Areas I Reopened Status

Text of Proposal/ Requested Action

I Reopened Status. A growing area temporarily placed in the closed status as provided in (b) above, shall be returned to the open status only when:

(i) The emergency situation or condition has returned to normal and sufficient time has elapsed to allow the shellstock to reduce pathogens or poisonous or deleterious substances that may be present in the shellstock to acceptable levels. Studies establishing sufficient elapsed time shall document the interval necessary for reduction of contaminant levels in the shellstock to pre-closure levels. In addressing pathogen concerns, the study may establish criteria for reopening based on coliform levels in the water~~; or~~ **and;**

(ii) For emergency closures (not applicable for conditional closures) of harvest areas caused by the occurrence of sewage contamination events, such as sewage collection system failures, the analytical sample results shall not exceed background levels or a level of 50 male-specific upernata per 100 grams from shellfish samples collected no sooner than 7 days after contamination has ceased and from representative locations in each growing area potentially impacted; or

~~(ii)~~ **(iii)** The requirements for biotoxins or conditional area management plans as established in §.04 and §.03, respectively, are met; and

~~(iii)~~ **(iv)** Supporting information is documented by a written record in the central file.

NOTE: An analytical method for shellfish meats and a draft laboratory checklist are separately proposed to enable the use of the new, optional re-opening criteria.

Public Health Significance:

The absence of bacterial pathogens such as *Salmonella* species can be reliably determined using the coliform bacterial indicators of the National Shellfish Sanitation Program (NSSP). However, when growing areas are implicated as the source of shellfish causing illness consistent with viral etiology, the NSSP requires closure for a minimum of 21 days.[1] That is because viruses and bacteria persist differently in growing waters and in shellfish[2,3,4,5], it takes considerably longer for shellfish to eliminate viruses[2,3,4,5], and the coliform bacterial indicators of contamination currently stipulated in the NSSP do not index risks from enteric viral pathogens very well[3,6,7]. This means that if open harvest areas become unexpectedly contaminated, the likelihood exists that viral pathogens may remain viable in shellfish long after growing waters appear safe according to the NSSP bacteriological criteria. Recognizing these facts, and lacking an alternative viral indicator or any other reasonable way to judge, the NSSP has stipulated 3 weeks as the criterion for achieving safe shellfish when viral pathogens are known or suspected to be involved.[1]

The NSSP needs an alternative viral indicator. Coliform bacteria do not reliably reflect the presence or absence of viral pathogens such as Noroviruses and hepatitis A[2,3,6]. Events such as sewage spills and bypasses, sewer pipe breaks, sewage pumping and lift station leaks, and wastewater treatment plant failures can heavily contaminate shellfish areas, at

least temporarily. Every harvest area even remotely impacted by sewage effluent is at risk for a lengthy closure if and when a mishap in the sewage collection system or at the sewage treatment plant occurs. Already some State Shellfish Control Authorities (SSCA) have issued 3 week emergency closures to harvest areas after power failures disrupted sewage treatment and following pipe ruptures and sewage spills. When such contamination events occur, there currently is no choice. In the absence of an alternative viral indicator, there exists no way under the NSSP to judge whether a lengthy closure can be avoided. Logically, if the NSSP had an indicator that better resembled the enteric viral pathogens of concern, then lengthy closures might be averted.

Male-specific □upernata:
Studies[4,7,8,9,10] demonstrate that a group of bacterial viruses called male-specific □upernata (MSC) appear to be good candidates as an alternative viral indicator in the NSSP. That is, MSC appear to be conservative indicators of sewage-borne enteric viral pathogens in shellfish and can be relied upon to indicate the virtual absence of these viral pathogens when derived from sewage. MSC occur universally in sewage in large numbers[6]. They only replicate in F+ (palliated) *E. coli* cells but do not reproduce below 30°C[5,,11,12]. Quantitative analysis for MSC is easy, inexpensive, and takes only 18-24 hours[13,14]. MSC persist in waters and in shellfish much like the infectious hepatitis and Norwalk-like viruses of concern to the NSSP[2,3,4,8]. The physical size and shape of most MSC closely resemble those of these pathogens as well, and they all contain RNA as their genetic material[5]. More importantly, studies further show that when shellfish are contaminated with sewage, male-specific □upernata provide a better measure of the potential presence of enteric viruses than do coliform bacteria[4,8].

To establish an alternative safety standard for shellfish based on MSC, a quantitative relationship between measurable levels of the indicator and the absence of viral pathogens is needed. Researchers in the United Kingdom studied shellfish harvested over a 2 year period, and their findings[7] show that when mean levels of F+ RNA bacteriophage remained below 50 per 100 grams; Norwalk-like viruses were not detected in any samples. Conversely, when the mean level of MSC exceeded 125 per 100 gram, 37% of the same shellfish samples were positive for enteric viruses. These data provide a scientific basis for establishing a level of MSC, one that is readily detectable and that provides a measurable indication that the sewage contamination levels in shellfish are below the threshold for containing enteric viral pathogens. Applied as an indicator following sewage contamination events in the

U.S., if the levels of MSC are found below 50 per 100 grams throughout the harvest area, then those shellfish should be as safe from sewage-borne enteric viral pathogens as they are under normal conditions.

Limitations of MSC:
Though abundant in sewage, sewerage collections systems, and most wastewater effluents, the MSC group is not reliably detected in fresh human waste, small point sources, vessel discharges, and vomit, all of which can transmit viral pathogens.[5,10] Therefore, it is not proposed as an index of enteric viral pathogens from these sources. Nonetheless, MSC are a useful alternative indicator for signaling the presence of sewage contamination in shellfish, and provide a science-based means for determining whether shellfish areas are safe from viral pathogens, such as Noroviruses and hepatitis A viruses, following sewage contamination events.

Proposal of Male-specific Coliphage as an Indicator:
It is proposed that MSC can, at the discretion of the SSCA, be used as an alternative to the

3 week period for re-opening shellfish areas to harvest activities after a sewage contamination event causes emergency closure. This re-opening option is based upon analytical results from shellfish samples collected at least 7 days after contamination has ceased and from representative locations in the growing area, whereby no samples exceed background levels or a level of 50 MSC per 100 grams.

Benefits:
The MSC option for re-opening could decrease currently practiced closure periods by as many as 13 days.
References:
[1] NSSP Model Ordinance, Chapter II @.01 H (2).
[2] Sobsey, M.D., A.L. Davis, and V.A. Rullman. 1987. Persistence of hepatitis A virus and other viruses in depurated eastern oysters. Proc. Oceans '87, Halifax, Nova Scotia. 5:1740-1745.
[3] Richards, G.P. 1988. Microbial purification of shellfish: a review of depuration and relaying. J. Food Prot. 51:218-251.
[4] Burkhardt, W., III, S.R. Rippey, and W.D. Watkins. 1992. Depuration rates of northern quahogs, *Mercenaria mercenaria* (Linnaeus, 1758), and eastern oysters, *Crassostrea virginica* (Gmelin, 1791), in ozone- and ultraviolet light-disinfected seawater systems. J. Shellfish Res. 11:105-109.
[5] Dore, W.J. and D.N. Lees. 1995. Behavior of *Escherichia coli* and male-specific bacteriophage in environmentally contaminated bivalve □upernat before and after depuration**.** Appl. Environ. Microbiol. 61: 2830-2834.
[6] Goyal, S.M., C.P. Gerba, and G. Britton (Eds). 1987. Phage Ecology. John Wiley & Sons, New York.
[7] Dore, W.J., K. Henshilwood, and D.N. Lees. 2000. Evaluation of F-Specific RNA Bacteriophage as a candidate human enteric virus indicator for bivalve molluscan shellfish. Appl. Environ. Microbiol. 66(4):1280-1285.
[8] 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.
[9] 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.
[10] Calci, K.R., W. Burkhardt III, W.D. Watkins, and S.R. Rippey. 1998. Occurrence of male-specific bacteriophage in feral and domestic animal waste, human feces, and human-associated wastes. Appl. Environ. Microbiol. 64:5027-5029.
[11] Novotny, C. P. and K. Lavin. 1971. Some effects of temperature on the growth of F pili. J. Bacteriology. 107: 671- 682.
[12] Woody, M.A. and D.O. Cliver. 1995. Effects of temperature and host cell growth phase on replication of F-specific RNA □upernata Qβ. Appl. Environ. Microbiol. 61:1520-1526.
[13] 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.
[14] Method 1601: Male-specific (F+) and Somatic Coliphage in Water by Two-step Enrichment Procedure, USEPA, EPA 821-R-01-030, April 2001.

Cost Information (if available):

The proposal provides optional re-opening criteria that are not required. Therefore, there are no added costs to State programs or industry. However, State laboratories that do not have requisite equipment already would incur such costs if the State chooses to implement the optional re-opening criteria described.

Action by 2005 Task Force I Recommended referral of Proposal 05-105 to the appropriate committee as determined by the Conference Chairperson.

Action by 2005 General Assembly Adopted recommendation of 2005 Task Force I.

Action by USFDA Concurred with Conference action.

Action by 2007 Growing Area Classification Committee Recommended referral of Proposal 05-105 to the appropriate Committee as determined by the Conference Chairman. Additionally, the Committee recommended the Executive Board establish a work group to evaluate the appropriateness of MSC as a viral indicator, and to identify appropriate applications. The workgroup should report its findings at the March 2008 Board meeting.

Action by 2007 Task Force I Recommended adoption of the Growing Area Classification Committee recommendation on Proposal 05-105.

Action by 2007 General Assembly Adopted recommendation of 2007 Task Force I.

Action by USFDA December 20, 2007
Concurred with Conference action.

Action by 2009 Growing Area Classification Committee Recommended adoption of Proposal 05-105 as amended.

I Reopened Status. A growing area temporarily placed in the closed status as provided in (b) above, shall be returned to the open status only when:

(i) The emergency situation or condition has returned to normal and sufficient time has elapsed to allow the shellstock to reduce pathogens or poisonous or deleterious substances that may be present in the shellstock to acceptable levels. Studies establishing sufficient elapsed time shall document the interval necessary for reduction of contaminant levels in the shellstock to pre-closure levels. In addressing pathogen concerns, the study may establish criteria for reopening based on coliform levels in the water or;

(ii) For emergency closures (not applicable for conditional closures) of harvest areas caused by the occurrence of ~~sewage contamination events, such as sewage collection system failures~~ raw untreated sewage discharged from a large community sewage collection system or wastewater treatment plant, the analytical sample results shall not exceed background levels or a level of 50 male-specific □upernata per 100 grams from shellfish samples collected no sooner than 7 days after contamination has ceased and from representative locations in each growing area potentially impacted; or

(iii) The requirements for biotoxins or conditional area management plans as established in §.04 and §.03, respectively, are met; and

(iv) Supporting information is documented by a written record in the central file.

Add the following definition in Section II.B "Male-specific Coliphage are a group of bacterial viruses that infect and lyse E. coli Famp and produce plaques within 18±2 hours at 35-37±0.5°C".

NOTE: An analytical method for shellfish meats and a draft laboratory checklist are separately proposed to enable the use of this new, optional re-opening criteria.

The committee further encourages continued effort by the ISSC members to further develop applications for use of the Male Specific Coliphage and requests that any information on the results of the use of this method be referred to the appropriate committee as determined by the Conference Chairman.

Action by 2009 Task Force I

Recommended adoption of Proposal 05-105 as amended.

I Reopened Status. A growing area temporarily placed in the closed status as provided in (b) above, shall be returned to the open status only when:

(i) The emergency situation or condition has returned to normal and sufficient time has elapsed to allow the shellstock to reduce pathogens or poisonous or deleterious substances that may be present in the shellstock to acceptable levels. Studies establishing sufficient elapsed time shall document the interval necessary for reduction of contaminant levels in the shellstock to pre-closure levels. In addressing pathogen concerns, the study may establish criteria for reopening based on coliform levels in the water or;

(ii) For emergency closures (not applicable for conditional closures) of harvest areas caused by the occurrence of raw untreated sewage discharged from a large community sewage collection system or wastewater treatment plant, the analytical sample results shall not exceed background levels or a level of 50 male-specific □upernata per 100 grams from shellfish samples collected no sooner than 7 days after contamination has ceased and from representative locations in each growing area potentially impacted; or

Note: The ISSC and USFDA should discourage the use of this standard for use as a Market standard or for use in Water Quality Classification

(iii) The requirements for biotoxins or conditional area management plans as established in §.04 and §.03, respectively, are met; and

(iv) Supporting information is documented by a written record in the central file.

Add the following definition in Section II.B "Male-specific Coliphage are a group of bacterial viruses that infect and lyse E. coli Famp and produce plaques within 18±2 hours at 35-37±0.5°C".

NOTE: An analytical method for shellfish meats and a draft laboratory checklist are separately proposed to enable the use of the new, optional re-opening criteria.

Action by 2009 General Assembly

Adopted recommendation of 2009 Task Force I on Proposal 05-105.

Proposal Subject: Real Time PCR Methods for Determining Levels of *V. parahaemolyticus* and *V. vulnificus*

Specific NSSP Guide Reference: Section IV Guidance Document, Chapter II, Growing Areas .10 Approved National Shellfish Sanitation Program Laboratory Tests: Microbiological and Biotoxin Analytical Methods

Text of Proposal/ Requested Action

Real time PCR methods provide additional options to currently used methods for identification of *V. parahaemolyticus* and *V. vulnificus* in shellfish. The use of real time PCR could reduce the time and cost of analysis while providing more reliability for detection and identification of these organisms in the environment as well as in Post Harvest Processed (PHP) products. The following methods are submitted for consideration, under Procedure XVI, by the ISSC Laboratory Methods Review Committee for identification and characterization of suspect bacterial isolates and direct analysis of APW enrichments from MPN analysis.

Real time PCR assays for total V. vulnificus (SYBR green and taqman) developed at the University of Florida (2,3,4) and at the University of Alabama-Birmingham (7).

Real time multiplex PCR assays (taqman) for total and pathogenic *V. parahaemolyticus* developed at FDA GCSL (8) and at the University of Alabama-Birmingham (1,6,9).

Real time PCR offers rapid, quantitative analysis for detection of a number of food-borne pathogens. The University of Florida and the University of Alabama at Birmingham have developed real time PCR assays for detection of the *V. vulnificus* vvh gene (2,3,4,7). The proposed methods can be used either in a Taqman or SYBR green format and could be applied in an MPN format as an alternate confirmation tool for identification of bacterial isolates in the validation and verification of PHP oysters. There is also the potential to significantly reduce the workload and time needed for analysis by using the PCR methods directly on APW enrichments, thus eliminating the need for streaking for isolation on selective media.

FDA has developed a multiplex real time PCR assay for *V. parahaemolyticus* species identification (*tlh*), virulence characterization (*tdh* and *trh*) and an internal amplification control to detect false negatives that could result from the presence of PCRinhibitors in the sample matrix (9). Currently the ISSC uses a DNA probe colony hybridization assay for quantifying total and pathogenic *V. parahaemolyticus* in oysters at harvest for the Interim Control Plan. The same method is also used for determining levels of *V. parahaemolyticus* after PHP. While this method can be completed in 24h, variations in signal strength sometimes complicate interpretations, especially at low colony numbers where this method is normally applied. Conversion of the *V. parahaemolyticus* procedure to an MPN format would also permit utilization of the same validation and verification procedures as used with *V. vulnificus*.

The University of Alabama at Birmingham has developed a method for the detection of *V. parahaemolyticus* in oysters using multiplexed real time PCR with taqman fluorescent probes (1,6,9). The current ISSC adopted procedure uses a colorimetric DNA probe colony hybridization assay for the detection of total and pathogenic *V. parahaemolyticus* targeting *tlh* and *tdh* genes. However, with this multiplex method, targeting additional genes, it is possible to achieve a comprehensive detection of all pathogenic forms of *V. parahaemolyticus* known to date in a single reaction tube by real-time Taqman-PCR method. The proposed method developed by the University of Alabama – Birmingham uses a multiplexed Taqman PCR-based detection of total (targeting *tlh* gene), pathogenic (targeting *tdh* and *trh* genes), and pandemic strains of *V. parahaemolyticus* O3:K6 serotype

(targeting ORF8 gene) in a single reaction that could potentially be applied for routine monitoring of molluscan shellfish for this pathogen. This method appears to be specific and can be used for the detection of <10 cfu *V. parahaemolyticus* following overnight enrichment in T_1N_1 broth. Further, this method has the potential to confirm MPN enrichment method of detection of this pathogen by direct amplification of the targeted genes without further culture-based confirmation. The multiplexed PCR method of detection of total and pathogenic strains including the pandemic strain of *V. parahaemolyticus* is rapid; detection can be achieved in real-time amplification of the targeted genes; specific for the targeted pathogen; and sufficiently sensitive in enriched oyster homogenate to consider as an alternate method of detection of this important pathogen.

Each of the proposed PCR methods were designed for use on the Cepheid Smart Cycler and would require some modifications to be used on other instruments. A review package including performance attributes will be provided for each method prior to the 2005 Conference.

References:

1. Bej, A.K., D.P. Patterson, C.W. Brasher, M.C.L. Vickery, D.D. Jones, C.A. Kaysner. 1999. Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tl*, *tdh*, and *trh*. *Journal of Microbiological Methods* 36:215-225.
2. Campbell, M.S. and A.C. Wright. 2003. Real-time PCR analysis of *Vibrio vulnificus* from oysters. Appl.Enviro.Microbiol. 69:7137-7144.
3. Calero, A. G. (Wright, A. C., advisor). 2003. Application of molecular detection methods to most probable number (MPN) enumeration of *Vv* in oysters. M.S. Thesis, University of FL http://etd.fcla.edu/UF/UFE0002740/calero_a.pdf.
4. Harwood V. J., Gandhi, J. P., and Wright, A. C. 2004. Methods for Isolation and Confirmation of *Vibrio vulnificus* from Oysters and Environmental Sources: A Review. *J. Microbiol. Methods*. 59: 301-16.
5. Mead, P.S., L. Slutsker, V.Dietz., L.F. McGaig, J.S. Bresee, C. Shapiro, P.M. Griffin, and R.V. Tauxe. 1999. Food-related illness and death in the United States. Emerg.Infect.Dis. 5:607-625.
6. Myers, M., G. Panicker, A.K. Bej. 2003. Detection of newly emerged pandemic *Vibrio parahaemolyticus* O3:K6 pathogen in pure cultures and seeded Gulf waters using PCR. *Applied and Environmental Microbiology* 69:2194-2200.
7. Panicker, G., M.L. Myers, and A.K. Bej. 2004. Rapid detection of *Vibrio vulnificus* in shellfish and Gulf of Mexico water by real-time PCR. Appl.Enviro.Microbiol. 70:498-507.
8. Vickery, M.C.L., G.M. Blackstone, J.L. Nordstrom, and A. DePaola. 2003. Detection and quantification of total and potentially virulent *Vibrio parahaemolyticus* using a 4-channel multiplex real-time PCR targeting the *tl, tdh*, and *trh* genes and a novel PCR internal control. ASM Annual Meeting, Washington, DC, May 18-22, 2003.
9. Ward, L.N. and A. K. Bej. Detection of total and pathogenic *V. parahaemolyticus* in shellfish using multiplexed real-time pCR with Taqman fluorescent probes. (In preparation).

Public Health Significance:

V. parahaemolyticus is the leading cause of bacterial gastroenteritis and *V. vulnificus* is the leading cause of death associated with seafood consumption in the US (5). ISSC has an ICP for Vp and has developed validation and verification of PHP for both organisms. Real time PCR is faster and more reliable than current methods but is not yet approved by ISSC. Approval of one or more of the proposed real time PCR methods would provide a faster

and more reliable means of enumerating *V. vulnificus* and *V. parahaemolyticus* while offering an equivalent level of public health protection for consumers of raw molluscan shellfish.

Cost Information (if available): None

Action by 2005 Laboratory Methods Review Committee Recommended Proposal 05-107 be referred to the appropriate committtee as determined by the Conference Chairman, with further direction to the Executive Office to organize a meeting of the Laboratory Methods Committee within six (6) months of the conclusion of this Biennial Meeting.

Action by 2005 Task Force I Recommended adoption of the Laboratory Methods Review Committee recommendation on Proposal 05-107.

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 05-107. Rationale – Inadequate data submission. The methods proposed in Proposal 05-107 would be very useful to the NSSP. The submitter will be requested to provide additional data to the Executive Office for approval consistent with Procedure XVI.

Action by 2007 Task Force I Recommended adoption of Laboratory Methods Review Committee recommendation of no action on Proposal 05-107.

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.

Action by 2009 Laboratory Methods Review Committee

Recommended no action on Proposal 05-107. Rationale: Addressed by Proposals 09-102, 09-103.

Action by 2009 Task Force I

Recommended adoption of Laboratory Methods Review Committee recommendation of Proposal 05-107.

Action by 2009 General Assembly

Adopted recommendation of 2009 Task Force I on Proposal 05-107.

Proposal Subject: Real Time PCR Method for Determining Levels of *V. parahaemolyticus*

Specific NSSP Guide Reference: Section IV Guidance Document, Chapter II, Growing Areas .10 Approved National Shellfish Sanitation Program Laboratory Tests: Microbiological and Biotoxin Analytical Methods

Text of Proposal/ Requested Action With the advent of real-time PCR assays, it is now possible to conduct more rapid and accurate screening for *Vibrio parahaemolyticus* within 24 hour time frame. Real-time PCR assays are generally less labor intensive and less time consuming then the traditional biochemical assays that have been used to detect total *Vibrio parahaemolyticus.*

The State of Washington Department of Health has developed a multiplex real time PCR assay for the detection of *Vibrio parahaemolyticus* (VP) using the Applied Biosystem Taqman Platform. This assay targets two species identification markers (*tlh* and *gyrase B*) for total VP, the virulence marker (*tdh*), and a *16S* target that is specific for five species (V. *parahaemolyticus*, V. *alginolyticus*, V. *furnissii*, V. *harveyi*, V. *fluvialis*) within the genus.

Public Health Significance: *Vibrio parahaemolyticus* continues to cause food borne outbreaks globally due to the consumption of raw or undercooked oysters[i, ii]. Current molecular methods can of differentiate between pathogenic (tlh+, tdh+) and non-pathogenic (tlh+, tdh-) organisms but real-time PCR procedures are not fully approved by the ISSC. This real-time PCR assay, if approved, would improve the turn around time for results for public health protection and seafood safety.

[i]Wong, H.C., S. H. Liu, et al. (2000). Characteristics of Vibrio parahaemolyticus O3:K6 from Asia." Appl Environ Microbiol **66**(9): 3981-6.

[Ii]DePaola, A., C. A. Kaysner, et al. (2000). "Environmental investigations of Vibrio parahaemolyticus in oysers after outbreaks in Washington, Texas, and New York (1997 and 1998)." Appl Environ Microbiol **66**(11): 4649-54.

Cost Information (if available): None

Action by 2005 Laboratory Methods Review Committee Recommended Proposal 05-108 be referred to the appropriate committee as determined by the Conference Chairman, with further direction to the Executive Office to organize a meeting of the Laboratory Methods Committee within six (6) months of the conclusion of this Biennial Meeting.

Action by 2005 Task Force I Recommended adoption of the Lab Methods Review Committee recommendation on Proposal 05-108.

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 05-108. Rationale – Inadequate data submission. The methods proposed in Proposal 05-108 would be very useful to the NSSP. The submitter will be requested to provide additional data to the Executive Office for approval consistent with Procedure XVI.

Action by 2007 Task Force I

Recommended referral of Proposal 05-108 to an appropriate committee as determined by the Conference Chairman.

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.

Action by 2009 Laboratory Methods Review Committee

Recommended no action on Proposal 05-108. Rationale: Not enough data provided for approval. Additional information requested by the Executive Office has not been provided.

Action by 2009 Task Force I

Recommended adoption of the Laboratory Methods Review Committee recommendation on Proposal 05-108.

Action by 2009 General Assembly

Adopted recommendation of 2009 Task Force I on Proposal 05-108.

Proposal Subject: Rapid Screening Method for ASP

Specific NSSP Guide Reference Section II. Model Ordinance Chapter III Laboratory @.02 Methods
ISSC Constitution, ByLaws, and Procedures
Procedure XVI. Procedure for Acceptance and Approval of Analytical Methods for the NSSP.

Text of Proposal/ Requested Action For many years, there has been an expression of need by regulatory agencies and industry to develop a test to monitor ASP levels with precision and accuracy.

The method developed by Jellett Rapid Testing Ltd has been presented to the ISSC and other regulatory bodies over the past several years. In cooperation with individuals, governments and those organizations, the analytical method has been refined and improved. The Rapid Test kits have been tested in several states and foreign countries, and JRT has some internal papers, including one done by Mike Quilliam, that are now in preparation and should be submitted/in press by the time of the ISSC meeting. There are some talks coming up ICMSS, CWHMA where the ASP test will be presented, and from which there will be proceedings later this year or early next year.

It should be noted that this test is built on the same platform by the same company, and uses a similar format to the Jellett Rapid Test for PSP that is already accepted by the ISSC.

The CONSTITUTION BY-LAWS and PROCEDURES of the INTERSTATE SHELLFISH SANITATION CONFERENCE allow the ISSC, through the Laboratory Methods Review Committee, to accept analytical methods that are sufficiently validated but are not AOAC or APHA methods. This is defined in the Constitution, PROCEDURE XVI. PROCEDURE FOR ACCEPTANCE AND APPROVAL OF ANALYTICAL METHODS FOR THE NSSP. Two possible reasons for considering a method are found in Subdivisions I and ii.

Subdivision i. Meets immediate or continuing need;

Subdivision ii. Improves analytical capability under the NSSP as an alternative to other approved or accepted method(s)

Currently, Table 4 of Chapter II.10 allows the use of any "Peer recognized HPLC Methods with or without clean up." For ASP analysis. The need for standard methods has been expressed by regulatory agencies, governmental organizations and industry for many years. The Jellett Rapid Test for ASP has been validated over a wide geographic area to demonstrate its simplicity, reliability, precision and accuracy. As a result of ongoing improvements and demonstrations of efficacy, and the need that has been expressed by industry and state agencies, the Jellett Rapid Test for ASP is presented as a screening method for the NSSP as a Type III or Type IV method.

Please see attached additional information.

Suggested wording:
Section II, Chapter III Laboratory @.02 Methods

C. Biotoxin. Methods for the analyses of shellfish and shellfish harvest waters shall be:

(1) The current AOAC and APHA methods used in bioassay for paralytic shellfish poisoning toxins; and

(2) The current APHA method used in bioassay for Karemia breve toxins.

(3) The Jellett Rapid Test for ASP may be used as a screening method for ASP toxins by regulatory and industry laboratories.

Public Health Significance:

Currently, only data from certified laboratories conducting ASP analyses using any "Peer recognized HPLC Methods with or without clean up" are considered reliable and acceptable. Because of many significant constraints, in practical terms, this means that only state laboratories (in the US, governmental laboratories in other countries) can provide acceptable data at this time using methods not specifically defined by the ISSC. Acceptance of the Jellett Rapid Test for ASP would allow harvesters, processors, and regulatory agencies to screen for ASP with an accepted standardized method that provides valid useable data.

The Jellett Rapid Test for ASP was developed over several years in answer to the oft-stated need for a rapid, reliable, non-animal analytical method. The Jellett Rapid Test for ASP is not meant to be a definitive "Standard Method", but rather to augment "Peer recognized HPLC Methods…" by providing an additional tool that is currently not available.

Possible applications for The Jellett Rapid Test for ASP include:

- as a method of screening out negative samples in shellfish regulatory labs;
- as a harvest management tool at aquaculture facilities or in wild shellfish harvest areas (especially nearshore areas) to determine if shellfish are free of ASP and safe to harvest; as a quality control tool for shellfish processing plants, distributors and wholesalers to ensure incoming shellfish are free of ASP toxins before processing or further distribution (this test could become part of the plant's HACCP program);
- as a tool for water classification for biotoxins;
- to assist in site selection for aquaculture activity;
- as a screening tool for toxic phytoplankton in seawater to provide an early warning for shellfish growers; and
- as a research tool for broad scale ecological monitoring.

The rationale for using the Jellett Rapid Test for ASP is that the kits provide a cost-effective screen (especially in low-volume laboratories) for ASP that can provide a standardized test for screening and substantially reduce the cost of analyses. The same extract is used for the Rapid Test that is used for HPLC, so the Jellett Rapid Method extract can easily be sent for a confirmation in another lab if necessary. As a harvest management tool, the use of the Jellett Rapid Test for ASP will supplement regulatory agency efforts and help prevent the harvest of contaminated product. Having the ability to conduct tests using an accepted standardized method will allow those processors who choose to use this test to demonstrate that they are truly controlling for ASP hazards in the harvested shellfish.

The Jellett Rapid Test for ASP could be used to build long-term databases on a broader scale than a regulatory lab can afford and, by using a standardized method, will provide consistent results. These databases could be supplemented with industry testing in areas where there is no testing currently. This would extend, augment and strengthen the current food safety system broadening and refining the food safety net by increasing the number of testing sites and generating long term data in more areas.

HPLC is expensive and highly technical, requiring a large capital and personnel investment. HPLC machines, like other analytical equipment, also break down regularly.

Therefore there needs to be backup HPLC machines OR other methods available.

A simple, rapid, effective, reliable test, available to all harvesters, regulators, and processors, would increase the monitoring and reduce the chance that shellfish containing ASP toxins above the regulatory limit would be harvested or marketed.

Cost Information (if available): Each test kit costs $20 (**€18)**. It has been reported that each analysis using the HPLC costs approximately $140 per test. History has shown that large numbers of ASP monitoring samples are negative. The costs cited do not take into account the costs associated emergency closures, recalls, or providing medical care to those affected by toxic shellfish. Also, some states are interested in the test because they do not have to invest in HPLC technology if they have the Rapid Test as an alternative.

Action by 2005 Laboratory Methods Review Committee Recommended that Proposal 05-109 be referred to the appropriate committee as determined by the Conference Chairman.

Action by 2005 Task Force I Recommended adoption of the Laboratory Methods Review Committee recommendation on Proposal 05-109.

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 05-109. Rationale – Method needs modification because of changes to the antibody. In addition, there is insufficient data to demonstrate acceptability to the Conference. The submitter is requested to provide data to the Executive Office for approval.

Action by 2007 Task Force I Recommended referral of Proposal 05-109 to an appropriate committee as determined by the Conference Chairman.

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.

Action by 2009 Laboratory Methods Review Committee

Recommended no action on Proposal 05-109. Rationale: Requested additional information has not been submitted.

Action by 2009 Task Force I

Recommended adoption of Laboratory Methods Review Committee recommendation on Proposal 05-109.

Action by 2009 General Assembly

Referred Proposal 05-109 to the Laboratory Methods Review Committee.

Laycock, Maurice V., Joanne F. Jellett, W. Hywel Morgan. 2004. Characteristics and Applications of the Jellett Rapid Tests for PSP and ASP. *In:* Holland, Patrick and Michael A. Quilliam, (Eds.) Proceedings 2nd HABTech 2003 Workshop, Nelson, New Zealand. Nov 26-30, 2003.

Characteristics and Applications of the Jellett Rapid Tests for PSP and ASP

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Abstract
The Jellett Rapid Tests for PSP and ASP toxins were tested with calibration standards to investigate sensitivities to individual toxins spiked into mussel extracts at concentrations around the regulatory limits. PSP test strips showed their highest sensitivity to saxitoxin (Stx) and gonyautoxins-2 and -3 (Gtx2/3) and were least sensitive to Gtx1/4 and neosaxitoxin (Neo). Sensitivities were intermediate to mixtures of Stx with Neo and to Gtx1/4 with Gtx2/3, which are more typical of naturally occurring PSP toxin profiles. All of the PSP toxins that were tested gave positive responses at or below the regulatory limit. The ASP test detected domoic acid at around 5 $\mu g.g^{-1}$, well below the regulatory limit. Uses for the Rapid Tests for screening in regulatory laboratories and testing in field conditions for PSP toxins and domoic acid in shellfish and phytoplankton are discussed.

Key words
Paralytic shellfish poisoning (PSP), amnesic shellfish poisoning (ASP), lateral flow immunochromatography (LFI), saxitoxin, domoic acid, test kits.

Introduction

Shellfish toxicity and food safety have been monitored successfully by mouse bioassays (AOAC, 1999) for more than fifty years. The current trend toward replacement methods has resulted in the development of more sophisticated methods such as liquid chromatography with mass spectrometric or fluorescence detectors. They not only provide a higher degree of accuracy and sensitivity but individual toxins can be identified in complex mixtures. However, aside from the high capital cost of the instruments, their maintenance and requirement for a well equipped laboratory and trained staff, sample clean up has been an on going problem. Antibody methods, such as ELISA require little sample preparation and equipment is relatively inexpensive. However, ELISA methods are slow and cannot be easily carried out outside the laboratory, or in unskilled hands.

Lateral flow immunochromatography (LFI) is an alternative format for antibody detection of shellfish toxins. The self-contained simplicity and reliability of these test strips has found applications in many areas such as screening for illicit drugs and home pregnancy testing. They are essentially yes/no tests engineered to indicate a specific analyte concentration. We have developed LFI tests for PSP and ASP toxins and one for DSP toxins is being developed. The absence of a coloured test line on the strip indicates that the sample contained the toxin at a concentration around half the regulatory limit. Because most samples tested by regulatory agencies are negative, LFI tests can be used to screen a large number of samples quickly and only those with toxin concentrations above or approaching regulatory limits need to be tested further, thereby speeding through-put, reducing costs and the number of mice used in bioassays. In addition to growing acceptance of the PSP and ASP test strips by regulatory agencies, they are also being tested in isolated communities, by shellfish farmers and for phytoplankton monitoring.

The Jellett Rapid Test for PSP (formerly, MIST Alert) is based on antibodies that □upernata all of the saxitoxin (Stx) and neosaxitoxin (Neo) analogues, but not equally. Our first publication (Laycock et al., 2001) describing the characteristics of the PSP test showed relative sensitivities to a range of purified PSP

toxins. All fell within the regulatory limit. Sensitivities to Neo and its 11-sulphated gonyautoxin analogues (Gtx1/4) were about five fold less than to Stx and its analogues. Detection levels for the sulfamate analogues of Stx (C1/2 and B1) fell between the two (Gtx2/3 and Gtx1/4) extremes. The PSP test has been subjected to extensive field trials (Jellett et al., 2002; MacIntosh et al., 2002) which showed no false negatives in over two thousand samples. Extracts containing only Gtx1/4 or Neo are rare but if encountered at concentrations close to the regulatory limit, would they fall within the detection limit of the test? We have examined this question with spiked samples containing only Gtx1/4 and Neo and the effect of the presence of other PSP toxins in the profile.

The ASP test has also been subjected to independent testing and shown to be easy to use and reliable (MacIntosh and Smith, 2002). The detection limits of the ASP test were examined in a similar manner to the PSP test with a calibration standard and the data are presented.

Materials and Methods

The LFI test strips are manufactured by Jellett Rapid Testing Ltd. With stringent quality control to ensure reproducibility. Test strips are contained in plastic cassettes with a sample well and a window. A test line (T-line) and a control line (C-line) can be seen in the window about 15 min after applying a sample. In the absence of toxin, both lines can be seen. For samples containing toxin in concentrations greater than the regulatory limit, no T-line appears, and only the C-line is seen. No clean-up is necessary but extracts must be diluted to 20% (1:5) for PSP and to 10% (1:10) for ASP with a buffer solution supplied with the tests to ensure the proper solution conditions for the test to function. This is indicated by the formation of a visible C-line.

Non-toxic mussels were □upernatant and extracted by the AOAC extraction procedures for PSP with 0.1 N HCl (AOAC, 1999). Samples of this control extract were spiked with purified PSP toxin calibration solutions obtained from the National Research Council of Canada. The total molar concentration of separate or mixed toxins was the same for each spiked extract. A series of dilutions was prepared from the highest concentration of 3200 nM with control extract. The prepared samples were then diluted 1:5 with buffer solution. Test units were removed from their sealed pouches and 100 µl of the buffered samples was applied to each sample well. After 15 min, test and control lines were fully developed and the results □upernata using a conventional computer scanner. T-line intensities were measured using Softmax Pro software (Molecular Devices, CA). Five replicate measurements were taken and each converted to percent of the maximum line intensity at zero toxin concentration.

For ASP, a non-toxic mussel homogenate was extracted into four volumes (1:5) of 50% aqueous methanol. A sample of this methanolic extract was spiked with a calibration standard of domoic acid to equivalent of 20 $\mu g.g^{-1}$ tissue and a dilution series was prepared by serial dilution using the non-toxic, control extract. A running buffer solution designed for the ASP test was then added (1:10) to the different concentrations in the series. Samples (100 µl) at each concentration were applied to the test strips and the results recorded by scanning.

Results

PSP

The five values for T-line colour were plotted against toxin concentration in spiked extracts before dilution 1:5 with the running buffer. The slopes and positions of the different curves reflect the proportions of toxins □upernatan differently by the antibodies. Plots of T-line intensities against toxin concentrations showed a lower sensitivity to Neo than to Stx, so that a weak T-line persisted with samples containing Neo alone at 1300 nM. This is approximately at the PSP regulatory limit of 80 µg per 100 g tissue (calculated for Stx as the free base) in an AOAC extract. The test showed the highest sensitivity to Stx and the plot from samples containing only Stx is shown together with that for Neo in Fig. 1A to illustrate the range of sensitivities.

Data for the sensitivities to Gtx2/3 and Gtx1/4 are plotted together in Fig. 1B. The PSP test had the lowest sensitivity to Gtx1/4. At the regulatory limit for Stx (1300 nM), T-line intensity was reduced to about 60% of that obtained with a non-toxic sample and 90% at twice that concentration. At 1300 nM Gtx2/3 reduced the T-line by 95%. Responses to equimolar mixtures of Stx with Neo and Gtx1/4 with Gtx2/3 are shown in Fig. 1C. Both curves indicate 90% reduction of T-line intensity for total toxin concentrations at the regulatory limit. A reduction of T-line intensity of 50% is interpreted as positive. Toxin concentrations at 50% decrease in T-line intensity are shown on the graphs by narrow vertical lines.

ASP

The sensitivity of the ASP test was well within the regulatory limit of 20 $\mu g.g^{-1}$. Figure 2 shows that in samples containing 5 $\mu g.g^{-1}$ in a methanol extract, the T-line intensity was 80% reduced, and 90% at 10 $\mu g.g^{-1}$, from that obtained with non-toxic extracts. The domoic acid concentration in methanolic extracts that resulted in a 50% decrease in T-line intensity, which is interpreted as positive, was 2.5 $\mu g.g^{-1}$. Spiked AOAC extracts were also tested. The tissue concentration in an AOAC extract is 2.5 times that in a methanolic extract and the 50% T-line was around 1.0 $\mu g.g^{-1}$. The ASP test was found to be more susceptible to a matrix effect with higher concentrations of tissue causing a decrease in C and T-line intensities. This difference between extraction methods was common with 1:5 dilutions in running buffer but not at with 1:10 dilutions. The latter dilution therefore was adopted for the ASP test.

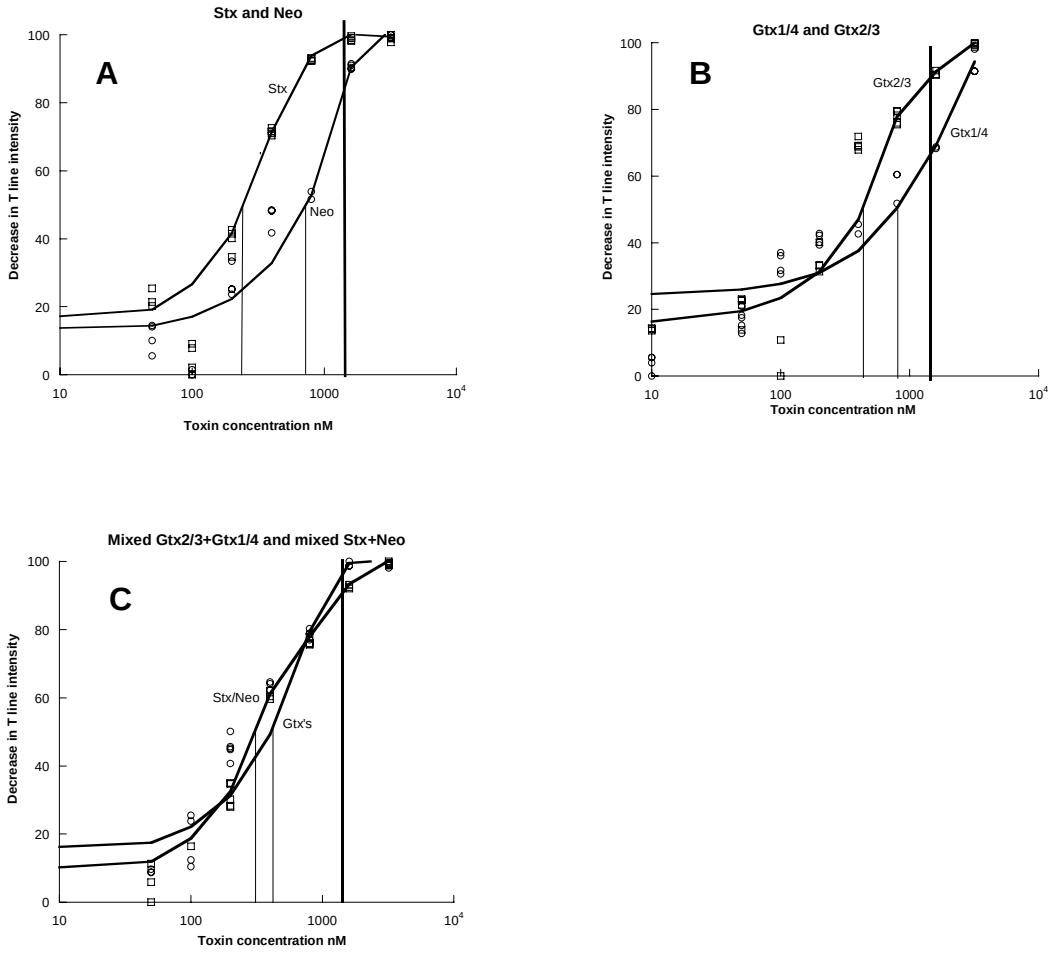


Figure 1.

Non-toxic mussel homogenate was extracted by the AOAC method into an equal volume of 0.1 M HCl. Samples were spiked with NRC certified toxin standards to 3200 nM. Dilution series were prepared by mixing with non-toxic extract. The extracts containing different toxin concentrations were then mixed 1:5 with PSP running buffer solution and 100 µl applied to the test strips. After 20 min. T line intensities were measured by scanning into a computer and supernatan (Softmax, Molecular devices, CA). The regulatory limit of 80 µg/100 g is indicated by the heavy vertical line and fine vertical lines indicate toxin concentrations at 50% decrease in T-line intensity.

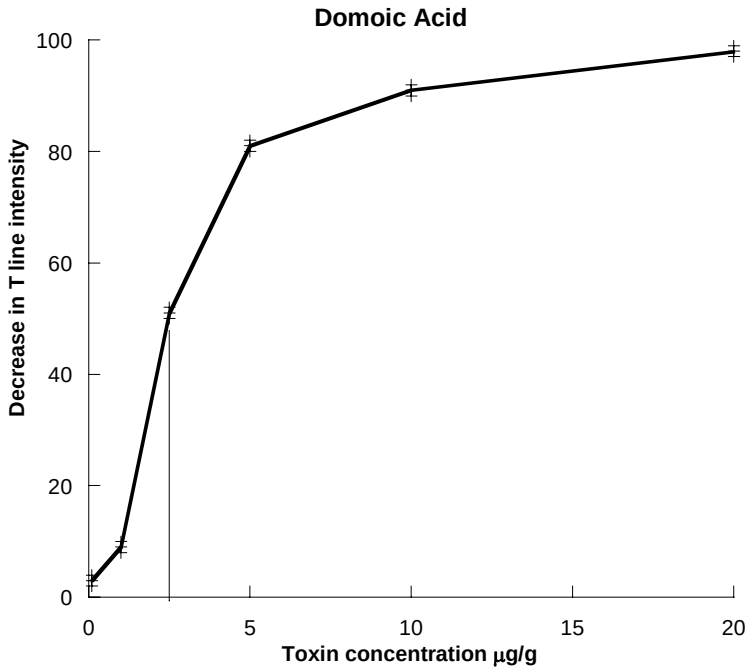


Figure 2.
Non-toxic mussel homogenate was extracted into four volumes of 50% methanol a sample spiked with domoic acid to 20 µg/g homogenate. Serial dilutions were made with non-toxic extract and mixed with ASP running buffer solution. A sample (100 µl) of each solution was applied to each test strip. Line intensities were measured as described in the legend to Fig. 1. The regulatory limit for ASP is 20 µg/g. The vertical line indicates the toxin concentration at 50% decrease in T-line intensity.

Discussion
The Jellett Rapid Tests for PSP and ASP are designed to indicate the presence of toxins in shellfish and phytoplankton at concentrations around half the regulatory limit for Stx and domoic acid in shellfish. Experiments with purified PSP toxins show that responses to different analogues are not equal (Laycock, et al., 2001). Also, at toxin concentrations around the regulatory limit T-line intensities may be intermediate. At lower and higher concentrations the T-line is either equal in intensity to the control line or it is absent. The recommended way to interpret tests that show T-lines of intermediate intensity is by comparison with the C line. In the absence of toxin T and C-line intensities are equal. If the T-line appears to be 50% or less intense than the C-line the test is considered to be positive, indicating that the

extract contained significant amounts of the toxin. If no T-line appears, toxin concentrations may be well above the regulatory limit. In this case, concentrations may be estimated by making serial dilutions with non-toxic extract. The recommended dilution with running buffer solution (1:5 for PSP and 1:10 for ASP) should be maintained and serial dilutions are prepared with non-toxic extract. A lower ratio of buffer to extract will increase the concentration of toxin in the sample but, depending on the extracted tissue, a matrix effect may be seen by diminished control line intensity.

The PSP test is least sensitive to Gtx1/4 and Neo. However, these analogues rarely occur in the absence of Stx, and more especially Gtx2/3, which is the most common of all the PSP toxins found in shellfish. The Rapid Test for PSP has shown the highest sensitivity for both of these toxins. Experiments to examine test responses to samples containing toxin profiles such as those for which the test is least sensitive were possible only with samples spiked with purified toxins of known concentrations. The results presented here show that only for extracts containing Gtx1/4 alone, at concentrations close to the regulatory limit, the test response may be intermediate between clearly positive or negative. The effect of mixed toxins increased sensitivity to samples containing Gtx1/4 and Neo. This is illustrated in Fig. 3 in which equimolar concentrations of Gtx2/3 with Gtx1/4 and Stx with Neo resulted in responses well within the regulatory limit. In an earlier publication (Laycock et al., 2001) the test was called MIST Alert but is now the Jellett Rapid Test for PSP. It should be noted that the earlier data were presented as toxin concentration before dilution (1:5) with running buffer solution. Current test strips are similar to those produced earlier with comparable sensitivities to the different PSP toxin analogues. Sensitivities to the sulfamate toxins C1/2 and B1 are not presented here but as shown earlier they fall between Neo and Stx. The decarbamoyl analogues of Stx have also been tested and responses were very similar to their corresponding carbamates.

Both the PSP and ASP tests have been subjected to extensive independent field trials (Jellett et al., 2002; MacIntosh et al., 2002; MacIntosh and Smith, 2002) with naturally occurring toxic shellfish. Based on the encouraging results of these trials the Rapid Tests for shellfish toxins are being adopted for routine use in monitoring programs. The test strips provide a reliable screening tool for regulatory agencies, costing significantly less than alternatives for shellfish monitoring, such as the mouse bioassay or HPLC. Screening out the high proportion of negative samples to be tested further not only reduces the overall cost it also increases the rate at which samples can be monitored. In addition to testing for toxins in shellfish the Rapid Tests can be used to test for toxicity in samples from plankton nets. *Alexandrium* and *Pseudo-nitzschia* cells were easily extracted into 0.1 M acetic acid without mechanical disruption providing a simple and sensitive field method for phytoplankton monitoring (Rafuse et al., 2002).

The Rapid Tests are essentially self-contained and extracts can be tested without laboratory equipment, allowing their use at shellfish farms, on boats, beaches or camps. However, for use in field conditions the preparation of shellfish extracts is more difficult than in a laboratory. Ineffective extraction could lead to false negatives, especially for samples with toxin concentrations close to the test strip detection limit. Kits are supplied with detailed instructions about making extracts from shellfish or plankton as extraction is a crucial part of the test procedure.

Acknowledgements
The authors thank Dr. Michael Quilliam for the toxin standards used in this study and for his continuing support. Dorothy Easy and Mary Anne Donovan provided technical help.

References

Association of Official Analytical Chemists International, 1999. Official Method 959.08-paralytic shellfish poison, biological method, 17th ed., Official Methods of Analysis, vol.1 Arlington, VA.

Jellett, J.F., Roberts, R.L., Laycock, M.V., Quilliam, M.A., Barrett, R.E., 2002. Detection of paralytic shellfish poisoning (PSP) toxins using MIST Alert™, a new rapid test in parallel with the regulatory AOAC® mouse bioassay. Toxicon. 40, 1407-1425.

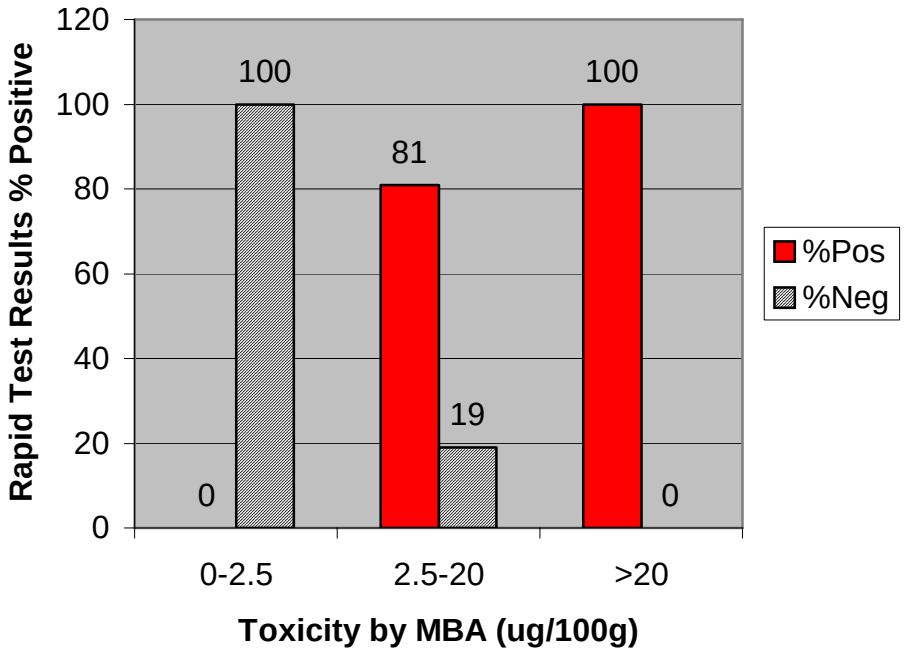
Laycock, M.V., Jellett, J.F., Belland, E.R., Bishop, P.C., Theriault, B.L., Russell-Tattrie, A.L., Quilliam, M.A., Cembella, A.D., Richards, R.C., 2001. MIST Alert™: A Rapid Assay for Paralytic Shellfish Poisoning Toxins. Proceedings of the 9th International Conference on Harmful Algal Blooms, Hobart, Australia, 7-11 February 2000, Hallegraeff, G.M. Blackburn, S.I. Bolch, L.J., Lewis, R.J. (Eds) IOC of UNESCO, 2001.

Mackintosh, F.H., Gallacher, S., Shanks A.M., Smith, E.A., 2002. Assessment of MIST Alert™, a Commercial Qualitative Assay for Detection of Paralytic Shellfish Poisoning Toxins in Bivalve Molluscs. Journal of AOAC International. 85, 632-641.

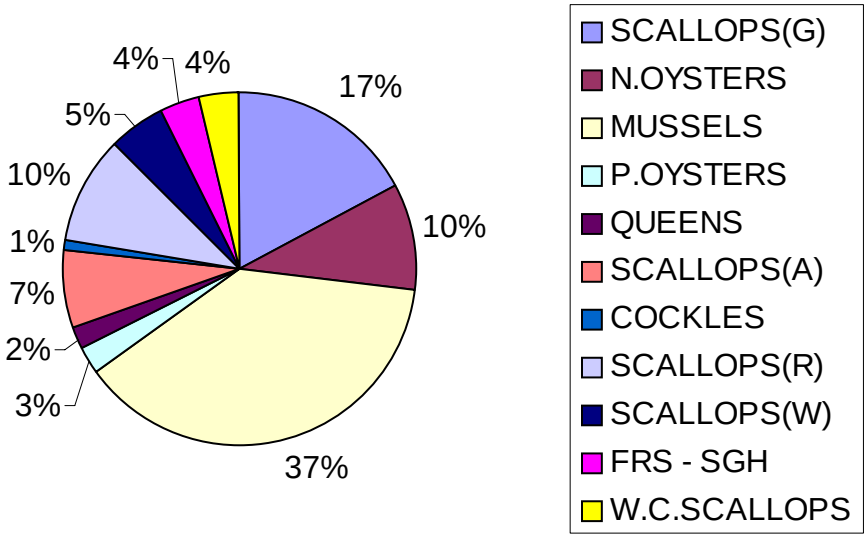
Mackintosh F.H., Smith, E.A., 2002. Evaluation of MIST Alert™ Rapid Test Kits For the Detection of Paralytic and Amnesic Shellfish poisoning Toxins in Shellfish. Journal of Shellfish Research. 21, 455-460.

Rafuse, C., Cembella, A.D., Laycock, M.V., Jellett, J.F., 2002. Rapid Monitoring of Toxic Phytoplankton and Zooplankton with a Lateral-Flow Immunochromatographic Assay for ASP and PSP Toxins. In: Steidinger, K. (Ed) Proceedings of the 10th International Conference on Harmful Algal Blooms St. Petersburg, Florida. Oct 21-25, 2002. Intergov. Oceanogr. Comm, UNESCO, Paris, in press.

Detection of ASP in Shellfish Tissue
from UK



Tissue Types from UK
(n= 111)



Proposal Subject: Rapid Extraction Method for PSP and ASP

Specific NSSP Guide Reference: Section II. Model Ordinance Chapter III Laboratory @.02 Methods
ISSC Constitution, ByLaws, and Procedures
Procedure XVI, Procedure for Acceptance and Approval of Analytical Methods for the NSSP.

Text of Proposal/ Requested Action

Marine biotoxins affect farmed and wild fish and shellfish, as well as having a deleterious effect on humans. Jellett Rapid Testing has designed and developed rugged tests for the presence of Paralytic Shellfish Poison, Amnesic Shellfish Poison and Diarrhetic Shellfish Poison (under development at the time of this submittal). To facilitate the use of these tests in the field (for aquaculturists, campers, regulatory officials, etc.), Jellett Rapid Testing has developed a "low-tech" rugged alternative to the standard AOAC method designed to extract the toxins in the field as well as the laboratory. The AOAC method requires the sample to be boiled in acid at low pH and the pH adjusted with strong acids. This requires a fully equipped laboratory and significant safety precautions. The JRT Rapid Extraction Method was designed for use in remote areas, with little sophisticated backup support, by average individuals with little training and education. It is faster, less labor-intensive and less expensive than the other available method.

The rapid extraction method requires vinegar and rubbing alcohol to extract the toxins. A simple, rapid, safe method such as this would make rapid tests for marine biotoxins available in remote areas, to fishermen, aquaculturists, and regulatory officials on an instant basis.

The method developed by Jellett Rapid Testing Ltd has been presented to regulatory bodies over the past several years. In cooperation with individuals, governments and those organizations, the analytical method has been refined and improved. The Rapid Extraction Method is being tested in several states and foreign countries. Publications will be forthcoming.

The CONSTITUTION BY-LAWS and PROCEDURES of the INTERSTATE SHELLFISH SANITATION CONFERENCE allows the ISSC, through the Laboratory Methods Review Committee, to accept analytical methods that are sufficiently validated but are not AOAC or APHA methods. This is defined in the Constitution, PROCEDURE XVI. PROCEDURE FOR ACCEPTANCE AND APPROVAL OF ANALYTICAL METHODS FOR THE NSSP. Two possible reasons for considering a method are found in Subdivisions I and ii.

Subdivision i. Meets immediate or continuing need;
Subdivision ii. Improves analytical capability under the NSSP as an alternative to other approved or accepted method(s)

Currently, only the AOAC extraction for PSP and ASP are accepted. The need for a simple safe extraction method has been expressed by regulatory agencies, governmental organizations and industry for many years. The Jellett Rapid Extraction Method is being validated over a wide geographic area to demonstrate its simplicity, reliability, precision and accuracy. As a result of demonstrations of efficacy and the need that has been expressed by industry and state agencies, the Jellett Rapid Extraction Method is presented as an alternative extraction method for PSP and ASP for the NSSP as a Type III or Type IV method.

Please see attached additional information.

Suggested wording:
Section II, Chapter III Laboratory @.02 Methods

C. Biotoxin. Methods for the analyses of shellfish and shellfish harvest waters shall be:
(1) The current AOAC and APHA methods used in bioassay for paralytic shellfish poisoning toxins; and
(2) The current APHA method used in bioassay for *Karemia breve* toxins.
(3) The Jellett Rapid Extraction Method may be used for extracting PSP and ASP toxins from Shellfish by regulatory and industry laboratories.

Public Health Significance:

Currently, only the AOAC extraction for PSP and ASP analyses are accepted. Because of many significant constraints, in practical terms, this means that analyses can be conducted only in laboratories, and then under dangerous conditions. Acceptance of the Jellett Rapid Extraction Method for PSP and ASP would allow harvesters, processors, and regulatory agencies to screen for PSP and ASP with an accepted standardized method that provides valid useable data.

The Jellett Rapid Extraction Method for PSP and ASP was developed over several years in answer to the oft-stated need for a rapid, reliable, rugged, simple and safe sample preparation method. The Jellett Rapid Extraction Method for PSP and ASP is not meant to be a definitive "Standard Method", but rather to provide a supplementary extraction method that can be used in the field as well as in the lab.

Possible applications for The Jellett Rapid Extraction Method for PSP and ASP include:

- as a supplement to analytical methods of screening out negative samples in shellfish regulatory labs;
- as a harvest management tool at aquaculture facilities or in wild shellfish harvest areas (especially near shore areas) to supplement available methods to determine if shellfish are free of PSP or ASP and safe to harvest;
- as a supplement to quality control methods for shellfish processing plants, distributors and wholesalers to ensure incoming shellfish are free of PSP and ASP toxins before processing or further distribution (this test could become part of the plant's HACCP program);
- as a supplement to analytical methods for water classification for biotoxins; and
- as a supplement to analytical methods for broad scale ecological monitoring.

The rationale for using the Jellett Rapid Extraction Method for PSP and ASP is that the method provides a rapid, reliable, rugged, simple, safe and cost-effective extraction method (especially in low-volume laboratories) for PSP and ASP that can supplement accepted tests and substantially reduce the cost of analyses. Used in conjunction with other rapid methods, the Jellett Rapid Extraction Method for PSP and ASP will supplement regulatory agency efforts and help prevent the harvest of contaminated product. Having the ability to conduct tests using an accepted rapid extraction method will allow those processors who choose to use this test to demonstrate that they are truly controlling for PSP and ASP hazards in the harvested shellfish.

The Jellett Rapid Extraction Method for PSP and ASP could contribute to building long-term databases on broader scales than a regulatory lab can afford and, by using an accepted standardized method, will provide consistent results. These databases could be

supplemented with industry testing in areas where there is no testing currently. This would extend, augment and strengthen the current food safety system broadening and refining the food safety net by increasing the number of testing sites and generating long term data in more areas.

A simple, rapid, rugged, effective, reliable, safe and cost-effective extraction method, available to all harvesters, regulators, and processors, would increase the monitoring and reduce the chance that shellfish containing ASP toxins above the regulatory limit would be harvested or marketed.

Cost Information (if available): It is difficult to determine exact costs because many government cost models do not consider capitol costs. Both extraction methods are the same through puree step, the chemicals used in both cases are minimal, as is the cost of incidental equipment (blender, pipettes, etc.). However, a comparison of time required using the Rapid Extraction Method (Add rapid liquid; Filter) with the time required using the AOAC Extraction (Add HCL; Boil; Wait; Filter; Pour in tube; Check PH) shows a significant difference. Our experience shows that it takes about 22 minutes for this portion of the AOAC extraction while it takes less than 2 minutes to complete the Jellett Rapid Extraction Method. At a salary of $33 / hour, that is a savings of $11.00 per sample extract.

Action by 2005 Laboratory Methods Review Committee Recommended referral of Proposal 05-111 to the appropriate committee as determined by the Conference Chairman.

Action by 2005 Task Force I Recommended adoption of the Laboratory Methods Review Committee recommendation of Proposal 05-111.

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 05-111. Rationale – Alternative extraction method for JRT PSP should be adopted to expand utility of the test; however there are insufficient data for acceptance at this time. The submitter will send data to the Executive Office for Conference approval.

Action by 2007 Task Force I Recommended referral of Proposal 05-111 to an appropriate committee as determined by the Conference Chairman.

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.

Action by 2009 Laboratory Methods Review Committee

Recommended no action on Proposal 05-111. Rationale: Requested additional information has not been submitted.

Action by 2009 Task Force I

Recommended adoption of Laboratory Methods Review Committee recommendation of Proposal 05-111.

Action by 2009 General Assembly

Referred Proposal 05-111 to the Laboratory Methods Review Committee.

Lab #	CFIA Sample #	CFIA Result HPLC (µg/g)	Jellett Result Approx. (µg/g)
04-01847	1	24.1	16-24
04-02156	2	1.4	0-4
04-01784	3	70.0	72-80
04-01968	4	71.9	72-92
04-01647	5	8.9	12-16
04-02328	6	9.3	6.4-11.2
04-02467	7	4.2	6.0-7.2
04-01646	8	31.2	40-64
04-02351	9	9.4	9.6-12
04-02238	10	4.7	4-5.6
04-01862	11	96.7	60-80
04-02240	12	10.3	12-20
04-01750	13	30.7	24-32
04-02231	14	2.5	0-4
04-01969	15	40.1	64-72

Jellett Rapid Testing Ltd.: NOAA Study – JREM Trial
Sample Record Sheet – Homogenate
State of Alaska – Department of Environmental Conservation

	Collection		Homogenization			Jellett Test						MBA Test					
Sample ID	**Date**	**Species**	**Field / Site / Lab Name**	**Date**	**Size of Sample (mL)**	**Field / Site / Lab Name**	**Date**	**Batch # - Test**	**Batch # - Buffer**	**Result (1=Pos, 0=Neg)**	**Intensity of C Line as % of T**	**Lab Name**	**Date**	**Toxin Standard Used**	**# of Mice Dead**	**Result (µg/100g)**	**# of Mice Sick**
20053168-C	3/06/05	Geoduck Viscera	ADEC-EHL	3/14/05	66[2]	ADEC-EHL	3/14/05	40000-13Aug04	40005-05Nov04	1	0%	ADEC-EHL	03/15/05	FDA	3	71	0
20053169-C	3/06/05	Geoduck Viscera	ADEC-EHL	3/14/05	495	ADEC-EHL	3/14/05	40000-13Aug04	40005-05Nov04	1	<10%	ADEC-EHL	03/15/05	FDA	3	39	0
20053170-C	3/06/05		ADEC-EHL	3/14/05	650	ADEC-EHL	3/14/05	40000-13Aug04	40005-05Nov04	1	0%	ADEC-EHL	03/15/05	FDA	3	71	0
20053183-C	3/13/05	Geoduck	ADEC-EHL	3/15/05	416	ADEC-EHL	3/15/05	40000-13Aug04	40005-05Nov04	1	>0%, <25%	ADEC-EHL	03/15/05	FDA	3	70	0
20053184-C	3/13/05	Geoduck	ADEC-EHL	3/15/05	632	ADEC-EHL	3/15/05	40000-13Aug04	40005-05Nov04	1	0%	ADEC-EHL	03/15/05	FDA	3	54	0
20053185-C	3/14/05	Geoduck	ADEC-EHL	3/15/05	561	ADEC-EHL	3/15/05	40000-13Aug04	40005-05Nov04	1	0%	ADEC-EHL	03/15/05	FDA	3	72	0
20053186-C	3/15/05	Geoduck	ADEC-EHL	3/15/05	301	ADEC-EHL	3/15/05	40000-13Aug04	40005-05Nov04	1	0%	ADEC-EHL	03/15/05	FDA	3	90	0
20053137	03/06/05	Oyster	ADEC-EHL	03/08/05	150	ADEC-EHL	03/08/05	40000-13Aug04	40005-05Nov04	INV	C <25% T	ADEC-EHL	03/08/05	FDA	0	NDT	0
20053136	03/06/05	Oyster	ADEC-EHL	03/08/05	500	ADEC-EHL	03/08/05	40000-13Aug04	40005-05Nov04	N/A INV	C <25% T	ADEC-EHL	03/08/05	FDA	0	NDT	0
20053138	03/05/05	Oyster	ADEC-EHL	03/08/05	500	ADEC-EHL	03/09/05	40000-13Aug04	40005-05Nov04	INV	C <25% T	ADEC-EHL	03/08/05	FDA	0	NDT	0
20053142	03/06/05	Oyster	ADEC-EHL	03/09/05	50	ADEC-EHL	03/09/05	40000-13Aug04	40005-05Nov04	INV	C <50% T	ADEC-EHL	03/09/05	FDA	0	NDT	0
20053124-C	3/5/05	Geoduck	ADEC-EHL	3/7/05	495	ADEC-EHL	3/7/05	40000-13Aug04	40005-05Nov04	1	0%	ADEC-EHL	03/07/05	FDA	3	117	0
20053125-C	3/5/05	Geoduck	ADEC-EHL	3/7/05	404	ADEC-EHL	3/7/05	40000-13Aug04	40005-05Nov04	1	75%	ADEC-EHL	03/07/05	FDA	3	58	0
20053006	2/29/05	Oyster	ADEC-EHL	3/3/05	125	ADEC-EHL	3/3/05	40000-13Aug04	40005-05Nov04			ADEC-EHL	3/3/05	FDA	0	NDT	0
20053040-C	03/01/05	Geoduck Viscera	ADEC-EHL	03/02/05	545	ADEC-EHL	03/02/05	40000-13Aug04	40009-06Oct04	1	50%	ADEC-EHL	03/02/05	FDA	3	86	0
20053039-C	03/01/05	Geoduck Viscera	ADEC-EHL	03/02/05	340	ADEC-EHL	03/02/05	40000-13Aug04	40009-06Oct04	1	10%	ADEC-EHL	03/02/05	FDA	3	175	0
20053007-C	02/26/05	Geoduck Viscera	ADEC-EHL	02/28/05	750	ADEC-EHL	03/01/05	40000-13Aug04	40009-06Oct04	1	25%	ADEC-EHL	02/28/05	FDA	3	59	0
20053010-C	02/26/05	Geoduck Viscera	ADEC-EHL	02/28/05	750	ADEC-EHL	03/01/05	40000-13Aug04	40009-06Oct04	1	<25%	ADEC-EHL	02/28/05	FDA	3	65	0
2005301-C	02/27/05	Geoduck Viscera	ADEC-EHL	02/28/05	750	ADEC-EHL	03/01/05	40000-13Aug04	40009-06Oct04	1	0%	ADEC-EHL	02/28/05	FDA	3	151	0

Jellett Rapid Testing Ltd.: NOAA Study
JREM Trial Sample Record Sheet – Homogenate
California – Microbial Disease Lab

	Collection		Homogenization			Jellett Test						MBA Test					
Sample ID	**Collection Date**	**Species**	**Field / Site / Lab Name**	**Date**	**Size of Sample (mL)**	**Field / Site / Lab Name**	**Date**	**Batch # - Test**	**Batch # - Buffer**	**Result (1=Pos, 0=Neg)**	**Intensity of C Line as % of T**	**Lab Name**	**Date**	**Toxin Standard Used**	**# of Mice Dead**	**Result µg/100g**	**# of Mice Sick**
05E-00110	02/05/05	LBMU	CA-DHS-EMDS	02/09/05	>130	CA-DHS-EMDS	02/09/05	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	2/09/05	FDA	0	<36	0
05W-00099	02/01/05	SSMU	CA-DHS-EMDS	02/02/05	>130	CA-DHS-EMDS	02/02/05	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	02/02/05	FDA	0	<34	0
05E-00096	02/28/05	CBMU	CA-DHS-EMDS	02/02/05	>130	CA-DHS-EMDS	02/02/05	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	02/02/05	FDA	0	<36	0
05W-00093	02/01/05	SBMU	CA-DHS-EMDS	02/02/05	>130	CA-DHS-EMDS	02/02/05	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	02/02/05	FDA	0	<36	0
05W-00079	01/25/05	SSMU	CA-DHS-EMDS	01/26/05	>130	CA-DHS-EMDS	01/26/05	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	1/26/05	FDA	0	<35	0
05W-00076	01/22/05	CBMU	CA-DHS-EMDS	01/26/05	>130	CA-DHS-EMDS	01/26/05	40000-8/13/04	40005-9/7/04	1	50%	CA-DHS-EMDS	01/26/05	FDA	3	39	0
05W-00069	01/24/05	SBMU	CA-DHS-EMDS	01/26/05	>130	CA-DHS-EMDS	01/26/05	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	01/26/05	FDA	0	<36	3
05W-00059	01/18/05	SSMU	CA-DHS-EMDS	01/19/05	>130	CA-DHS-EMDS	01/19/05	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	1/19/05	FDA	0	<35	3
05W-00055	01/14/05	CBMU	CA-DHS-EMDS	01/18/005	>130	CA-DHS-EMDS	01/18/05	40000-8/13/04	40005-9/7/04	1	25%	CA-DHS-EMDS	01/18/05	FDA	3	37	
05W-00052	01/17/05	SBMU	CA-DHS-EMDS	01/18/05	>130	CA-DHS-EMDS	01/18/05	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	01/18/05	FDA	0	<36	0
05W-00025	1/10/05	SBMU	CA-DHS-EMDS	1/12/05	>130	CA-DHS-EMDS	1/12/05	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	1/12/05	FDA	0	<35	0
05W-00023	1/11/05	SSMU	CA-DHS-EMDS	1/12/05	>130	CA-DHS-EMDS	1/12/05	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	1/12/05	FDA	0	<36	0
05W-00020	1/7/05	CBMU	CA-DHS-EMDS	01/11/05	>130	CA-DHS-EMDS	01/11/05	40000-8/13/04	40005-9/7/04	1	25%	CA-DHS-EMDS	1/11/05	FDA	3	44	0

Jellett Rapid Testing Ltd.: NOAA Study
JREM Trial Sample Record Sheet – Homogenate
California – Microbial Disease Lab (CONTINUED)

	Collection		Homogenization			Jellett Test						MBA Test					
Sample ID	**Collection Date**	**Species**	**Field / Site / Lab Name**	**Date**	**Size of Sample (mL)**	**Field / Site / Lab Name**	**Date**	**Batch # - Test**	**Batch # - Buffer**	**Result (1=Pos, 0=Neg)**	**Intensity of C Line as % of T**	**Lab Name**	**Date**	**Toxin Standard Used**	**# of Mice Dead**	**Result µg/100g**	**# of Mice Sick**
05W-00011	1/3/05	SBMU	CA-DHS-EMDS	1/5/05	>130	CA-DHS-EMDS	1/5/05	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	1/5/05	FDA	0	<34	0
05W-00007	¼/05	SSMU	CA-DHS-EMDS	1/5/05	>130	CA-DHS-EMDS	1/5/05	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	1/5/05	FDA	0	<34	0
05W-00002	12/30/04	CBMU	CA-DHS-EMDS	1/04/05	>130	CA-DHS-EMDS	1/04/05	40000-8/13/04	40005-9/7/04	0	75%	CA-DHS-EMDS	1/04/05	FDA	2	36	1
04W-01458	12/28/04	SSMU	CA-DHS-EMDS	12/29/04	>130	CA-DHS-EMDS	12/29/04	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	12/29/04	FDA	0	<36	0
04W-01454	12/27/04	SBMU	CA-DHS-EMDS	12/29/04	>130	CA-DHS-EMDS	12/29/04	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	12/29/04	FDA	0	<36	0
04W-01457	12/24/04	CBMU	CA-DHS-EMDS	12/28/04	>130	CA-DHS-EMDS	12/28/04	40000-8/13/04	40005-9/7/04	1	<25%	CA-DHS-EMDS	12/28/04	FDA	3	42	0
04W-1446	12/21/04	SSMU	CA-DHS-EMDS	12/22/04	>130	CA-DHS-EMDS	12/22/04	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	12/22/04	FDA	0	<34	0
04W-01436	12/20/04	SBMU	CA-DHS-EMDS	12/21/04	>130	CA-DHS-EMDS	12/21/04	40000-8/13/04	40005-9/7/04	0	75%	CA-DHS-EMDS	12/21/04	FDA	0	<34	3
04W-01399	12/13/04	SBMU	CA-DHS-EMDS	12/14/04	>130	CA-DHS-EMDS	12/15/04	40000-8/13/04	40005-9/7/04	1	50%	CA-DHS-EMDS	12/15/04	FDA	2	35	0
04W-01421	12/11/04	CBMU	CA-DHS-EMDS	12/15/04	>130	CA-DHS-EMDS	12/15/04	40000-8/13/04	40005-9/7/04	1	0%	CA-DHS-EMDS	12/15/04	FDA	3	48	0
04W-01424	12/14/04	SSMU	CA-DHS-EMDS	12/15/04	>130	CA-DHS-EMDS	12/15/04	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	12/15/04	FDA	0	<35	0

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 and Section IV. Guidance Documents, 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

Action by 2009 Laboratory Quality Assurance Committee

Recommended a substitute checklist for the Male-Specific Coliphage in Proposal 05-113 with the Male-Specific Coliphage Laboratory Method recommendation for acceptance by the Laboratory Methods Review Committee with the changes recommended by the Laboratory Quality Assurance Committee (Changes are denoted in bold).

(1) Request that the ISSC Executive Board appoint a workgroup to review the current format of the checklists on the ISSC Website and report their findings back to the Laboratory Quality Assurance Committee via email and conference call set by the ISSC Executive Office. Laboratory Quality Assurance Committee will report to the Executive Board with revisions to the checklists posted on the website.

(2) Request that the ISSC Executive Board charge the Laboratory Quality Assurance Committee to review the SLV Protocol for Acceptance of a New Method for compliance with quality assurance requirements and specifically when a developer of a newly accepted method by the ISSC is required to submit a checklist for the method to the Laboratory Quality Assurance Committee for review.

(3) Request the ISSC Executive Office make available on the ISSC website the step-by-step procedures for newly accepted lab methods for use in the NSSP.

(4) Request the ISSC Executive Board to change the structure of the Laboratory Quality Assurance Committee to a subcommittee of the Laboratory Methods Review Committee for better use of the member's expertise.

Action by 2009 Task Force I Recommended adoption of Laboratory Quality Assurance Committee recommendation on Proposal 05-113 with additional recommendations.

Actionby 2009 General Assembly Adopted recommendation of 2009 Task Force I on Proposal 05-113.

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		
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* F_{amp} 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**		**10. Immediately after blending , 33 grams of the homogenized elution mixture are weighed into centrifuge tubes.**
C	**28**		**11. The homogenized elution mixture is centrifuged for 15 minutes at 9000 x g at 4°C.**
C	**27, 28**		**12. The supernatant is pipetted off, weighed and the weight recorded.**
C	**27, 28**		**13. The supernatant is allowed to warm to room temperature about 20 to 30 minutes.**
K	27, 28		14. The autoclaved soft agar is tempered and held at 50 – 52°C throughout the period of sample analysis.
K	27, 28		15. 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		16. The sample supernatant is shaken or vortexed before being added to the tempering soft agar.
C	**27, 28**		**17. 2.5 ml of sample supernatant is added to each tube of tempering soft agar.**
C	**27, 28**		**18. The soft agar/sample supernatant/host cell mixture is gently rolled between the palms of the hands to mix.**
C	**27, 28**		**19. 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**		**20. 10 plates are used, 2.5 ml per plate for a total of 25 ml of supernatant analyzed per sample.**
K	27, 28		21. Negative and positive control plates are prepared and accompany each set of samples analyzed.
K	27, 28		22. Growth broth is used as the negative control or blank.
K	27, 28		23. Type strain MS2 (ATCC 15597) male specific upernata is used as the positive control.
K			24. A negative control plate is plated at the beginning and end of each set of samples analyzed.
K	27, 28		25. The positive control is plated after all the samples are analyzed and immediately prior to the final negative control.
C	**27, 28**		**26. 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		The formula used for determining the density of MSC in PFU/100 gm is: $(0.364)(N)(W_s)$, where N = total number of plaques counted on all 10 plates and W_s = weight of the supernatant used.
O	9		3. The MSC count is rounded off conventionally to give a whole number.

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 for the Control of Molluscan Shellfish*. 1997. FDA/ISSC.
21.	*Microbiological Methods for Monitoring the Environment, Water and Wastes*. 1978. US EPA/600/8/78/017.
22.	Furfari, Santo. March 21, 1972. Personal Communication to Dan Hunt, FDA.
23.	United States Environmental Protection Agency, *Improved Enumeration Methods for the Recreational Water Quality Indicators: Enterococci and Escherichia coli*. EPA/821/R-97-004, EPA, Washington, D.C.
24.	Rippey, Scott, R, Adams, Willard, N, Watkins, William, D. Enumeration of fecal coliforms and *E. coli* in marine and estuarine waters: an alternative to the APHA-MPN approach, Journal WPCF, 59, 8 (1987).
25.	FDA Manual of Interpretations, National Shellfish Sanitation Program *Guide for the Control of Molluscan Shellfish*, 2003 Revision, Interpretation Number 03-IV-@.02-102.
26.	*Membrane Filtration: A Users Guide And Reference Manual*, Thomas D. Brock, Science Tech Inc., Madison WI, 1983.
27.	Proceedings of the Male-specific Bacteriophage (MSC) Workshop, Gloucester, MA, March 9-12, 2004.
28.	MSC Method and SLV write-up, Spinney Creek Shellfish, Inc., September, 2009.

Proposal Subject:	Method to Determine the Presence of Male Specific Coliphage in Shellfish Meats and the Microbiology
Specific NSSP Guide Reference:	Section IV. Guidance Documents Chapter II. Growing Areas .10 Approved Laboratory Tests
Text of Proposal/ Requested Action	The MSC method must be reviewed and adopted prior to use in determining the acceptability of shellfish growing waters for reopening.
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). The MSC method must be reviewed and adopted prior to use in determining the acceptability of shellfish growing waters for reopening.
Cost Information (if available):	Not available.
Action by 2005 Laboratory Methods Review Committee	Recommended referral of Proposal 05-114 to the appropriate committee as determined by the Conference Chairman.
Action by 2005 Task Force I	Recommended adoption of the Laboratory Methods Review Committee recommendation on Proposal 05-114.
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 05-114. Rationale – The data necessary to approve the method is not available. The submitter will send data to the Executive Office for Conference approval consistent with Procedure XVI.
Action by 2007 Task Force I	Recommended referral of Proposal 05-114 to an appropriate committee as determined by the Conference Chairman.
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.

Enumeration of Male- specific bacteriophage in water and shellfish tissue

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What are male- specific (f-specific) bacteriophage?

•Lytic viruses of bacteria- (killing of host bacteria)

E. coli and S. typhimurium

(production of E. coli pili*)*

•Requires a piliated host cell for adsorption, they do not attach to cell surface (somatic).

•Requires host cell in log- phase of growth- cells do not produce pili at < 30°C

•Optimal growth temperature: 35- 37°C.

•Plaque size is generally self- limiting

Two Predominant Host Strains

•*E. coli* HS(pFamp)RR

Section IV. Resistant to Streptomycin and Ampicillin

•*Salmonella typhimurium* WG49

Result of mating: *E. coli* WG27 (piliated)

x

S. typhimurium WG45

-Resistant to Naladixic acid and Kanamycin

--Pili production in each strain is plasmid mediated

Media Composition
E. coli Famp

Bottom Agar

•Tryptone 10.0 g
•Dextrose 1.0 g
•NaCl 5.0 g
•Agar 15.0 g
••Water 1000 ml

•Autoclave 121℃ 15 min
- temper to 50°C.
•Add 0.05 g Streptomycin sulfate
0.05 g Ampicillin (aseptically)

DS Soft Agar

•Tryptone 10.0 g
•Dextrose 1.0 g
•NaCl 5.0 g
•1M $CaCl_2$ 0.5 ml
•Agar 7.0 g
••Water 500 ml
••Boil- Dispense in 2.5 ml aliquots (16 x 100 ml tubes) and freeze (-20°C)
••Autoclave prior to use;
temper to 50- 52° C

Growth broth- same formulation as Bottom Agar w/o agar or antibiotics

Media Composition
S. typhimurium WG49
Bottom Agar

•Trypticase Peptone 10.0 g
•Yeast Extract 1.0 g
•Dextrose 1.0 g
•NaCl 8.0 g
•Agar 15.0 g
••Water 1000 ml

•Autoclave 121℃ 15 min
- temper to 50°C.
•Add 0.10 g Naladixic Acid
0.02 g Kanamycin sulfate (aseptically)

DS Soft Agar

•Trypticase Peptone 10.0 g
•Yeast Extract 1.0 g
•Dextrose 1.0 g
•NaCl 5.0 g
•1M $CaCl_2$ 0.5 ml
•Agar 7.0 g
••Water 500 ml
••Boil- Dispense in 2.5 ml aliquots (16 x 100 ml tubes) and freeze (-20°C)
••Autoclave prior to use;

temper to 50- 52° C

Growth broth- same formulation as Bottom Agar w/o agar or antibiotics

Differentiation of RNA and DNA Bacteriophage

•RNAse Type I-A Sigma # R4875
••Final conc= 100ug/ ml of media
•Stock concentration= 10 mg/ml (100X)
••Dissolve at a conc. Of 10 mg/ml in 0.01 M Sodium Acetate (pH 5.2); Boil for 15 min and allow to cool to RT; PH by adding 0.1 vol of 1M Tris HCl (pH 7.4)
••Store @ -20C

Propagation of *E. coli* Famp

Bottom Agar Streak plate-
Transfer preferable < 1 week old.
•Broth Growth medium tempered to 35- 37°C- vortex to aerate.
•Using 10ul loop collect material from of several colonies and transfer to broth medium.
•Shake briefly to mix, then incubate at 35- 37°C for 4-6 hours
(turbidity ≈ 10^7cells/ ml; O.D @540 nm= 0.4)

% RECOVERY OF BACTERIOPHAGE F-2W/ VARIOUS AGES OF FAMP CULTURE

Age of a 10 ml host cell culture(h)	% Recovery (Mean ± SD)3.0
	105.4 ± 2.5
3.5	97.4 ± 2.2
4.0	96.0 ± 2.8
4.5	95.0 ± 3.3
5.0	92.5 ± 1.2
6.0	90.9 ± 1.8

Adapted from DeBartolomeis, 1999

<u>For MSB density determinations in shellfish tissue</u>

1. Homogenize by blending 12 shellfish for 1 min at high speed.
2. Aliquot 30- 50 g from each sample into centrifuge bottle.
3. Centrifuged for 15 min. @ 9,000 x g; 4°C.
4. Collect and weigh supernatant in a sterile container.
5. Allow supernatant to warm to RT (20- 30 min)
6. Combine 2.5 ml aliquot of supernatant, 2.5 ml DS Soft agar (tempered to 52°C) and 0.2 ml of *E. coli* HS(pFamp)RR
7. Overlay onto a tryptone agar plate containing streptomycin/ ampicillin (50 µg/ml final).
8. Plates are inverted and incubated for 18- 24 h @ 35- 37°C

Information needed for

Bacteriophage density determinations:

Plate counts of plaques

g Shellfish homogenate centrifuged
g Shellfish supernatant recovered

Calculations

MSB/ 100 grams=

1) Ave PFU/ plate ÷ number of ml added/ plate= Average PFU/ml

2) Average PFU/ml x grams of supernatant x 100 g . = PFU/ 100 g

g homogentate

Example: Plate counts- 75, 73,80; 2.5 ml/ plate

50 g homogenate; 33 g supernatant

76 ÷ 2.5 x 33 g supernatant x 100 grams = 2006 PFU/ 100 grams

50 g homogenate

To determine level of sensitivity

3 plates containing 0, 0, 0; 2.5 ml/ plate
50 g Homogenate; 33 g Supernatant
Assume 1 plaque on 1 plate then calculate
1÷ 3 plates ÷ 2.5 ml x 33 x (100 ÷ 50) =
Reported as < 9 pfu/ 100 grams

For MSB density determinations in low contaminated water- Concentration technique

1. Weigh 100 ml of water in a sterile container centrifuge bottle.
2. Allow water to warm to RT (20- 30 min).
3. Add 1g tryptone and 1 g beef extract to water aliquot, shake to dissolve.
4. Add 10 ml of *E. coli* Famp culture- Do not shake
5. Incubate at 35- 37°C for 50 min – rotate at 100 rpm.
6. Centrifuged for 15 min. @ 9,000 x g; 4°C.

For MSB density determinations in highly contaminated water (> 100 pfu/ 100 ml)

1. Allow an aliquot of water to warm to RT (20- 30 min)
2. Combine 2.5 ml aliquot of supernatant, 2.5 ml DS Soft agar (tempered to 52^{o} C), and 0.2 ml of *E. coli* HS(pFamp)RR
3. Overlay onto a tryptone agar plate containing streptomycin/ ampicillin (50µg/ml final).
4. Plates are inverted and incubated for 18- 24 h @ 35- 37°C

Problems that may arise

Multiple layers are formed after centrifugation

Reason- glycogen- lipids associated w/ shellfish physiological state

Sliding pellet- not solid

Reason- waited too long to remove supernatant

Clumping Agar

Reason- sample was too cool

Runny plaques

Reason- wet plates; too much condensation

No plaques/ individual bacterial colonies on agar plates

Reason- no phage present or inadequate amount host cell

Ways of Enhancing Plaque Visibility

Addition of 2,3,5- triphenyl tetrazolium chloride (TTC), 1% solution in ethanol
65 ul / tube of tempered DS soft agar
Assuming: 2.5 ml of DS agar and
2.5 ml sample
or
Grams Safrin 1:100 in water- differentiates lawn from plaque

Storage of *E. coli* Famp

Selective pressure- Streptomycin and Ampicillin

Bottom Agar Streak Plate
Storage: Refrigerator (2-3 weeks)
•Tryptic Soy Agar Deep w/ Mineral oil overlay
Storage: Room temperature in Dark (2-5 years +)
•Addition of glycerol (10% final) into broth culture. Storage: Freeze at – 80°C (Indefinite?)

Source of Bacterial Host Strains

•*E. coli* HS(pFamp)R; ATCC #700891
•*Salmonella choleraesuis* subsp. *Choleraesuis* (Smith) Weldin serotype Typhimurium aka WG49; ATCC #700730

Types and Sources of Positive MSB Controls

Bacteriophage MS2; ATCC# 15597-B1

Bacteriophage Fd; ATCC# 15669 –B2

Municipal Wastewater

Bacteriophage Stability in Shellfish Homogenate

		Time (h)		
Temperature	**Addition**	**0**	**4**	**10**
1- 3 °C	**-**	**2.57**	**2.58**	**2.42**
25°C	**-**	**3.81**	**3.64**	
25°C	**Log Famp[a]**	**3.81**	**3.86**	**3.89 (24h)**
35°C		**3.81**	**3.45[b]**	

[a]Famp added at a density of 270 cells/ g
[b]Significant decrease at 95% Confidence limit

Bacteriophage Stability in Shellfish Supernatant

		Time (h)		
Temperature	**Addition**	**0**	**4**	**24**
25°C	**-**	**3.81**	**3.74**	**2.60[b]**
25°C	**Log Famp[a]**	**3.81**	**3.13[b]**	**2.90[b]**
35°C	**-**	**3.81**	**3.73**	**2.90[b]**
35°C	**Log Famp[a]**	**3.81**	**3.56[b]**	**5.51[c]**

[a]Famp added at a density of 270 cells/ g
[b]Significant decrease at 95% Confidence limit
[c]Significant increase at 95% Conifidence limit

Action by 2009 Laboratory Methods Review Committee Recommended adoption of the substitute MSC method as a Type IV method for analysis of soft shell clam and oyster tissue to determine impacts of wastewater treatment plant effluent spills.

Action by 2009 Task Force I Recommended adoption of Laboratory Methods Review Committee recommendation on Proposal 05-114.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force I on Proposal 05-114.

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

This method for determining levels of male-specific upernata in soft shelled clam and American oyster meat is based on the method described by DeBartolomeis and Cabelli[1,2]. FDA has refined the method for oyster and hard clam meats as described in the workshop instructions, *Male-specific Bacteriophage (MSB) Workshop*, conducted in Gloucester, Massachusetts on March 9-12, 2004[3]. This original FDA (2004) method was submitted as ISSC Proposal 05-114.

Modification of the FDA (2004) Method

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

A. Apparatus and Materials.

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

Laboratory Equipment:
Centrifuge with rotor for 50 ml conical (or larger) tubes, 9000 x *g* performance capability, 4°C
Water bath, 50-52°C
Air Incubator, 35-37°C
Balance
Stir plate and magnetic stirring bars, sterile
Mini vortexer
Blender
Autoclave, 121°C
Refrigerator, 0–4° C
Freezer, -20°C
Thermometers, range -20–121°C
pH meter
Erlenmeyer flasks, 1 L and 2 L
Graduated cylinders, 100 ml, 500 ml and 1000 ml
600 ml beaker
500 ml jars, autoclavable with caps

Inoculating loops (3 mm in diameter or 10 □L volume)
Bacti-cinerator
Sterile swabs
Sterile, disposable filters, 0.22 or 0.45 µm pore size
Syringes, sterile disposable; 5, 10 or 20 ml
Scrub brushes, sterile
Knives, sterile
Blender jars, sterile
Sterile plastic cups 250 ml
Pipets- 5 ml, 10 ml
Pipet-aid
Micro-Pipettors, 100 µL, 200 µL, 1000 µL, 2500 µL
Micro-Pipet tips 200 µL, 1000 µL, 2500 µL
Pipetor Stand
Centrifuge tubes, sterile disposable 50 ml or larger
Petri dishes, sterile disposable 100 x 15 mm
Petri dish racks
Test tubes 16 x 100 mm (for soft agar)
Test tubes 16 x 150 mm, with screw caps
Test tube racks–size to accommodate tubes
Freezer vials, sterile 30 ml with screw caps
Baskets with tops to hold freezer vials
Parafilm tape
Aluminum foil

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

Antibiotic stocks:
Ampicillin sodium salt (Sigma A9518)
Streptomycin sulfate (Sigma S6501)
Streptomycin and Ampicillin stock solutions (50 µg/ml each). Note: Antibiotics must always be added to liquids and media after these have been autoclaved and cooled.

Media:
Bottom Agar
DS Soft Agar
Growth Broth

Bacterial Host Strain:
E.coli $F_{amp}^{\sim}$ *E. coli* HS(pFamp)RR (selected by Dr. Victor J. Cabelli, University of Rhode Island, Kingston, RI, USA, frozen stock ATCC # 700891).

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

B. Media Composition.

Bottom Agar:

Tryptone	10.0 g
Dextrose	1.0 g
NaCl	5.0 g
Agar	15.0 g
DI water	990 ml
Final pH	6.7 ± 0.2 at 25°C

1. With gentle mixing, add all the components to 990 ml of dH_2O in a 2000 ml flask. Dissolve, heat until clear.
2. Sterilize at 121°C ± 2°C for 15 minutes.
3. Temper to 50°C in the water bath.
4. Add 5 ml of Streptomycin sulfate/Ampicillin solution, aseptically to the flask (50 µg/ml each in final) and mix. Transfer to 2 – 500ml sterile jars (easier to pour plates from jars).
5. Pipet (or pour) 15 ml aliquots aseptically into sterile 100 x 15 mm Petri dishes and allow the agar to harden. Tip Petri dish lids off slightly to reduce condensation.
6. Store bottom agar plates inverted at 4°C and warm to room temperature for 1 hour before use.
7. Plates stored sealed at 4°C can be used up to 3 months.

Streptomycin sulfate/Ampicillin Solution:

1. Dissolve 0.5g of streptomycin sulfate and 0.5g of ampicillin in 50 ml of dH_2O with a sterile 100 ml graduated cylinder in sterile 600 ml beaker with sterile stir bar.
2. Stir for 2 to 3 minutes, no heat.
3. Filter through sterile 0.22 µm filter.
4. Store in 5 ml aliquots in sterile 30 ml capped freezer vials at -20°C for up to one year. Label and date.
5. Allow to come to room temperature before adding and mixing in tempered bottom agar at 50°C.

DS Soft Agar:

Tryptone	10.0 g
Dextrose	1.0 g
NaCl	5.0 g
1M $CaCl_2$	0.5 ml
Agar	7.0 g
DI water	500 ml
Final pH	6.7 ± 0.2

1. With gentle mixing, add all the components to 500 ml of dH_2O in a 1000 ml flask.
2. Bring flask contents to a boil.
3. Dispense in 2.5 ml aliquots into 16 x 100 ml tubes, cover and freeze (-20°C) for up to three months.
4. Sterilize prior to use at 121°C ± 2°C for 15 minutes, then temper to 50-52°C for no longer than 2 hours

1M $CaCl_2$ Solution:

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

Growth Broth:

Tryptone	10.0 g
Dextrose	1.0 g
NaCl	5.0 g
DI water	1000 ml

1. With gentle mixing, add all the components to 1000 ml of dH_2O water in a 2000 ml flask.

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

Storage Slants: Tryptic Soy Agar.

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

Storage:
1. Lab stock culture – Frozen at – 80°C indefinitely (most desirable method) in broth culture containing 10% glycerol under no selective pressure. Selective pressure is reapplied when the culture is retrieved, by streaking onto Bottom Agar plates containing the two antibiotics.
2. Long-term working stock culture – Grown tryptic soy agar slant with sterile mineral oil overlay under no selective pressure and stored at room temperature in the dark for up to 2 years.
3. Long-term working stock – 6-hour grown tryptic soy agar slant and deep stab with sterile mineral oil overlay containing the two antibiotics, Ampicillin and Streptomycin (least desirable method).
4. Short-term working stock culture – Grown Bottom Agar streak plate stored at 4°C up to 3 weeks.
5. Short-term working stock culture – Grown in Growth broth and used within 6-12 hours (same day).

Glycerol Solution, 10%: Add 9 ml of distilled water to 1 ml of undiluted glycerol. Autoclave resulting 10% glycerol solution at 121°C for 15 minutes and use at room temperature. For storage, add $1/5^{th}$ volume of 10% glycerol solution, let stand for 30 minutes, dispense 1 ml aliquots in 2 ml cryo-vials and store at -70 to –80°C (best) or at –20°C.

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

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

D. Control Plates.
1. Negative Control – Add 2.5 ml of Growth Broth and 0.2 ml host to the 2.5 ml DS Soft Agar tube.
2. Positive Control – Make serial dilutions using growth broth of the concentrated MS2 control (to grow approximately 50-100 PFU per 2.5 ml), and add 2.5 ml of appropriate MS2 dilution and 0.2 ml of host to 2.5 ml DS Soft agar.

E. MSC Density Determinations in Soft Shelled Clam and American Oyster Tissues.

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

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

alcohol and allow to air dry. Shellfish are shucked and the meats and liquors are saved into a sterile 250 ml cups.

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

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

Calculations of Results

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

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

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

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

F. Sample Collection and Storage.

2. Record all pertinent information on the collection form.
3. During transportation store samples in a cooler at 0 to 10°C.
4. At laboratory, store samples in a refrigerator at 0 to 4 °C.
5. Maximum holding times for shellfish samples is up to 24 hours.

G. Quality Assurance.

1. Positive and negative control plates are run with MSC analyses each day.
2. Media sterility checks are made per batch and records are maintained.
3. Media log book is maintained (pH, volume, weights of each components, lot numbers, etc.).
4. An intra- and inter-laboratory performance program is developed.
5. Circular zones of clearing (typically 1 to 10 mm in diameter) in lawn of host bacteria after 16- 20 hours of incubation are counted as plaques. (Count the number of plaques on each plate.)

6. MSC determinations are reported as plaque forming unit (PFU) per 100 grams.
7. The desired range for counting is 0 to 100 PFU per plate. If the count exceeds the upper range or if the plaques are not discrete, results should be recorded as "too numerous to count" (TNTC) or >10,000 PFU of MSC/100gm.
8. Temperatures incubators are checked twice daily (at least 4 hours apart) to ensure operation within the stated limits of the method, and results are recorded in a logbook.
9. Check thermometers at least annually against a NIST-certified thermometer.
10. Calibrate the balance monthly using ASTM-certified Class 1 or 2 or NIST Class S reference weights.
11. Laboratory analysts adhere to all applicable quality control requirements set forth in the most recent version of FDA's *Shellfish Laboratory Evaluation Checklist*.
12. Calibration of micro-pipettors needs to be checked quarterly and records kept. Micro-pipettors used for handling MSC control and transferring host cells need to have a barrier tip or be dedicated to the specific use to prevent contamination

H. Safety.

Samples, reference materials, and equipment known or suspected to have Coliphage attached or contained must be sterilized prior to disposal.

I. Technical Terms.

°C - degrees Celsius
µL - microliter
g - gram
L - liter
M - molar
ml - milliliter
rpm - revolutions per minute
Ave. - average
MSC - Male-specific Coliphage, Male-specific Bacteriophage, F+ Bacteriophage
NIST - National Institute of Standards and Technology
PFU - plaque forming units
RT - room temperature
TNTC - too numerous to count
LOD - Limit of Detection
LOQ - Limit of Quantitation
Host Strain - *E.coli* F_{amp} bacteria (*E.coli* HS(pFamp)RR)
Male-specific Coliphage – Viruses that infect coliform bacteria only via the F-pili.
Plaque - Clear circular zones (typically 1 to 10 mm in diameter) in lawn of host cells after incubation.

References:

1. Cabelli, V.J. 1988. Microbial indicator levels in shellfish, water, and sediments from the upper Narragansett Bay conditional shellfish-growing area. Report to the Narragansett Bay Project, Providence, RI.
2. DeBartolomeis, J. and V.J. Cabelli. 1991. Evaluation of an *Escherichia coli* host strain for enumeration of F male-specific Coliphages. Appl. Environ. Microbiol. 57(4):1201-1205.
3. U.S. Food and Drug Administration. 2004. Male-specific Coliphage (MSC) Workshop, conducted in Gloucester, Massachusetts on March 9-12, 2004.

Other Information:

This method for the enumeration of male-specific upernata in soft-shelled clams and American oysters is inexpensive, easy to perform, and rapid, providing results within 24 hours. The cost of laboratory glassware, plastic-ware, agars, and reagents is approximately $25 per shellfish sample. In a well set-up laboratory, the method requires 6 hours of time from initiating host to pouring plates. Hands on technician time to perform

this test is significantly less on the order of 1-4 hours per test depending upon how many tests are done per day. The most expensive piece of equipment is a refrigerated centrifuge plus rotor, which costs approximately $10,000. There are no special skill sets required beyond those required to operate a state-approved shellfish laboratory under the NSSP.

Proposal Subject:	Thermazyme™ ACP Test
Specific NSSP Guide Reference:	NSSP Section IV Guidance Documents Chapter II. Growing Areas .10 Approved Laboratory Tests
Text of Proposal/ Requested Action	Advanced Instruments, Inc. request ISSC adoption of this method for use in the National Shellfish Sanitation Program
Public Health Significance:	Thermazyme™ ACP Test will provide the basis for determining if shellfish have been thermally processed. This test will allow decisions to be based on a rapid, quantitative method rather than sensory related methods.
Cost Information (if available):	Not available
Action by 2005 Laboratory Methods Review Committee	Recommended the Conference direct the ISSC Executive Office to continue to investigate the issue of standards and pursue the development of standards and report back to the Laboratory Methods Committee with progress on the issue in six (6) months.
Action by 2005 Task Force I	Recommended adoption of the Laboratory Methods Review Committee recommendation for Proposal 05-115.
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 referral of Proposal 05-115 to the Executive Board for consideration for interim approval. Insufficient data at this time to approve this method under Procedure XVI. Need AP curves at 145 for 15 seconds for each type of shellfish.
Action by 2007 Task Force I	Recommended adoption of the Laboratory Methods Review Committee recommendation on Proposal 05-115.
Action by 2007 General Assembly	Adopted recommendation of 2007 Task Force I.
Action by USFDA	December 20, 2007 Concurred with Conference action.
Action by 2009 Laboratory Methods Review Committee	Recommended referral of Proposal 05-115 to the appropriate Committee as determined by the Conference Chairman to review new data as it becomes available.
Action by 2009 Task Force I	Recommended adoption of Laboratory Methods Review Committee recommendation on Proposal 05-115.
Action by 2009 General Assembly	Adopted recommendation of 2009 Task Force I on Proposal 05-115.

ADVANCED INSTRUMENTS, INC.

RECEIVED JUN 2 8 2004

June 25, 2004

Ken Moore
Executive Director
Interstate Shellfish Sanitation Conference
209-2 Dawson Drive
Columbia, SC 29223

Dear Mr. Moore:

Due to the advice of the USFDA Office of Seafood, I am writing this letter to request a review and approval of the Thermazyme™ ACP Test for use on thermally processed (cooked versus raw) shellfish products in order to make decisions based on a rapid, quantitative method rather than sensory related methods.

I have enclosed some literature and materials to demonstrate how the Fluorophos® ALP Test and Thermazyme ACP Test have brought value to the dairy and meat processing industries as an assessment tool for determining lethality of the kill step and finished product analysis.

Catherine Cutter, Ph.D documents the current situation in the seafood industry and has scientifically demonstrated that the Thermazyme ACP Test could also be utilized to advance the cause of food safety, thereby protecting consumers by minimizing the potential of under processed products making it into distribution channels.

Please have this method reviewed and approved for its use by seafood processors and agencies interested in maintaining the highest level of public safety.

I will be out of the office from June 28-July 6th. For assistance you may contact Eileen Garry, R&D Lab Manager, Advanced /Instrument, Inc. at 781-320-9000 X2118 or email eileeng@aicompanies.com or Gary Wolf, Regional Shellfish Specialist, FDA Office of Seafood, Vorhees, NJ, at 856-783-1420 X13 or Email - gwolf@ora.fda.gov.

I look forward to speaking with you about this exciting opportunity for the industry and thank you for your attention to this important development.

Sincerely,

Kenneth F. Micciche
Director of Marketing
Advanced Instruments, Inc.
Office – 781-471-2145
Facsimile 781-320-8181
Cell 781-354-9739

RECEIVED JUN 2 8 2004

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TECHNICAL REPORT

NUMBER: TR203 DATE: 04 May 24

TITLE: Performance characteristics of the ThermaZyme® acid phosphatase ("ACP") measurement system on seafood.

AUTHOR: R. A. LaBudde

ABSTRACT: Data from a recent study of the use of the ThermaZyme® acid phosphatase measurement system on seafood was analyzed to assess relevant performance characteristics such as accuracy and precision, false positive and false negative error rates and other parameters. Although the data in the study were limited, some quantitative assessment of these parameters was possible.

KEYWORDS: 1) THERMAZYME 2) ACP 3) EPT

REL.DOC.:

REVISED: 04 May 28

INTRODUCTION

The use of heat-labile enzymes in the determination of cook endpoint temperatures has a long history in the food industry [1-13]. Heat lethality in bacteria is believed to be due primarily to denaturation of cellular enzymes, so verification of destruction of such enzymes is highly correlated to bacterial destruction.

Catalase (meat), alkaline phosphatase (milk) and acid phosphatase (various foods) have been used as surrogates to verify post-process that adequate pasteurization has taken place [2-12].

The ThermaZyme® system, distributed by Advanced Instruments, is based on the fluorometric measurement of acid phosphatase ("ACP") enzyme. Several validation studies have been published for the system involving a variety of food products.

Recently, a study by Cutter and Miller [1] has investigated ACP for endpoint temperature verification in seafood.

BACKGROUND ASSUMPTIONS RELATED TO ACP IN SEAFOOD

The performance characteristics and inferences with respect to ACP in seafood are based entirely on the work done by Cutter and Miller [1]. In particular, the population of inference is limited to those sources of supply locally available to these authors. However, in recommended use, the method may be calibrated using samples of control raw material and cook EPT determination to establish a standard curve for the relevant population of inference.

PERFORMANCE CHARACTERISTICS IN SEAFOOD

The ThermaZyme system may be used to verify endpoint temperatures in two different ways:

1. INFERENTIAL: Was the food processed to a specific minimum endpoint temperature ("EPT") or higher?
2. ESTIMATION: What was the highest equivalent endpoint temperature to which the food was exposed?

A. ACCURACY:

The ThermaZyme Test System can detect as low as 0.1 U/kg of sample, based on a 1:3 initial dilution.

Based on the Cutter and Miller data [1], estimation of endpoint temperature is subject to the following precisions, based on each test comprising the average of 5 replications:

PRECISION OF ENDPOINT TEMPERATURE			
Seafood	*Range*	*Standard error of fit*	*95% Confidence Interval of EPT*
Clams	130-165 F	9.1 F	+/- 8.6 – 14.9 F
Lobster	140-165 F	5.4 F	+/- 6.1 – 13.9 F
Oysters	140-175 F	3.8 F	+/- 3.4 – 5.9 F
Shrimp	140-165 F	5.4 F	+/- 6.1 – 13.9 F

B. SPECIFICITY:

For the inference that raw seafood has been cooked to a specified minimum EPT:

RAW SEAFOOD COMPARED TO MINIMUM ENDPOINT TEMPERATURE		
Seafood	*Minimum EPT*	*False Positive Rate*
Clams	130 F	0.0064%
Lobster	140 F	0.0987%
Oysters	150 F	1.7385%
Shrimp	140 F	0.3711%

C. PRECISION:

Based on the Cutter and Miller data [1], estimation of endpoint temperature is subject to the following precisions, based on each test comprising the average of 5 replications:

PRECISION OF ENDPOINT TEMPERATURE			
Seafood	*Range*	*Standard error of fit*	*95% Confidence Interval of EPT*
Clams	130-165 F	9.1 F	+/- 8.6 – 14.9 F
Lobster	140-165 F	5.4 F	+/- 6.1 – 13.9 F
Oysters	140-175 F	3.8 F	+/- 3.4 – 5.9 F
Shrimp	140-165 F	5.4 F	+/- 6.1 – 13.9 F

D. SENSITIVITY:

The ThermaZyme Test System can detect as low as 0.1 U/kg of sample, based on a 1:3 initial dilution.

E. SELECTIVITY:

The test is specific for the ACP enzyme involved and has no interferences from other compounds.

F. ASSAY INTERVAL:

Each test involves comminution of the bulk sample, possible draining, weighing of a 0.8 g specimen, dilution with standard reagents, homogenization and measurement in the fluorometer. Total time expended per sample is less than 10 minutes for one replicate and an additional 5 minutes for each further replicate.

G. ASSAY COST:

Reagent costs per replicate are approximately $3.00 with approximately 1/6 hr of analyst time.

H. COMPARABILITY:

Alternative methods of verifying EPT are limited. The most obvious being Aerobic Plate Count ("APC") microbial determination. In this case, the analysis cost is approximately $1.00-$2.00 in supplies and 1/6 hr of analyst time per replicate. For viral determinations, the cost would be significantly higher ($30-$100).

I. OTHER STUDIES:

See references [2-13] for studies based on acid or alkaline phosphatase as a means of cook endpoint temperature determination in various meat and dairy products.

J. REGULATORY APPROVALS:

1. AOAC First Action, 1991.
2. AOAC Final Action, 1995. Method 979.13.
3. International Dairy Federation, 1992.
4. Interstate Milk Shippers, 1993.
5. ISO/DIS 11816-2, 2001.
6. FDA, 1995. (Cheese)
7. NCIMS, 2001. (Cream)

REFERENCES

1. Cutter CN and Miller BJ. 2003. Use of an acid phosphatase assay to detect deviations in thermal processing of seafood. J Assoc Food and Drug Officials 67(4):1-14.
2. Davis CE. 1998. Fluorometric determination of acid phosphatase in cooked, boneless, nonbreaded broiler breast and thigh meat. J AOAC Inter 81(4):887-906.
3. Davis CE and Townsend WE. 1994. Rapid fluorometric analysis of acid phosphatase activity in cooked poultry meat. J Food Prot 57:1094-1097.
4. Lyon BG et al. 2001. Acid phosphatase activity and color changes in consumer-style griddle-cooked ground beef patties. J Food Prot 64:1199-1205.
5. Wang SF et al. 1996. Proteins as potential endpoint temperature indicators for ground beef patties. J Food Sci 61(1):5-7.
6. Wilson CE et al. 2002. Influence of Listeria spp. contamination on end-point temperature determination in broiler breast patties by a fluorometric acid phosphatase assay.
7. Cohen EH. 1969. Determination of acid phosphatase activity in cooked hams as an indicator of temperature attained during cooking. Food Tech 23:101-104.
8. Kormendy L et al. 1987. Determination of the extent of heat treatment in canned hams by use of the phosphatase test. Meat Sci 19:77-?.
9. Lint J. 1965. The determination of the acid phosphatase activity in canned hams. Report 25/65A, Danish Meat Products Laboratory, Royal Veterinary and Agricultural College, Denmark.
10. Rocco RM. 1990. Fluorometric analysis of alkaline phosphatase in fluid dairy products. J Food Prot 53:588-591.
11. Rocco RM. 1990. Fluorometric determination of alkaline phosphatase in fluid dairy products: collaborative study. J AOAC 73:842-849.
12. Anonymous. 1986. Determination of internal cooking temperature (acid phosphatase activity). Revised Basic Chemistry Laboratory Guidebook (rev. March 1986), USDA-FSIS No. 3.018:3-49.
13. Jones DR et al. 2002. Variations in levels of acid phosphatase present in chicken whole leg meat. Poultry Sci 81(10):1567-1570.

Proposal Subject: Laboratory Methods *(Vibrio vulnificus and Vibrio parahaemolyticus)*

Specific NSSP Guide Reference: Section II Model Ordinance
Chapter XVI. Post Harvest Processing

Text of Proposal/ Requested Action

A. If a dealer elects…

(1) Have a HACCP plan approved by the Authority for the process that ensures that the target pathogen(s) are at safe levels for the at risk population in product that has been subjected to the process.

(a) The dealer must demonstrate that the process reduces the level of *Vibrio vulnificus* in the processed product to non-detectable (<30 MPN/gram) and the process achieves a minimum 3.52 log reduction, to be determined by use of the *Vibrio vulnificus* FDA approved EIA procedure of Tamplin, et al, as described in Chapter 9 of the FDA *Bacteriological Analytical Manual*, 7th Edition, 1992, or ~~other method approved for NSSP use~~ **the MPN method in Chapter 9 of the FDA Bacteriological Analytical Manual, 7th Edition, May 2004 revision, followed by confirmation using biochemical analyses or by the DNA alkaline phosphatase labeled gene probe (vvhA).**

(b) The dealer must demonstrate that the process reduces the level of *Vibrio parahaemolyticus* in the processed product to non-detectable (<30 MPN/gram) and the process achieves a minimum 3.52 log reduction **Vibrio parahaemolyticus levels are to be determined using the MPN format with confirmation by biochemical analysis, gene probe methodology, or PCR methods as they are listed in Chapter 9 of the FDA Bacteriological Analytical Manual, 7th Edition, May 2004 revision, or a method that a State can demonstrate is equivalent.**

Public Health Significance:

Cost Information (if available):

Action by 2007 Task Force I Recommended referral of Proposal 07-103 to an appropriate committee as determined by the Conference Chairman.

Action by 2007 General Assembly Adopted recommendation of 2007 Task Force I.

Action by USFDA December 20, 2007
Concurred with Conference action.

Action by 2009 Laboratory Methods Review Committee Recommended no action on Proposal 07-103. Rationale: Adequately addressed by Proposal 09-229.

Action by 2009 Task Force I Recommended adoption of Laboratory Methods Review Committee recommendation on Proposal 07-103.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force I on Proposal 07-103.

Proposal Subject: Adoption of ELISA as a Type I NSSP Analytical Method to Replace the Mouse Bioassay for Monitoring NSP-Causing Toxins in Molluscan Shellfish

Specific NSSP Guide Reference: Section IV Guidance Documents
Chapter II Growing Areas
.10 Approved NSSP Laboratory Tests

Text of Proposal/ Requested Action Request adoption of enzyme linked immunosorbent assay (ELISA) as a Type I NSSP analytical method for neurotoxic shellfish poisoning (NSP) toxins in molluscan shellfish, under NSSP Guidance Documents Chapter II.10 Approved National Shellfish Sanitation Program Laboratory Tests: Microbiological and Biotoxin Analytical Methods.

An AOAC collaborative study is planned for the ELISA method. Drs. Jerome Naar and Francie Coblentz at UNCW will be the Principle Investigators. A single lab validation of the method is nearing completion, prior to submission to the AOAC Methods Committee for approval to run the collaborative trial. Results of the AOAC collaborative study will be provided to the ISSC for review by the Laboratory Methods Review Committee.

Public Health Significance: Accumulation of the breve toxins, the toxins responsible for Neurotoxic Shellfish Poisoning (NSP) in shellfish can cause illness in human consumers. Monitoring for NSP toxicity is essential to assure the safety of bivalves harvested for food and to protect the industry by sustaining consumer confidence.

The mouse bioassay for NSP has served well since it was developed in the 1970s. The assay is relatively simple, able to detect dangerous levels of toxicity, and appears to be an accurate measure of human oral potency. Nevertheless, there has long been a need for detection methods that are more sensitive and more precise, that do not require live test animals, while still providing an accurate measure of human oral potency. Motivation for finding alternatives includes the ethical concerns and negative public perceptions focused on test methods that use live animals.

The ELISA for NSP provides an excellent alternative to the mouse bioassay, offering far greater sensitivity, greater accuracy, and a reliable measure of toxin contamination in shellfish. In the format developed at the UNCW, it offers very high throughput.

Because of the higher throughput, the use of the ELISA as screening method will allow monitoring programs to increase their capacity to monitor shellfish beds after blooms of breve toxin-producing algae while minimizing the use of live animals. This will allow for shellfish to be tested at shorter time intervals to potentially expedite reopenings.

The ELISA in its current mode is best suited to use in a central lab to which samples are sent. Since this is the way in which most toxin monitoring is now conducted, the ELISA can, with suitable equipment and training, be used where mouse bioassays are currently conducted. In Florida, the state that is the most routinely affected by Karenia brevis red tides, shellfish testing is conducted by the Fish and Wildlife Conservation Commission at the Fish and Wildlife Research Institute (FWRI), which is already equipped and familiar with the use of the ELISA. Researchers from FWRI have been involved in the development of this assay and its current validation.

Implementation:
Progress in implementation of the ELISA has been greatly facilitated by the support from NOAA and the Fish and Wildlife Research Institute, which has funded projects to assist the development and the validation of this assay. Drs. Naar and Coblentz are planning an

AOAC collaborative study of the ELISA with the technical support form various investigators from UNCW, FWRI, FDA and US Army. The AOAC task force on marine □upernat detection methods, led by Dr. James Hungerford, has identified AOAC validation of the ELISA as a high priority.

Some comparisons of the ELISA with:

Receptor Binding Assay:

A preliminary study performed by several investigators under the lead of Dr Robert Dickey FDA, demonstrated ELISA provides similar results as the receptor binding assay; however, the ELISA does not require the use of any radioactive material.

HPLC/MS:

Side by side analysis of shellfish extracts by ELISA and HPLC-MS was conducted by the FDA and reveal good correlation betweens both methods
However, HPLC/MS require careful filtration of the sample, which is a significant cost, and provide a single path, so throughput per instrument is dependent on run time. Equipment cost and operator skill requirements are also much higher.

Cost Information (if available): None

Action by 2007 Laboratory Methods Review Committee Recommended referral of Proposal 07-104 to an appropriate committee as determined by the Conference Chairman.

Action by 2007 Task Force I Recommended adoption of the Laboratory Methods Review Committee recommendation on Proposal 07-104.

Action by 2007 General Assembly Adopted recommendation of 2007 Task Force I.

Action by USFDA December 20, 2007
Concurred with Conference action.

Action by 2009 Laboratory Methods Review Committee Recommended no action on Proposal 07-104. Rationale: Adequate data has not been submitted.

Action by 2009 Task Force I Recommended adoption of the Laboratory Methods Review Committee recommendation on Proposal 07-104.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force I on Proposal 07-104.

Proposal Subject: Adoption of LC-MS as a Type I NSSP Analytical Method to Replace the Mouse Bioassay for Monitoring NSP

Specific NSSP Guide Reference: Section IV Guidance Documents
Chapter II Growing Areas
.10 Approved NSSP Laboratory Tests

Text of Proposal/ Requested Action Request adoption of liquid chromatrography-mass spectrometry (LC-MS) as a Type I NSSP analytical method for neurotoxic shellfish poisoning (NSP) toxins in molluscan shellfish, under NSSP Guidance Documents Chapter II.10 Approved National Shellfish Sanitation Program Laboratory Tests: Microbiological and Biotoxin Analytical Methods.

An AOAC collaborative study is planned for the LC-MS method. Dr. Steven M. Plakas at FDA will be the Principle Investigator. A single lab validation of the method is nearing completion, prior to submission to the AOAC Methods Committee for approval to run the collaborative trial. Results of the AOAC collaborative study will be provided to the ISSC for review by the Laboratory Methods Review Committee.

Public Health Significance: Neurotoxic shellfish poisoning (NSP) is caused by consumption of shellfish contaminated with algal brevetoxins. Monitoring for NSP toxicity is essential to assure the safety of bivalves harvested for food and to protect the industry by sustaining consumer confidence.

The mouse bioassay for NSP toxic shellfish has served well since it was developed in the 1960s. The assay is relatively simple and detects dangerous levels of toxins. However, there has long been a need for detection methods that are more sensitive and precise, that do not require live test animals, while still providing a reliable measure of human oral potency. Motivation for finding alternatives includes ethical concerns and negative public perceptions focused on test methods that use live animals.

The LC-MS method provides an excellent alternative to the mouse bioassay, offering far greater sensitivity and specificity. Greater sensitivity provides a higher level of assurance that growing areas can be closed before violative product is harvested, and enable growers to harvest product while still safe in anticipation of a closure. Greater specificity enables unambiguous identification of toxins present as indicators of human oral potency.

The LC-MS in its current mode is best suited to use in a central lab to which samples are sent. Since this is the way in which most toxin monitoring is now conducted, LC-MS can, with suitable equipment and training, be used as a direct replacement for the mouse bioassay in many existing □upernat management programs. The principal limitation of LC-MS is the high initial cost of capital equipment.

Implementation:
A single lab validation of the LC-MS method is now in progress. An AOAC collaborative study of the method is planned. The AOAC task force on marine □upernat detection methods, led by Dr. James Hungerford, has identified validation of the LC-MS method as a high priority.

Validity:
The idea that the LC-MS provides a valid measure of toxicity of brevetoxin-contaminated shellfish arose from a systematic study of the fate of these toxins in the Eastern oyster, along with comparison of alternative methods to that of mouse bioassay of field samples. LC-MS and ELISA data correlated well with other, and with those of mouse bioassay. LC-MS provides unambiguous identification of brevetoxins, while other in vitro methods and

mouse bioassay cannot.

Some comparisons of the LC-MS method with:

Mouse bioassay:
The mouse bioassay gives a useful, approximate answer quickly and will reliably detect a dangerously toxic sample. However, LC-MS offers high specificity and is much more sensitive (by several orders of magnitude). Field studies in Eastern oyster have provided a useful approximation of the levels of toxin by LC-MS equivalent to the toxicity guidance level by mouse bioassay.

Immunoassays:
In field studies of Eastern oyster, LC-MS data were highly correlated with those of enzyme-linked immunoassay (ELISA). ELISA, as performed, measures a composite of brevetoxins present in the sample that share common structural features, while LC-MS offers a higher level of specificity. However, ELISA can be portable and performed by persons with little training, under field conditions.

Receptor binding assay (RBA):
RBAs are generally believed to reflect toxin potencies better than the structurally-based methods (LC-MS and ELISA). However, in field studies with Eastern oyster, mouse bioassay data were more highly correlated with LC-MS, compared with RBA. RBA also has the disadvantage of requiring the use of radioactive materials, which adds considerable costs. Appropriate procedures for the receipt, use and disposal of radioactive materials must be implemented to satisfy regulatory requirements.

Cost Information (if available):	None
Action by 2007 Laboratory Methods Review Committee	Recommended referral of Proposal 07-105 to an appropriate committee as determined by the Conference Chairman.
Action by 2007 Task Force I	Recommended adoption of the Laboratory Methods Review Committee recommendation on Proposal 07-105.
Action by 2007 General Assembly	Adopted recommendation of 2007 Task Force I.
Action by USFDA	December 20, 2007 Concurred with Conference action.
Action by 2009 Laboratory Methods Review Committee	Recommended no action on Proposal 07-105. Rationale: Additional information requested has not been submitted.
Action by 2009 Task Force I	Recommended adoption of Laboratory Methods Review Committee recommendation on Proposal 07-105.
Action by 2009 General Assembly	Adopted recommendation of 2009 Task Force I on Proposal 07-105.

Proposal Subject: Receptor Binding Assay (rba) for PSP as a Type I NSSP Analytical Method

Specific NSSP Guide Reference: Section IV Guidance Documents
Chapter II Growing Areas
.10 Approved NSSP Laboratory Tests

Text of Proposal/ Requested Action Adopt the receptor binding assay (rba) as a Type I NSSP analytical method for PSP testing for use as an alternative to and improvement over the AOAC mouse bioassay under NSSP Guidance Documents Chapter II.10 Approved National Shellfish Sanitation Program Laboratory Tests: Microbiological and Biotoxin Analytical Methods.

An AOAC collaborative study is planned for the rba. Dr. Fran Van Dolah at NOAA will be the Principle Investigator. Dr. Van Dolah is nearing completion of a single lab validation, using a HOAc extraction, prior to submission to the Method Committee for approval to run the collaborative trial. Results of the AOAC collaborative study will be provided to the ISSC for review by the Laboratory Methods Review Committee.

Public Health Significance: Accumulation of the saxitoxins, the toxins responsible for Paralytic Shellfish Poisoning (PSP) in shellfish can cause illness and death in human consumers. Monitoring for PSP toxicity is essential to assure the safety of bivalves harvested for food and to protect the industry by sustaining consumer confidence.

The mouse bioassay for paralytic shellfish poison (PSP) has served well since it was developed in the 1930s. The assay is relatively simple, quickly detects dangerous levels of toxicity, and appears to be an accurate measure of human oral potency. Nevertheless, there has long been a need for detection methods that are more sensitive, more precise, do not require live test animals, while still providing an accurate measure of human oral potency. Motivation for finding alternatives includes the ethical concerns and negative public perceptions focused on test methods that use live animals.

The receptor binding assay (rba) for PSP provides an excellent alternative to the mouse bioassay, offering far greater sensitivity, greater accuracy, and a reliable measure of human oral potency. In the format developed at the NOAA/Charleston laboratory, it offers very high throughput.

The greater sensitivity of the rba will allow monitoring programs to detect the arrival of a PSP event earlier than is possible with the mouse bioassay. By providing more latitude between the detection limit and regulatory limit this will provide a higher level of assurance that growing areas can be closed before violative product is harvested and will also allow growers to get product out of the water while still safe in anticipation of a closure.

The rba in its current mode is best suited to use in a central lab to which samples are sent. Since this is the way in which most toxin monitoring is now conducted, the rba can, with suitable equipment and training, be used as a direct replacement for the mouse bioassay in many existing □upernat management programs.

The principal limitation of the rba is that, in its current form, it requires the use of radioactive material. Although the amounts of radioactivity are miniscule and the risk negligible, appropriate procedures for the receipt, use, and disposal of radioactive materials must be implemented to satisfy regulatory requirements. This is a small cost, but must still be recognized. While efforts are underway to develop methods that have the benefits of the receptor assay without requiring radioactive materials, they have not advanced sufficiently to justify delaying implementation of the rba in its current format.

Implementation:
Progress in implementation of the rba has been greatly facilitated by the support of the International Atomic Energy Agency, which has funded several technical cooperation projects to assist developing nations in both setting up the rba and in establishing the necessary infrastructure to ensure that its employment will be useful. As a part of the IAEA program, Dr. Fran Van Dolah is planning an AOAC collaborative study of the rba. The AOAC task force on marine □upernat detection methods, led by Dr. James Hungerford, has identified AOAC validation of the rba as a high priority.

Molecular basis for validity:
The idea that the rba was a fundamentally valid measure of toxicity of the saxitoxins to mammals arose from a systematic study of structure/activity relationships among carefully purified and characterized saxitoxins aimed at understanding the reasons for differences in observed toxicity to mice and, ultimately, the nature of the highly selective interaction with the binding site. In the course of this work it was found that the mouse intraperitoneal potencies of the various saxitoxins corresponded well with their binding affinities in the rba.

Some comparisons of the rba with:

Mouse bioassay:
The mouse bioassay gives a useful, approximate answer more quickly and will reliably detect a dangerously toxic sample, while the rba produces more results per day, can produce a large number of precise results much more quickly, and is much more sensitive. The limit of sensitivity for the rba is ca 0.5nM STX, vs 0.5 micromolar STX for the mouse bioassay. As usually applied, the rba is 10x to 100x more sensitive than the mouse bioassay.

Immunoassays:
The response spectrum of the rba is better matched to human oral potency than the immunoassays now available so, while the rba can be considered an accurate measure of human oral potency, the accuracy of an immunoassay depends on which toxins are present in the sample and may not accurately reflect toxicity to consumers. On the other hand, some immunoassays can be portable and can be performed by persons with little training, under field conditions.

HPLC, LC/MS:

Both methods are analyses, rather than assays, and thus determine the concentrations of individual toxins. This information can be vital for research and can be useful in regulatory applications. However, HPLC and LC/MS require careful filtration of the sample, which is a significant cost, and provide a single path, so throughput per instrument is dependent on run time. Equipment cost and operator skill requirements are also much higher, particularly for LC/MS.

Cost Information (if available):

Action by 2007 Laboratory Methods Review Committee

Recommended referral of Proposal 07-106 to an appropriate committee as determined by the Conference Chairman.

Action by 2007 Task Force I	Recommended adoption of the Laboratory Methods Review Committee recommendation on Proposal 07-106.
Action by 2007 General Assembly	Adopted recommendation of 2007 Task Force I.
Action by USFDA	December 20, 2007 Concurred with Conference action.
Action by 2009 Laboratory Methods Review Committee	Recommended no action on Proposal 07-106. Rationale: Adequate data has not been submitted. If new data becomes available a new proposal will be submitted in the SLV format.
Action by 2009 Task Force I	Recommended adoption of Laboratory Methods Review Committee recommendation on Proposal 07-106.
Action by 2009 General Assembly	Adopted recommendation of 2009 Task Force I on Proposal 07-106.

Proposal Subject: Deletions and Additions to Table 1 Action Levels, Tolerances and Guidance Levels for Poisonous or Deleterious Substances in Seafood

Specific NSSP Guide Reference: Section IV Guidance Documents
Chapter II. Growing Areas
.04 Action Levels, Tolerances and Guidance Levels for Poisonous or Deleterious Substances in Seafood

Text of Proposal/ Requested Action Delete arsenic, cadmium, chromium, lead, and nickel from Table 1 – Action Levels, Tolerances and Guidance Levels for Poisonous and Deleterious Substances in NSSP Section IV Guidance Documents Chapter II.04.

Add the following chemicals and chemotherapeutic drugs and the associated safety level to Table 1 – Action Levels, Tolerances and Guidance Levels for Poisonous and Deleterious Substances in NSSP Guidance Documents Chapter II.04:

Carbaryl in oysters – 0.25 ppm
Endothall and its monomethyl ester in all fish – 0.1 ppm
Chloramphenicol in all fish – no residue
Clenbuterol in all fish – no residue
Diethylstilbestrol (DES) in all fish – no residue
Demetridazole in all fish – no residue
Ipronidazole and other nitroimidazoles in all fish – no residue
Frazolidone and other nitrofurans in all fish – no residue
Fluoroquinilones in all fish – no residue
Glycopeptides in all fish – no residue

Delete the less than symbol in front of 20 MU/100 g for Neurotoxic Shellfish Poisoning (NSP) in Table 1 – Action Levels, Tolerances and Guidance Levels for Poisonous and Deleterious Substances in NSSP Guidance Documents Chapter II.-04.

Public Health Significance: Acceptable levels established in the FDA guidance documents for each of the five elements to be deleted were intended only as general guidance. Use of these Guidance Documents as a general formula for calculating levels of concern is somewhat subjective based on the particular circumstances under which they are applied, for example, the rate of consumption. Furthermore, in the 14 years since their publication, new scientific data and information has rendered them somewhat obsolete and in need of revision. Until such time as they can be updated with current information and science, FDA toxicologists have determined the safe levels set forth in them for molluscan shellfish are inappropriate.

Addition to the NSSP of the two named chemicals and the nine chemotherapeutic drugs is in keeping with establishment of FDA and EPA safety levels for their presence in shellfish meats and with their citation in the FDA Seafood HACCP Fish and Fisheries Products Hazards and Controls Guide.

Cost Information (if available): None

Action by 2007 Task Force I Recommended referral of Proposal 07-107 to an appropriate committee as determined by the Conference Chairman.

Action by 2007 General Assembly Adopted recommendation of 2007 Task Force I.

Action by USFDA December 20, 2007
Concurred with Conference action.

Action by 2009 Chemical Contamination Committee

(1) (a) Recommended the guidance levels for these heavy metals listed above should be removed from Table 1 as proposed by FDA. (b) The Conference should recommend that FDA work to expeditiously update the heavy metals guidance documents based on current science and set standards for national and international commerce.

(2) The Conference should reach out to FDA's National Shellfish Team for information on standards on heavy metals used by foreign countries to help assure consistency in our approach.

(3) The Chemical contaminants listed for addition in the FDA proposal should be added to Table 1 Action Levels, Tolerances and Guidance Levels for Poisonous or Deleterious Substances in Seafood.

(4) The less than symbol in front of 20 MU/100 g for Neurotoxic Shellfish Poisoning (NSP) in Table 1 should be removed as proposed by FDA in 07-107.

(5)

Action by 2009 Task Force I Recommended adoption of Chemical Contamination Committee recommendations (1) (a), (3), and (4) and recommended no action on recommendations (1) (b) and (2).

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force I on Proposal 07-107.

Proposal Subject: Remote Status of Shellfish Growing Areas

Specific NSSP Guide Reference: Section II Model Ordinance Definitions B. Definition of Terms
Section II Model Ordinance Chapter IV. @.03 A. (5) (e)

Text of Proposal/ Requested Action Revise Section II, B. Definition of Terms.

(86) **Remote status** means a designation applied to **an Approved** shellfish growing area that has no human habitation**, or has sparse human habitation** and is not impacted by any actual or potential pollution sources.

Revise Section II, Chapter IV@.03A.(5)

(e) Remote Status. A growing area may be placed in the remote status if~~:~~

~~(i)~~ ~~A~~**a** sanitary survey determines that the area ~~has no human habitation, and~~ is not impacted by any actual or potential pollution sources; ~~and~~

~~(ii)~~ ~~T~~the area is in the approved classification~~.~~**; and the area:**

(i) Has no human habitation; or

(ii) Has sparse human habitation and other factors that provide protection equivalent to areas having no human habitation. Equivalent protection must be provided by factors such as overwhelming marine water dilution and dispersion between the potential pollution sources and the harvest area, human habitation that is well removed from the shoreline or aquaculture sites that are a significant distance (more than one mile) from the shoreline.

Public Health Significance: Shellfish sanitation control authorities must use their limited resources to focus on sanitation issues that provide the greatest public health benefit. This proposed change to the remote status could eliminate unneeded marine water sampling in areas not threatened by pollution and allow limited resources to be redirected to more significant shellfish sanitation issues.

The proposal would expand the remote status designation only to sparsely populated areas that have additional factors that impart equivalent public health protection provided by areas where there is no human habitation. When an area is designated as remote, the only change in the sanitation requirements is the reduced frequency of water sampling. The frequency and thoroughness of shoreline surveys, sanitary survey reports, triennial or annual report updates remain unchanged.

Cost Information (if available): No cost information is available.

Action by 2009 Task Force I Recommended no action on Proposal 09-100.
Rationale: Adequately addressed in Model Ordinance.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force I on Proposal 09-100.

Proposal Subject: Correction of the wording for the action level for NSP toxins and the incorporation of action levels for AZP and DSP toxins in shellfish in the Guide.

Specific NSSP Guide Reference: Section II. Model Ordinance Chapter IV. Shellstock Growing Areas
@.04 Marine Biotoxin Control C. (1)

Section IV. Guidance Documents Chapter II. Growing Areas
.04 Action Levels, Tolerances and Guidance Levels for Poisonous or Deleterious Substances in Seafood

Text of Proposal/ Requested Action In Section II Model Ordinance, Chapter IV. Shellstock Growing Areas @.04 Marine Biotoxin Control C. (1), correct the wording for NSP toxins and add the action levels for azaspiracids (AZP) and DSP toxins, as follows:

C. Closed Status of Growing Areas.

(1) A growing area, or portion(s) thereof as provided in §A.(4), shall be placed in the closed status for the taking of shellstock when the Authority determines that the number of toxin-forming organisms in the growing waters and/or the level of upernat present in shellfish meats is sufficient to cause a health risk. The closed status shall be established based on the following criteria:

PSP – cells/L n/a; 80 µg/100 grams
NSP – 5,000 cells/L or 20 MU/**100 grams** (~~approximate as 80 µg/100 g~~**0.8 mg brevetoxin-2 equivalents/kg**)
AZP – cells/L n/a; 0.16 mg AZA-1 equivalents/kg (0.16 ppm)
DSP – cells/L n/a; 0.16 mg OA equivalents/kg (0.16 ppm)
ASP – cells/L n/a; 2 mg/100 grams (20 ppm)

(a) The concentration of paralytic shellfish poison (PSP) equals or exceeds 80 micrograms per 100 grams of edible portion of raw shellfish; or

(b) For neurotoxic shellfish poisoning (NSP), the harvesting of shellstock shall not be allowed when:
(i) The concentration of NSP equals or exceeds 20 mouse units per 100 grams of edible portion of raw shellfish; or
(ii) The cell counts for *Karenia brevis* organisms in the water column exceed 5,000 per liter; or

I For domoic acid, the toxin concentration shall not be equal to or exceed 20 ppm in the edible portion of raw shellfish.

(d) For azaspiracid shellfish poisoning (AZP), the concentration of azaspiracids shall not be equal to or exceed 0.16 mg/kg (AZA-1 equiv.) in the edible portion of raw shellfish.

(e) For diarrhetic shellfish poisoning (DSP), the concentration of DSP toxins shall not be equal to or exceed 0.16 mg/kg (OA equiv.) in the edible portion of raw shellfish.

And under the Natural Toxins section of Table 1 of the Guidance Documents: Chapter II-Growing Areas; .04 Action Levels, Tolerances and Guidance Levels for Poisonous or Deleterious Substances in Seafood, correct and insert the following:

Substance	Level	Food Commodity[a]	Reference
Neurotoxic Shellfish Poisoning (NSP) toxins	20 MU/100g	Clams, mussels, oysters, fresh frozen or canned	NSSP MO
Azaspiracid Shellfish Poisoning (AZP) toxins	0.16 mg/kg	Clams, mussels, oysters, fresh frozen or canned	NSSP MO
Diarrhetic Shellfish Poisoning (DSP) toxins	0.16 mg/kg	Clams, mussels, oysters, fresh frozen or canned	NSSP MO

Public Health Significance:

NSP Toxins

Neurotoxic shellfish poisoning (NSP) is caused by consumption of shellfish contaminated with brevetoxins. Brevetoxins are a group of lipophilic neurotoxins produced by the marine dinoflagellate *Karenia brevis* and other algal species (e.g., *Chattonella* spp.). Brevetoxins are accumulated and extensively metabolized in filter-feeding molluscan shellfish. Toxicity of shellfish has been historically assessed by mouse bioassay, while efforts are underway to validate alternative methods of analysis (e.g., LC-MS, immunoassay). Shellfish exhibiting any detectable level of toxicity by mouse bioassay are considered potentially unsafe for human consumption. In practice, a value of 20 MU/100 g shellfish tissue has been considered the regulatory limit by the States. Expressed in brevetoxin-2 (PbTx-2) equivalents, this level is 0.8 mg/kg in shellfish tissue. Method alternative to mouse bioassay must provide an equivalent level of public health protection.

The requested action is editorial corrections to the Guide with respect to the current action level.

AZP Toxins

Azaspiracids (AZA) are a group of lipophilic marine algal toxins that accumulate in various shellfish species (Twiner et al., 2008). Consumption of AZA-contaminated shellfish causes the acute illness azaspiracid shellfish poisoning (AZP). AZP is characterized by severe gastrointestinal disturbances; symptoms include nausea, vomiting, diarrhea, abdominal pain and cramps. AZA were first discovered in 1995 following an outbreak linked to consumption of Irish mussels. Since then, several documented outbreaks of AZP have been reported in Europe, and AZA have been isolated from shellfish along the European Atlantic coast from Norway to Portugal, and in Morocco. In 2008, the first recognized cases of AZP in the U.S. were reported, and linked to consumption of imported mussels from Ireland (Klontz et al., 2009). The finding of AZA in the imported product highlights the concern for the consumer safety of molluscan shellfish marketed internationally.

The first risk assessment for AZA was conducted by the Food Safety Authority of Ireland (FSAI) in 2001. In 2002, the European Commission set the regulatory limit for AZA (AZA-1, -2, and -3) at 0.16 mg/kg, based on the FSAI data and the limit believed to be detectable by mouse bioassay (EC, 2002). This regulatory limit was strengthened by a second risk assessment conducted by the FSAI (FSAI, 2006). The latter incorporated new data with respect to tissue distribution of AZA in mussels, ratios of different analogues, and the effects of cooking. The calculated median acute reference dose (ArfD, 0.63 g/kg b.w.) was comparable to the intake value for a 60 kg individual consuming 250 g mussels contaminated with AZA at the 0.16 mg/kg regulatory limit.

EC regulation allows for the use of alternative methods (e.g., LC-MS, immunoassay) to

the reference test (mouse bioassay) for AZA in shellfish (EC,2005). These methods must be capable of detecting the AZA analogues AZA-1, -2, and -3. And they must provide an equivalent level of public health protection to the biological method. The EU-harmonized mouse bioassay and LC-MS methods were recently demonstrated equivalent in their effectiveness in implementation of this regulatory limit (Hess et al., 2009).

The FSAI risk assessment did recognize the uncertainties inherent in its outcome, particularly relating to limitations in the available epidemiological data. Moreover, the toxicity of AZA analogues, and their distribution and metabolism in various shellfish species, have not been well characterized. Chronic and low dose effects of AZA are unknown. Refinement of the risk assessment and revision of regulatory limit may be necessary when additional toxicological and epidemiological data become available.

The requested action is adoption of a regulatory limit for azaspiracids (AZA) of 0.16 mg/kg in molluscan shellfish, in accordance with that set by the European Commission (EC, 2002). By using LC-MS, this limit is based on the sum of the individual azaspiracid toxin analogues AZA-1, -2, and -3, expressed in AZA-1 equivalents. AZA-1 is the only certified analytical standard presently available. AZA-1 equivalents of AZA-2 and -3 are calculated by weighting their relative response factor (RRF)-corrected concentrations with their toxic equivalence factors (TEFs). TEF multipliers derived from initial studies on mice are 1, 1.8, and 1.4 for AZA-1, -2, and -3, respectively (Ofuji et al., 1999).

DSP Toxins

Diarrhetic shellfish poisoning (DSP) is caused by consumption of molluscan shellfish contaminated with toxins of the okadaic acid (OA) group, the origin of which is principally marine dinoflagellates (e.g., *Dinophysis, Prorocentrum* spp.) DSP is characterized by acute gastrointestinal disturbance (e.g., diarrhea, nausea, vomiting, abdominal pain). Toxins responsible are primarily okadaic acid (OA) and the related dinophysistoxins (DTXs) and their acyl esters. Pectenotoxins (PTX) and yessotoxins (YTX) may co-occur, the former of similar toxic potency.

DSP outbreaks were first reported in 1976 in Japan, and in the 1980s in Europe. The first documented outbreak in N. America occurred in 1990, in eastern Canada (Qulliam et al., 1993). There have been no reported cases of DSP to date in the U.S. However, in 2008, toxin-producing *Dinophysis*, and DSP toxins in shellfish above the proposed action levels, were recorded for the first time in the Gulf of Mexico (Deeds, pers. uper.). *Dinophysis* has been found along the east and west coast of the U.S. Since DSP toxin-producing organisms occur throughout the world, DSP toxins in molluscan shellfish are a significant public health concern.

DSP toxins in shellfish have been assessed traditionally by mouse bioassay, and more recently by instrumental methods (LC-FTD, LC-MS), immunoassay, and pharmacology-based assays (protein phosphatase assay). Current EU regulatory limit is 0.16 mg OA equivalents/kg shellfish meat (EC, 2002, 2005). This level represents the sum of that of OA, DTXs, and PTXs. Methods alternative to mouse bioassay incorporate a base hydrolysis step for conversion of DTX acyl esters to free acid forms.

The requested action is adoption of a regulatory limit for DSP toxins of 0.16 mg/kg (OA equivalents) in molluscan shellfish. This limit is based on the sum of OA, DTXs (including acyl esters), and PTXs. Revision of regulatory limit may be necessary when additional toxicological and epidemiological data become available.

References

EC, 2002. Commission decision 2002/225/EC of 15 March 2002 laying down detailed rules for the implementation of Council Directive 91/492/EEC as regards the maximum levels and the methods of analysis of certain marine biotoxins in bivalve mollusks, echinoderms, tunicates and marine gastropods. Off. J. Eur. Comm. L75:62-64.

EC, 2005. Commission Regulation (EC) No 2074/2005 of 5 December 2005 laying down implementing measures for certain products under Regulation (EC) No 853/2004 of the European Parliament and of the Council and for the □upernatant□ of official controls under Regulation (EC) No 854/2004 of the European Parliament and of the Council and Regulation (EC) No 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004. Off. J. Eur. Union. L338:27-59.

FSAI. 2006. Risk assessment of azaspiracids (AZAs) in shellfish. August 2006. A Report of the Scientific Committee of the Food Safety Authority of Ireland (FSAI), Dublin, Ireland.

Hess, P., Butter, T., Peterson, A., Silke, J., McMahon, T. 2009. Performance of the EU-harmonized mouse bioassay for lipophilic toxins for the detection of azaspiracids in naturally contaminated mussel (*Mytilus edulis*) hepatopancreas tissue homogenates characterized by liquid chromatography coupled to tandem mass spectrometry. Toxicon 53:713-722.

Klontz, K.C., Abraham, A., Plakas, S.M., Dickey, R.W. 2009. Mussel-associated azaspiracid intoxication in the United States. Ann. Int. Med. 150:361.

Ofuji, K., Satake, M., McMahon, T., Silke, J., James, K.J., Naoki, H., Oshima, Y., Yasumoto, T. 1999. Two analogs of azaspiracid isolated from mussels, Mytilus edulis, involved in human intoxication in Ireland. Nat. Toxins 7:99-102.

Quilliam, M., Gilgan, M., Pleasance, S., Defreitas, A., Douglas, D., Friz, L., Hu, T., Marr, J., Smyth, C., Wright, J. 1993. Confirmation of an incident of diarrhetic shellfish poisoning in Eastern Canada. In: Smayda and Shimizu (eds.). Toxic Phytoplankton Blooms in the Sea, pp. 547-552.

Twiner, M.J., Rehmann, N., Hess, P., Doucette, G.J. 2008. Azaspiracid shellfish poisoning: review on the chemistry, ecology, and toxicology with an emphasis on human health impac Mar. Drugs 6:39–72.

Cost Information (if available):

Action by 2009 Task Force I

Recommended referral of Proposal 09-101 to an appropriate committee as determined by the Conference Chairman. The Committee should be directed to gather more information on the standards, methods and costs.

Action by 2009 General Assembly

Adopted recommendation of 2009 Task Force I on Proposal 09-101.

Proposal Subject:	Alternative analytical method for *Vibrio vulnificus, Vibrio cholerae, Vibrio parahaemolyticus*
Specific NSSP Guide Reference:	Section IV. Guidance Documents Chapter II Growing Areas .10 Approved National Shellfish Sanitation Program Laboratory Tests: Microbiological and Biotoxin Analytical Methods. (5) Interim Approval by ISSC Executive Board August 2007
Text of Proposal/ Requested Action	Text of proposal: See attached proposal Requested actions: Accept the adoption of DuPont Qualicon BAX ® Real Time *Vibrio* Test Kit as an alternative analytical protocol to determine the levels of *Vibrio vulnificus, V. cholerae, V.parahaemolyticus*
Public Health Significance:	Proposed method will greatly improve the speed of analysis to help the industry to increase the amount of PHP products in the market. For details see attached proposal
Cost Information (if available):	See attached proposal.

Proposed Specific Research Need/Problem to be Addressed:

Improve the speed of analysis to help the industry to increase the amount of PHP products in the market.

How will addressing this research support/improve the mission/role of the ISSC/NSSP/Industry? Support need with literature citations as appropriate.

See attached description

Relative Priority Rank in Terms of Resolving Research Need:

Immediate	☒	**Important**	☐
Required	☐	**Other**	☐
Valuable	☐		

Estimated Cost:

Proposed Sources of Funding/Support:

Time Frame Anticipated: 2009-2010

Action by 2009 Laboratory Methods Review Committee	Recommended referral of Proposal 09-102 to appropriate committee as determined by Conference Chairman. Rationale: Additional data under development.
Action by 2009 Task Force I	Recommended adoption of Laboratory Methods Review Committee recommendation on Proposal 09-102.
Action by 2009 General Assembly	Adopted recommendation of 2009 Task Force I on Proposal 09-102.

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

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Methods Review (LMR) Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

Name of the New Method	**QPCR-MPN Assay using DuPont Qualicon BAX ® Real Time *Vibrio* Test Kit for Rapid Detection of *Vibrio* speices in seafood**
Name of the Method Developer	Anita Wright et. Al.
Developer Contact Information	Anita Wright 461 AFPL bldg. Newell Dr. Gainesville, FL 32611 352-392-1991 ext. 311

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.	Y	An alternative method to confirm upern bacteria in shellfish
2. What is the intended purpose of the method?	Y	Replace confirmation step in MPN determination of Vibrios in shellfish
3. Is there an acknowledged need for this method in the NSSP?	Y	End users are requiring faster more economical alternatives to the current approved method
4. What type of method? i.e. chemical, molecular, culture, etc.	Y	Quantitative PCR
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title	Y	
Method Scope	Y	
References	Y	
Principle	Y	
Any Proprietary Aspects	Y	
Equipment Required	Y	
Reagents Required	Y	
Sample Collection, Preservation and Storage Requirements	Y	
Safety Requirements	Y	
Clear and Easy to Follow Step-by-Step Procedure	Y	
Quality Control Steps Specific for this Method	Y	
C. Validation Criteria		
1. Accuracy / Trueness	Y	
2. Measurement Uncertainty	Y	
3. Precision Characteristics (repeatability and reproducibility)	Y	
4. Recovery	n/a	
5. Specificity	Y	
6. Working and Linear Ranges	Y	
7. Limit of Detection	Y	
8. Limit of Quantitation / Sensitivity	Y	
9. Ruggedness	Y	
10. Matrix Effects	Y	

11. Comparability (if intended as a substitute for an established method accepted by the NSSP)	Y	
D. Other Information		
1. Cost of the Method	Y	
2. Special Technical Skills Required to Perform the Method	Y	
3. Special Equipment Required and Associated Cost	Y	
4. Abbreviations and Acronyms Defined	N/A	
5. Details of Turn Around Times (time involved to complete the method)	Y	
6. Provide Brief Overview of the Quality Systems Used in the Lab	Y	
Submitters Signature	Date:	
Submission of Validation Data and Draft Method to Committee	Date:	
Reviewing Members	Date:	
Accepted	Date:	
Recommendations for Further Work	Date:	
Comments: See attached application document.		

DEFINITIONS

1. **Accuracy/Trueness** - Closeness of agreement between a test result and the accepted reference value.
2. **Analyte/measurand** - The specific organism or chemical substance sought or determined in a sample.
3. **Blank** – Sample material containing no detectable level of the analyte or measurand of interest that is subjected to the analytical process and monitors contamination during analysis.
4. **Comparability** – The acceptability of a new or modified method as a substitute for an established method in the NSSP. Comparability must be demonstrated for each substrate or tissue type by season and geographic area if applicable.
5. **Fit for purpose** – The analytical method is appropriate to the purpose for which the results are likely to be used.
6. **HORRAT value** – HORRAT values give a measure of the acceptability of the precision characteristics of a method.[4]
7. **Limit of Detection** – the minimum concentration at which the analyte or measurand can be identified. Limit of detection is matrix and analyte/measurand dependent.[4]
8. **Limit of Quantitation/Sensitivity** – the minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.
9. **Linear Range** – the range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.
10. **Measurement Uncertainty** – A single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.
11. **Matrix** – The component or substrate of a test sample.
12. **Method Validation** – The process of verifying that a method is fit for purpose.[1]
13. **Precision** – the closeness of agreement between independent test results obtained under stipulated conditions.[1, 2] There are two components of precision:
    a. **Repeatability** – the measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.
    b. **Reproducibility** – the measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.
14. **Quality System –** The laboratory's quality system is the process by which the laboratory conducts its activities so as to provide data of known and documented quality with which to demonstrate regulatory compliance and for other decision–making purposes. This system includes a process by which appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.
15. **Recovery –** The fraction or percentage of an analyte or measure and recovered following sample analysis.
16. **Ruggedness** – the ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]
17. **Specificity** – the ability of a method to measure only what it is intended to measure.[1]
18. **Working Range –** the range of analyte or measure and concentration over which the method is applied.

REFERENCES:

1. Eurachem Guide, 1998. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. LGC Ltd. Teddington, Middlesex, United Kingdom.
2. IUPAC Technical Report, 2002. Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, Pure Appl. Chem., Vol. 74, (5): 835-855.
3. Joint FAO/IAEA Expert Consultation, 1999. Guidelines for Single-Laboratory Validation of Anilytical Methods for Trace-Level Concentrations of Organic Chemicals.

4. MAF Food Assurance Authority, 2002. A Guide for the Validation and Approval of New Marine Biotoxin Test Methods. Wellington, New Zealand.
5. National Environmental Laboratory Accreditation. , 2003. Standards. June 5.
6. EPA. 2004. EPA Microbiological Alternate Procedure Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Methods: Guidance. U.S. Environmental Protection Agency (EPA), Office of Water Engineering and Analysis Division, 1200 Pennsylvania Avenue, NW, (4303T), Washington, DC 20460. April.

Title: QPCR-MPN Assay using DuPont Qualicon BAX ® Real Time *Vibrio* Test Kit for Rapid Detection of *Vibrio* species in seafood

JUSTIFICATION FOR NEW METHOD

This protocol is submitted for approval to the Laboratory Methods Review Committee. This proposal was prepared to support the use of a new molecular detection method: **DuPont Qualicon BAX ® Real Time *Vibrio* Test Kit for** rapid detection of *Vibrio cholerae, V. parahaemolyticus, and V. vulnificus* It will be used in conjunction with current Vibrio MPN assay and will substitute for the use of DNA probe colony hybridization for confirmation of the presence of *Vibrio* species (8). Method was developed by collaborative efforts of Dr. Anita Wright, Dr. Steve Otwell, Victor Garrido, Charlene Burke, and Melissa Evans, University of Florida, Gainesville, Florida and DuPont Qualicon Laboratories. The QPCR method was recently approved for American Organization of Analytical Chemists (AOAC) and has been accepted for publication by the Journal of AOAAC: Morgan Wallace, Anita Wright, Tim Dambaugh, Monica Kingsley, Chris Malota, Bridget Andaloro, Dawn Fallon, Daniel Delduco, George Tice and, DuPont Qualicon BAX ® Real Time *Vibrio* Test Kit for the Detection of *Vibrio cholera, parahaemolyticus and vulnificus* from Tuna, Shrimp and Oysters, *AOAC Performance Tested Methods* (15)

The QPCR-MPN method described herein provided increased assay sensitivity and reduced both time and labor costs. Detection of *Vibrio* species was achieved at levels < 30 CFU/g as required for validation protocols (2, 10, 16). For these reasons we propose acceptance of the application of QPCR-MPN for improved assessment of validation and verification protocols related to oyster post harvest processing. The oyster industry's livelihood will be determined by their ability to adapt to FDA demands, and evolving technological breakthroughs. Until this demand has abated, the industry and the scientific community will continue to work in conjunction to learn more and thus protect the public from Vibrio disease.

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Wilmington, DE 19880

Date of Submission
Proposal submission date is June 20, 2009.

Purpose and Intended Use of the Method. *Vibrio* species are responsible for 75% of seafoodborne bacterial infections and 95% of related fatalities (7). *V. vulnificus* the leading cause of death in the US related to seafood consumption and is predominantly associated with consumption uncooked Gulf Coast oysters. *V. parahaemolyticus* is the most common source of outbreaks of infectious disease related to seafood, and *V.*

cholerae contamination threatens the safety of imported seafood products. The proposed method will benefit the seafood industry and the consumer by providing improved, faster, and more supernat deteiction of these pathogens in oysters and other seafood products. This method is being proposed for use in screening potential contamination of seafood products and for validation of Post Harvest Processing (PHP) protocols, as well as for future applications to assure the public of a safer product.

Need for the New Method in the NSSP

QPCR-MPN assay described herein is proposed as an alternative to the standard MPN assay for enumeration of *Vibrio* species using most probable number (MPN) end-point titration of replicate samples in enrichment broth cultures (4, 17). The current standard protocols described in the FDA Bacteriological Analytical Manual (BAM) use growth in enrichment broth, followed by isolation of typical colonies on selective agar medium with subsequent confirmation of each species by DNA probe (16), PCR, or biochemical profiling (8). This method is laborious cost prohibitive, labor intensive, and time consuming (6, 8). Enumeration of multiple *Vibrio* species requires isolation on different selective agars followed by separate confirmation tests that are different for each species. Furthermore, users of this protocol have upernata difficulty with DNA probe product reliability and plating problems related to "spreading" colonies that upernata with the assay. Total amount of time to perform the traditional MPN method with DNA colony blot hybridization as a confirmatory method is at least 4 days, with numerous steps; additionally, technician requires a great deal of experience in performing this assay for successful quantification to be possible. QPCR-MPN method reduces working time half and offers greater sensitivity for detection of *V. vulnificus*; with detection of 1 bacterium per gram post enrichment in alkaline peptone water (APW) overnight (1, 4, 9, 10, 11, 17).

Although PHP methods are currently employed on < 10% of all domestic raw oyster sales in the United States, the industry continues to examine and employ new technologies and take initiative on expanding acceptance and knowledge regarding these treated oyster products (5). The industry is investing money and resources to ensure a market acceptance by educated oyster public, in addition to mitigating risk potential for the at risk consumers of fresh oysters. ISSC mandated that 25% of oysters upernata from the Gulf of Mexico receive some type of validated post uperna processing. Thus, there is an urgent need for improved and more rapid validation methods.

The University of Florida has partnered with several dealers who are using ISSC methods for validation of oyster PHP. Work supporting this proposal was upernata in 2007-2009 working with mild heat treatment (Panama City), nitrogen freezing (Leavin's seafood) and blast freezing (Buddy Ward's Seafood). Throughout the validation, samples were randomly selected for side-by-side comparisons of standard MPN described by the FDA BAM (8) to MPN using the DuPont Bax QPCR for MPN species-specific identification. Test results support the application of QPCR-MPN for improved assessment of validation and verification protocols related to oyster PHP, which was described in a publication by Wright et al., 2007.

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

This method is specific to applications testing growth of *V. cholerae, V. parahaemolyticus, and V. vulnificus* in MPN enrichment of oyster homogenates. This QPCR method does not claim to differentiate between pathogenic and nonpathogenic *Vibrio* species. Method was found to be appropriate for up to 1g of oyster tissues. QPCR-MPN provided more sensitive detection than standard MPN, as enriched samples that were PCR positive but negative on selective media were falsely negative on mCPC, as indicated by agreement of positive mCPC and QPCR results in more diluted inocula of the same sample (16). The result is an increase in sensitivity and a reduction in time and labor costs while still permitting detection of *Vibrios* at levels < 30 CFU/g as required for validation protocols (2, 10, 16). For these reasons we propose acceptance of the application of QPCR-MPN for improved assessment of validation and verification protocols related to oyster post harvest processing.

METHOD DOCUMENTATION

Method Title
QPCR-MPN Assay using DuPont Qualicon BAX ® Real Time *Vibrio* Test Kit

Method Scope
This method is designed for MPN analysis of validation trials for oyster PHP and for detection of *Vibrio* species in seafood and monitoring shellfish harvesting waters.

Principle
QPCR-MPN will be substituted as an alternative to the officially recognized NSSP method for MPN analysis of validation trials for oyster PHP (3). Specifically QPCR will be substituted for microbiological/DNA probe confirmation of *V. cholerae, V. parahaemolyticus, and V. vulnificus* following growth in MPN enrichment. Since the FDA and the ISSC have mandated postharvest processing (PHP) of oysters harvested from Gulf Coast states in order to reduce *V. vulnificus* infections validation and verification are necessary in order to ensure that the process will substantially reduce numbers of *V. vulnificus* bacteria to levels to below the predicted threshold for disease. QPCR-MPN is a rapid and reliable method to accomplish agency mandates and industry goals. Validation criteria was recently expanded to include reduction of *V. parahaemolyticus* in PHP oysters. Application to evaluation of other seafood products is also anticipated, especially imported products that may be a greater risk for *V. cholerae* contamination

Proprietary Aspects
Ingredients in **DuPont Qualicon BAX ® Real Time *Vibrio* Test Kit** are proprietary information.

Equipment
Applied Biosystems Inc real-time thermocycler 7500S

Reagents
- **DuPont Qualicon BAX ® Real Time *Vibrio* Test Kit**
- SYBR green I (Invitrogen)
- Autoclaved molecular grade water

Media (Media are specified in FDA BAM, reference 8)
- Modified colistin polymyxin cellobiose (mCPC) agar
- T1N1 agar
- Alkaline peptone water (APW) enrichment broth
- Phosphate buffered saline (PBS)

Matrix or Matrices of Interest
The validation of post harvest processing for raw gulf coast oysters is performed on oyster homogenate. Thus the matrix is dilutions of oyster homogenate, consisting of oyster meats and PBS.

Sample Collection, Preservation, Preparation, Storage, Cleanup, Test Procedures:
Sample collection will follow procedures described by NSSP for validation of oyster PHP.
Preservation, preparation, storage, cleanup and test procedures follow manufacture's recommendations

Cost of the Method
The cost of the **DuPont Qualicon BAX ® Real Time *Vibrio* Test Kit** platform costs approximately $9 per PCR reaction.

Special Technical Skills Required to Perform the Method
Only basic laboratory skills are required.

Special Equipment Required and Associated Cost

Equipment	Approximate Cost
Dupont Bax thermocycler	$45,000 + accessories
Incubator	$3,000 - $6,000
Centrifuge	$2,000
Heat block	$500

Abbreviations and Acronyms

- PHP –post harvest processing
- DNA- deoxyribonucleic acid
- QPCR- quantitative polymerase chain reaction
- APW- alkaline peptone water
- PBS- phosphate buffered saline
- MPN- most probable number

Test Procedures and Quality Control

MEDIA: Dehydrated media is commercially dehydrated. Media must be sterilized according to manufacturer's instructions. Prepared culture media, dehydrated media and media components must be stored in a cool, clean, dry space unless refrigeration is required as per manufacturer instruction. Stored media is labeled with batch number, expiration date and sterilization date. Storage of prepared culture media at room temperature does not exceed 7 days. Refrigerated storage of prepared media with loose fitting closures does not exceed 1 month; screw-cap closures do not exceed 3 months. All prepared media stored under refrigeration are held at room temperature overnight prior to use. To determine the pH of prepared media, a pH meter with a standard accuracy of 0.1 units is used. The pH meter is calibrated with each use and a minimum of two standard buffer solutions (ph 4, 7 and 10) are used to calibrate the pH meter. Standard buffer solutions are used once and discarded.

COLD STORAGE: Refrigerator temperature must be monitored daily; temperature is maintained between 0˚C to 4˚C. Freezer temperature must be monitored at least once daily, freezer temperatures is maintained at -20˚C (DNA storage) and –80˚C (strain storage).

INCUBATOR: Temperature of incubators must be maintained at 30˚C (+/-0.5), 37˚C (+/-0.5), and 40˚C (+/-0.5). Thermometers must be graduated no greater than 0.5˚C increments. Temperatures are taken twice daily.

SUPPLIES: Utensils and containers made of clean borosilicate glass, stainless steel or other non-corroding material. Culture tubes made of a suitable size to accommodate the volume for broth and samples. Sample containers made of glass or other inert material. Dilution bottles and tubes are made of plastic and closed with attached snap-lock lids. Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method is used to ensure appropriate volumes. Reusable sample containers must be capable of being properly washed and sterilized. Hardwood applicator transfer sticks, utilized for streaking and picking positive colonies, and Whatman # 3 and #541 filter papers, utilized in colony blot hybridization, are sterilized prior to use and stored in sterile, airtight containers. Pipettes used to inoculate the sample deliver accurate aliquots, have unbroken tips and are appropriately graduated. Pipettes larger than 10ml are not used to deliver 1ml; nor, are pipettes larger than 1ml used to deliver 0.1ml. Reagents for DNA extraction and PCR reaction are included in **DuPont Qualicon BAX ® Real Time *Vibrio* Test Kit**

MAINTENANCE: Routine autoclave maintenance must be performed and serviced annually or as needed by a qualified technician and records maintained. Autoclave provides a sterilizing temperature of 121˚C (tolerance 121 +/- 2˚C) as determined daily. Spore suspensions or strips must be used monthly to evaluate the effectiveness of the autoclave sterilization process, with results recorded. Heat sensitive tape must be used with each autoclave

batch. Autoclave sterilization records including length of sterilization, total heat exposure time and chamber temperature must be maintained in an autoclave log.

SHELLSTOCK SAMPLES: A representative sample of shellstock is collected. Shellstock is collected in clean, waterproof, puncture resistant containers. Shellstock labeled with collector's name, type of shellstock, the source, the harvest area, time, date and place of collection. Shellstock are maintained in dry storage between 0 and 10˚C until examined. Examination of the sample is initiated as soon as possible after collection, and does not exceed 24 hours after collection. Shucking knives, scrub brushes and blender jars are sterilized for 35 minutes prior to use. Blades of shucking knives free from debris corrosion. Prior to scrubbing and rinsing debris off shellstock, the hands of the technician are thoroughly washed with soap and water. Shellstock are scrubbed with a stiff, sterile brush and rinsed under water of drinking water quality. Shellstock are allowed to drain in a clean container or on clean towels prior to opening. Prior to opening, the technician washes hands and rinses with 70% alcohol. Shellstock are not shucked directly through the hinge.

FDA-MPN PREPARATION AND METHOD: Contents of shellstock are shucked into a sterile, tared blender jar. At least 12 animals (100 g of meat) are used for analysis. The sample is weighted to the nearest 0.1 gram and an equal amount by weight of sterile PBS diluent is added. Samples are blended at high speed for 90 seconds. Immediately after blending, the homogenized sample is diluted in a multiple dilution series with 3 replicas and inoculated into tubes of APW presumptive media for MPN analysis. Positive and negative controls cultures accompany samples throughout the procedure. Inoculated media are incubated at 37 +/- 0.5˚C. Presumptive tubes are read at 24+/- 2 hours of incubation and transferred if positive. Transfers are made to mCPC plates by sterile hardwood applicator sticks from presumptive positive APW tubes and confirmed by DNA probe.

QPCR-MPN PREPARATION: Prior to DNA extraction and preparing Cepheid© unit for QPCR, all micro-centrifuge tubes and pipette tips are sterilized for 35 minutes. The technician's hands are washed with soap and water. Gloves are worn and rinsed with 70% alcohol. All Pipetteman and Eppendorf pipettes are calibrated semi-annually and prior to use are wiped down with 70% alcohol. All working areas, centrifuge racks, and equipment are wiped down with 70% alcohol. Proper sterile technique is observed throughout the procedure to ensure contamination free samples. 1ml of sample from each positive MPN tube is used for the boil extraction procedure (appendix 1) to extract DNA to be used as template for Sybr green 1 QPCR-MPN assay as described in appendix 2. Cepheid© thermocycler cycle threshold is set at 30 and factory default is utilized for melt curve analysis regarding peak height.

VALIDATION CRITERIA

Ruggedness of Assay

DuPont Qualicon BAX ® Real Time *Vibrio* Test Kit for detection of *V. vulnificus, V. parahaemolyticus* and *V. cholerae* was recently accepted for AOAC approval (15). Proposed method will extend applications to MPN analysis of oyster PHP. Validity of MPN assay for detection of *V. vulnificus* has been previously established by ISSC and FDA. The ruggedness of reagents used for PCR is determined by manufacturer and meets specifications. Method uses a bead format that incorporates all reagents on bead to eliminate common pipetting and cross-contamination errors.

Data Comparability and Statistical Analysis

Quantitative PCR was previously applied to most probable number (QPCR-MPN) for validation of PHP and single specie detection of *V. vulnificus in* oysters (17). Published results by Wright et al., 2007 showed that immediately following inoculation of APW (pre-enrichment with either 0.1 or 0.01 g oyster homogenate detection *V. vulnificus* was 100 to 1000 fold more sensitive by QPCR than by growth on selective agar. Following O.N. growth in enrichment, both assays were equally as sensitive. For PHP oysters received nitrogen immersion, side by side comparison of standard MPN vs. QPCR-MPN showed excellent correlation (R^2=0.97 by Pearson's correlation co-efficient) and no significant differences between the two assays (Table 2). Results were comparable for untreated oysters and for PHP oysters at both 1 and 7 days post treatment. In this study results were also examined side by side for both Nitrogen Immersion and Nitrogen Tunnel PHP treatments and statistical

comparison of this data, utilizing both JMP from SAS and Minitab, both one way ANOVA and Tukeys post hoc tests show no significant differences ($p < 0.05$) between detection methods.

The AOAC evaluation of the DuPont Bax Vibrio QPCR test kit described application of the assay on five food types; raw shrimp, cooked shrimp, oysters, raw ahi tuna, and raw scallops (See attached draft of publication in appendix). Results supported the applicability of the BAX ® system for detecting *Vibrio* in foods. Samples were analyzed using the BAX ® system method and the FDA-BAM methods for detecting *Vibrio*. One food type, ahi tuna, was tested by an external independent laboratory (the State of Texas Department of Public Health, Consumer Microbiology Division) as a shared matrix. Results were in nearly complete concordance with only two cases where the test kit yielded a result that could not be confirmed by culture. Inclusivity and exclusivity of the assay was determined with all tested isolates (n = 126 target *Vibrio* strains and n = 55 non-*Vibrio* and non-target *Vibrio* species strains) demonstrating expected results and an assessment of test kit stability, lot to lot variability, and assay ruggedness was also performed demonstrating robustness of the assay.

During 2007 summer PHP validation trials were conducted by The University of Florida Aquatic Food Products group in a partnership with the oyster industry in Apalachicola FL. Side by side field trials compared the FDA-MPN to the QPCR-MPN assay are described below (Table 1). Side-by-side sample comparisons of the two assays support application of QPCR technology for validation oyster processing protocols. Samples (n=3), consisting of 12 oysters each, were obtained from untreated oysters (25IS, 29IS); temperature abused (26 TA, 30TA) by incubation O.N. at room temp; PHP heat treated oysters (65.5 for 5 min) after 7 days storage at -20C (26HSD7, 30HSD7); or Blast frozen oyster (-50C) after 42 days storage (26BLD42). The mean MPN/g for the two assay were nearly identical with R^2=0.99.

Table 1: Comparison of MPN Protocols

OYSTER LOT:	Log MPN/g	
	FDA MPN	BAX-QPCR MPN
25IS25,	2.0±0.56	2.0±0.62
29IS	2.0±0.6	2.0±1.03
26TA	4.0±0.64	4.0±0.40
30TA	6.0±0.11	6.0±0.22
26HSD7	<3.0	<3.0
30HSD7	1.0±0.66	1.1±0.58
26BLD42	2.0±0.43	2.1±0.51

Limit of Quantitation and Specificity

The attached AOAC draft manuscript details the limits of quantitation and specificity.
Inclusivity testing (n=50 strains) was performed at ~10^5 cfu/ml, while exclusivity testing (n= 50 strains) was performed at ~10^8 cfu/ml from broth cultures. Additional strains were tested by Wright Lab (see attached Table 2, 3, 4 in appendix)

For AOAC approval for spiked foods, Vibrio strains were inoculated to yield fractional positive results for plus/minus screening, or at levels informative of method performance for MPN-based approaches. Samples were

tested with the FDA-BAM culture-based method and by PCR using the BAX® system. Ahi tuna was spiked at three levels with Vc and tested for presence or absence of target in sets of twenty 25g sub-samples and five unspiked sub-samples, with PCR testing from the BAM enrichments. Similarly, scallops were spiked with Vv at a level giving fractional results for the (how many samples?) 1g samples, and each MPN tube was tested by the BAM method and PCR as were five 25g samples enriched in a comparable manner. Naturally occurring low-level Vc in raw shrimp was also tested using twenty 25g samples with both the BAM method and PCR testing from the same enrichments. All inclusivity/exclusivity testing demonstrated expected results. For effectiveness testing, comparing PCR and culture, results for the spiked ahi tuna (36 positive of 65 samples tested) and shrimp (5 positive of 20 samples tested) were identical with no false negative or false positive results by PCR. Scallop data gave identical MPN results for test and reference methods and 25g enrichments were all positive by PCR.

Additional seeding studies conducted by Wright lab utilized known concentrations of *Vibrio* species to spike APW with or without oyster homogenates. Samples were assayed by QPCR immediately without growth using various combinations of high (10^6), mediun (10^4), low (10^2) concentrations of the three species. All samples were positive for all species with the exception of samples with High Vp and low or medium concentrations of Vv. In these cases, Vv was not detected. However, samples where growth was permitted (O.N. incubation at 37C), all species were detected in all samples.

References

1. **Blackstone, G. M., J. L. Nordstrom, M. C. L. Vickery, M.D. Bowen, R. F. Meyer, A. Depaola.** 2003. Detection of pathogenic *Vibrio parahaemolyticus* in oyster enrichments by real time PCR. J. Microbiol. Methods. 53: 149-155.
2. **Campbell, M. S. and A. C. Wright.** 2003. Real-time PCR analysis of *Vibrio vulnificus* from oysters. Appl. Environ. Microbiol. **69:**7137-7144.
3. **Food and Drug Administration** (FDA). 2005. National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish. Guidance Documents Chapter IV Naturally Occurring Pathogens, Section .04 www.cfsan.fda.gov/~ear/nss3-44.html#p04
4. **Gordon K.V.**, **M.C. Vickery**, **A. DePaola**, **C. Staley**, **V.J. Harwood**. 2008. Real-time PCR assays for quantification and differentiation of Vibrio vulnificus strains in oysters and water. Appl. Environ. Microbiol. **74**: 1704-9.
5. **Gulf Oyster industry Council (G.O.I.C).** 2001. Gulf Oyster Industry Leads the Way Toward Providing Less Risky Oysters for Certain At Risk Consumers Through Post-Harvest Treatment. Online: November 21, 2007. www.gulfoysters.org/postharvest.cfm
6. **Harwood, V. J., J. P. Gandhi, and A. C. Wright**. 2004. Methods for isolation nd confirmation of Vibrio vulnificus from oysters and environmental sources: a review. J. Microbiol. Methods 59:301–316.
7. **Interstate Shellfish Sanitation Conference** (ISSC). 2003. Issue relating to a *Vibrio vulnificus* risk management plan for oysters. ISSC, Columbia, SC.
8. **Kaysner, C. A. and A. DePaola.** 2004. *Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and Other *Vibrio* spp., Food and Drug Administration Bacteriological Analytical Manual, 8 ed. Revision A, 1998. Chapter 9. Substantially rewritten and revised May 2004. www.cfsan.fda.gov/~ebam/bam-9.html
9. **Paniker, G. and A. K. Bej.** 2005. Real-time PCR detection of *Vibrio vulnificus* in oysters: comparison of oligonucleotide primers and probes targeting vvhA. Appl. Environ. Microbiol. **71**: 5702-5709.
10. **Panicker, G., M. L. Myers, and A. K. Bej.** 2004a. Rapid detection of *Vibrio vulnificus* in shellfish and Gulf of Mexico water by real-time PCR. Appl. Environ. Microbiol. **70**: 498–507.
11. **Panicker, G., M. L. Myers, and A. K. Bej.** 2004b. Multiplex PCR detection of clinical and environmental strains of *Vibrio vulnificus* in shellfish. Can. J. Microbiol. **50:**911-922.
12. **Randa, M. A., M. F. Polz, and E. Lim.** 2004. Effects of temperature and salinity on *Vibrio vulnificus* population dynamics as assessed by quantitative PCR. Appl. Environ. Microbiol. **70:**5469–5476.
13. **Ririe, K. M., R. P. Rasmussen, and C. T. Wittwer**. 1997. Product differentiation by analysis of DNA melting curves during the polymerase chainreaction. Anal. Biochem. 245:154–160.
14. **Shapiro, R. L., S. Altekruse, L. Hutwagner, R. Bishop, R. Hammond, S. Wilson, B. Ray, S. Thompson, R. V. Tauxe, P. M. Griffin, and the Vibrio Working Group.** 1998. The role of Gulf Coast

oysters harvested in warmer months in *Vibrio vulnificus* infections in the United States, 1988-1996. J. Infect. Dis. **178**:752-759

15. **Wallace, M., A.C. Wright, T. Dambaugh, M. Kingsley, C. Malota, B. Andaloro, D. Fallon, D. Delduco[1], G. Tice**. 2009. DuPont Qualicon BAX ® Real Time *Vibrio* Test Kit for the Detection of *Vibrio cholera, parahaemolyticus and vulnificus* from Tuna, Shrimp and Oysters, *AOAC Performance Tested Methods* (In Press).
16. **Wright, A. C., G. A. Miceli, W. L. Landry, J. B. Christy, W. D. Watkins, and J. G. Morris, Jr.** 1993. Rapid identification of *Vibrio vulnificus* on nonselective media with an alkaline phosphatase-labeled oligonucleotide probe. Appl. Environ. Microbiol. **59:**541-6.
17. **Wright, A.C.**, **V. Garrido, G. Debuex, M. Farrell-Evans, A.A. Mudbidri, W.S. Otwell .** 2007. Evaluation of postharvest-processed oysters by using PCR-based most-probable-number enumeration of Vibrio vulnificus bacteria. Appl. Environ. Microbiol. **73**: 7477-81

APPENDIX 1

Table 2 QPCR analysis for *V. cholerae* strains

***V. cholerae* Strains**	**Clinical (C) vs. Environmental (E): Description**	***Vjp***	***Vv***	***Vc***	**CT**	**CFU/ml**
1. Vc598	**C:** classical Inaba O1	-	-	+	23.7	>1.0E+09
2. Vc NRT 36S	C: non O1, O139, NAG-ST (Japan)	-	-	+	23.1	>1.0E+09
3. Vc JVY212		-	-	+	25.7	5.50E+08
4. Vc JVB52		-	-	+	27.2	1.60E+08
5. Vc 5439/62		-	-	+	28.2	7.00E+07
6. Vc 569B	**C:** O1, classical, inaba (India)	-	-	+	24.6	>1.0E+09
7. Vc S171		-	-	+	24.7	>1.0E+09
8. Vc NAG12		-	-	+	25.5	6.50E+08
9. Vc ATCC25874		-	-	+	22.4	>1.0E+09
10. Vc 8		-	-	+	24.8	>1.0E+09
11. Vc B1307 Dacca		-	-	+	24.8	>1.0E+09
12. Vc A5		-	-	+	26.3	3.50E+08
13. Vc I10		-	-	+	25.2	8.20E+08
14. Vc 646	**C:** O1, Ogawa,	-	-	+	24	>1.0E+09
15. Vc 395	**C:** Classical Ogawa O1 (India)	-	-	+	25.6	6.10E+08
16. Vc 6337		-	-	+	35.4	1.90E+05
17. Vc T2001		-	-	+	34.7	3.40E+05
18. Vc T5957		-	-	+	34.7	3.60E+05
19. Vc 2076-79	C: non O1, O139, NAG-ST (oysters, US)	-	-	+	33.7	7.60E+05
20. Vc BA312		-	-	+	34.4	4.30E+05
21. Vc 569B	**C: O1 classical Inaba (India)**	-	-	+	33.9	6.60E+05
22. Vc AI1837		-	-	+	34	6.20E+05
23. Vc Arg-3	**E:**	-	-	+	34.4	4.40E+05
24. Vc C6706	**C: O1, el tor, Inaba (Peru)**	-	-	+	33.9	6.50E+05
25. Vc CA385	**C: O1**	-	-	+	34.6	3.80E+05
26. Vc CO603		-	-	+	34.2	5.00E+05
27. Vc CO845		-	-	+	33.8	7.10E+05
28. Vc N16961	**C: 01 eltor Inaba**	-	-	+	33.9	6.40E+05
29. Vc NG288/36	**C: O139 (Thailand)**	-	-	+	33.7	7.50E+05
30. Vc NRT36S		-	-	+	36.6	7.20E+04
31. Vc PS15		-	-	+	36.2	1.00E+05
32. Vc V5C		-	-	+	34.3	5.00E+05
33. Vc ATCC 25873		-	-	+	0	0
34. Vc SO47W		-	-	+	33.9	6.40E+05
35. Vc RB1		-	-	+	34.9	3.00E+05
36. Vc J31W		-	-	+	39.3	<1.0E+04
37. Vc T96W		-	-	+	36.6	7.00E+04
38. Vc 6358		-	-	+	0	0
39. Vc 5057		-	-	+	34.5	4.20E+05
40. Vc 7261		-	-	+	34	6.40E+05
41. Vc 7165		-	-	+	33.2	1.20E+06
42. Vc 9115		-	-	+	0	0
43. Vc 5145		-	-	+	34.3	4.80E+05
Blank		-	-	-	0	0

Table 3 QPCR analysis for *V. parahaemolyticus* strains

V. parahaemolyticus Strains	Clinical (C) vs. Environmental (E): Description	*VP*	*Vv*	*Vc*	CT	CFU/ml
1. Vp TX2103		+	-	-	25.6	3.50E+08
2. Vp TX3547		+	-	-	26	2.60E+08
3. Vp DAL1094		+	-	-	26.2	2.30E+08
4. Vp 17802		+	-	-	24.6	6.80E+08
5. Vp 43996		+	-	-	25.5	3.70E+08
6. Vp 10290		+	-	-	25.5	3.70E+08
7. Vp Y 9398		+	-	-	23.7	>1.0E+09
8. Vp 5E-3		+	-	-	34.1	1.40E+06
9. Vp 205-757		+	-	-	33.4	2.30E+06
10. Vp AQ3810		+	-	-	33.5	2.10E+06
11. Vp AQ4235		+	-	-	33.1	2.80E+06
12. Vp EDL896		+	-	-	33.4	2.30E+06
13. Vp VP2		+	-	-	33.5	2.20E+06
14. Vp VP250		+	-	-	33	3.00E+06
15. Vp VP331		+	-	-	33.2	2.70E+06
16. Vp VP356		+	-	-	33.3	2.40E+06
17. Vp VP381		+	-	-	33.4	2.30E+06
18. Vp VP53		+	-	-	33.2	2.50E+06
19. Vp VP81		+	-	-	31.7	6.70E+06
20. Vp WP-1		+	-	-	34.2	1.40E+06
21. Vp S162-71		+	-	-	33.5	2.10E+06
22. Vp 3D-38		+	-	-	33.5	2.10E+06
23. Vp EDL 1044		+	-	-	33.1	2.80E+06
24. Vp SN36		+	-	-	33.8	1.80E+06
25. Vp 14d13		+	-	-	33.4	2.30E+06
26. Vp AAG9574		+	-	-	33.3	2.50E+06
27. Vp P125		+	-	-	33.5	2.20E+06
28. Vp HMG38		+	-	-	33.2	2.60E+06
29. Vp P29		+	-	-	34.1	1.40E+06
30. Vp VV104		+	-	-	33.7	1.90E+06
31. Vp VV27-1		+	-	-	33.5	2.10E+06
32. Vp VV27-2		+	-	-	33.9	1.60E+06
33. Vp A602		+	-	-	32.9	3.20E+06
34. Vp FC1011		+	-	-	34.7	9.70E+05
35. Vp B10576		+	-	-	33.4	2.30E+06
36. Vp EDL1041		+	-	-	34	1.60E+06
Blank		-	-	-	0	0

Table 4 QPCR analysis for *V. vulnificus* strains:

vulnificus rains	Clinical (C) vs. Environmental (E): Description	*Vjp*	*Vv*	*Vc*	CT	cfu/ml
Vv MO6-24/O	C	-	+	-	24.3	5.20E+08
Vv FLA 109	C	-	+	-	27	5.90E+07
Vv FLA141	C	-	+	-	27.3	4.80E+07
Vv FLA126	C	-	+	-	28.5	1.90E+07
Vv FLA134	E: oyster	-	+	-	26.5	8.90E+07
Vv FLA129	C	-	+	-	26.8	6.80E+07
Vv FLA127	E: oyster	-	+	-	26.5	9.10E+07
Vv FLA135	E: oyster	-	+	-	26.8	7.00E+07
Vv FLA 115	E: oyster	-	+	-	27.1	5.60E+07
Vv FLA 149	C	-	+	-	28.5	1.90E+07
Vv B3-313/98	E: fish	-	+	-	27.3	4.70E+07
Vv FLA121	E: oyster	-	+	-	26.9	6.20E+07
Vv FLA137	E: oyster	-	+	-	26.4	9.40E+07
Vv B3-302/99	E: fish	-	+	-	26.7	7.60E+07
Vv FLA119	E: oyster	-	+	-	26.7	7.30E+07
Vv FLA116	C	-	+	-	26.5	8.80E+07
Vv FLA102	C	-	+	-	26.7	7.60E+07
Vv B2-2	E: fish	-	??	-	0	0
Vv FLA108	C	-	+	-	26	1.30E+08
ank		-	-	-	0	0

APPENDIX 2: Draft manuscript for AOAC approval:

DuPont Qualicon BAX ® Real Time *Vibrio* Test Kit for the Detection of *Vibrio cholera, parahaemolyticus and vulnificus* from Tuna, Shrimp and Oysters

AOAC Performance Tested Methodsm YYMMXX

ABSTRACT
An evaluation was conducted on five food types; raw shrimp, cooked shrimp, oysters, raw ahi tuna, and raw scallops to demonstrate the applicability of the BAX ® system for detecting *Vibrio* in foods. Samples were analyzed using the BAX ® system method and the FDA-BAM methods for detecting *Vibrio.* One food type, ahi tuna, was tested by an external independent laboratory (the State of Texas Department of Public Health, Consumer Microbiology Division) as a shared matrix. Results were in nearly complete concordance with only two cases where the test kit yielded a result that could not be confirmed by culture. Inclusivity and exclusivity of the assay was determined with all tested isolates (n = 126 target *Vibrio* strains and n = 55 non-*Vibrio* and non-target *Vibrio* species strains) demonstrating expected results and an assessment of test kit stability, lot to lot variability, and assay ruggedness was also performed demonstrating robustness of the assay.

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Scope of method
1.1 Target organisms – Vibrio cholera, parahaemolyticus, and vulnificus. A wide range of *Vibrio* and non-*Vibrio* strains was used for inclusivity/exclusivity testing.
1.2 Matrices – Specific foods tested included shrimp, oysters, tuna, and scallops.
1.3 Performance claims – Sensitivity and specificity equivalent to the official FDA-BAM culture-based method.

Definitions

- From the AOAC International Official Methods of Analysis Program Manual Appendix X [1]: Sensitivity rate (p+) for a food type and inoculation level - The probability that the method, alternative or reference, will classify a test sample as positive, given that a test sample is a known positive. A known positive refers to the confirmation of upernatan analyte.

Sensitivity rate is defined as: Total number of confirmed positive test portions by the method divided by total number of confirmed positive test portions by both the alternative and reference methods.
Specificity rate (p-) for a food type and inoculation level - The probability that the method will classify the test sample as negative, given that the test sample is a known negative. A known negative refers to a confirmed negative test portion.
Specificity rate is defined as: Total number of analyzed negative test portions by the method divided by total number of confirmed negative test portions by both the alternative and reference methods. For microbiological methods involving a confirmation step, a presumptive positive result is taken through the cultural procedure and

confirmed to be a positive or determined to be a negative. In other words, the confirmation procedure allows the sample to be reclassified as a known positive or a known negative. As such, the specificity rate of results after confirmation is always 100%.
False negative rate (pf-) for a food type and inoculation level – The probability that a test sample is a known positive, given that the test sample has been classified as negative by the method. Pf- is the number of misclassified known positives divided by the total number of positive test samples (misclassified positives plus the number of correctly classified known positives) obtained with the method. Incidence of false negatives equals 100 minus the sensitivity rate.
False positive rate (pf+) for a food type and inoculation level – The probability that a test sample is a known negative, given that the test sample has been classified as positive by the method. Pf+ is the number of misclassified known negatives divided by the total test samples (misclassified positives plus the number of correctly classified known negatives) obtained with the method.
Incidence of false positives equals 100 minus the specificity rate.

Principle

The BAX® system uses the Polymerase Chain Reaction (PCR) to amplify specific DNA fragments, which are stable and unaffected by growth conditions [2]. Each fragment is a genetic sequence that is unique to the targeted organism, thus providing a highly reliable indicator that the organism is present. The BAX® system simplifies the PCR process by combining the requisite PCR reagents into a stable, dry, manufactured tablet already packaged inside the PCR tubes. After hydrating these tablets with prepared samples, the tubes remain sealed to reduce the potential for contamination.

In a typical PCR application, sample DNA is combined with DNA polymerase, nucleotides and primers that are specific for a given nucleotide sequence. The mixture then undergoes a series of timed heating and cooling cycles. Heating denatures the DNA, separating it into single strands. As the mixture cools, the primers recognize and anneal (bind) to the targeted DNA sequence. DNA polymerase then uses nucleotides to extend the primers, thus creating two copies of the targeted fragment (amplification). Repeating cycles of denaturing, annealing and extending produces an exponential increase in the number of target DNA fragments, creating millions of copies in a very short time. If the target sequence is not present, no detectable amplification takes place [2]. Inhibitors to PCR are present in some food matrices. In particular, phenolic compounds found in some spices and other plant-based materials such as high purity cocoa can cause the PCR reaction to shut down. Because of this, each BAX reagent tablet is formulated with a low level control DNA molecule and associated primers. This Internal Positive Control (INPC) must be shown to amplify in the absence of specific pathogen target amplification product for the BAX ® instrument to report a negative result. In the absence of any target or INPC associated product, the instrument reports an indeterminate result.

The BAX® system PCR tablets used in real-time assays also contain multiple dye-labeled probes. Intact probes are short oligonucleotides with quencher dye at one end that absorbs the signal from fluorescent reporter dye at the opposite end. During PCR cooling cycles, probes bind to a specific area within the targeted fragment. During extension, DNA polymerase encounters the probe in its path and breaks the probe apart. This releases the reporter dye, resulting in increased fluorescent signal [3]. In multiplex reactions such as in this test kit, each species specific probe is labeled with a different fluorescent reporter dye, allowing independent detection of the presence or absence of each target. The BAX® system Q7 instrument uses multiple filters to measure specific signal resulting from the presence of each target at the end of each cycle and report results for the presence or absence of *Vibrio cholera*, *vulnificus*, or *parahaemolyticus* in less than 90 minutes.

General information

Vibrio is a gram-negative genera consisting of 65 known species [4]. It can cause seafood and water-borne illnesses and infections in humans. It is most commonly found in marine and freshwater environments and is transmitted to humans mainly through the consumption of raw or undercooked shellfish, particularly oysters, or through contaminated drinking water [5].

The risk of *Vibrio*-caused illness is increased following a natural disaster leading to disruption of water and sanitation systems or massive displacement of a population to inadequate and overcrowded temporary housing. Such an effect was seen in the aftermath of Hurricane Katrina in 2005, where surveillance identified 22 new cases of *Vibrio* illness, including five deaths [5].

The three species of Vibrio that cause the majority of human illness and infection are *Vibrio cholera*, *parahaemolyticus*, and *vulnificus* [6].

Cholera is a major disease that occurs when *Vibrio cholera* colonizes the small intestine and releases enterotoxin(s) leading to a secretory diarrhea that without supportive oral rehydration and replacement of salts can prove fatal. The disease is currently endemic in many countries in South Asia, Africa and the Americas and remains a global threat to public health [6].

Vibrio parahaemolyticus is an invasive organism that primarily affects the colon. It is estimated that up to 4500 cases of *Vibrio parahaemolyticus* infection occur annually in the United States [7]. These illnesses are mainly due to the consumption of undercooked oysters and other seafood.

Vibrio vulnificus is an emerging human pathogen that can cause illnesses such as gastroenteritis and can cause wound infections that can progress to septicemia. Though the total number of cases of *V. vulnificus* infection is small, it is highly pathogenic in certain populations, and thus is responsible for an estimated 1% of all foodborne deaths in the United States [8].

Test Kits Information

5.1 *Test kit name* – BAX® System Real-Time PCR Assay for Screening *Vibrio cholerae*, *parahaemolyticus*, *vulnificus*

5.2 *Test kits catalog numbers* – D12863877

5.3 Ordering information –

5.3.1 DuPont Qualicon, Experimental Station, Bldg. 400, P.O. Box 80400, Rt. 141 & Henry Clay Road, Wilmington, DE 19880-0400, USA, Phone 800-863-6842 or 302-695-5300, Fax 302-695-5301, Internet www.qualicon.com

5.3.2 DuPont Qualicon Europe, Ltd Wedgwood Way, Stevenage Herts SG1 4QN, UK

5.3.3 DuPont Qualicon, Asia/Pacific DuPont Company (Singapore) Pte, Ltd. 1 Harbour Front Place #11-01, Harbour Front Tower One, Singapore 098633

5.4 Test kit components –

5.4.1 PCR tubes with tablets (twelve 8-tube strips, each tube containing 1 PCR tablet)

5.4.2 Flat optical caps for PCR tubes (twelve 8-cap strips)

5.4.3 Lysis buffer (two 12-ml bottles)

5.4.4 Protease (one 400-µl vial)

5.4.5 Package insert (1)

Additional reagents

Protease reagent – Using test kit reagents, pipette 150 µL of protease into one 12-mL bottle of lysis buffer. Label bottle with the date prepared. Reagent will remain stable for up to two weeks if stored at 2-8ºC.

Apparatus

7.1 Incubators – Static incubators at 35 ± 2ºC, 39-40ºC, and a heated water bath capable of maintaining a temperature of 41± 0.2ºC.

7.2 Stomacher, Blender, and Scissors – For sample preparation. Seward model 400 or equivalent stomacher, Blender with blending jars, and autoclavable scissors.

7.3 BAX® system Q7 apparatus (all components listed in this section are included with the *BAX®* Q7 System Start Up package. Components 7.3.3 – Cluster tubes with caps, and 7.3.6 – Pipette tips; after the initial boxes included with the start-up package are used; must be purchased by the test kit user).

7.3.1 BAX® System cycler/detector with computer workstation

7.3.2 BAX® System application software

7.3.3 Cluster tubes with caps and racks for lysis

7.3.4 Capping/de-capping tools – for removing and sealing cluster tube caps and PCR tube caps without jarring the contents

7.3.5 Heating blocks with inserts and thermometers – for maintaining lysis tubes at 37ºC ± 1ºC, 55ºC ± 1ºC and 95ºC ± 1ºC

7.3.6 Pipettes – for transferring reagents; two adjustable mechanical pipettes covering 20-200 µl and 5-50 µl; one repeating pipette; and one multi-channel pipette covering 8 channels and 5-50 µl. Pipettes should be calibrated to deliver required volumes within 10%.

7.3.7 Pipette tips with barriers: 0.5-250 µl, 0.5-100 µl extended barrier; 2.5 ml and 5 ml repeater pipette tips

7.3.8 Cooling block assemblies – for keeping lysate tubes and PCR tubes chilled at 2-8ºC during sample preparation

7.3.9 PCR tube holders – for transferring a rack of tubes from the cooling block to the cycler/detector

7.3.10 Printer

Standard Reference Materials

8.1 DuPont Qualicon culture collection (DD) – proprietary

8.2 *American Type Culture Collection* (ATCC) – *American Type Culture Collection* (ATCC) – www.atcc.org, American Type Culture Collection (ATCC), P.O. Box 1549, Manassas, VA 20108, USA.

Standard solutions, consumables, and media

Media – where applicable FDA-BAM designations listed in parentheses.

Alkaline peptone water (APW) (M10)
AKI medium (M7)
Arginine glucose slants (AGS) (M16)
Blood agar (5% sheep red blood cells) (M20)
Casamino acids yeast extract (CAYE) broth (M34)
modified Cellobiose polymyxin colistin (mCPC) agar (M98)
Cellobiose colistin (CC) agar (M189)
Motility test medium-1% NaCl (M103)
Oxidase reagent (1% N,N,N,N'-tetramethyl-p-phenylenediamine.2HCl in dH2O) (R54)
Phosphate buffered saline (PBS) (R59)
Polymyxin B disks, 50 U (Difco or equivalent) (R64)
Saline soln – 0.85% in dH2O (R63)
2% NaCl soln (R71)
Sodium desoxycholate – 0.5% in sterile dH2O (R91)
Thiosulfate citrate bile salts sucrose (TCBS) agar (M147)
T_1N_1 and T_1N_3 agars (1% tryptone and either 1% or 3% NaCl) (M163)

T_1N_0, T_1N_3, T_1N_6, T_1N_8, T_1N_{10} broths (M161)
Tryptic soy agar-magnesium sulfate- 3% NaCl (TSAMS) (32) Trypticase (or tryptic) soy broth (TSB), agar (TSA)(M152) (with added NaCl, 2%)
TSB-1% NaCl-24% glycerol
Urea broth (M171) (or Christensen's urea agar (M4+0) with added NaCl (2%) (R71)
Vibrio parahaemolyticus sucrose agar (VPSA) (M191)
Vibrio vulnificus agar (VVA) (M190)
Chromagar *Vibrio* (DRG International Mountainside, NJ Product number VB912)
API 20E diagnostic strips and reagents (BioMerieux, Hazelwood, Mo.)
All microbiological media was prepared by autoclaving at 121°C at 15 psi for 15 min if preparing $\leq$ 4 L of media and 20 min if preparing > 4 L of media.

Safety Precautions

10.1 *Kits* – The reagents used in the BAX® system should pose no hazards when used as directed. Dispose of lysate, PCR mixture and other waste according to your site practices.
10.2 *Cycler/detector* – Only qualified laboratory personnel should operate the cycler/detector. Do not attempt to repair the instrument. Live power may still be available inside the unit even when a fuse has blown or been removed. Refer to the *User Guide* for maintenance procedures when cleaning the unit or changing a fuse. The heating block can become hot enough during normal operation to cause burns or cause liquids to boil. Wear safety glasses or other eye protection at all times during operation.
10.3 Enrichment Broths- All enrichment broths whether testing positive or negative for this assays targets, may contain enriched pathogens and should be autoclaved following any culture-based confirmatory steps.

General Preparation / Sample preparation and recovery

- 11.1 *Selection of strains for testing*- Strains were taken from the DuPont/Qualicon culture collection (samples tested by Qualicon) (see Table 2), collaborators' culture collections (the University of Florida and the Texas State Department of Public Health), and the American Type Culture Collection (ATCC).
11.2 *Culture preparation for artificially contaminated food – Vibrio* were grown to stationary phase in APW and serially diluted in APW to final concentrations likely to give fractional recovery (based on preparatory studies).
11.3 *Food samples* – Five food types were included in this study; raw ahi tuna, raw shrimp, cooked shrimp, oysters, and raw scallops.
Raw tuna was artificially inoculated with *V. cholera*, cooked shrimp were artificially inoculated with *V. parahaemolyticus*, and raw scallops were artificially inoculated with *V. vulnificus*, while naturally occurring flora was tested in raw shrimp and raw oysters. Reference method enrichment varied according to the sample type examined. Tuna and raw shrimp were tested on a plus/minus basis according to the FDA-BAM protocols for *V. cholera*. Though much of the FDA-BAM *Vibrio* chapter is MPN-based, and thus the MPN-based methods were used to validate the effectiveness of the assay, it is anticipated that the BAX ® test kit will primarily be used to screen on a presence/absence basis so additional samples were tested to validate this type of screening. That is, samples were tested using the FDA-BAM enrichment conditions and culture confirmation with BAX ® testing from each of the MPN replicates, but with additional unpaired 25g samples enriched in 225 ml of enrichment media before BAX ® testing as a complement. Each 25g sample enrichment was also culture confirmed using the FDA-BAM methodology.

Analysis – BAX® system methods

12.1 *Prepare equipment* – Turn on heating blocks (37ºC and 95ºC). Check that cooling blocks have been refrigerated overnight. Turn on power to cycler/detector, then to computer. Launch BAX® system

application. If instrument diagnostics recommends verification, follow Verification Wizard screen prompts for procedure.

12.2 *Create rack file* – Follow prompts in the Rack Wizard to enter identifying data on the entire rack and on the individual samples.

12.3 *Perform lysis* –Add 5 µL of enrichment from the top of each enrichment to 200 µL of protease reagent in a cluster tube. Place in heating block at 37±1°C for 30 minutes. Transfer tubes to 95°C heating block for 10 minutes. Transfer to cooling block (2–8°C) for 5 minute.

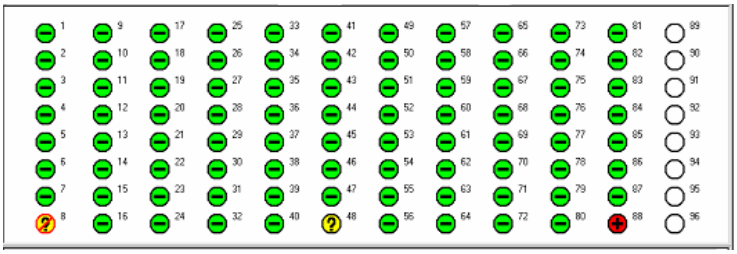
12.4 *Warm up cycler/detector* – Select RUN FULL PROCESS from the menu bar of the application window to heat the instrument to the set temperature (90ºC for the block, 100ºC for the lid).

12.5 *Hydrate PCR tablets with lysate* – Place PCR tube holder over insert of the PCR cooling block (solid side in rear). Place one PCR tube per sample into the holder. Loosen all caps, and remove caps from a row of tubes. Using a multi-channel pipette, transfer 30 µL of lysate to the row of PCR tubes for the *Vibrio* assay. Seal tubes with replacement optical caps. Using new tips, repeat transfer for each row until all samples have been transferred into PCR tubes.

12.6 *Amplify and detect* – Follow screen prompts at the PCR Wizard for loading samples into the cycler/detector and begin the program. The Full Process program takes about 75 min to complete. When finished, the PCR Wizard will prompt you to unload the samples and will automatically display the results.

Interpretation and test result report

Review results on screen as a grid of wells



Negative – Circle with (-) symbol
Positive – Circle with (+) symbol
Indeterminate – Circle with (?) symbol
Error (low signal) – Circle with (?) symbol and slash (/)

Food method comparison studies

Methodology – In accordance with an AOAC-RI approved study design, DuPont Qualicon compared the BAX® system method to the FDA-BAM [9] method for detecting *Vibrio* species in food samples.

Tuna (*V. cholera*) – Internal Qualicon and Independent Laboratory Shared Matrix

For tuna testing, a strain of *V. cholera* was taken from the DuPont Qualicon culture collection and struck for purity on a T_1N_1 agar plate. A single colony was inoculated into a tube containing 10 ml of APW broth, and incubated 18 hrs at 35ºC. The stationary phase culture was enumerated by plating dilutions on T_1N_3 and TSA agar plates. Based on preparatory studies, a dilution factor was established to give inoculation levels appropriate for achieving fractional positive results for the tuna matrix. Samples were inoculated as a master sample of sliced tuna, and mixed well by shaking and hand massaging in a biohazard bag. Samples were divided into analytical size portions into blender jars if they were to be blended or stomacher bags if they were to be processed by scissors and held at 4°C for 48-72 hours before enrichment (Qualicon tested by scissors processing while the independent laboratory tested by blending). Following this cold stress/acclimation, if processing with scissors, portions of tuna were removed and processed with scissors which were decontaminated with ethanol and allowed to air dry before preparation of another sample. Samples prepared in this way were cut into approximately 1g pieces (~25 pieces per analytical unit). If processing with blending, portions were blended at high speed for 1 min. If processing with blending, portions were blended at high speed for 1 min. Three each samples of 100g, 10g and 1g were also prepared from this mix for MPN analysis.

Tuna portions were mixed as described above in 225 ml of APW and incubated at 35°C for 22 +/- 2 hrs total with reference method plating performed at 6-8 hrs and concurrently with BAX® testing after 16-20 hrs of incubation.

At each reference culture sample point, a 3 mm loop was used to streak for isolation onto dried plates of TCBS, mCPC, and CHROMagar *Vibrio* agar plates. Three or more typical colonies from each agar media when present were struck onto T_1N_3 agar plates and subjected to the initial biochemical screenings specified in the FDA BAM. Colonies which were phenotypically consistent with *Vibrio* (with a preference for *V. cholera* for this spiked study) were subjected to API-20E testing as described in the FDA BAM. If PCR positive samples' culture results had been inconsistent with *V. cholera*, up to 24 additional colonies would have been picked for characterization, but this was not needed for this matrix.

Raw Shrimp (*V. cholera*)

For raw frozen shrimp in an ongoing retail survey, Qualicon found shrimp with a low enough level of naturally occurring *V. cholera* to give fractionally positive results. Twenty samples of 25g each were removed from this batch and blended at high speed for 2 min at high speed in 225 ml of APW and incubated at 35°C overnight (18 +/- 2 hrs) with reference method plating performed at 6-8 hrs and concurrently with BAX® testing after overnight incubation onto TCBS, mCPC, and CHROMagar. Plates were incubated at 35-37°C overnight.

At each reference culture sample point, a 3 mm loop was used to streak for isolation onto dried plates of TCBS, mCPC, and CHROMagar *Vibrio* agar plates. Three or more typical colonies from each agar media were struck onto T_1N_3 agar plates and subjected to the initial biochemical screenings specified in the FDA BAM. Presumptive *V. cholera* was given preference for selection, despite the fact that there were many more colonies consistent with *V. parahaemolyticus,* and most enrichments (11/20) in this study were PCR positive for the presence of this species. Though not part of this study, all *V. parahaemolyticus* PCR positive enrichments did culture confirm for the presence of this species, and none of the PCR negative samples were culture positive. Colonies which were consistent with *Vibrio* in initial screening were subjected to API-20E testing as described in the FDA BAM. In two of the BAX ® positive enrichments, no culture confirmed isolates were initially obtained. Additional isolates were picked (up to 24 per plating media where available) and characterized. In both cases one or more *V. cholera* isolates were recovered. Samples from which one or more confirmed *V. cholera* isolates were obtained were considered reference method positive in this study.

Cooked Shrimp (*V. parahaemolyticus*)

Frozen, cooked shrimp were tested for artificially introduced *V. parahaemolyticus*. Cooked refrigerated shrimp were spiked as master samples at two levels with *V. parahaemolyticus* strain TD3129 in which at least one level was likely to be informative of method performance when compared to the reference MPN method. Shrimp were held at 4°C for 48-72 hrs to acclimate the introduced *Vibrio*. For the FDA BAM method, from the spiked master samples, five replicates of 50g of shrimp were weighed into blender jars and homogenized at high speed for 90 sec and used for analysis. The entire animal was used for blending. PBS (450 ml) was added and blended for 1 min at 8,000 RPM. This constituted the 1:10 dilution. Two further serial dilutions were prepared in PBS for final 1:100 and 1:1000 dilutions (in testing of artificially contaminated product, since very low spike levels were used, no further dilutions were performed). Since this was a cooked product, 3 x 10 ml portions of the 1:10 dilution were transferred into 3 tubes containing 10 ml of 2X APW. This represented the 1 g portion. Similarly, 3 x 1 ml portions of the 1:100 and 1:1000 dilutions were inoculated into 10 ml of single-strength APW. APW enrichments were incubated overnight at 35 ±2°C (18 +/- 2 hrs). A 3-mm loopful from the top 1 cm of each APW tube was struck for isolation onto TCBS, mCPC, and *Vibrio* Chromagar plates. Concurrently with plating, a BAX ® PCR assay was performed from each MPN tube. TCBS and Chromagar plates were incubated at 35 ±2°C and mCPC at 39-40 °C overnight.

Additionally, five 25g samples from the same master sample were directly stomached (2 min at 100 rpm) with APW. For enrichment and plating, the 25g enrichments were treated as described above for MPN analysis.

V. parahaemolyticus appear as round, opaque, green or bluish colonies (usually), 2 to 3 mm in diameter on TCBS agar. Interfering, competitive *V. alginolyticus* colonies are, large, opaque, and yellow (usually). Isolates were struck for purity on T_1N_3 agar plates and subjected to initial screening by oxidase and string tests. Isolates giving expected reactions were subjected to further screening using the API 20E test kit as modified in the FDA-BAM by using 2% NaCl as the diluent.

Raw Scallops (*V. vulnificus*)

Raw scallops were spiked with *V. vulnificus* strain TD3149 at a level likely to be informative of method performance (in which at least one dilution of the MPN analysis was fractionally positive) when compared to the reference MPN method. For the FDA BAM method, from the spiked master samples, five replicates of 50g of scallops were weighed into blender jars and homogenized at high speed for 90 sec and used for analysis. Scallops were held at 4°C for 48-72 hrs to acclimate the introduced *Vibrio*. PBS (450 ml) was added and blended for 1 min at 8,000 RPM. This constituted the 1:10 dilution. One further serial dilution was prepared in PBS for a final 1:100 dilution (in testing of artificially contaminated product, since very low spike levels were used, no further dilutions were performed). 3 x 10 ml portions of the 1:10 dilution were transferred into 3 tubes containing 10 ml of 2X APW. This represented the 1 g portion. Similarly, 3 x 1 ml portions of the 1:10 and 1:100 dilutions were inoculated into 10 ml of single-strength APW. APW enrichments were incubated overnight at 35 ± 2°C (18 +/- 2 hrs). A 3-mm loopful from the top 1 cm of each APW tube was struck for isolation onto TCBS, mCPC, and *Vibrio* Chromagar plates. Concurrently with plating, a BAX ® PCR assay was performed from each MPN tube. TCBS and Chromagar plates were incubated at 35 ±2°C and mCPC at 39-40 °C overnight (18 +/- 2 hrs).

Additionally, five 25g samples from the same master sample were directly stomached (2 min at 100 rpm) with APW. For enrichment and plating, the 25g enrichments were treated as described above for MPN analysis.

V. vulnificus appear as purple colonies on mCPC agar. Isolates were struck for purity on T_1N_3 agar plates and subjected to initial screening by oxidase and string tests. Isolates giving expected reactions were subjected to further screening using the API 20E test kit as modified in the FDA-BAM by using 2% NaCl as the diluent.

Oysters (*V. parahaemolyticus* and *V. vulnificus*)

BAX ® lysates were prepared as described above for scallops (with the exception that dilutions were carried out to 10^{-6}) from samples tested using the MPN procedures of the FDA-BAM in collaboration with the FDA Dauphin Island Seafood Laboratory. The FDA-BAM protocol with *tlh* (thermo-labile hemolysin) pcr based isolate confirmation for *V. parahaemolyticus* and with *vvh*-a (cytolysin) pcr based isolate confirmation for *V. vulnificus* was used for these studies. BAX ® results were compared to the results from the appropriate species specific FDA-BAM PCR for the presence of *V. parahaemolyticus* and *V. vulnificus* in the MPN tubes. To demonstrate the utility of the protocol across a wide level of contamination density, three sets of oysters were examined. One set was stored overnight after harvest at 3°C, another set at 25°C overnight, and a third set at 35°C. For molluscan shellfish, ~12 animals were pooled and blended 90 sec with an equal vol of PBS (1:2 diln). A 1:10 dilution was prepared by weighing (weighing is recommended because air bubbles in the 1:2 dilution prevent accurate volumetric transfer) of the 1:2 homogenate to 4 X ml of PBS. Additional 10-fold dilutions were prepared volumetrically (i.e. 1ml of 1:10 to 9.0ml of PBS for a 1:100 dilution).

Three 100 ml portions (the 10g samples) were added to 100 ml 2X APW. Three 10 ml portions of the 1:10 dilution were inoculated into 3 tubes containing 10 ml of 2X APW. This represented the 1 g

portions. Similarly, 3 x 1 ml portions of the 1:10, 1:100, 1: 1000, and 1:10,000 dilutions were inoculated into 10 ml of single-strength APW. APW was incubated overnight (18 +/- 2 hrs) at 35 ±2°C. A 3-mm loopful was struck from the top 1 cm of all APW tubes onto TCBS, mCPC, and CC agars.

1.1 TCBS plates were incubated at 35 ±2°C overnight (18 +/- 2 hrs) while mCPC and CC plates were incubated at 39-40°C. *V. parahaemolyticus* appear as round, opaque, green or bluish colonies, 2 to 3 mm in diameter on TCBS agar. Interfering, competitive *V. alginolyticus* colonies are, large, opaque, and yellow. Most strains of *V. parahaemolyticus* will not grow on mCPC or CC agar. On mCPC and CC agars, *V. vulnificus* colonies are round, flat, opaque, yellow, and 1 to 2 mm in diameter. Presumptive isolates (three typical isolates per species per MPN tube where available) were purified as described previously and inoculated onto T_1N_3 plates and into 96 well plates for freezing and subsequent FDA-BAM colony confirmation pcr testing.

1.1.1 Isolates with typical morphology from each MPN tube were identified as *V. parahaemolyticus* or *V. vulnificus* by pcr as described in the FDA-BAM and the following sections.

Confirmation of *V. vulnificus* by polymerase chain reaction

1. Isolates obtained by the MPN procedure plating were confirmed by PCR as described in the FDA-BAM.
2. Primers for PCR vvhA (519 base amplicon) are from base 785 to 1303 of the cytolysin gene. The following primers should be used:
   Vvh-785F 5’ ccg cgg tac agg ttg gcg ca 3’
   Vvh-1303R 5’cgc cac cca ctt tcg ggc c 3’
3. The follow reaction was used:

Reagent	Reaction vol.
dH2O	28.2 µl
10X Buffer.MgCl2	5.0 µl
dNTPs	8.0 µl
primer mix (6 primers)	7.5 µl
template	1.0 µl
Taq polymerase	0.3 µl
Total vol	50.0 µl

4. The following PCR conditions were used:
   PCR conditions :

denature	94°C 10 min	
denature	94° C 1 min	
anneal	62°C 1 min	25 cycles
extend	72°C 1 min	
final extend	72°C 10 min	
hold	8°C indefinite	

5. Agarose gel analysis of PCR products. For each isolate, 10 µl PCR product was combined with 2 µl 6X loading gel and loaded into wells of a 1.5% agarose gel containing 1 µg/ml ethidium bromide submerged in 1X TBE. A constant voltage of 5 to 10 V/cm was applied. Gels were illuminated with a UV transluminator (Gel Doc 1000 System, BioRad, Hercules, CA) and bands were visualized relative to molecular weight marker migration. Positive and negative culture controls and reagent controls were included with each PCR run. Isolates were confirmed with the presence of a 519 bp for the species specific pcr product.

Confirmation of *V. parahaemolyticus* by polymerase chain reaction

1. Isolates obtained by the MPN procedure plating were confirmed by PCR as described in the FDA-BAM.
2. The following primer sets were used (final concentration in each reaction for each primer 0.2µM):
   tlh gene species specific (450 bp)

L-TL 5’ aaa gcg gat tat gca gaa gca ctg 3’
R-TL 5’ gct act ttc tag cat ttt ctc tgc 3’

3. The following PCR reagents were used:

Reagent	Reaction vol.
dH2O	28.2 µl
10X Buffer.MgCl2	5.0 µl
dNTPs	8.0 µl
primer mix (6 primers)	7.5 µl
template	1.0 µl
Taq polymerase	0.3 µl
Total vol	50.0 µl

4. The following PCR conditions were used:

PCR conditions :

denature	94°C 3 min	
denature	94° C 1 min	
anneal	60°C 1 min	25 cycles
extend	72°C 2 min	
final extend	72°C 3 min	
hold	8°C indefinite	

5. Agarose gel analysis of PCR products. For each isolate, 10 µl PCR product was combined with 2 µl 6X loading gel and loaded into wells of a 1.5% agarose gel containing 1 µg/ml ethidium bromide submerged in 1X TBE. A constant voltage of 5 to 10 V/cm was applied. Gels were illuminated with a UV transluminator (Gel Doc 1000 System, BioRad, Hercules, CA) and bands were visualized relative to molecular weight marker migration. Positive and negative culture controls and reagent controls were included with each PCR run. Isolates were confirmed with the presence of the 450 bp band for the species specific pcr product.

Table 1. BAX vs. Reference Results for Presence/Absence Testing

Sample type	MPN or Spike Level	Samples	BAX pos	BAX Confirmed	Reference pos	Sensitivity[1]	Specificity[2]	Chi Square[3]
Tuna	0.5 MPN/25g (*V. cholerae*)	20	3	3	3	100%	100%	-
	1.9 MPN/25g (*V. cholerae*)	20	13	13	13	100%	100%	-
	3.75 MPN/25g (*V. cholerae*)	20	19	19	19	100%	100%	-
	0 cfu/25g	5	0	0	0		100%	
Tuna (Independent Laboratory)	6 MPN/25g (*V. cholerae*)	20	9	9	9	100%	100%	-
	0 cfu/25g	5	0	0	0		100%	
Frozen raw shrimp	Naturally contaminated (*V. cholerae*)	20	5	5	5	100%	100%	-

[1] Sensitivity – Total number of confirmed positive test portions by the method divided by total number of confirmed positive test portions by both the alternative and reference methods.

[2] Specificity – Total number of analyzed negative test portions by the method divided by total number of confirmed negative test portions by both the alternative and reference methods.

[3] McNemar Chi-Square test statistic used for calculating significance

Table 2. BAX System Results for Samples with Presence/Absence and MPN Testing								
	Presence/Absence in 25g sample			MPN (3 tube, 3 dilution – 1g, 0.1g, 0.01g)				
Sample type	Inoculation level	BAX positive / confirmed	Reference positive / confirmed	Sample	BAX positive (1g, 0.1g, 0.01g)	Reference positive (1g, 0.1g, 0.01g)	BAX MPN[1]	Reference MPN[1]
Cooked shrimp (*V. parahaemolyticus*)	1.8 cfu/g	5/5	5/5	1	1, 0, 0	1, 0, 0	0.36/g	0.36/g
				2	1, 0, 0	1, 0, 0	0.36/g	0.36/g
				3	1, 0, 0	1, 0, 0	0.36/g	0.36/g
				4	1, 0, 0	1, 0, 0	0.36/g	0.36/g
				5	1, 0, 0	1, 0, 0	0.36/g	0.36/g
Cooked shrimp (*V. parahaemolyticus*)	18 cfu/g	5/5	5/5	1	2, 0, 0	2, 0, 0	0.92/g	0.92/g
				2	2, 2, 0	2, 2, 0	2.1/g	2.1/g
				3	2, 0, 0	2, 0, 0	0.92/g	0.92/g
				4	3, 0, 0	3, 0, 0	2.3/g	2.3/g
				5	2, 1, 0	2, 1, 0	1.5/g	1.5/g
Scallops (*V. vulnificus*)	1.4×10^4 cfu/g	5/5	5/5	1	1, 0, 0	1, 0, 0	0.36/g	0.36/g
				2	0, 0, 0	0, 0, 0	<0.3/g	<0.3/g
				3	2, 0, 0	2, 0, 0	0.92/g	0.92/g
				4	0, 0, 0	0, 0, 0	<0.3/g	<0.3/g
				5	0, 0, 0	0, 0, 0	<0.3/g	<0.3/g

[1] MPN values determined using the FDA-BAM MPN tables.

Table 3. BAX System Results for Oysters with MPN Testing *V. parahaemolyticus* (3 tube, 8 dilution)				
Sample Set	BAX positive (10g, 1g, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6})	Reference positive (10g, 1g, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6})	BAX MPN[1]	Reference MPN[1]
3°C	3, 3, 3, 1, 0, 0, 0, 0	3, 3, 3, 1, 0, 0, 0, 0	42 MPN/g	42 MPN/g
25°C	3, 3, 3, 3, 3, 3, 3, 2	3, 3, 3, 3, 3, 3, 3, 2	1.1×10^6 MPN/g	1.1×10^6 MPN/g
35°C	3, 3, 3, 3, 3, 3, 3, 3	3, 3, 2, 3, 3, 3, 3, 3	$>1.1 \times 10^6$ MPN/g	$>1.1 \times 10^6$ MPN/g *

[1] MPN values determined using the FDA-BAM MPN tables.

***An MPN of 3,3,3 for the Reference MPN was used for the 10^{-4}, 10^{-5} and 10^{-6} replicates. This MPN calculation assumes that the one 10^{-1} g MPN tube from which no confirmed *V. parahaemolyticus* strain was recovered was a failure to pick a true typical isolate present in the background of non-*V. parahaemolyticus* which exhibited typical morphology for the target. Since all three**

replicates for the MPN tubes up to 5 orders of magnitude more dilute than the 10-1 tube were culture confirmed, it is unlikely that the culture result from this one discordant tube was correct.

Table 4. BAX System Results for Oysters with MPN Testing *V. vulnificus* (3 tube, 8 dilution)				
Sample Set	BAX positive (10g, 1g, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6})	Reference positive (10g, 1g, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6})	BAX MPN[1]	Reference MPN[1]
3°C	3, 3, 1, 0, 0, 0, 0, 0	3, 3, 1, 0, 0, 0, 0, 0	4.6 MPN/g	4.6 MPN/g
25°C	3, 3, 3, 3, 3, 1, 0, 0	3, 3, 3, 3, 3, 1, 0, 0	4,200 MPN/g	4,200 MPN/g
35°C	3, 3, 3, 3, 3, 2, 0, 1	3, 2, 3, 3, 3, 2, 0, 1	14,000 MPN/g	14,000 MPN/g *

[1] MPN values determined using the FDA-BAM MPN tables

*** An MPN of 2,0,1 for the Reference MPN was used for the 10^{-4}, 10^{-5} and 10^{-6} replicates. This MPN calculation assumes that the one 1 g MPN tube from which no confirmed *V. vulnificus* strain was recovered was a failure to pick a true typical isolate present in the background of non-*V. vulnificus* which exhibited typical morphology for the target. Since all three replicates for the MPN tubes up to 3 orders of magnitude more dilute than the 10-1 tube were culture confirmed, it is unlikely that the culture result from this one discordant tube was correct..**

Table 5. BAX vs. Reference Results Aggregate									
Sample type	Target Level by MPN or cfu per 25 gram	Samples or Number of MPN Tubes	BAX pos	Reference pos	Sensitivity %[1]	Specificity %[2]	False Pos %[3]	False Neg %[4]	Chi Square[5]
Tuna	0.5 MPN/25g	20	3	3	100	100	0	0	-
	1.9 MPN /25g	20	13	13	100	100	0	0	-
	3.75 MPN /25g	20	19	19	100	100	0	0	-
	0 cfu/25g	5	0	0		100	0	0	-
Tuna (Independent Laboratory Study)	MPN/25g	20	9	9	100	100	0	0	-
	0 cfu/25g	5	0	0		100	0	0	-
Frozen raw shrimp	Naturally contaminated	20	5	5	100	100	0	0	-
Cooked shrimp (MPN)	1.8 cfu/g	45	5	5	100	100	0	0	-
Cooked shrimp (25g)	1.8 cfu/g	5	5	5	100		0	0	-

Cooked shrimp (MPN)	18 cfu/g	45	14	14	100	100	0	0	-
Cooked shrimp (25g)	18 cfu/g	5	5	5	100		0	0	-
Frozen Scallops (MPN)	1.4 x 10^4 cfu/g	45	3	3	100	100	0	0	-
Frozen Scallops (25g)	1.4 x 10^4 cfu/g	5	5	5	100		0	0	-
Oysters 3°C	Naturally contaminated – *V. parahaemolyticus*	24	10	10	100	100	0	0	-
Oysters 25°C Abuse		24	23	23	100	100	0	0	-
Oysters 35°C Abuse		24	24	23	100	96	4	0	0
Oysters 3°C	Naturally contaminated – *V. vulnificus*	24	7	7	100	100	0	0	-
Oysters 25°C Abuse		24	16	16	100	100	0	0	-
Oysters 35°C Abuse		24	18	17	100	94	6	0	0

[1] Sensitivity – Total number of confirmed positive test portions by the method divided by total number of confirmed positive test portions by both the alternative and reference methods.
[2] Specificity – Total number of analyzed negative test portions by the method divided by total number of confirmed negative test portions by both the alternative and reference methods.
[3] False negative rate is calculated as BAX (-) Ref (+) BAX enrichment samples / Tot Ref (+) samples
[4] False positive rate is calculated as BAX (+) Ref (-) / Tot Ref (-) samples
[5] McNemar Chi-Square test statistic used for calculating significance of results

Results and Discussion of Food Studies

Data from these studies exhibits near complete equivalence between test and reference method results. In all studies except the oyster trials, complete equivalence was found. From two enrichments in the oyster studies, there was a discordant result, one for *V. parahaemolyticus* and one for *V. vulnificus*. In both of these cases the result occurred in an MPN tube that was well under the highest dilution that tested positive and was thus likely indicative of a failure to be able to isolate the target when it was truly present in the enrichment. Since selective and differential media for Vibrio do not give complete inhibition against many other genre there was likely a relatively high number of non-target similar appearing bacterial colonies on the plate, and none of the selected colonies were found to be the target species by phenotypic characterization from these two enrichment tubes.

Since the BAX ® test kit returns a result in about 24 hours versus the 3-5 days needed for culture based methods; the test kit can lead to a significantly faster increase in release of product.

Inclusivity / Exclusivity Study

Choice of Strains

V. cholera (n=46), *V. parahaemolyticus* (n=47), and *V. vulnificus* (n=33) strains were tested by the BAX ® assay for inclusivity. Most isolates were originally obtained from naturally contaminated food and environmental samples (many from the laboratory of Dr. Judy Johnson, collected when she was on faculty at the University of Maryland) and an effort is being made to more accurately determine source for non-ATTC isolates shown below. Additionally, 36 strains were obtained through an ongoing retail shrimp study at Qualicon. Identifications were confirmed biochemically using either the API 20E test kit as modified in the FDA-BAM or using the biochemical characterization scheme described in Table 1 of the FDA-BAM *Vibrio* chapter (9), some *V. cholera* isolates (see table 6) were also characterized by serology.

Culture Enrichment

For each inclusivity strain, one colony from an overnight T_1N_3 agar plate was inoculated into a tube containing alkaline peptone water (APW) and incubated at 37°C overnight, giving a cell density of approximately 10^8 cfu/ml. Isolates were diluted 1:1000 in APW to reach the target enrichment level of 10^5 cfu/mL before processing in the BAX® system.

Each non-*Vibrio* exclusivity strain was incubated at 37°C overnight in Brain Heart Infusion (BHI) broth. Isolates were diluted 1:10 in BHI before processing in the BAX® system. *Vibrio* strains in the exclusivity panel were grown at 35°C overnight in APW, then diluted 1:10 in APW before processing in the BAX® system.

Results

Table 6. Inclusivity Results for *Vibrio cholerae/parahaemolyticus/vulnificus*

Strain ID	Other strain designation	Source	Location of testing	Species (serotype)	Result *V. cholera*	Result *V. parahaemolyticus*	Result *V. vulnificus*
VcJVY212		Unknown	UF	*V. cholerae*	Pos	Neg	Neg
VcJVB52		Unknown	UF	*V. cholerae*	Pos	Neg	Neg
Vc5439/62		Unknown	UF	*V. cholerae*	Pos	Neg	Neg
Vc569B		Unknown	UF	*V. cholerae*	Pos	Neg	Neg
VcS171		Unknown	UF	*V. cholerae*	Pos	Neg	Neg
VcNAG12		Unknown	UF	*V. cholerae*	Pos	Neg	Neg
VcATCC25874		Unknown	UF	*V. cholerae*	Pos	Neg	Neg
Vc8		Unknown	UF	*V. cholerae*	Pos	Neg	Neg
VcB1307 Dacca		Unknown	UF	*V. cholerae*	Pos	Neg	Neg
VcA5		Unknown	UF	*V. cholerae*	Pos	Neg	Neg
VcI10		Unknown	UF	*V. cholerae*	Pos	Neg	Neg
Vc646 Ogawa01		Unknown	UF	*V. cholerae*	Pos	Neg	Neg
Vc395 Classical Ogawa01		Unknown	UF	*V. cholerae*	Pos	Neg	Neg
TD3192		Unknown	Qualicon	*V. cholerae*	Pos	Neg	Neg
TD7000	ATCC 9459	Unknown	Qualicon	*V. cholerae*	Pos	Neg	Neg
DD9892		Unknown	Qualicon	*V. cholerae*	Pos	Neg	Neg
DD13084	ATCC 14035	Unknown	Qualicon	*V. cholerae*	Pos	Neg	Neg
TD3161		Unknown	Qualicon	*V. cholerae (non-O1, O139)*	Pos	Neg	Neg
TD3162		Unknown	Qualicon	*V. cholerae (non-O1, O139)*	Pos	Neg	Neg
TD3163		Unknown	Qualicon	*V. cholerae (non-O1, O139)*	Pos	Neg	Neg
TD3164		Unknown	Qualicon	*V. cholerae (non-O1, O139)*	Pos	Neg	Neg
TD3165		Unknown	Qualicon	*V. cholerae (non-O1, O139)*	Pos	Neg	Neg
TD3167		Unknown	Qualicon	*V. cholerae (non-O1, O139)*	Pos	Neg	Neg
TD3170		Unknown	Qualicon	*V. cholerae (non-O1, O139)*	Pos	Neg	Neg
TD3171		Unknown	Qualicon	*V. cholerae (non-O1, O139)*	Pos	Neg	Neg
TD3173		Unknown	Qualicon	*V. cholerae (non-O1, O139)*	Pos	Neg	Neg
TD3180		Unknown	Qualicon	*V. cholerae O1*	Pos	Neg	Neg
TD3183		Unknown	Qualicon	*V. cholerae O1*	Pos	Neg	Neg
TD3185		Unknown	Qualicon	*V. cholerae O1*	Pos	Neg	Neg
TD3186		Unknown	Qualicon	*V. cholerae O1*	Pos	Neg	Neg

Table 6. Inclusivity Results for *Vibrio cholerae/parahaemolyticus/vulnificus*							
Strain ID	Other strain designation	Source	Location of testing	Species (serotype)	Result *V. cholera*	Result *V. parahaemolyticus*	Result *V. vulnificus*
TD3187		Unknown	Qualicon	*V. cholerae O1*	Pos	Neg	Neg
TD3858		Unknown	Qualicon	*V. cholerae O1*	Pos	Neg	Neg
TD3859		Unknown	Qualicon	*V. cholerae O1*	Pos	Neg	Neg
TD3860		Unknown	Qualicon	*V. cholerae O1*	Pos	Neg	Neg
TD3861		Unknown	Qualicon	*V. cholerae O1*	Pos	Neg	Neg
TD3862		Unknown	Qualicon	*V. cholerae O1*	Pos	Neg	Neg
TD3863		Unknown	Qualicon	*V. cholerae O1*	Pos	Neg	Neg
TD3864		Unknown	Qualicon	*V. cholerae O1*	Pos	Neg	Neg
TD3203		Unknown	Qualicon	*V. cholerae O139*	Pos	Neg	Neg
TD3211		Unknown	Qualicon	*V. cholerae O139*	Pos	Neg	Neg
TD3213		Unknown	Qualicon	*V. cholerae O139*	Pos	Neg	Neg
TD3214		Unknown	Qualicon	*V. cholerae O139*	Pos	Neg	Neg
VpTx2103		Unknown	UF	*V. parahaemolyticus*	Neg	Pos	Neg
VpTx3547		Unknown	UF	*V. parahaemolyticus*	Neg	Pos	Neg
VpDAL1094		Unknown	UF	*V. parahaemolyticus*	Neg	Pos	Neg
Vp17802		Unknown	UF	*V. parahaemolyticus*	Neg	Pos	Neg
Vp43996		Unknown	UF	*V. parahaemolyticus*	Neg	Pos	Neg
DD2633	ATCC 17802	Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
TD3129		Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
TD3130		Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
TD3131		Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
TD3132		Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
TD3133		Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
TD3134		Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
TD3135		Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
TD3153		Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
TD3154		Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
TD3155		Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
TD3156		Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
TD3157		Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
TD3159		Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
TD3160		Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg

Table 6. Inclusivity Results for *Vibrio cholerae/parahaemolyticus/vulnificus*							
Strain ID	Other strain designation	Source	Location of testing	Species (serotype)	Result *V. cholera*	Result *V. parahaemolyticus*	Result *V. vulnificus*
Vv FLA141		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
Vv FLA126		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
VvFLA134		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
Vv Fla 129		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
VvFLA127		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
VvFLA135		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
VvFLA115		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
VvFLA149		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
VvB3-313/98		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
VvFLA121		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
VvFLA137		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
VvB3-302/99		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
VvFLA119		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
VvFLA116		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
VvFLA102		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
VvB2-2		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
VvFLA108		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
TD3121		Unknown	Qualicon	*V. vulnificus*	Neg	Neg	Pos
TD3148		Unknown	Qualicon	*V. vulnificus*	Neg	Neg	Pos
TD3149		Unknown	Qualicon	*V. vulnificus*	Neg	Neg	Pos
TD3204		Unknown	Qualicon	*V. vulnificus*	Neg	Neg	Pos
TD3207		Unknown	Qualicon	*V. vulnificus*	Neg	Neg	Pos
TD3208		Unknown	Qualicon	*V. vulnificus*	Neg	Neg	Pos
TD3210		Unknown	Qualicon	*V. vulnificus*	Neg	Neg	Pos
TD3212		Unknown	Qualicon	*V. vulnificus*	Neg	Neg	Pos
TD3217		Unknown	Qualicon	*V. vulnificus*	Neg	Neg	Pos
TD3219		Unknown	Qualicon	*V. vulnificus*	Neg	Neg	Pos
TD4527	ATCC 27562	Unknown	Qualicon	*V. vulnificus*	Neg	Neg	Pos
DD13082	ATCC BAA-86	Unknown	Qualicon	*V. vulnificus*	Neg	Neg	Pos
DD13231		Shrimp	Qualicon	*V. cholera*	Pos	Neg	Neg
DD13232		Shrimp	Qualicon	*V. cholera*	Pos	Neg	Neg
DD13208		Shrimp	Qualicon	*V. cholera*	Pos	Neg	Neg

Table 6. Inclusivity Results for *Vibrio cholerae/parahaemolyticus/vulnificus*							
Strain ID	Other strain designation	Source	Location of testing	Species (serotype)	Result *V. cholera*	Result *V. parahaemolyticus*	Result *V. vulnificus*
DD13209		Shrimp	Qualicon	*V. cholera*	Pos	Neg	Neg
DD13212		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13216		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13217		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13218		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13211		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13222		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13223		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13224		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13225		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13226		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13228		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13229		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13230		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13233		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13234		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13235		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13236		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13204		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13207		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13200		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13202		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13201		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13203		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13211		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13214		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13215		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13210		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13205		Shrimp	Qualicon	*V. vulnificus*	Neg	Neg	Pos
DD13206		Shrimp	Qualicon	*V. vulnificus*	Neg	Neg	Pos
DD13227		Shrimp	Qualicon	*V. vulnificus*	Neg	Neg	Pos
DD13213		Shrimp	Qualicon	*V. vulnificus*	Neg	Neg	Pos

Table 7. Inclusivity Results for *Vibrio cholerae/parahaemolyticus/vulnificus*

Strain ID	Other strain designation	Source	Species	Result *V. cholera*	Result *V. parahaemolyticus*	Result *V. vulnificus*
DD2558		Unknown	*Citrobacter freundii*	Neg	Neg	Neg
DD383		Unknown	*Citrobacter freundii*	Neg	Neg	Neg
DD2560		Unknown	*Citrobacter kosei*	Neg	Neg	Neg
DD2561		Unknown	*Citrobacter kosei*	Neg	Neg	Neg
DD12835		Unknown	E. coli O157:H7	Neg	Neg	Neg
DD1450		Unknown	E. coli O157:H7	Neg	Neg	Neg
DD1979		Unknown	E. coli O157:H7	Neg	Neg	Neg
TD8136		Unknown	E. coli O157:H7	Neg	Neg	Neg
DD2554		Unknown	*Enterococcus faecalis*	Neg	Neg	Neg
DD6523		Unknown	*Klebsiella oxytoca*	Neg	Neg	Neg
DD2546		Unknown	*Klebsiella pneumoniae*	Neg	Neg	Neg
DD1144		Unknown	*Listeria monocytogenes*	Neg	Neg	Neg
DD1283		Unknown	*Listeria monocytogenes*	Neg	Neg	Neg
DD1309		Unknown	*Listeria monocytogenes*	Neg	Neg	Neg
DD3572	ATCC 9459	Unknown	*Listeria innocua*	Neg	Neg	Neg
DD3376		Unknown	*Listeria ivanovii*	Neg	Neg	Neg
DD2874	ATCC 14035	Unknown	*Listeria seeligeri*	Neg	Neg	Neg
DD3354		Unknown	*Listeria welshimeri*	Neg	Neg	Neg
DD3411		Unknown	*Listeria welshimeri*	Neg	Neg	Neg
DD2357		Unknown	*Proteus mirabilis*	Neg	Neg	Neg
DD374		Unknown	*Proteus mirabilis*	Neg	Neg	Neg
DD13148		Unknown	*Pseudomonas aeruginosa*	Neg	Neg	Neg
DD3982		Unknown	*Pseudomonas aeruginosa*	Neg	Neg	Neg
DD3019		Unknown	*Salmonella ser.* Dublin	Neg	Neg	Neg
DD706		Unknown	*Salmonella ser.* Enteritidis	Neg	Neg	Neg
DD1261		Unknown	*Salmonella ser.* Newport	Neg	Neg	Neg
DD13060		Unknown	*Salmonella ser.* Senftenburg	Neg	Neg	Neg
DD586		Unknown	*Salmonella ser.* Typhimurium	Neg	Neg	Neg
DD1083		Unknown	*Shigella flexneri*	Neg	Neg	Neg
DD699		Unknown	*Shigella soneii*	Neg	Neg	Neg

Table 7. Inclusivity Results for *Vibrio cholerae/parahaemolyticus/vulnificus*

Strain ID	Other strain designation	Source	Species	Result *V. cholera*	Result *V. parahaemolyticus*	Result *V. vulnificus*
DD10156		Unknown	*Staphylococcus aureus*	Neg	Neg	Neg
DD7426		Unknown	*Staphylococcus aureus*	Neg	Neg	Neg
DD9775		Unknown	*Staphylococcus aureus*	Neg	Neg	Neg
DD11233		Unknown	*Vibrio alginolyticus*	Neg	Neg	Neg
TD3146		Unknown	*Vibrio alginolyticus*	Neg	Neg	Neg
TD3195		Unknown	*Vibrio alginolyticus*	Neg	Neg	Neg
TD3200		Unknown	*Vibrio alginolyticus*	Neg	Neg	Neg
TD3658		Unknown	*Vibrio alginolyticus*	Neg	Neg	Neg
TD4501		Unknown	*Vibrio anguillarum*	Neg	Neg	Neg
TD4498		Unknown	*Vibrio carchariae*	Neg	Neg	Neg
TD3194		Unknown	*Vibrio damsela*	Neg	Neg	Neg
TD4524		Unknown	*Vibrio damsela*	Neg	Neg	Neg
DD2631		Unknown	*Vibrio fluvialis*	Neg	Neg	Neg
TD4526		Unknown	*Vibrio fluvialis*	Neg	Neg	Neg
TD4497		Unknown	*Vibrio harveyi*	Neg	Neg	Neg
DD11232		Unknown	*Vibrio mimicus*	Neg	Neg	Neg
DD13083		Unknown	*Vibrio mimicus*	Neg	Neg	Neg
TD3137	ATCC 17802	Unknown	*Vibrio mimicus*	Neg	Neg	Neg
TD3147		Unknown	*Vibrio mimicus*	Neg	Neg	Neg
TD3216		Unknown	*Vibrio mimicus*	Neg	Neg	Neg
TD4500		Unknown	*Vibrio natriegens*	Neg	Neg	Neg
TD4528		Unknown	*Vibrio pelagia*	Neg	Neg	Neg
TD4523		Unknown	*Vibrio tubiashii*	Neg	Neg	Neg
DD2399		Unknown	*Yersinia aldovae*	Neg	Neg	Neg
DD592		Unknown	*Yersinia enterocolitica*	Neg	Neg	Neg

Results – ALL TARGET *VIBRIO* ISOLATES GAVE EXPECTED POSITIVE RESULTS AND ALL NON-*VIBRIO* AND NON-TARGET *VIBRIO* SPECIES GAVE EXPECTED NEGATIVE RESULTS.

Stability Study

Methodology – BAX ® system test kits were evaluated in experiments to determine a reasonable shelf-life using both accelerated and non-accelerated storage conditions (see table below). *V. cholera* TD 3858, *V. cholera* TD 3192, *V. parahaemolyticus* TD 3129, *V. parahaemolyticus* TD 4496, and *V. vulnificus* DD 13082 were assayed using purified DNA at a level equivalent to one order of magnitude over the product's claimed sensitivity level (i.e. 10^5 cfu/mL) by the BAX ® assay. Additionally, two non-target *Vibrio* and non-*Vibrio* strains, *Pseudomonas aeruginosa* DD 962 and *Vibrio mimicus (non-target Vibrio species)* DD 13083 were tested using purified DNA at levels corresponding to 10^8 cfu/ml in an enriched sample. Three replicates of each strain at each temperature condition at each time point were assayed. Also, for each condition, for each time point, three non-spiked lysis buffer controls were tested.

Results – All results except for one *V. vulnificus* test at the 23 day 25°C treatment gave the expected result (tests spiked with positive target tested BAX ® positive while non-target and non-spiked tests tested BAX ® negative). It is not known why this one result was atypical, though it is possible this was due to a procedural error such as a pipette tip not properly affixed during the 5 µl lysate preparation step or an accidental loading of a non-target replicate into what was supposed to be a target reaction. The results of the accelerated stability study showed no difference in the performance of this test kit after being stored for 122 days at 25°C and 37ºC as compared to the 4ºC control. Based on these results and applying the Q_{10} rule of the Arrhenius equation, a 32 month shelf life has been assigned to these test kits.

Predicted Stability = Accelerated Stability X $2^{\Delta t/10}$
For example: Stability of a product at 50°C is 32 days.
Recommended storage temperature is 25°C and n = (50 – 25)/10 = 2.5
Qn = (2)2.5 = 5.66 The predicted shelf life is 32 days X 5.66 = 181 days

Accelerated stability studies are continuing and it is anticipated that the next lot of test kits will be assigned a 36 month shelf life. Real-time testing at 4ºC has shown stability for 122 days and is continuing.

Table 8. Summary of stability study

Time Point (days)	Storage Temp (°C)	BAX ® Positive *Vibrio cholera/parahaemolyticus/ vulnificus*	BAX ® Positive non-target organisms and non-spiked controls
23	4	15/15	0/9
	25	14/15	0/9
	37	15/15	0/9
60	4	15/15	0/9
	25	15/15	0/9
	37	15/15	0/9
122	4	15/15	0/9
	25	15/15	0/9
	37	15/15	0/9

Lot-to-lot study

Methodology –BAX® system test kits from three lots with different expiration dates were tested in an experiment to determine any evidence of lot-to-lot performance differences. *V. cholera* TD 3858, *V. cholera* TD 3192, *V. parahaemolyticus* TD 3129, and *V. vulnificus* DD 13082 were assayed using dilutions of overnight cultures at levels equivalent to approximately one order of magnitude over the product's claimed sensitivity level (i.e. ~10^5 cfu/mL) by the BAX ® assay. Additionally, two non-target *Vibrio* and non-*Vibrio* strains, *Pseudomonas aeruginosa* DD 962 and *Vibrio mimicus (non-target Vibrio species)* DD 13083 were tested using cells at levels of approximately 10^8 cfu/ml. Two replicates of each strain at each temperature condition at each time point were assayed. Also, for each condition, for each time point, two non-spiked lysis buffer controls were tested.

Table 9. Lot to Lot Test Kit Comparison

Lot #	Expiration Date	*Vibrio* spiked positives	Non-*Vibrio* spiked positives
030508	12/05/2010	8/8	0/6
061008	02/09/2011	8/8	0/6
8263	08/23/2011	8/8	0/6

Results – This lot to lot comparison study found no evidence of performance differences.

Ruggedness Study

Methodology –The BAX® system was evaluated to determine whether it performs as expected despite variations in operational parameters. Since the entire amplification and detection phases are fully automated, independent variables were selected from the enrichment and sample preparation phases. Eight variables believed to have the largest potential for impact on performance were selected, as shown in Table 10 with associated low and high levels:

Table 10. Variables in ruggedness study

Variable	Normal level	Low level	High level
1) Sample volume	5 µL	4	6
2) Incubation temperature (lysis)	37ºC	34	40
3) Incubation time (lysis)	20 minutes	15	30
4) Inactivation temperature (lysis)	95ºC	91	99
5) Inactivation time (lysis)	10 minutes	8	12
6) Total hydration volume	30 µL	27	33
7) Enrichment temperature	35ºC	32	38

For assay factors (1-6) each factor was varied, both high and low level as well as a normal level, for three replicates of 6 strains (4 different *Vibrio target strains* and 2 different non-*target strains*). Additionally, two non-inoculated samples were assayed for each variable/level studied.

For inoculated samples, *V. cholera* TD 3858, *V. cholera* TD 3192, *V. parahaemolyticus* TD 3129, and *V. vulnificus* TD 3121 were serially diluted to just above the product's claimed sensitivity level (i.e. 10^5 cfu/ml of enriched culture) and prepared for the BAX® assay. Additionally, two non-target *Vibrio* and non-*Vibrio* strains, *Vibrio mimicus (non-target Vibrio species)* TD 3147 and *Salmonella* Newport DD 1261 were grown and diluted to attain inoculation levels of ~10^7-10^8 cfu/ml as described in the lot to lot study. Uninoculated samples were freshly prepared with APW and treated in an analogous manner to the inoculated samples.

For the enrichment factor (7) low levels of ~10 cfu of *V. cholera* TD 3858, *V. cholera* TD 3192, *V. parahaemolyticus* TD 3129, and *V. vulnificus* TD 3121 and high levels (~10^5 cfu) of non-target strains *Salmonella* Newport DD 1261 and *Vibrio mimicus (non-target Vibrio species)* TD 3147 were added to 225 ml aliquots of APW with replicates for each variable for each strain and assayed for high (n=2), low (n=2), and normal (n=2) conditions.

Table 11. Results of ruggedness study

Variable	Normal level	Positive *Vibrio*	Positive Non- *Vibrio*	Positive Uninoc.	Low level	Positive *Vibrio*	Positive Non- *Vibrio*	Positive Uninoc.	High level	Positive *Vibrio*	Positive Non- *Vibrio*	Positive Uninoc.
1) Sample volume	5 µL	12/12	0/6	0/2	4	12/12	0/6	0/2	6	12/12	0/6	0/2
2) Incubation temperature	37ºC	12/12	0/6	0/2	34	12/12	0/6	0/2	40	12/12	0/6	0/2
3) Incubation time	20 min	12/12	0/6	0/2	15	12/12	0/6	0/2	30	12/12	0/6	0/2
4) Inactivation temperature	95ºC	12/12	0/6	0/2	91	12/12	0/6	0/2	99	12/12	0/6	0/2
5) Inactivation time	10 min	12/12	0/6	0/2	8	12/12	0/6	0/2	12	12/12	0/6	0/2
6) Total hydration volume	30 µL	12/12	0/6	0/2	27	12/12	0/6	0/2	33	12/12	0/6	0/2
7) Enrichment temperature	35ºC	8/8	0/4	0/2	32	8/8	0/4	0/2	38	8/8	0/4	0/2

Results – The results of the ruggedness study are shown in Table 11. All *Vibrio*-inoculated samples returned positive results. All non-*Vibrio* inoculated and un-inoculated samples were negative. These results indicate that the variables studied did not affect the performance of the BAX® system assay within the ranges tested.

Discussion

In initial development studies, some enriched samples were found to test positive by the BAX® pcr assay but negative by the reference culture method. Often, this is the case when non-target competitive flora, either non-*Vibrio*, or non-target *Vibrio* species are present in an enrichment with cell densities at a much higher level than the target organism. In such cases, an additional plating media, CHROMagar *Vibrio*, has been found to be useful. For each sample tested for most studies (with the exception of the oyster studies performed at Dauphin Island), a CHROMagar *Vibrio* plate was also struck from each enriched sample to reflect this fact. In one study (the naturally contaminated frozen raw shrimp work) two samples were found to be pcr positive/culture negative. For these samples that tested pcr positive, but from which no confirmed colonies of a positive species were found from the FDA-BAM media, more colonies than required by the FDA BAM procedure were picked from the TCBS, mCPC and CHROMagar *Vibrio* plates into cluster tubes containing 500 µl APW (up to 24 per sample per media where available). Individual isolates were allowed to grow in the cluster tubes overnight at room temperature and tested by BAX® assay. Presumptive positive cluster tubes were struck onto TCBS or T_1N_3 agar and confirmed using the FDA-BAM methods. Both of these samples were then found to be positive using this enhanced protocol, yielding at least one confirmed *V. cholera* isolate. Qualicon has also demonstrated the presence of atypical *V. parahaemolyticus* strains (confirmed by DNA sequence-based characterization) that do not present with typical characteristics on *Vibrio* selective and differential agars. All enrichments which tested positive by PCR, with the exception of two MPN tubes from the oyster study, were also positive for typical confirmed colonies on one or more of the three agars above. In the oyster studies, only three typical colonies per MPN tube were selected as per the FDA-BAM protocols, and a greater number of colonies selected per tube would have made the experiment unmanageable. This highlights a potential issue with the reference method in that typical colony morphology on plates is a critical step in the reference method and the complex microbial ecology of an oyster can potentially lead to less than optimal results when non-target isolates with a typical phenotype on *Vibrio* selective agars are present in significant numbers relative to the levels of target *Vibrio*. In other non-AOAC studies conducted at Qualicon some instances of PCR positive enrichments have yielded phenotypically atypical isolates that test positive by PCR. These isolates have been characterized by sequence-based identification (microSeq ®, Applied Biosystems, Foster City, CA) as target *Vibrio* species and are being shared with the community of *Vibrio* experts for further characterization (data not shown). The above described work supports continued work on the natural phenotypic and genetic variation of pathogenic species of *Vibrio* occurring in foods.

Conclusion

The data in these studies, within their statistical uncertainty, support the product claims of the BAX® System PCR Assay for Detecting *Vibrio cholera, parahaemolyticus, and vulnificus* with the tested foods, including raw frozen shrimp, cooked shrimp, raw oysters, raw ahi tuna, and raw scallops.

References

1. Feldstine, P., Abeyta, C., & Andrews, W.H. (2002) AOAC International Committee Guidelines for Validation of Qualitative and Quantitative Food Microbiological Official Methods of Analysis. AOAC INTERNATIONAL
2. Innis, M.A., & Gelfand, D.H. (1989) *PCR Protocols: A Guide to Methods and Applications*, Academic Press, Burlington, MA
3. Livak, K.J., Flood, S.J.A., Marmaro, J., & Mullah, K.B. (inventors), Perkin-Elmer Corporation, Foster City, CA (assignee) (1999) Hybridization assay using self-quenching fluorescence probe. U.S. patent 5,876,930
4. Thompson, F., Iida T., & Swings, J., (2004) Biodiversity of *Vibrios*. Microbiol. Mol. Biol. Rev. 68:403-431.
5. Heilpern, K., & Borg K. (2006) Update on emerging infections: news from the Centers for Disease Control and Prevention. *Vibrio* illness after Hurricane Katrina—multiple states, August–September 2005. Ann Emerg Med. 47(3):255–258
6. Daniels, N., & Shafaie, A., (2002) A Review of Pathogenic Vibrio Infections for Clinicians Infect Med 17(10):665-685.

7. U.S. Food and Drug Administration (2005) Quantitative Risk Assessment on the Public Health Impact of Pathogenic *Vibrio parahaemolyticus* in Raw Oysters. Available at: http://www.cfsan.fda.gov/~acrobat/vpra.pdf Date of access 2/15/09.
8. Mead, P., Slutsker L., Dietz V., McCaig L., Bresee J., Shapiro C., Griffin P., & Tauxe R.. (1999) Food-related illness and death in the United States. Emerg Infect Dis 5(5), pp. 607-625.
9. U.S. Food and Drug Administration, FDA Bacteriological Analytical Manual, http://www.cfsan.fda.gov/~ebam/bam-9.html, date of access 2/15/09.

Proposal Subject:	Alternative Analytical Method for *Vibrio vulnificus*
Specific NSSP Guide Reference:	Section IV. Guidance Documents, Chapter II. Growing Areas .10 Approved National Shellfish Sanitation Program Laboratory Tests: Microbiological and Biotoxin Analytical Methods (5) Interim Approval by ISSC Executive Board August 2007
Text of Proposal/ Requested Action	Accept the adoption of Quantitative Real-Time PCR as an alternative analytical protocol to determine the levels of *Vibrio vulnificus*. Rename "Sec IV Chapter II.10 (5) Interim Approval by ISSC Executive Board August 2007" to reflect the methods committee's action on these methods and to include QPCR as an alternative method for analysis of Vv in PHP products
Public Health Significance:	Improve the speed of analysis to help the industry to increase the amount of PHP products in the market.
Cost Information (if available):	See attached application report
Action by 2009 Laboratory Methods Review Committee	Recommended acceptance of Proposal 09-103 SYBR Green 1 QPCR-MPN in conjunction with the PHP of oysters as a Type IV method provided the information on the step-by-step procedures and the manufacturer's ruggedness data are submitted within 30 days.
Action by 2009 Task Force I	Recommended adoption of Proposal 09-103 as submitted.
Action by 2009 General Assembly	Adopted recommendation of 2009 Task Force I on Proposal 09-103.

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

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Methods Review (LMR) Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

Name of the New Method	Sybr Green I QPCR-MPN for Rapid Detection of *Vibrio vulnificus*
Name of the Method Developer	Anita Wright et. Al.
Developer Contact Information	Anita Wright 461 AFPL bldg. Newell Dr. Gainesville, FL 32611 352-392-1991 ext. 311

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.	Y	Give the shellfish industry, regulatory and analytical labs an alternative method to confirm upern bacteria in shellfish
2. What is the intended purpose of the method?	Y	Replace confirmation step in MPN determination of Vibrios in shellfish
3. Is there an acknowledged need for this method in the NSSP?	Y	End users are requiring faster more economical alternatives to the current approved method
4. What type of method? i.e. chemical, molecular, culture, etc.	Y	Quantitative PCR
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title	Y	
Method Scope	Y	
References	Y	
Principle	Y	
Any Proprietary Aspects	Y	
Equipment Required	Y	
Reagents Required	Y	
Sample Collection, Preservation and Storage Requirements	Y	
Safety Requirements	Y	
Clear and Easy to Follow Step-by-Step Procedure	Y	
Quality Control Steps Specific for this Method	Y	
C. Validation Criteria		
1. Accuracy / Trueness	Y	
2. Measurement Uncertainty	Y	
3. Precision Characteristics (repeatability and reproducibility)	Y	
4. Recovery	n/a	
5. Specificity	Y	
6. Working and Linear Ranges	Y	
7. Limit of Detection	Y	
8. Limit of Quantitation / Sensitivity	Y	
9. Ruggedness	Y	
10. Matrix Effects	Y	

11. Comparability (if intended as a substitute for an established method accepted by the NSSP)	Y	
D. Other Information		
1. Cost of the Method	Y	
2. Special Technical Skills Required to Perform the Method	Y	
3. Special Equipment Required and Associated Cost	Y	
4. Abbreviations and Acronyms Defined	N/A	
5. Details of Turn Around Times (time involved to complete the method)	Y	
6. Provide Brief Overview of the Quality Systems Used in the Lab	Y	

Submitters Signature	Date:
Submission of Validation Data and Draft Method to Committee	Date:
Reviewing Members	Date:
Accepted	Date:
Recommendations for Further Work	Date:

Comments:

See attached application document.

DEFINITIONS

1. **Accuracy/Trueness** - Closeness of agreement between a test result and the accepted reference value.
2. **Analyte/measurand** - The specific organism or chemical substance sought or determined in a sample.
3. **Blank** – Sample material containing no detectable level of the analyte or measurand of interest that is subjected to the analytical process and monitors contamination during analysis.
4. **Comparability** – The acceptability of a new or modified method as a substitute for an established method in the NSSP. Comparability must be demonstrated for each substrate or tissue type by season and geographic area if applicable.
5. **Fit for purpose** – The analytical method is appropriate to the purpose for which the results are likely to be used.
6. **HORRAT value** – HORRAT values give a measure of the acceptability of the precision characteristics of a method.[4]
7. **Limit of Detection** – the minimum concentration at which the analyte or measurand can be identified. Limit of detection is matrix and analyte/measurand dependent.[4]
8. **Limit of Quantitation/Sensitivity** – the minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.
9. **Linear Range** – the range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.
10. **Measurement Uncertainty** – A single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.
11. **Matrix** – The component or substrate of a test sample.
12. **Method Validation** – The process of verifying that a method is fit for purpose.[1]
13. **Precision** – the closeness of agreement between independent test results obtained under stipulated conditions.[1, 2] There are two components of precision:
    a. **Repeatability** – the measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.
    b. **Reproducibility** – the measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.
14. **Quality System –** The laboratory's quality system is the process by which the laboratory conducts its activities so as to provide data of known and documented quality with which to demonstrate regulatory compliance and for other decision–making purposes. This system includes a process by which appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.
15. **Recovery –** The fraction or percentage of an analyte or measure and recovered following sample analysis.
16. **Ruggedness** – the ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]
17. **Specificity** – the ability of a method to measure only what it is intended to measure.[1]
18. **Working Range –** the range of analyte or measure and concentration over which the method is applied.

REFERENCES:

7. Eurachem Guide, 1998. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. LGC Ltd. Teddington, Middlesex, United Kingdom.
8. IUPAC Technical Report, 2002. Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, Pure Appl. Chem., Vol. 74, (5): 835-855.
9. Joint FAO/IAEA Expert Consultation, 1999. Guidelines for Single-Laboratory Validation of Anilytical Methods for Trace-Level Concentrations of Organic Chemicals.

10. MAF Food Assurance Authority, 2002. A Guide for the Validation and Approval of New Marine Biotoxin Test Methods. Wellington, New Zealand.
11. National Environmental Laboratory Accreditation. , 2003. Standards. June 5.
12. EPA. 2004. EPA Microbiological Alternate Procedure Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Methods: Guidance. U.S. Environmental Protection Agency (EPA), Office of Water Engineering and Analysis Division, 1200 Pennsylvania Avenue, NW, (4303T), Washington, DC 20460. April.

QPCR-MPN Assay for Rapid Detection of *Vibrio vulnificus* in Oysters

Justification for New Method

This proposal was prepared to support the use of a new molecular detection method: Sybr Green I QPCR-MPN for Rapid Detection of *Vibrio vulnificus* to be substituted for the use of DNA probe colony hybridization for confirmation of the presence of *V. vulnificus* growth in the MPN enrichment protocol described in the FDA Bacteriological Analytical Mannual (8). This protocol is submitted for approval to the Laboratory Methods Review Committee. Method was developed by collaborative efforts of Dr. Anita Wright, Dr. Steve Otwell, Victor Garrido, Charlene Burke, and Melissa Evans, University of Florida, Gainesville, Florida.

Developer Contact Information:
Anita Wright, Ph.D. (Method Developer)
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352-392-1991 x 311

Date of Submission
Proposal submission date is June 30, 2009.

Purpose and Intended Use of the Method
V. vulnificus the leading cause of death in the US related to seafood consumption and is predominantly associated with consumption uncooked Gulf Coast oysters (7). The proposed method will benefit the seafood industry and the consumer by providing improved, faster, and more upernat deteiction of this pathogen in oysters. This method is being proposed for use in validation of Post Harvest Processing protocols, as well as for future applications to assure the public of a safer product.

Need for the New Method in the NSSP
V. vulnificus QPCR-MPN is proposed as an alternative to the standard MPN assay using most probable number (MPN) end-point titration of replicate samples in enrichment broth cultures (4). In the current protocol, species-specific growth in enrichment is determined by isolating typical *V. vulnificus* colonies on selective medium with subsequent confirmation by DNA probe (15). This method is laborious cost prohibitive, labor intensive, and time consuming (6, 8). Furthermore, users of this protocol have upernata difficulty with DNA probe product reliability and plating problems related to "spreading" colonies that upernata with the assay. Total amount of time to perform the traditional MPN method with DNA colony blot hybridization as a confirmatory method is at least 4 days, with numerous steps; additionally, technician requires a great deal of experience in performing this assay for successful quantification to be possible. QPCR-MPN method reduces working time half and offers greater sensitivity for detection of *V. vulnificus*; with detection of 1 bacterium per gram post enrichment in alkaline peptone water (APW) overnight (1, 4, 9, 10, 11, 16).

Although these post harvest processing methods are currently employed on < 10% of all domestic raw oyster sales in the United States, the industry continues to examine and employ new technologies and take initiative on expanding acceptance and knowledge regarding these treated oyster products (5). The industry is investing money and resources to ensure a market acceptance by educated oyster public, in addition to mitigating risk potential for the at risk consumers of fresh oysters. ISSC mandated that 25% of oysters upernata from the Gulf of Mexico receive some type of validated post uperna processing. Thus, there is a continued need for improve validation methods.

The University of Florida has partnered with several dealers who are using ISSC recommended for validation of post-harvest processing methods. Work was performed in 2004, working with Leavin's seafood (nitrogen

freezing) and Tommy Ward Seafood (blast freezing). Throughout the validation, samples were randomly selected for side-by-side comparisons of standard MPN (FDA BAM) to QPCR-MPN. Test results support the application of QPCR-MPN for improved assessment of validation and verification protocols related to oyster PHP, which was described in a publication by Wright et al., 2007 (Appendix 3).

QPCR-MPN method increased assay sensitivity and reduced both time and labor costs. Detection of *V. vulnificus* was achieved at levels < 30 CFU/g as required for validation protocols (2, 10, 15). For these reasons we propose acceptance of the application of QPCR-MPN for improved assessment of validation and verification protocols related to oyster post harvest processing. The oyster industry's livelihood will be determined by their ability to adapt to FDA demands, and evolving technological breakthroughs. The scientific community works to discover the most expedient, accurate detection methods and the most effective treatments for the eradication of naturally occurring Vibrio as the public continues to demand a fresh, uncooked product. Until this demand has abated, the industry and the scientific community will continue to work in conjunction to learn more and thus protect the public from Vibrio disease.

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

This method is specific to applications testing growth of *V. vulnificus* in MPN enrichment of oyster homogenates at concentrations of 1.0 g or less. This QPCR method does not claim to differentiate between pathogenic and nonpathogenic *V. vulnificus*. QPCR-MPN provides more sensitive detection than standard MPN, as enriched samples that were PCR positive but negative on selective media were falsely negative on mCPC, as indicated by agreement of positive mCPC and QPCR results in more diluted inocula of the same sample (15). The result is an increase in sensitivity and a reduction in time and labor costs while still permitting detection of *V. vulnificus* at levels < 30 CFU/g as required for validation protocols (2, 10, 15). For these reasons we propose acceptance of the application of QPCR-MPN for improved assessment of validation and verification protocols related to oyster post harvest processing.

Method Documentation

Method Title

Sybr Green I QPCR-MPN for Rapid Detection of *Vibrio vulnificus*

Method Scope

This method is designed for MPN analysis of validation trials for oyster PHP.

Principle

QPCR-MPN will be substituted as an alternative to the officially recognized NSSP method for MPN analysis of validation trials for oyster PHP (3). Specifically QPCR will be substituted for microbiological/DNA probe confirmation of *V. vulnificus* growth in MPN enrichment. Since the FDA and the ISSC have mandated postharvest processing (PHP) of oysters harvested from Gulf Coast states in order to reduce *V. vulnificus* infections validation and verification are necessary in order to ensure that the process will substantially reduce numbers of *V. vulnificus* bacteria to levels below the predicted threshold for disease. QPCR-MPN is a rapid and reliable method to accomplish agency mandates and industry goals.

Proprietary Aspects

Ingredients in Smartmix beads (Cepheid©) containing PCR reagents for use with Cepheid© Smartcycler are proprietary information.

Equipment

Cepheid© Smartcycler

Reagents

- SmartMix beads (Cepheid)
- SYBR green I (Invitrogen)
- VvhA forward and reverse primers (Geno-mechanix, Gainesville, FL)
- Autoclaved molecular grade water

Media (Media are specified in FDA BAM, reference 8)

- Modified colistin polymyxin cellobiose (mCPC) agar
- T1N1 agar
- Alkaline peptone water (APW) enrichment broth
- Phosphate buffered saline (PBS)

Matrix or Matrices of Interest

The validation of post harvest processing for raw gulf coast oysters is performed on oyster homogenate. Thus the matrix is dilutions of oyster homogenate, consisting of oyster meats and PBS.

Sample Collection, Preservation, Preparation, Storage, Cleanup, Test Procedures

- A boiling lysis was used for DNA preparation. APW cultures (1 ml) were centrifuged (15,000 x g,10 min), resuspended in 1 ml phosphate-buffered saline (PBS, boiled for 10 min, and subsequently centrifuged to removeparticulates. Supernatants were stored at -20°C.
- VvhA Primers (Geno-mechanix, Gainesville, FL) were stored at -20°C.
- DNA templates (2 µl) and water were added to QPCR reactions for a total volume of 25 µl.

Cost of the Method

The cost of the method varies depending on the Q-PCR platform chosen; however, the Cepheid© smartcycler platform costs approximately $5 per PCR reaction.

Special Technical Skills Required to Perform the Method

Only basic laboratory skills are required.

Special Equipment Required and Associated Cost

Equipment	Approximate Cost
Cepheid© thermocycler	$30,000 + accessories
Incubator	$3,000 - $6,000
Centrifuge	$2,000
Heat block	$500

Abbreviations and Acronyms

- PHP –post harvest processing
- DNA- deoxyribonucleic acid
- QPCR- quantitative polymerase chain reaction
- APW- alkaline peptone water
- PBS- phosphate buffered saline
- MPN- most probable number
- VVAP- Vibrio vulnificus alkaline-phosphatase probe
- mCPC- modified colistin polymyxin cellobiose

Test Procedures and Quality Control

MEDIA:

Dehydrated media is commercially dehydrated. Media must be sterilized according to manufacturer's instructions. Prepared culture media, dehydrated media and media components must be stored in a cool, clean, dry space unless refrigeration is required as per manufacturer instruction. Stored media is labeled with batch number, expiration date and sterilization date. Storage of prepared culture media at room temperature does not exceed 7 days. Refrigerated storage of prepared media with loose fitting closures does not exceed 1 month; screw-cap closures do not exceed 3 months. All prepared media stored under refrigeration are held at room temperature overnight prior to use.

To determine the pH of prepared media, a pH meter with a standard accuracy of 0.1 units is used. The pH meter is calibrated with each use and a minimum of two standard buffer solutions (ph 4, 7 and 10) are used to calibrate the pH meter. Standard buffer solutions are used once and discarded.

COLD STORAGE:

Refrigerator temperature must be monitored daily; temperature is maintained between 0˚C to 4˚C. Freezer temperature must be monitored at least once daily, freezer temperatures is maintained at -20˚C (DNA storage) and –80˚C (strain storage).

INCUBATOR:

Temperature of incubators must be maintained at 30˚C (+/-0.5), 37˚C (+/-0.5), and 40˚C (+/-0.5). Thermometers must be graduated no greater than 0.5˚C increments. Temperatures are taken twice daily.

SUPPLIES:

Utensils and containers made of clean borosilicate glass, stainless steel or other non-corroding material. Culture tubes made of a suitable size to accommodate the volume for broth and samples. Sample containers made of glass or other inert material.

Dilution bottles and tubes are made of plastic and closed with attached snap-lock lids. Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method is used to ensure appropriate volumes. Reusable sample containers must be capable of being properly washed and sterilized.

Hardwood applicator transfer sticks, utilized for streaking and picking positive colonies, and Whatman # 3 and #541 filter papers, utilized in colony blot hybridization, are sterilized prior to use and stored in sterile, airtight containers.

Pipettes used to inoculate the sample deliver accurate aliquots, have unbroken tips and are appropriately graduated. Pipettes larger than 10ml are not used to deliver 1ml; nor, are pipettes larger than 1ml used to deliver 0.1ml.

MAINTENANCE:

Routine autoclave maintenance must be performed and serviced annually or as needed by a qualified technician and records maintained. Autoclave provides a sterilizing temperature of 121˚C (tolerance 121 +/- 2˚C) as determined daily. Spore suspensions or strips must be used monthly to evaluate the effectiveness of the autoclave sterilization process, with results recorded. Heat sensitive tape must be used with each autoclave batch. Autoclave sterilization records including length of sterilization, total heat exposure time and chamber temperature must be maintained in an autoclave log.

SHELLSTOCK SAMPLES:

A representative sample of shellstock is collected. Shellstock is collected in clean, waterproof, puncture resistant containers. Shellstock labeled with collector's name, type of shellstock, the source, the harvest area, time, date and place of collection. Shellstock are maintained in dry storage between 0 and 10˚C until examined. Examination of the sample is initiated as soon as possible after collection, and does not exceed 24 hours after collection.

Shucking knives, scrub brushes and blender jars are sterilized for 35 minutes prior to use. Blades of shucking knives free from debris corrosion. Prior to scrubbing and rinsing debris off shellstock, the hands of the technician are thoroughly washed with soap and water. Shellstock are scrubbed with a stiff, sterile brush and rinsed under water of drinking water quality. Shellstock are allowed to drain in a clean container or on clean towels prior to opening. Prior to opening, the technician washes hands and rinses with 70% alcohol. Shellstock are not shucked directly through the hinge.

FDA-MPN PREPARATION AND METHOD:
Contents of shellstock are shucked into a sterile, tared blender jar. At least 12 animals (100 g of meat) are used for analysis. The sample is weighted to the nearest 0.1 gram and an equal amount by weight of sterile PBS diluent is added. Samples are blended at high speed for 90 seconds.

Immediately after blending, the homogenized sample is diluted in a multiple dilution series with 3 replicas and inoculated into tubes of APW presumptive media for MPN analysis. Positive and negative controls cultures accompany samples throughout the procedure. Inoculated media are incubated at 37 +/- 0.5˚C. Presumptive tubes are read at 24+/- 2 hours of incubation and transferred if positive. Transfers are made to mCPC plates by sterile hardwood applicator sticks from presumptive positive APW tubes and confirmed by DNA probe.

QPCR-MPN PREPARATION:
Prior to DNA extraction and preparing Cepheid© unit for QPCR, all micro-centrifuge tubes and pipette tips are sterilized for 35 minutes. The technician's hands are washed with soap and water. Gloves are worn and rinsed with 70% alcohol. All Pipetteman and Eppendorf pipettes are calibrated semi-annually and prior to use are wiped down with 70% alcohol. All working areas, centrifuge racks, and equipment are wiped down with 70% alcohol. Proper sterile technique is observed throughout the procedure to ensure contamination free samples.

1ml of sample from each positive MPN tube is used for the boil extraction procedure (appendix 1) to extract DNA to be used as template for Sybr green 1 QPCR-MPN assay as described in appendix 2. Cepheid© thermocycler cycle threshold is set at 30 and factory default is utilized for melt curve analysis regarding peak height.

Validation Criteria

Ruggedness of Assay

Validity of MPN assay for detection of *V. vulnificus* has been previously established by ISSC and FDA. The ruggedness of reagents used for PCR is determined by manufacturer and meet specifications. Method uses a bead format that incorporates all reagents on bead to eliminate common pipetting and cross-contamination errors.

Data Comparability and Statistical Analysis

During 2004 summer PHP validation trials were conducted by The University of Florida Aquatic Food Products group in a partnership with the oyster industry in Apalachicola FL. Side by side field trials compared the FDA-MPN (8), which consists of selection of typical colonies on mCPC and confirmation by VVAP DNA probe, to the QPCR-MPN assay described herein. Results of a side by side sample comparison support application of QPCR technology for validation oyster processing protocols.

Quantitative PCR was applied to most probable number (QPCR-MPN) for validation of PHP methods for reduction of *V. vulnificus in* oysters for Day 1 and Day 7. Published results by Wright et al., 2007 showed that immediately following inoculation of APW (pre-enrichment with either 0.1 or 0.01 g oyster homogenate detection *V. vulnificus* was 100 to 1000 fold more sensitive by QPCR than by growth on selective agar (Table 1). Following O.N. growth in enrichment, both assays were equally as sensitive.

For PHP oysters received nitrogen immersion, side by side comparison of standard MPN vs. QPCR-MPN showed excellent correlation (R^2=0.97 by Pearson's correlation co-efficient) and no significant differences between the two assays (Table 2). Results were comparable for untreated oysters and for PHP oysters at both 1 and 7 days post treatment.

Samples results were also examined side by side for both Nitrogen Immersion and Nitrogen Tunnel PHP treatments (Figure 1). In statistical comparison of this data, utilizing both JMP from SAS and Minitab, both one way ANOVA and Tukeys post hoc tests show no significant differences ($p < .05$) between detection methods; utilizing mCPC (presumptive positive) and VVAP (confirmed positive) known as the FDA-MPN, and QPCR-MPN. Field trials indicated that QPCR offered an improved confirmatory assay compared to the standard method, given that it reduced time and labor costs while still permitting detection of *V. vulnificus* without the risk of false positives.

Inocula (log CFU/ml)	Pre-enrichment Positive APW (%)		Post-enrichment Positive APW (%)		Post- enrichment QPCR (C_t)	
	mCPC	QPCR Melt	mCPC	QPCR Melt	SYBR	TaqMan
0.01 g Oyster Homogenate:						
5.40	100	100	100	100	17.94±0.56	16.74□0.44
4.40	100	100	100	100	18.40±0.40	16.97□0.34
3.40	100	100	100	100	17.90±0.58	17.64□0.20
2.40	33	100	100	100	17.90±0.56	16.83□0.09
1.40	0	100	100	100	17.70±0.60	18.56□0.32
0.40	0	33	100	100	18.70±0.21	17.86□0.30
0.04	0	0	100	100	20.01±2.09	19.87□2.44
Uninoculated	0	0	0	0	35.38±0.25	0.00
0.10 g Oyster Homogenate:						
5.40	100	100	100	100	15.84±0.27	16.99□0.77
4.40	100	100	100	100	16.40±0.07	16.76□0.10
3.40	33	100	100	100	16.29±0.02	17.57□0.21
2.40	0	33	100	100	17.01±1.61	17.44□1.41
1.40	0	0	100	100	17.93±2.89	18.35□1.63
0.40	0	0	100	100	15.55±0.70	16.92□0.23
0.04	0	0	100	100	20.72±1.27	20.27□1.43
Uninoculated	0	0	0	0	33.18±2.43	0.00

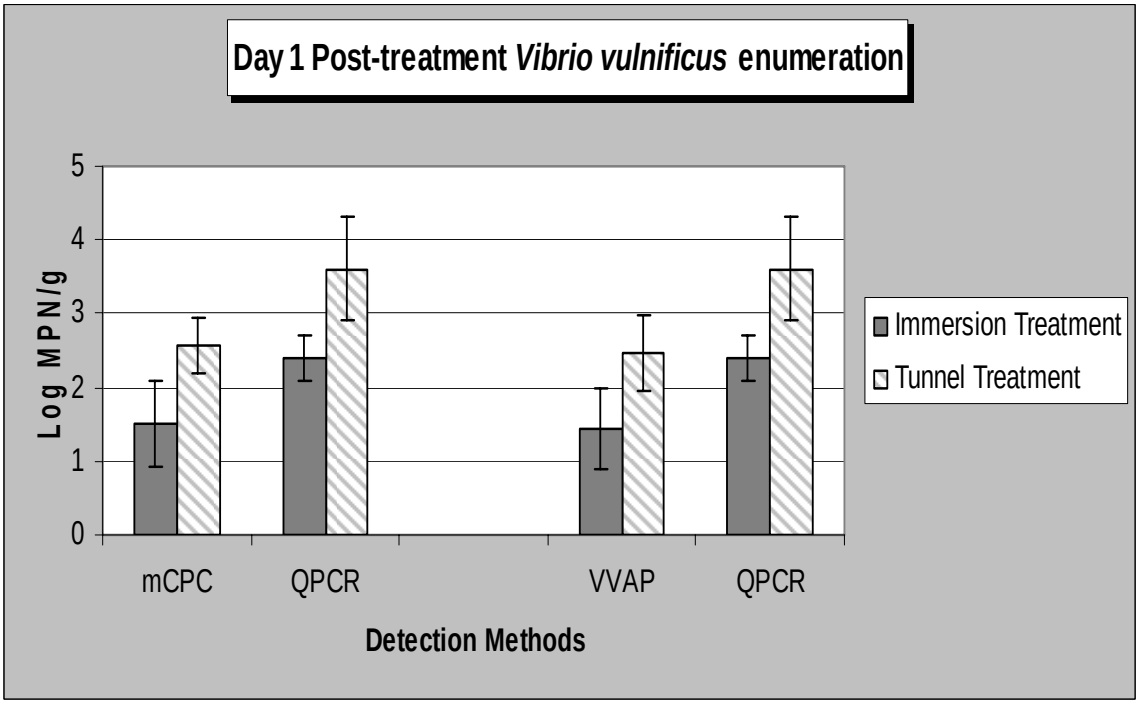
Table 1. Detection of *V. vulnificus* in artificially inoculated APW enrichment

a) *V. vulnificus* inocula (logCFU/ml determined by plate count) for seeding of MPN enrichment of PHP oyster homogenates (0.01 or 0.10 g).
b) Detection of *V. vulnificus* in APW determined as percent (%) positive samples from three tubes based on either observation of *V. vulnificus* typical colonies on selective agar (mCPC) or on melt peak analysis (QPCR) for pre- and post-enrichment. Values are the mean of duplicate experiments with identical results.

Table 2. Comparison of standard MPN to **QPCR-MPN Analysis of PHP Oyster Samples**

Oysters	Treatment[a]	Average LogMPN/g[b]	
		FDA MPN	**QPCR-MPN**
Lot 1	Pre-PHP	2.7 ± 1.5	3.2 ± 0.3
Lot 2	Pre-PHP	4.4 ± 0.4	4.8 ± 0.2
Lot 3	Pre-PHP	4.1 ± 1.0	4.3 ± 0.5
Lot 1	PHP (1 D)	0.9 ± 0.5	1.7 ± 1.1
Lot 2	PHP (1 D)	1.9 ± 0.6	2.3 ± 0.3
Lot 3	PHP (1 D)	3.7 ± 0.3	3.8 ± 0.2
Lot 1	PHP (21 D)	1.5 ± 0.4	2.0 ± 0.1
Lot 2	PHP (21 D)	0.6 ± 0.3	0.6 ± 0.3
Lot 3	PHP (21 D)	0.5 ± 0.0	0.5 ± 0.0
Lot 4	PHP (21 D)	1.1 ± 0.2	0.9 ± 0.3

a) Individual oyster lots (n=4) were heat abused by incubation at 26°C for 24h (Pre-PHP), followed by processing with ultralow freezing in liquid nitrogen and frozen storage at -10°C for 1 (PHP 1D) and 21 days (PHP 21D) following PHP.
b) For each lot, oysters (n=12) were sampled in triplicate, and average logMPN/g ± standard deviation determined by standard BAM method (FDA MPN) or by MPN using QCPR confirmation with SYBR Green I (QPCR-MPN), as described in text. Lots 1 to 3 were examined before and after PHP, and lot 4 was examined only at 21D after PHP.



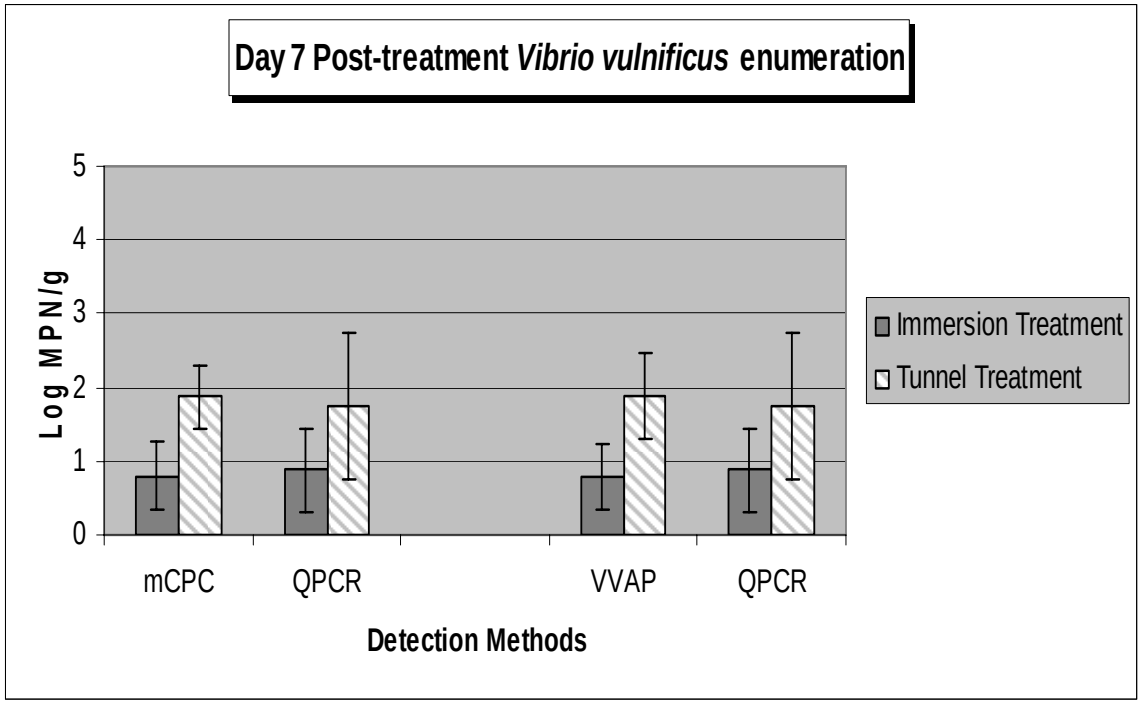


Figure 1. MPN's of temperature abused nitrogen treated samples. Comparison of detection methods, mCPC, VVAP and QPCR. Immersion treatment day 1 $p<0.09$, Immersion treatment day 7 $p<0.95$, Tunnel treatment day 1 $p<0.8$, and Tunnel treatment day 7 $p<0.95$.

Limit of Quantitation and Specificity

Wright et al., 2007, entitiled "Evaluation of Postharvest-Processed Oysters by Using PCR-Based Most-Probable-Number Enumeration of *Vibrio vulnificus* Bacteria", details the limits of quantitation and specificity. Seeding studies, utilizing known concentrations of *V. vulnificus* to spike oyster homogenates, were performed for a side by side comparison of mCPC selective media with QPCR results (Table 1). Although some loss of sensitivity was observed with the addition of 0.10 g oyster tissue at lower inocula, as compared to 0.01 g tissue, confirmation of positive samples in seeded homogenates prior to growth in APW still was about 100-fold more sensitive by QPCR melt peak than by recovery on mCPC. However, after 24 h of enrichment all concentrations of seeded homogenates were positive, as indicated by both growth on mCPC and SYBR QPCR melt peak for both 0.10g and 0.01g homogenates (16). Thus, results confirmed that approximately one cell in the original inoculum could be detected by QPCR-MPN, in agreement with previous reports (1, 9, 10, 11). Positive and negative controls were included with each thermocycler run.

QPCR examination of DNA from *V. vulnificus* (n=25) and non-*V. vulnificus* (n=28) strains (Table 3) showed SYBR Green I detection was 100% sensitive to all *V. vulnificus* strains and species-specific for *V. vulnificus.* Results were confirmed by previously described TaqMan assay using identical primers with an additional TaqMan probe (2). C_t values (number of cycles required to reach threshold for detection) for SYBR Green I detection of *V. vulnificus* strains was comparable to TaqMan QPCR with mean C_t=16.48 ±0.79 and 16.61± 0.87, respectively. All *V. vulnificus* strains were positive by TaqMan assay while non-target species were all negative, including "false positives" stains (shown in bold in Table 1) described in the prior report. Although SYBR detected C_t values above threshold for non-target strains, detection only occurred after extended PCR cycling (mean number of cycles=34.86 ± 2.28), and is a consequence of artifactual signal (22). First derivative analysis of melting curves provides sensitive discrimination of nucleotide differences in the DNA sequence of amplicons (20, 22), and species-specific detection of PCR product by SYBR green I was confirmed by single melt peak with consistent values (mean= 88.02 ± 0.26) from *V. vulnificus* strains (Table 1). In contrast, melt peak values for non-target species averaged >22 standard deviations apart from the mean of positive controls. Melt peak analysis is recommended for confirmation of positive samples.

Table 1. Specificity and sensitivity of *V. vulnificus* QPCR detection with SYBR Green I and TaqMan detection.

T arget	QPCR[b]			Non-Target	QPCR		
Strains[a]:	**TaqMan (C_t)**	**SYBR (C_t)**	**Melt Peak**	**Strains:**	**TaqMan (C_t)**	**SYBR (C_t)**	**Melt peak**
Vibrio vulnificus				***Aeromonas***			
1009	16/46	16.15	88.29	***hydrophila* 7965**	0	34.77	70.76
MO6-24/O	16.14	16.17	88.10	*Escherichia coli*			
MLT365	ND[b]	18.29	88.15	JM109	0	37.42	82.68
6353	16.45	15.92	87.91	HB101	0	35.12	79.43
MLT367	17.21	17.6	88.42	*Listeria*			
CVD752	15.94	14.87	88.26	*monocytogenes*	0	36.11	78.15
345/T	15.60	16.36	87.77	*Pseudomonas*			
BO6312	17.56	16.44	88.12	*aeruginosa*	0	35.15	86.4
5C1326	ND	16.16	88.14	***shigelloides*14029**	0	35.12	76.69
NJMSA	15.91	15.65	87.83	*Salmonella enterica*			
UNCC1015	15.92	15.98	87.98	Cholerasius10708	0	36.04	77.97
CVD737	ND	16.13	87.93	Enterica10112	0	37.64	62.4
LC4	15.62	16.27	87.86	Enteridis13076	0	39.38	63.37
UNCC9	ND	16.32	88.02	Enteridis14050	0	38.99	62.66
85A667	ND	15.61	87.92	*V. cholerae*			
1015	16.16	15.87	88.13	JVY212	0	34.7	79.47
345/O	16.56	16.64	87.91	JVB 52	0	33.38	74.24
80363	15.72	16.09	88.78	JVY210	0	28.3	73.88

LC4/T	16.95	17.29	88.13	JVB 25	0	30.36	74.9
E4125	16.49	15.62	87.83	2076	0	35.06	79.59
2400112	18.2	17.34	88.08	A5	0	35.44	79.59
52785	ND	17.71	87.46	***V. alginolyticus***	0	33.18	77.16
EDL174	ND	16.5	87.91	*V. fisherii* ES114	0	38.44	63.17
MLT403	17.13	17.31	87.77	***V. fluvialis* 1959-2**	0	33.14	78.04
LL728	17.69	17.29	87.81	***V. furnissii* 1958-83**	0	34.35	78.76
				***V. hollisae* 89[a]7053**	0	31.37	78.07
				V. parahaemolyticus			
				LM 5674	0	31.93	72.51
				10290	0	34.32	72.71
				LM 4892	0	36.31	78.76
				N4 3483R	0	39.27	78.9
				NY3547	0	33.06	71.91
				NVY3483	0	33.06	86.8
				TX2103	0	33.14	86.35
Average:	16.61	16.48	88.02	Average:	0	34.81	75.90
	±0.87	±0.79	±0.26			±2.66	±6.75

a) Strains in bold were reported to be positive by prior study (16).
b) C_t values are shown for QPCR as described in text with melt peak analysis for SYBR Green I assay.

APPENDIX 1

DNA extraction:
For boiling lysis DNA extraction, 1 ml of APW IS centrifuged (15,000g) for 10 min. The supernatant is discarded and the pellet resuspended in 400 µl of phosphate-buffered saline (PBS). Sample is boiled for 10 min, and subsequently centrifuged for three minutes to remove particulates. Supernatants are stored at -20°C.

APPENDIX 2

Q-PCR method:
After cleaning work surfaces as described previously, a mastermix is prepared by conbining primers, sterile molecular grade PCR water, SYBR green I dye, and Cepheid© smartmix beads, according to manufacturer's specifications. Reagents are pippetted into Cepheid© tubes that have sterilized. The mastermix aliquot equals 23µl in volume for each sample. These tubes are then centrifuged for 5 seconds to ensure all reagents are in the bottom of the tube in the chamber. Primers were obtained from Geno-mechanix, Gainesville Florida. 1x SYBR green I dye (Invitrogen) is added to mastermix containing, vvhA primers (2, 16) and Cepheid© smartmix beads (1 bead per 2 reactions). DNA template (2 µl) or water are added to QPCR reactions for a total volume of 25 µl. The program utilized the following parameters, a hold for 2 min at 95°C followed by 40 cycles of 15 s at 95°C and 60°C for 1 min. Cycle threshold (*Ct*) values, the number of cycles required to reach threshold for detection, were compared to standard curve values to enumerate for SYBR green I detection of *V. vulnificus* strains. Analysis of melting peaks, curves representative of melting temperature, provide a sensitive discrimination of non target sequences in the DNA sequence of amplicons (12, 13), and species-specific detection of PCR product by SYBR green I was confirmed by single melt peaks in the target range.

References

18. **Blackstone, G. M., J. L. Nordstrom, M. C. L. Vickery, M.D. Bowen, R. F. Meyer, A. Depaola.** 2003. Detection of pathogenic *Vibrio parahaemolyticus* in oyster enrichments by real time PCR. J. Microbiol. Methods. 53: 149-155.
19. **Campbell, M. S. and A. C. Wright.** 2003. Real-time PCR analysis of *Vibrio vulnificus* from oysters. Appl. Environ. Microbiol. **69:**7137-7144.
20. **Food and Drug Administration** (FDA). 2005. National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish. Guidance Documents Chapter IV Naturally Occurring Pathogens, Section .04 http://www.cfsan.fda.gov/~ear/nss3-44.html#p04
21. **Gordon K.V.**, **M.C. Vickery**, **A. DePaola**, **C. Staley**, **V.J. Harwood**. 2008. Real-time PCR assays for quantification and differentiation of Vibrio vulnificus strains in oysters and water. Appl. Environ. Microbiol. **74**: 1704-9.
22. **Gulf Oyster industry Council (G.O.I.C).** 2001. Gulf Oyster Industry Leads the Way Toward Providing Less Risky Oysters for Certain At Risk Consumers Through Post-Harvest Treatment. Online: November 21, 2007. http://www.gulfoysters.org/postharvest.cfm
23. **Harwood, V. J., J. P. Gandhi, and A. C. Wright**. 2004. Methods for isolation and confirmation of Vibrio vulnificus from oysters and environmental sources: a review. J. Microbiol. Methods 59:301–316.
24. **Interstate Shellfish Sanitation Conference** (ISSC). 2003. Issue relating to a *Vibrio vulnificus* risk management plan for oysters. ISSC, Columbia, SC.
25. **Kaysner, C. A. and A. DePaola.** 2004. *Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and Other *Vibrio* spp., Food and Drug Administration Bacteriological Analytical Manual, 8 ed. Revision A, 1998. Chapter 9. Substantially rewritten and revised May 2004. http://www.cfsan.fda.gov/~ebam/bam-9.html
26. **Paniker, G. and A. K. Bej.** 2005. Real-time PCR detection of *Vibrio vulnificus* in oysters: comparison of oligonucleotide primers and probes targeting vvhA. Appl. Environ. Microbiol. **71**: 5702-5709.
27. **Panicker, G., M. L. Myers, and A. K. Bej.** 2004a. Rapid detection of *Vibrio vulnificus* in shellfish and Gulf of Mexico water by real-time PCR. Appl. Environ. Microbiol. **70**: 498–507.
28. **Panicker, G., M. L. Myers, and A. K. Bej.** 2004b. Multiplex PCR detection of clinical and environmental strains of *Vibrio vulnificus* in shellfish. Can. J. Microbiol. **50:**911-922.
29. **Randa, M. A., M. F. Polz, and E. Lim.** 2004. Effects of temperature and salinity on *Vibrio vulnificus* population dynamics as assessed by quantitative PCR. Appl. Environ. Microbiol. **70:**5469–5476.
30. **Ririe, K. M., R. P. Rasmussen, and C. T. Wittwer**. 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Anal. Biochem. 245:154–160.
31. **Shapiro, R. L., S. Altekruse, L. Hutwagner, R. Bishop, R. Hammond, S. Wilson, B. Ray, S. Thompson, R. V. Tauxe, P. M. Griffin, and the Vibrio Working Group.** 1998. The role of Gulf Coast oysters harvested in warmer months in *Vibrio vulnificus* infections in the United States, 1988-1996. J. Infect. Dis. **178**:752-759
32. **Wright, A. C., G. A. Miceli, W. L. Landry, J. B. Christy, W. D. Watkins, and J. G. Morris, Jr.** 1993. Rapid identification of *Vibrio vulnificus* on nonselective media with an alkaline phosphatase-labeled oligonucleotide probe. Appl. Environ. Microbiol. **59:**541-6.
33. **Wright, A.C.**, **V. Garrido, G. Debuex, M. Farrell-Evans, A.A. Mudbidri, W.S. Otwell .** 2007. Evaluation of postharvest-processed oysters by using PCR-based most-probable-number enumeration of Vibrio vulnificus bacteria. Appl. Environ. Microbiol. **73**: 7477-81.

Proposal Subject: Method for the Determination of Paralytic Shellfish Toxins (PST) in Shellfish

Specific NSSP Guide Reference: Section IV. Guidance Documents Chapter II. Growing Areas .10 Approved National Shellfish Sanitation Program Laboratory Tests: Microbiological and Biotoxin Analytical Methods.

Text of Proposal/ Requested Action I am submitting for your review and consideration a method for the determination of Paralytic Shellfish Toxins (PST) in shellfish. This method provides an alternative to the current AOAC methods of analysis for the determination of PST in shellfish that is sensitive, robust and accurate.

This post-column oxidation (PCOX) method was developed to provide a rapid, high throughput chemical assay for PST which would eliminate the need to sacrifice animals, using the AOAC mouse bioassay (MBA), for toxin detection. The shellfish tissues are blended with dilute acid, heated, and the supernatant is purified. The PST are separated chromatographically using ion pair chromatography and oxidized to a fluorescent derivative post column using a periodic acid, phosphate oxidant. The derivatized toxins are monitored using fluorescence detection. The method has been validated following guidelines recommended by the IUPAC Harmonized Guidelines for Single-laboratory Validation of Analytical Methods. Results were also compared to those obtained using the AOAC MBA Method and those obtained using the AOAC pre-column oxidation method (AOAC Official Method 2005.06). The method development and single laboratory validation studies have been peer reviewed and accepted for publication in the Journal of the AOAC International.

The PCOX method is simple, robust and provides repeatable precise and accurate results. I would like the Laboratory Methods Review Committee to approve the PCOX method as a suitable National Shellfish Sanitation Program laboratory test for the analysis of Paralytic Shellfish Toxins in shellfish.

Public Health Significance: The method was developed to provide a rapid, high throughput chemical assay for Paralytic Shellfish Toxins (PST) which would eliminate the need to sacrifice animals, using the AOAC mouse bioassay (MBA), for toxin detection. There is a worldwide move to replace assays which use live animals as test subjects.

Cost Information (if available): Total consumable costs for the analysis is estimated at $10/sample. A chemistry laboratory will usually be equipped with an LC system and will only require a post column system to be equipped to carry out the analysis at a cost of approximately $20,000. Total capital costs for the instrumentation required for the analysis is approximately $100.000.

Action by 2009 Laboratory Methods Review Committee Recommended adoption of Proposal 09-104 post-column oxidation HPLC method to detect PST for mussels, clams, oysters, scallops as a Type IV method.

Action by 2009 Task Force I Recommended adoption of Laboratory Methods Review Committee recommendation on Proposal 09-104.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force I on Proposal 09-104.

Dartmouth Laboratory
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Dartmouth, Nova Scotia
Canada
B3B 1Y9

June 12, 2009

Laboratory Methods Review Committee
INTERSTATE SHELLFISH SANITATION CONFERENCE
209-2 Dawson Road
Columbia, SC 29223

Dear Colleagues;

I am submitting for your review and consideration a method for the determination of Paralytic Shellfish Toxins (PST) in shellfish. This method provides an alternative to the current AOAC methods of analysis for the determination of PST in shellfish that is sensitive, robust and accurate.

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The PCOX method is simple, robust and provides repeatable precise and accurate results. I would like the Laboratory Methods Review Committee to approve the PCOX method as a suitable National Shellfish Sanitation Program laboratory test for the analysis of Paralytic Shellfish Toxins in shellfish. If you require further information or have questions please contact me, my contact information is included below;

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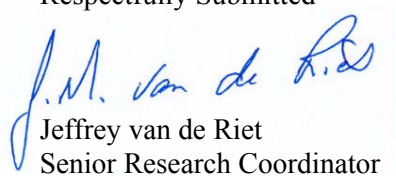
Respectfully Submitted



Jeffrey van de Riet
Senior Research Coordinator

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

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Methods Review (LMR) Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

Name of the New Method	**Rapid Post-column Oxidation Method for the Determination of Paralytic Shellfish Toxins in Mussels, Clams, Oysters and Scallops.**
Name of the Method Developer	**Jeffrey van de Riet- Senior Research Coordinator, Dartmouth Laboratory**
Developer Contact Information	**1992 Agency Drive Dartmouth, Nova Scotia Canada B3B 1Y9**

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.	Y	The method was developed to provide a rapid, high throughput chemical assay for Paralytic Shellfish Toxins (PST) which would eliminate the need to sacrifice animals, using the AOAC mouse bioassay (MBA), for toxin detection.
2. What is the intended purpose of the method?	Y	This method is validated for the determination of PST in mussels, clams, oysters and scallops. The method provides an alternative methodology to the AOAC MBA for the analysis of PST in shellfish.
3. Is there an acknowledged need for this method in the NSSP?	Y	There is a worldwide move to replace assays which use live animals as test subjects.
4. What type of method? i.e. chemical, molecular, culture, etc.	Y	Chemical. The PST are separated chromatographically using ion pair chromatography. The separated toxins are then oxidized to a fluorescent derivative post column using a periodic acid, phosphate oxidant. The derivatized toxins are monitored using fluorescence detection.
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title	Y	Rapid Post-Column Oxidation Method for the Determination of Paralytic Shellfish Toxins in Mussels, Clams, Oysters and Scallops.
Method Scope	Y	This method is validated for the determination of Paralytic Shellfish toxins (PST) in mussels, clams, oysters and scallops.
References	Y	Rourke, W.A., Murphy, C.J., Pitcher, G., van de Riet, J.M., Burns, B.G., Thomas, K.M., Quilliam, M.A. (2008) J.AOAC Int 91(3), 589-597. Van de Riet, J.M., Gibbs, R.S., Chou, F.W., Muggah, P.M., Rourke, W.A., Burns, B.G., Thomas, K. and Quilliam, M.A. (2009) J.AOAC Int, In Press. Additional references are included with the SOP in Appendix II
Principle	Y	The PST are extracted from the edible portion of supernat by heating with dilute acid for 5 minutes in a boiling water bath. The deproteinized supernatant is adjusted to pH-4. The toxins are separated using ion pair chromatography and are oxidized post column to produce purines by breakage of a C4-C12 bond in a complex 3-ring structure characteristic of PSP toxins. The resulting products monitored with fluorescent detection.
Any Proprietary Aspects	N	None

Equipment Required	Y	- Liquid Chromatograph with a solvent selection valve, column switching valve, and fluorescence detector - Two post column pumps and a heater capable of maintaining 85 C - 1 mL reaction coil and miscellaneous PEEK tubing - General laboratory apparatus A detailed list of the required equipment can be found in the attached SOP
Reagents Required	Y	A detailed list of the required reagents can be found in the attached SOP, Appendix II.
Sample Collection, Preservation and Storage Requirements	Y	A detailed SOP, Appendix II is attached and includes all steps on the sample collection, preservation and storage requirements..
Safety Requirements	Y	All safety precautions are laid out in the method protocol.
Clear and Easy to Follow Step-by-Step Procedure	Y	A detailed SOP is attached and includes all steps on the sample analysis procedure. See Appendix II
Quality Control Steps Specific for this Method	Y	-Full Instrument calibration curve is analysed weekly -Calibration checks are run within each batch of injections after every 20 injections. - QC and recovery sample is analysed with each batch of extracts
C. Validation Criteria		
1. Accuracy / Trueness	Y	Accuracy/Trueness was assessed by recovery experiments, as recommended in Section A4.3.4 of the IUPAC Harmonized Guidelines for Single-laboratory Validation of Analytical Methods. Results were also compared to those obtained using the AOAC MBA Method and those obtained using the AOAC pre-column oxidation method (AOAC Official Method 2005.06).
2. Measurement Uncertainty	Y	- The combined Measurement Uncertainty for the four matrices was determined to be 0.16 at the regulatory limit
3. Precision Characteristics (repeatability and reproducibility)	Y	- Repeatability and Reproducibility (Intermediate Precision) results are summarized in Appendix I Tables 1-3
4. Recovery	Y	- Recovery for the method ranged from 94 to 106 % over the three levels and 4 matrices. The data are summarized in Appendix I Table 4
5. Specificity	Y	- Specificity of the LC method is increased due to a number of characteristics of the method over the MBA. Summary of the specificity comparison to the AOAC MBA is found in Appendix I Table 8.
6. Working and Linear Ranges	Y	- The method has been validated at 0.4., 0.8 and 1.6 mg STX•diHCl eq/ kg (40, 80 and 160 ug STX•diHCl eq/100g). The linear range of the method is greater with an upper limit in excess of 2000 ug STX•diHCl eq/100g. A summary of the estimated linear range of the individual toxins is shown in Appendix I Table 7.
7. Limit of Detection	Y	Appendix I Table 5 summarizes the estimated limits of detection and quantitation for the individual PST according to the validated species.
8. Limit of Quantitation / Sensitivity	Y	Appendix I Table 5 summarizes the estimated limits of detection and quantitation for the individual PST according to the validated species.
9. Ruggedness	Y	A ruggedness study was conducted and the factors investigated had no observable effect. The studied factors are shown in Appendix I Table 6
10 Matrix Effects	Y	The validation data have demonstrated that the method is 'blind' to the matrix.
11 Comparability (if intended as a substitute for an established method accepted by the NSSP)	Y	Comparison of the PCOX method to the AOAC MBA, Lawrence and Oshima methods of analysis are shown in Appendix I Figure 1 to 4.
D. Other Information		
1. Cost of the Method	Y	The cost of consumables in the method is less than $10 per sample
2. Special Technical Skills Required to Perform the Method	Y	Competence in the operation and maintenance of a basic Liquid Chromatographic system.
3. Special Equipment Required and Associated Cost	Y	- Liquid Chromatograph- Isocratic LC with a solvent selection valve or binary or quaternary system with a fluorescence detector- $50000-100,000 CAN - Post-column derivitization system- $25000 CAN
4. Abbreviations and Acronyms Defined	Y	A detailed SOP is attached and includes all various abbreviations and acronyms used in the procedure..
5. Details of Turn Around Times (time involved to complete the method)	Y	A single LC system has the capacity to analyse 24 samples/24 hour period. If the analysis of C-toxins is not required capacity is

		50 samples/24 hour period.
6. Provide Brief Overview of the Quality Systems Used in the Lab	Y	CFIA laboratories are accredited to ISO 17025 by the Standards Council of Canada and maintain an internal QA system consistent with the IUPAC Harmonized Guidelines for Internal Quality Control in Analytical Laboratories (Pure & Applied Chemistry, **67**: 649-666 (1995).
Submitters Signature	Date:	
Submission of Validation Data and Draft Method to Committee	Date:	
Reviewing Members	Date:	
Accepted	Date:	
Recommendations for Further Work	Date:	
Comments:		

DEFINITIONS

1. Accuracy/Trueness - Closeness of agreement between a test result and the accepted reference value.

2. Analyte/measurand - The specific organism or chemical substance sought or determined in a sample.

3. Blank – Sample material containing no detectable level of the analyte or measurand of interest that is subjected to the analytical process and monitors contamination during analysis.

4. Comparability – The acceptability of a new or modified method as a substitute for an established method in the NSSP. Comparability must be demonstrated for each substrate or tissue type by season and geographic area if applicable.

5. Fit for purpose – The analytical method is appropriate to the purpose for which the results are likely to be used.

6. HORRAT value – HORRAT values give a measure of the acceptability of the precision characteristics of a method.[4]

7. Limit of Detection – the minimum concentration at which the analyte or measurand can be identified. Limit of detection is matrix and analyte/measurand dependent.[4]

8. Limit of Quantitation/Sensitivity – the minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.

9. Linear Range – the range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.

10. Measurement Uncertainty – A single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.

11. Matrix – The component or substrate of a test sample.

12. Method Validation – The process of verifying that a method is fit for purpose.[1]

13. Precision – the closeness of agreement between independent test results obtained under stipulated conditions.[1, 2] There are two components of precision:

- **a. Repeatability** – the measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.
- **b. Reproducibility** – the measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.

14. Quality System – The laboratory's quality system is the process by which the laboratory conducts its activities so as to provide data of known and documented quality with which to demonstrate regulatory compliance and for other decision–making purposes. This system includes a process by which appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.

15. Recovery – The fraction or percentage of an analyte or measurand recovered following sample analysis.

16. Ruggedness – the ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]

17. Specificity – the ability of a method to measure only what it is intended to measure.[1]

18. Working Range – the range of analyte or measurand concentration over which the method is applied.

REFERENCES:

1. Eurachem Guide, 1998. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. LGC Ltd. Teddington, Middlesex, United Kingdom.
2. IUPAC Technical Report, 2002. Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, Pure Appl. Chem., Vol. 74, (5): 835-855.

3. Joint FAO/IAEA Expert Consultation, 1999. Guidelines for Single-Laboratory Validation of Analytical Methods for Trace-Level Concentrations of Organic Chemicals.
4. MAF Food Assurance Authority, 2002. A Guide for the Validation and Approval of New Marine Biotoxin Test Methods. Wellington, New Zealand.
5. National Environmental Laboratory Accreditation. , 2003. Standards. June 5.
6. EPA. 2004. EPA Microbiological Alternate Procedure Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Methods: Guidance. U.S. Environmental Protection Agency (EPA), Office of Water Engineering and Analysis Division, 1200 Pennsylvania Avenue, NW, (4303T), Washington, DC 20460. April.

Appendix I Validation Data.

Accuracy and Trueness

Currently there are no materials available that are considered as Certified Reference Materials for PST. Analytical standards were obtained from NRCC, with supporting documentation. Accuracy/Trueness was assessed by recovery experiments, as recommended in Section A4.3.4 of the IUPAC Harmonized Guidelines for Single-laboratory Validation of Analytical Methods, Pure & Applied Chemistry, **74**: 835-855 (2002). The recoveries obtained by this methodology are shown in Table 4. Results from samples analysed by this method were also compared to those obtained using the AOAC MBA Method and those obtained using the AOAC pre-column oxidation method (AOAC Official Method 2005.06). Comparison to a reference method is also recommended in the IUPAC guideline cited above. The comparisons of the PCOX method to the AOAC and other methods of analysis are shown in Figures 1 to 4.

Repeatability and Intermediate Precision

Materials for the repeatability and intermediate precision were prepared by blending of blank materials (mussels, clams, scallops or oysters, respectively) with a highly contaminated mussel material. The materials were blended using a ratio of 1 part contaminated mussel to 100, 50 or 25 parts each of the respective blank study matrices to achieve concentrations that result in a total toxicity equivalent to ½ MRL, MRL and 2 MRL (0.40, 0.80, 1.60 µg STX•diHCl eq/kg) for each of the four matrix materials, as described above. The materials were extracted and analyzed according to the method, as described. The concentration of each detected toxin was determined and corrected for the method dilution. The repeatability is determined by conducting 5 replicate analyses, repeated over three days, for a total of 15 determinations for each matrix at each concentration.

The intermediate precision (repeatability) was determined on the same materials as were used for the repeatability. Sufficient material was preserved to allow for a second analyst to extract and analyse the same tissues on a second instrument. The second analyst reproduced the work conducted previously by the first analyst, by conducting analyses of 5 replicates for each matrix and concentration on each of 3 days, for a total of 15 determinations for each test material by each analyst. The data is summarized in Tables 1-3.

Table 1. Repeatability for Gonyautoxins and Saxitoxins

	Matrix	Average Concentration of PST (3 days, 5 replicates/day n=15 mg STX eq/kg ± %RSD)						
		Total	**GTX4**	**GTX1**	**GTX3**	**GTX2**	**NEO**	**STX**
0.40 mg/kg	Clams	0.42 ± 2.2%	**0.016 ± 17%**	**0.051 ± 7.7%**	0.050 ± 6.6%	0.067 ± 3.9%	0.065 ± 9%	0.17 ± 3.2%
	Mussels	0.41 ± 6%	**0.019 ± 16%**	**0.049 ± 24%**	0.051 ± 2.2%	0.061 ± 15%	0.063 ± 8.4%	0.17 ± 5.5%
	Scallops	0.45 ± 3.5%	**0.021 ± 16%**	**0.048 ± 10%**	0.060 ± 2.7%	0.081 ± 3.7%	0.061 ± 8.6%	0.18 ± 4.5%
	Oysters	0.38 ± 7.2%	**0.017 ± 48%**	**0.072 ± 35%**	0.047 ± 2.8%	0.066 ± 2.7%	0.050 ± 8.8%	0.13 ± 11%
0.80 mg/kg	Clams	0.83 ± 2.2%	**0.032 ± 4.6%**	0.099 ± 6.9%	0.099 ± 2%	0.13 ± 2.9%	0.13 ± 7.4%	0.35 ± 3.1%
	Mussels	0.79 ± 3.9%	**0.040 ± 5.8%**	0.097 ± 3.7%	0.096 ± 1.7%	0.12 ± 8.3%	0.12 ± 4.9%	0.32 ± 4.2%
	Scallops	0.84 ± 1.9%	**0.032 ± 10%**	0.090 ± 5.5%	0.11 ± 1.3%	0.14 ± 2.3%	0.11 ± 5.7%	0.35 ± 2.9%
	Oysters	0.67 ± 3.7%	**0.029 ± 34%**	0.11 ± 17%	0.089 ± 3.9%	0.12 ± 4.1%	0.082 ± 8.7%	0.24 ± 17%
1.60 mg/kg	Clams	1.660 ± 2%	0.065 ± 6%	0.20 ± 6.2%	0.20 ± 1.1%	0.26 ± 1.5%	0.24 ± 2.9%	0.69 ± 3.4%
	Mussels	1.650 ± 3.1%	0.064 ± 4.4%	0.20 ± 2%	0.20 ± 1.7%	0.26 ± 4.8%	0.25 ± 6.5%	0.68 ± 3.6%
	Scallops	1.670 ± 2%	0.063 ± 6.4%	0.19 ± 3.5%	0.21 ± 2%	0.26 ± 3.4%	0.22 ± 9%	0.71 ± 2.6%
	Oysters	1.380 ± 6.2%	0.063 ± 25%	0.20 ± 11%	0.18 ± 3.9%	0.24 ± 6.3%	0.18 ± 11%	0.51 ± 13%

Values in **BOLD and Underlined** are below the LOQ for one of the matricies tested
Materials were pooled tissues, analysed in replicate (5 reps/day), repeated on three days (n=15)

The repeatability of the method for the N-sulfocarbamoyl-gonyautoxin C1 and C2 was a challenge as these toxins are not prevalent in materials that were available for use in this study. For this reason the repeatability for the C1 and C2 toxins was only determined in a single material at a single concentration. The analysis of this material was replicated (5 times) each day and repeated over 3 three days. The concentrations of the toxins were calculated and the results are summarized in Table 2.

Table 2. Repeatability for C-toxins

Day	Replicate	C-1	C-2	Total
1	1	0.021	0.17	0.21
	2	0.022	0.17	0.21
	3	0.022	0.17	0.21
	4	0.022	0.18	0.22
	5	0.022	0.17	0.21
	Average	0.020	0.170	0.210
	STD Dev	0.0004	0.0016	0.0033
	RSD	1.8%	0.9%	1.6%
2	1	0.023	0.17	0.21
	2	0.023	0.17	0.22
	3	0.023	0.17	0.21
	4	0.024	0.17	0.23
	5	0.023	0.17	0.22
	Average	0.020	0.170	0.220
	STD Dev	0.0005	0.0030	0.0058
	RSD	2.3%	1.8%	2.7%
3	1	0.021	0.16	0.22
	2	0.022	0.16	0.22
	3	0.023	0.16	0.22
	4	0.022	0.16	0.22
	5	0.022	0.16	0.22
	Average	0.020	0.160	0.220
	STD Dev	0.0004	0.0010	0.0030
	RSD	2.2%	0.6%	1.4%
Combined	Average	0.02	0.17	0.22
	SD	0.0	0.0	0.0
	%RSD	3.1%	2.7%	2.4%

The relative standard deviation under repeatability conditions for the toxins that were present in the samples above the limit of quantitation was below 13% in all cases. This is within the acceptable range as indicated by AOAC International. The relative standard deviation for the C-toxins as determined is below 5% and is within the acceptable range as indicated by the AOAC. The repeatability for all toxins in all matrices were relatively consistent. The one exception was STX in oysters, which was observed to show the greatest variation but is within acceptable ranges.

Table 3. Intermediate Precision (within-lab reproducibility) of the PCOX method for the analysis of PST in shellfish.

	Matrix	Analyst	3-day Average Concentration of PST (n=15, mg STX eq/kg ± %RSD)						
			Total	**GTX4**	**GTX1**	**GTX3**	**GTX2**	**NEO**	**STX**
0.40 mg/kg	Clams	1	0.42 ± 2.2%	**0.016 ± 17%**	**0.051 ± 7.7%**	0.050 ± 6.6%	0.067 ± 3.9%	0.065 ± 9.0%	0.17 ± 3.2%
		2	0.44 ± 3.0%	**0.022 ± 32%**	**0.075 ± 9.6%**	0.050 ± 3.9%	0.055 ± 9.6%	0.058 ± 10%	0.17 ± 4.0%
		Avg.	0.43 ± 3.0%	**0.019 ± 32%**	**0.063 ± 22%**	0.050 ± 5.3%	0.061 ± 12%	0.062 ± 11%	0.17 ± 3.6%
		HorRat	**0.17**	**1.12**	**0.90**	**0.21**	**0.51**	**0.45**	**0.18**
	Mussels	1	0.41 ± 6.0%	**0.019 ± 16%**	**0.049 ± 24%**	0.051 ± 2.2%	0.061 ± 15%	0.063 ± 8.4%	0.17 ± 5.5%
		2	0.38 ± 0.1%	**0.03 ± 0.3%**	**0.052 ± 0.2%**	0.052 ± 0.03%	0.040 ± 0.1%	0.063 ± 0.2%	0.15 ± 0.04%
		Avg.	0.39 ± 7.5%	**0.22 ± 27%**	**0.049 ± 22%**	0.050 ± 4.0%	0.052 ± 22%	0.061 ± 13%	0.16 ± 8.2%
		HorRat	**0.41**	**0.95**	**0.87**	**0.16**	**0.90**	**0.55**	**0.39**
	Scallops	1	0.45 ± 3.5%	**0.021 ± 16%**	**0.048 ± 10%**	0.060 ± 2.7%	0.081 ± 3.7%	0.061 ± 8.6%	0.18 ± 4.5%
		2	0.48 ± 5.5%	**0.14 ± 98%**	**0.064 ± 16%**	0.067 ± 11%	0.084 ± 5.9%	0.063 ± 19%	0.19 ± 3.6%
		Avg.	0.46 ± 5.7%	**0.015 ± 60%**	**0.056 ± 20%**	0.064 ± 10%	0.083 ± 5.3%	0.062 ± 15%	0.18 ± 5.3%
		HorRat	**0.32**	**2.00**	**0.81**	**0.41**	**0.23**	**0.61**	**0.26**
	Oysters	1	0.38 ± 7.2%	**0.017 ± 48\%**	**0.072 ± 35%**	0.047 ± 2.8%	0.066 ± 2.7%	0.050 ± 8.8%	0.13 ± 11%
		2	0.37 ± 3.9%	**0.015 ± 47%**	**0.049 ± 9.8%**	0.047 ± 4.4%	0.064 ± 9.4%	0.051 ± 5.5%	0.15 ± 3.1%
		Avg.	0.38 ± 5.8%	**0.016 ± 48%**	**0.060 ± 35%**	0.047 ± 3.8%	0.065 ± 7.0%	0.050 ± 7.2%	0.14 ± 10%
		HorRat	**0.32**	**1.62**	**1.47**	**0.15**	**0.29**	**0.29**	**0.48**
0.80 mg/kg	Clams	1	0.83 ± 2.2%	**0.032 ± 4.6%**	0.099 ± 6.9%	0.099 ± 2.0%	0.13 ± 2.9%	0.13 ± 7.4%	0.35 ± 3.1%
		2	0.84 ± 2.8%	**0.038 ± 12%**	0.13 ± 7.4%	0.10 ± 3.8%	0.12 ± 7.6%	0.11 ± 4.3%	0.34 ± 4.6%
		Avg.	0.84 ± 2.5%	**0.03.5 ± 13%**	0.11 ± 15%	0.10 ± 3.3%	0.12 ± 7.3%	0.12 ± 9.7%	0.34 ± 4.0%
		HorRat	**0.15**	**0.50**	**0.69**	**0.15**	**0.34**	**0.44**	**0.21**
	Mussels	1	0.79 ± 3.9%	**0.040 ± 58%**	0.097 ± 3.7%	0.096 ± 1.7%	0.12 ± 8.3%	0.12 ± 4.9%	0.32 ± 4.2%
		2	0.70 ± 3.8%	**0.039 ± 17%**	0.089 ± 8.3%	0.094 ± 4.4%	0.093 ± 3.3%	0.099 ± 9.0%	0.28 ± 2.9%
		Avg.	0.74 ± 6.9%	**0.40 ± 43%**	0.093 ± 7.3%	0.095 ± 3.5%	0.11 ± 15%	0.11 ± 11%	0.30 ± 7.0%
		HorRat	**0.41**	**1.66**	**0.32**	**0.15**	**0.67**	**0.51**	**0.37**
	Scallops	1	0.84 ± 1.9%	**0.032 ± 10%**	0.090 ± 5.5%	0.11 ± 1.3%	0.14 ± 2.3%	0.11 ± 5.7%	0.35 ± 2.9%
		2	0.93 ± 1.7%	**0.031 ± 26%**	0.11 ± 12%	0.13 ± 8.8%	0.16 ± 2.2%	0.12 ± 4.6%	0.39 ± 2.5%
		Avg.	0.88 ± 5.7%	**0.031 ± 20%**	0.10 ± 14%	0.12 ± 9.8%	0.15 ± 5.6%	0.11± 5.5%	0.37 ± 5.6%
		HorRat	**0.35**	**0.73**	**0.64**	**0.45**	**0.26**	**0.25**	**0.31**
	Oysters	1	0.67 ± 3.7%	**0.029 ± 33%**	0.11 ± 17%	0.089 ± 3.9%	0.12 ± 4.1%	0.082 ± 8.7%	0.24 ± 17%
		2	0.68 ± 2.6%	**0.029 ± 40%**	0.087 ± 3.6%	0.086 ± 2.5%	0.12 ± 3.9%	0.10± 15%	0.27 ± 2.0%
		Avg.	0.68 ± 3.2%	**0.029 ± 37%**	0.099 ± 19%	0.087 ± 3.6%	0.12 ± 4.0%	0.092 ± 16%	0.26 ± 12%
		HorRat	**0.19**	**1.35**	**0.83**	**0.16**	**0.18**	**0.73**	**0.61**
1.60 mg/kg	Clams	1	1.66 ± 2.0%	0.065 ± 6.0%	0.20 ± 6.2%	0.20 ± 1.1%	0.26 ± 1.5%	0.24 ± 2.9%	0.69 ± 3.4%
		2	1.69 ± 1.9%	0.069 ± 5.9%	0.24 ± 5.9%	0.20 ± 3.0%	0.26 ± 6.1%	0.22± 3.4%	0.69 ± 3.2%
		Avg.	1.67 ± 2.0%	0.067 ± 6.7%	0.22 ± 12%	0.20 ± 2.5%	0.26 ± 4.5%	0.23 ± 6.2%	69 ± 3.3%
		HorRat	**0.14**	**0.28**	**0.61**	**0.13**	**0.23**	**0.31**	**0.19**
	Mussels	1	1.65 ± 3.1%	0.064 ± 4.4%	0.20 ± 2.0%	0.20 ± 1.7%	0.26 ± 4.8%	0.25 ± 6.5%	68 ± 3.6%
		2	1.56 ± 2.4%	0.076 ± 14%	0.21 ± 7.1%	0.20 ± 3.5%	0.22 ± 1.9%	0.22 ± 6.1%	61 ± 2.0%
		Avg.	1.60 ± 4.1%	0.070 ± 13%	0.21 ± 6.4%	0.20 ± 2.7%	0.24 ± 8.1%	0.24 ± 8.8%	0.64 ± 6.0%
		HorRat	**0.28**	**0.57**	**0.32**	**0.13**	**0.41**	**0.45**	**0.35**
	Scallops	1	1.67 ± 2.0%	0.063 ± 6.4%	0.19 ± 3.5%	0.21 ± 2.0%	0.26 ± 3.4%	0.22± 9.0%	0.71 ± 2.6%
		2	1.81 ± 2.9%	0.060 ± 21%	0.226 ± 12%	0.24 ± 7.1%	0.30 ± 2.4%	0.22 ± 7.5%	0.76 ± 3.2%
		Avg.	1.74 ± 4.8%	0.061 ± 15%	0.20 ± 12%	0.23 ± 7.7%	0.28 ± 7.4%	0.22 ± 8.3%	0.74 ± 4.6%
		HorRat	**0.33**	**0.64**	**0.59**	**0.39**	**0.38**	**0.42**	**0.28**
	Oysters	1	1.38 ± 6.2%	0.063 ± 25%	0.20 ± 12%	0.18 ± 3.9%	0.24 ± 6.3%	0.18 ± 11%	0.51 ± 13%
		2	1.42 ± 1.4%	0.058 ± 18%	0.18 ± 3.2%	0.18 ± 1.3%	0.25 ± 3.6%	0.19 ± 2.5%	0.56 ± 1.7%
		Avg.	1.40 ± 4.6%	0.061 ± 22%	0.19 ± 10%	0.18 ± 3.0%	0.24 ± 5.3%	0.18 ± 8.0%	0.53 ± 9.6%
		HorRat	**0.30**	**0.92**	**0.51**	**0.15**	**0.27**	**0.39**	**0.55**

Values in **Bold and Underlined** are below the LOQ for one of the matricies tested
Materials were pooled tissues, analysed in replicate (5 reps/day), repeated on three days (n=15) by each analyst

As this is a report on single laboratory validation, true between-lab reproducibility can not be determined; however, within-lab intermediate precision was determined for this method. A second analyst analysed the same materials that were employed for the repeatability study on a separate instrument. These data were compiled and a Horwitz ratio (HorRat) was determined for each analyte concentration studied in each matrix. These data are summarized in Table 3. Ideal values for HorRat are between 0.3 and 1.2, and for this method all values are all below 1.0. A number of results are below 0.3, which is an indication of analyst bias. The analysts in question had some indication of the expected levels of the toxins in the tissues and this knowledge may have lead to results that were unexpectedly close in agreement resulting in lower than ideal HorRat values. In addition, both analysts, trained by the method developers, have a consistent approach to integration and interpretation of the chromatograms. These two factors may be expected to lead to analyst bias regardless of the proper intentions of each analyst and will therefore result in artificially low HorRat values. A true indication of the method repeatability will be determined by an inter-laboratory or full collaborative study at a later date.

Recovery

The method recovery, defined as the fraction or percentage of an analyte recovered following extraction and analysis of a blank sample to which the analyte has been added at a known concentration, was determined in mussels, clams, scallops and oysters, at concentrations designed to result in a total toxicity equivalent to ½ MRL, MRL and 2 MRL (0.40, 0.80, 1.60 mg STX•diHCl eq/kg). Recovery experiments were carried out at three levels for each species of shellfish, with the determinations being replicated (5 times) on each day and repeated over 3 three days, for a total of 15 determinations for each matrix at each concentration. Pre-weighed portions of blank tissue were fortified with aliquots (125, 250 or 500 µL) of a mixture of PST to achieve the desired concentrations of analytes in tissues. The fortification solution by the extraction and dilution of highly contaminated tissue and subsequent fortification with other PST standards to obtain a solution containing the most common and most toxic PST analogues available. The materials were extracted and analyzed according to the method, as described. The concentration of each detected toxin was determined and converted to STX equivalents, the total toxicity value was calculated by combining the STX equivalents of the individual toxins. The fortification solution was calibrated against the matrix fortified analytical standard and the concentration of each toxin was determined. The recovery was calculated by evaluating the amount of each toxin found as well as the total toxins recovered from the sample against the amount of toxins added. Table 4 summarizes the results obtained from the recovery experiments.

Table 4. Recovery of PST using the CFIA PCOX methodology.

Spike Level	Matrix		Percent Recovery (3 days, 5 replicates/day n=15)										
			Total	**GTX4**	**GTX1**	**dcGTX3**	**dcGTX2**	**GTX3**	**GTX2**	**NEO**	**STX**	**C-1**	**C-2**
Toxin Concentration mg/kg			**0.44**	**0.013**	**0.058**	**0.000**	**0.000**	**0.039**	**0.076**	**0.069**	**0.181**	**0.000**	**0.000**
0.4 mg/kg	Clams	Avg.	99	100	101	ND	ND	103	105	103	102	ND	ND
		SD	7	13	10			9	13	13	6		
	Mussels	Avg.	102	113	110	ND	ND	114	106	104	113	ND	ND
		SD	1	22	11			15	5	9	6		
	Scallops	Avg.	94	132	105	ND	ND	105	103	76	101	ND	ND
		SD	9	63	13			7	10	7	8		
	Oysters	Avg.	97	104	137	ND	ND	103	104	97	88	ND	ND
		SD	4	15	38			4	7	5	11		
Toxin Concentration mg/kg			**0.88**	**0.030**	**0.014**	**0.020**	**0.006**	**0.079**	**0.17**	**0.12**	**0.38**	**0.0002**	**0.0010**
0.8 mg/kg	Clams	Avg.	104	109	102	ND	ND	106	109	101	106	ND	ND
		SD	12	12	6			7	13	13	9		
	Mussels	Avg.	106	100	107	107	121	115	107	103	111	101	91
		SD	2	10	6	13	20	15	3	4	4	11	13
	Scallops	Avg.	97	138	107	131	70	108	107	75	105	ND	ND
		SD	8	14	9	10	29	7	9	5	9		
	Oysters	Avg.	106	90	126	ND	ND	110	116	99	92	127	105
		SD	5	21	16			4	10	3	9	40	34
Toxin Concentration mg/kg			**1.76**	**0.047**	**0.020**	**0.004**	**0.009**	**0.149**	**0.30**	**0.22**	**0.68**	**0.0003**	**0.0002**
1.6 mg/kg	Clams	Avg.	102	103	99	128	159	103	107	98	104	88	ND
		SD	7	9	5	6	47	5	10	11	7	20	
	Mussels	Avg.	106	103	104	105	119	115	108	105	110	98	90
		SD	2	14	3	9	20	16	3	3	4	13	8
	Scallops	Avg.	99	132	105	117	82	106	104	75	104	147	103
		SD	7	13	5	9	17	6	8	4	8	34	23
	Oysters	Avg.	104	104	114	151	186	107	109	99	89	126	98
		SD	3	18	7	14	26	3	3	5	7	42	27

Values in **Bold and Underlined** are below the LOQ for one of the matricies tested
Materials were pooled tissues, analysed in replicate (5 reps/day), repeated on three days (n=15)

At the concentrations evaluated in this study, an IAEA/FAO/IUPAC/AOAC expert consultation report on single laboratory validation recommended that an acceptable recovery range was 70-100%. The average total toxicity recoveries ranged from 94 to 106 % for the three levels studied. Individual toxin recoveries were between 90-110 %. The recovery of NEO in Scallops was determined to be approximately 75% which was significantly lower than the recovery of any of the other toxins. While this recovery is lower than expected, it still falls within the acceptable range specified by the expert consultation report and has been shown to be consistent between various analysts. The fortification levels for some of the toxins were below the LOQ for those compounds at one or more of the fortification levels. For this reason the recoveries for these toxins are higher than would be ideal (greater than 110%) and the RSDs are large. For toxins fortified at levels above the LOQ the RSDs were generally below 15%. Statistical analysis shows that although the toxin recovery from the various matrices differs, the recoveries are within acceptable values.

Limit of Determination and Quantification

The limit of determination (LOD), the lowest concentration of analyte that can be detected and limit of quantification (LOQ), the lowest concentration of analyte that can be quantified, are determined for each matrix by analysis of five replicate extracts of blank matrix, repeated over 6 days (n=30). The baseline signal to noise ratio was determined at the approximate retention time for all toxins. This noiise response (height units) was multiplied by a factor of 3 and converted to µmoles of toxin using the response from the working standard. The amount of toxin was corrected for method dilution and toxicity (relative to STX) to result in an LOD expressed as; mg STX•diHCl eg/kg for each toxin. The LOQ for the method was calculated by multiplying the LOD by a factor of 3.

Table 5. Estimated Limits of Detection and Quantitation for the individual PST in the validated matrices.

Toxin	Clams		Mussels		Scallops		Oysters	
	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ
GTX4	0.0120	0.036	0.0160	0.048	0.016	0.048	0.026	0.078
GTX1	0.0210	0.063	0.0240	0.072	0.024	0.072	0.037	0.111
dcGTX3	0.0025	0.008	0.0008	0.002	0.018	0.054	0.003	0.008
GTX5	0.0060	0.018	0.0032	0.010	0.007	0.021	0.008	0.024
dcGTX2	0.0070	0.021	0.0021	0.006	0.005	0.014	0.007	0.021
GTX3	0.0025	0.008	0.0012	0.004	0.003	0.008	0.003	0.009
GTX2	0.0310	0.093	0.0220	0.066	0.024	0.072	0.029	0.087
NEO	0.0250	0.075	0.0240	0.007	0.024	0.072	0.026	0.078
dcSTX	0.0096	0.029	0.0077	0.023	0.008	0.023	0.010	0.029
STX	0.0170	0.051	0.0130	0.039	0.013	0.039	0.014	0.042
C-1	0.0004	0.001	0.0002	0.001	0.001	0.003	0.000	0.001
C-2	0.0008	0.002	0.0008	0.002	0.001	0.004	0.009	0.028
Total	0.135	0.404	0.115	0.345	0.143	0.430	0.172	0.515

LOD = 3 xS/N
LOQ= 3 x LOD

While the total toxicity LOD and LOQ are calculated by summing the LOD or LOQ for each toxin, this assumes the presence of all toxins are at the respective limit. In reality, as the toxicity increases from zero, one will see one or two of the more predominant toxins at the low concentrations. As the toxicity increases, the abundance of the predominant toxins increases and the less predominant congeners begin to appear. The realistic LOD and LOQ are approximately 0.03 and 0.1 mg STX•diHCl eq/kg of tissue, respectively. This is a 4-fold improvement in the detection capability of the method over the conventional AOAC MBA. These improved limits provide a better early warning system from monitoring programs as well as better information about the toxin profiles in the concentration range of interest.

Ruggedness

The ruggedness of an analytical method is the resistance to change in the results produced by an analytical method when minor deviations are made from the experimental conditions described in the procedure. The ruggedness approach used in this validation was Youden's factorial approach, where seven variables were combined in a specific manner to determine the effects of all seven variables using eight combinations in a single experiment. Seven variables were tested using a partial factorial approach followed by statistical evaluation of significance using a two-sample t-test assuming equal variance. The experiment was carried out in its entirety twice on separate days, with mean values being used for statistical evaluation. The material used was an incurred mussel tissue that was established to contain 1.72 mg STX•diHCl eq/kg. The seven variables tested are listed in Table 6.

Ruggedness of the technique was studied and statistical analysis was carried out using a two sample t-test. The statistical analysis indicated that the single factor that showed a significant affect on the results was the type of filter membrane used in the analysis. The Teflon membrane showed a significantly higher result for the total toxicity as well as several individual toxins over the nylon membrane. When this factor was then further studied independently it was found not to have a significant impact on the results.

Table 6. Factors evaluated in ruggedness experiments

Variable #	Description	Original Condition		Alternate Condition	
1	Concentration of HCl for initial extraction	A-	0.1 M	a-	0.12 M
2	Delay after acid addition before boiling	B-	no delay	b-	10 min delay
3	Time in boiling water bath	C-	5 min	c-	10 min
4	Final pH	D-	3	d-	2.5
5	Volume of TCA added	E-	25 µL	e-	20 µL
6	Volume of NaOH added	F-	35 µL	f-	40 µL
7	Filter material	G-	nylon	g-	teflon

Sample #	Factor Combinations	Measurement
1	A B C D E F G	s
2	A B c D e f g	t
3	A b C d E f g	u
4	A b c d e F G	v
5	a B C d e F g	w
6	a B c d E f G	x
7	a b C D e f G	y
8	a b c D E F g	z

Other factors were investigated as part of the optimization of method performance, but were not part of the statistical design to determine ruggedness/robustness. Specifically, various post-column reactors and coils were evaluated. The reactor used in this study required modification from the original manufacturer's design. As purchased, the reactor system has a large amount of heat exchanger tubing included as part of the system. This plumbing results in peak broadening and loss of resolution for the various toxins. Once this tubing is excluded from the flow path, the peak resolution is restored. Other post column reactor systems have been evaluated and found to be suitable. Some systems such as the Pickering Pinnacle PCX have incorporated the pumps and reactor system. The Pinnacle system was evaluated and provided better sensitivity which was attributed to less baseline noise from the post column pumps.

Linearity and Analytical range

The matrix fortified calibration curves of the toxins are linear at all ranges examined in this study. The concentration of toxins chosen for study are close to or at the limits of detection ranging up to 5.00 mg STX•diHCl eq/kg. The equations for typical curves and correlations are shown in Table 7. All the correlation coefficients are greater than 0.99. The ranges examined effectively encompass the regulatory limit of 0.80 mg STX•diHCl eq/kg for a typical toxin profile.

Table 7. Linearity and Linear Range of the calibrations curves as determined by serial dilutions of the working standards.

	Range				Calibration Curve	
	ng injected		mg / kg		Equation	Correlation
	lower	upper	lower	upper		
GTX4	0.08	5.08	0.017	1.110	y = 92.341x - 0.09	R^2 =1.0
GTX1	0.31	21.1	0.068	4.590	y = 48.248x - 2.137	R^2 =0.9999
dcGTX3	0.02	1.11	0.36	24.200	y = 1096x - 13.021	R^2 =0.9987
GTX5	0.05	0.84	0.011	0.184	y = 238.59x - 0.5407	R^2 =0.9995
dcGTX2	0.05	1.61	0.011	0.350	y = 360.45x - 2.2149	R^2 =0.9998
GTX3	0.02	2.45	0.004	0.530	y = 622.69x - 14.544	R^2 = 0.9996
GTX2	0.13	4.16	0.021	0.910	y = 101.46x + 0.13	R^2 = 1.00
NEO	0.17	11.6	0.037	2.520	y = 62.793x - 6.2136	R^2 = 0.9994
dcSTX	0.16	2.56	0.034	0.560	y = 117.12x - 2.4566	R^2 = 0.9989
STX	0.18	5.88	0.039	1.280	y = 69.892x - 4.1551	R^2 = 0.999
C1	0.0026	0.162	0.0011	0.076	y = 2567.8x + 0.4421	R^2 = 0.9999
C2	0.0065	0.798	0.028	0.350	y = 1353.6x - 7.6822	R^2 = 0.999
C3	0.0015	0.483	0.00007	0.021	y = 2741.9x - 0.1585	R^2 = 0.9998
C4	0.0106	0.166	0.0046	0.072	y = 618.28x + 0.5843	R^2 = 0.9996

The method was developed as part of a project to compare results obtained from analytical methods of analysis to the results obtained from the AOAC MBA. A long term comparison study was carried out where extracts used for MBA analysis were also analyzed by the CFIA-PCOX method. The results of this long term study are presented in Figure 1 and show good correlation of the PCOX results to the results obtained by the MBA.

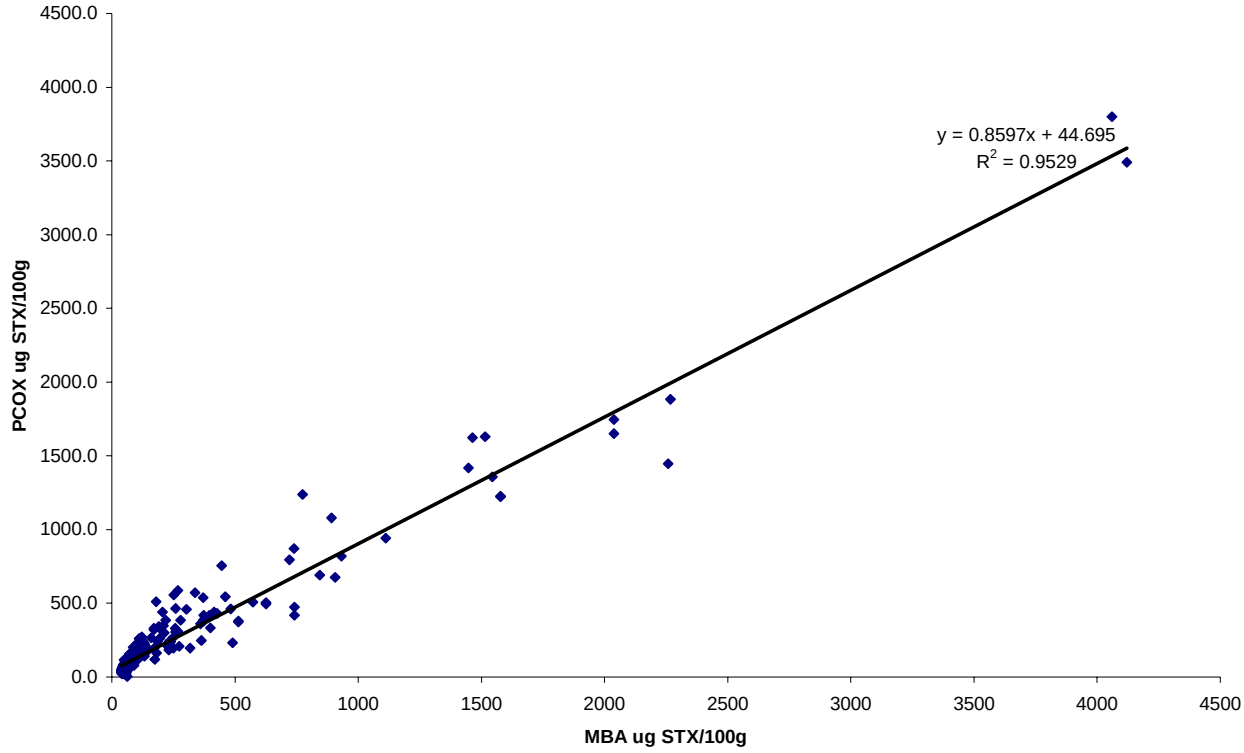


Figure 1. Comparison of the results obtained by the AOAC-MBA to the results obtained from the CFIA-PCOX methodology

This method has been employed to compare results obtained to those obtained in laboratories in other countries, which used other methods of analysis on samples collected in their monitoring programs. Samples or extracts were obtained, analysed by PCOX and results compared to those obtained from the method utilized in the source country; Norway {Oshima}, United Kingdom {AOAC Official Method 995.08 and AOAC Official Method 2005.06}, and New Zealand {AOAC Official Method 995.08}. The results obtained from those samples compared well to the results obtained from the source country using various methods (Figures 1 to 4). This indicates that the CFIA-PCOX method results are in agreement with other methods currently employed worldwide and that it therefore is a viable alternative to these various methods. The authors therefore have proposed that the method should be considered for further assessment by a full AOAC collaborative study.

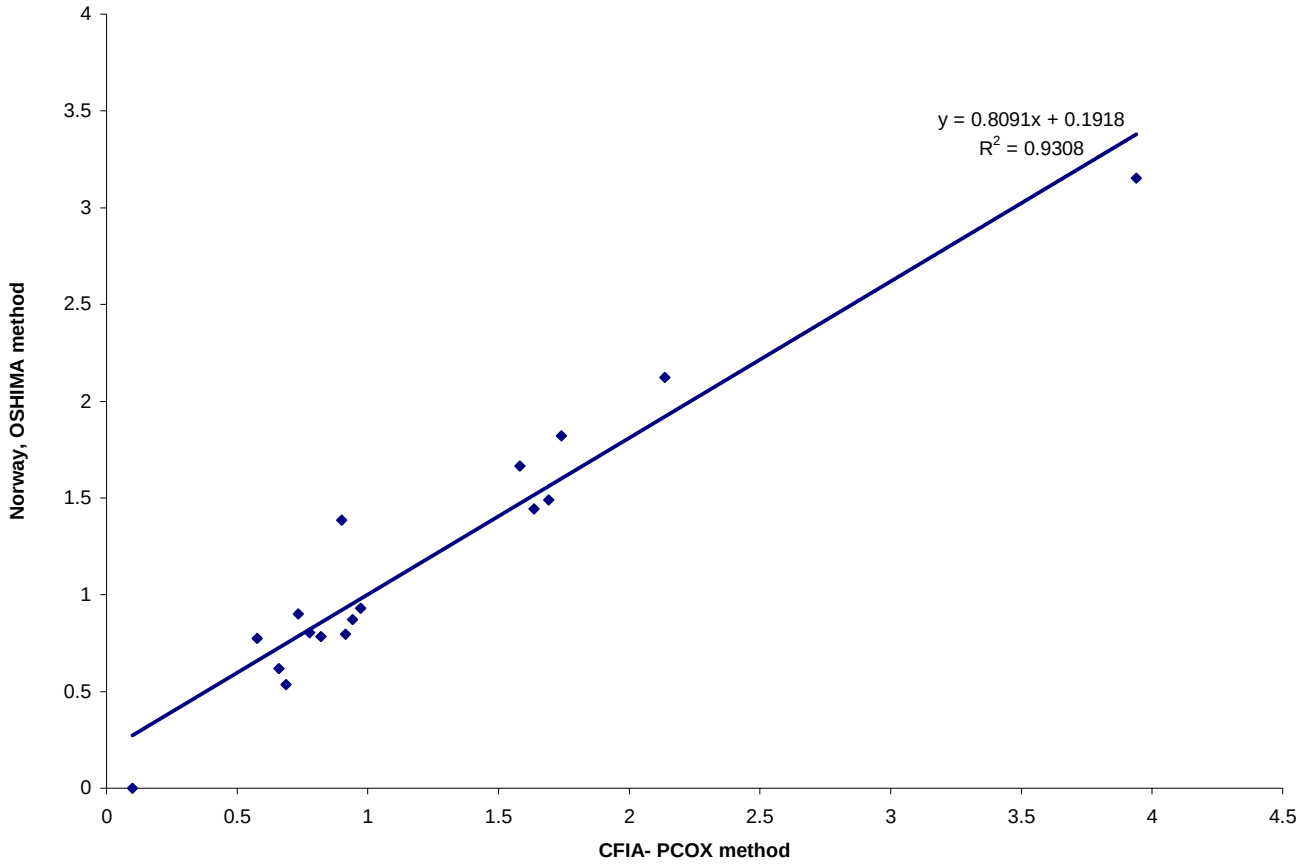
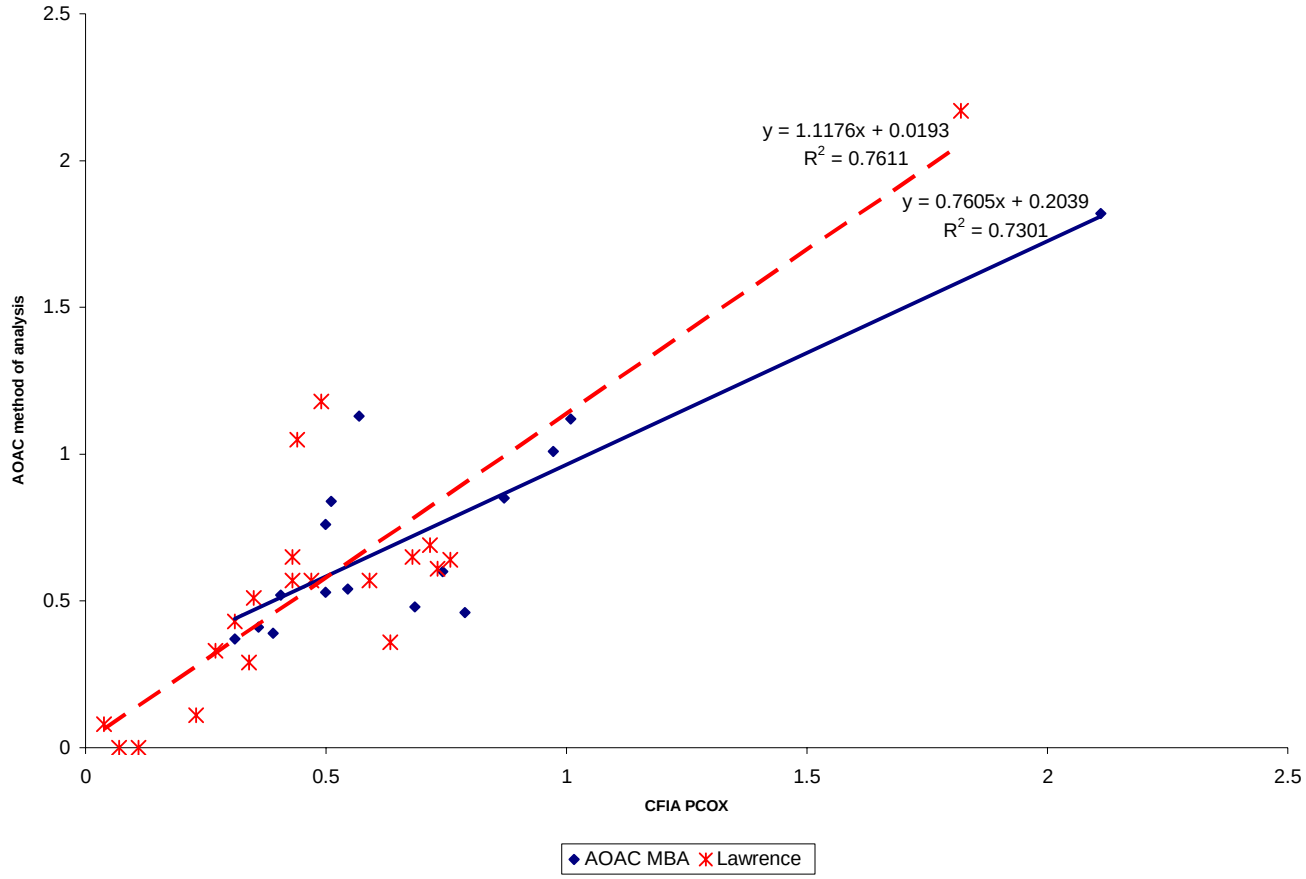


Figure 2. Comparison of PST results (mg STX•diHCl eq/kg) from tissues supplied and analysed by Norwegian School of Veterinary Science laboratory and the CFIA Dartmouth Laboratory



*Note: Twenty-four samples were provided however only the data from twenty were plotted as four of the samples were below the LOD of the MBA

Figure 3. Correlation of results obtained (mg STX•diHCl eq/kg) by CEFAS laboratories utilizing AOAC MBA and Lawrence methods of analysis to the CFIA-PCOX method for PST.

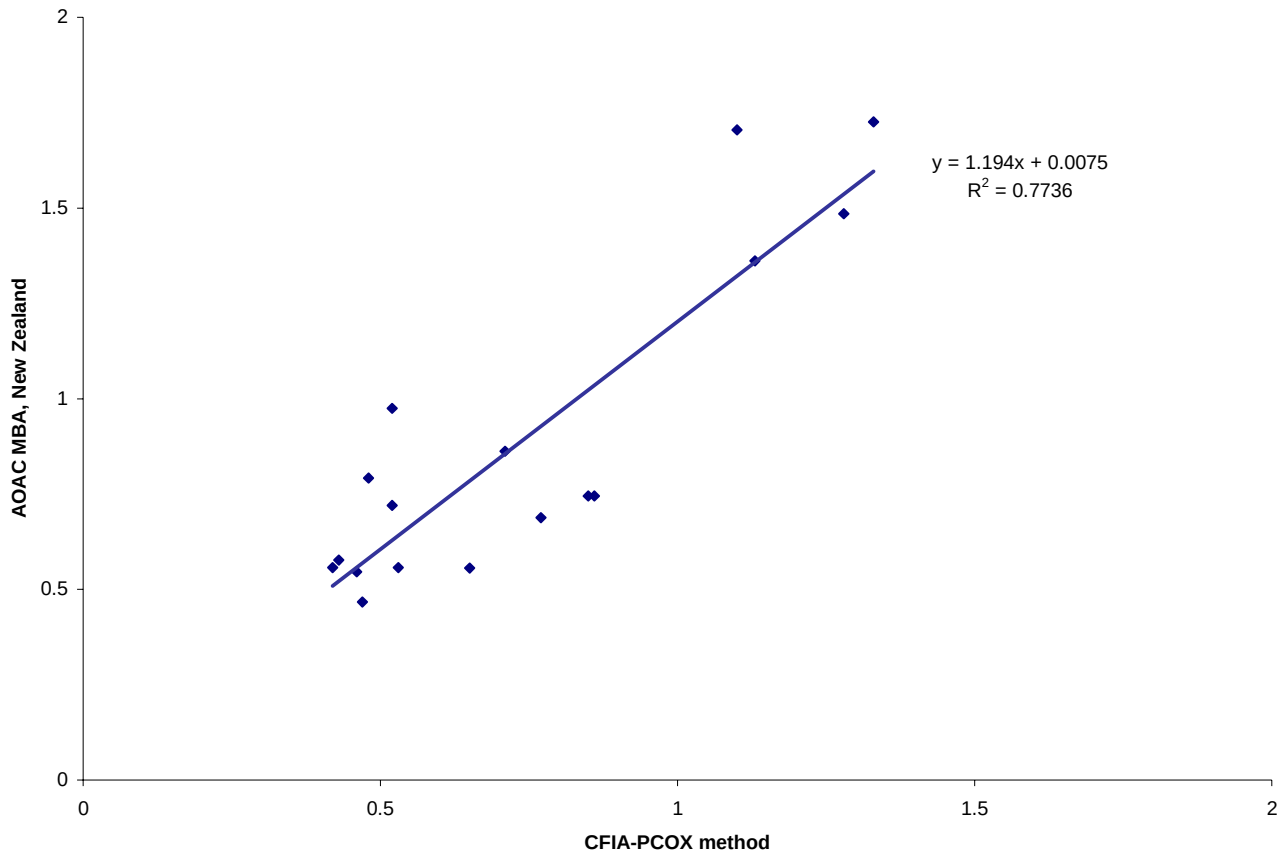


Figure 4. Correlation of PST toxicity (mg STX•diHCl eq/kg) in samples analysed by the MBA at the Cawthron Institute (NZ) and CFIA-PCOX.

Specificity

The current method of choice for the analysis of PST by most regulatory authorities is the AOAC mouse bioassay. This methodology employs and extraction of the toxins with a dilute acid and an injection in to the interperitoneal cavity of a mouse. The level of toxicity on the sample is inversely proportional to the time required for the mouse to die. This method is relatively non specific and has been shown to be subject to various interferences such as salts, high level of metals and pesticides. The CFIA-PCOX methodology utilizes a similar extraction technique as the MBA but a number of other steps are added into the process to provide greater specificity. Table 8 is a side by side comparison of the two methods (AOAC MAB and the CFIA-PCOX) highlighting the specificity created by the various steps in the procedure.

The table shows that the PCOX method adds specificity in to the analysis by building on the basis of the MBA extraction and adding steps such as protein precipitation, chromatographic separation of the toxins, oxidation of the toxins to a purine and detection of the purine by fluorescence at specific excitation and emission wavelengths

Table 8. Comparison of the specificity of the AOAC MAB and CFIA-PCOX methodologies

Method step	MBA Method	LC Method	Specificity
Data from the chemistry used in the extraction and clean-up procedure	1. Acid extraction: Acid extractables, <pH4	1. Acid extraction: Acid extractables, pH 2 to 4 2. De-proteination :-Proteins removed	1. Specificity is equivalent for both methods 2. Specificity of LC method is increased, due to removal of proteins (i.e., response in LC method cannot be caused by protein-based material)
Data from the subsequent chromatography	Not applicable	Substances must be initially retained on a reversed phase LC column and eluted by the mobile phase gradient conditions with characteristic retention times between 6 – 20 minutes	Specificity of LC method is increased by limiting the method to analytes retained and chromatographed on a LC column, additional selectivity is provided by the chromatographic separation and the characteristic retention times of the analytes which may be compared to reference standards.
Data from the detecting spectroscopy or electrochemistry	Not applicable	The LC method includes a post-column oxidation reaction which is specific to molecular structures which are oxidized by a phosphoric acid, periodic acid buffer solution to form purines by breakage of a C4-C12 bond in a complex 3-ring structure characteristic of PSP toxins, with formation of an aromatic ring structures which produces characteristic fluorescence.	The LC detection method is specific to compounds with complex 3-ring chemical structures which form fluorescent purines via the post-column reaction.
Detection	Mice respond to toxins, particularly neurotoxins.	Characteristic fluorescence (excitation: 330 nm, emission: 390 nm) associated with conversion of PSP toxins to purines.	MBA response is not specific to PSP, but is a general response to toxins, particularly neurotoxins; fluorescence associated with the LC method has been characterized by mass spectrometry to demonstrate it is from the reaction of PSP toxins with the periodic acid solution to form purines which exhibit native fluorescence [see Janacek, M., Quilliam, M.A. & Lawrence, J.F. *Journal of Chromatography*, **644 (**1993) 321-331]
data from the "blank" reagents	No positive response	No positive response	Methods have equivalent specificity in this regard.
Data from the "blank" samples	Method has an expected "false positive" rate	No interfering co-extractives have been detected to date.	Response of LC method is "compound specific", as individual analytes are separated by LC prior to detection (selectivity of LC separation plus specificity of detection method)
data from library searches for potential interferences or matches	Other known toxins elicit a positive response (eg., neurotoxins such as carbamate and organophosphate insecticides)	None identified to date	Available data suggest LC method is more specific than mouse bioassay.
Data and arguments why potential interferences in practice do not or likely will not interfere	MBA is specific to "toxins", not to PSP toxins	LC combines selectivity of clean-up and separation with specificity of detection reaction.	LC method has greater selectivity and specificity than the MBA, plus analytes in extracts may be confirmed by LC/MS/MS.
Other data i.e choice of matrix, other quality control data			Sample source, collection, transport and storage are equivalent – no additional specificity is associated with these factors for either method.

Rapid Post-column Oxidation (PCOX) Method for the Determination of Paralytic Shellfish Toxins in Mussels, Clams, Oysters and Scallops.

1. PURPOSE

1.1. To give specific information required to carry out the method of analysis for the determination of paralytic shellfish toxins in shellfish by the CFIA Post-column Oxidation (PCOX) method.

2. REFERENCES

2.1. Rourke, W.A., Murphy, C.J., Pitcher, G., van de Riet, J.M., Burns, B.G., Thomas, K.M., Quilliam, M.A. (2008). Rapid Post-column Methodology for Determination of Paralytic Shellfish Toxins in Shellfish Tissue. J.AOAC Int 91(3), 589-597.

2.2. van de Riet, J.M., Murphy C. J., Rourke, W.A., Burns, B.G., Thomas, K.M and Quilliam, M. A.(2006). Alternate validated methodology for regulatory analysis of PSP toxins in Canadian shellfish. 120th AOAC International Meeting and Exposition, Sept 17-21, Minneapolis, Minnesota.

2.3. AOAC. (1995b). Paralytic shellfish poison: Biological method. Sec. 35.1.37, Method 959.08. In Official Methods of Analysis of AOAC International, 16th ed., P.A. Cunniff (Ed.), p. 22-23. AOAC International, Gaithersburg, MD.

3. SCOPE

3.1. This method is validated for the determination of Paralytic Shellfish toxins (PST) in tissues of mussels, clams, oysters and scallops.

3.2. This method is an alternative to AOAC MBA methodology for the analysis of PST in molluscan shellfish

3.3. This method has been used to determine paralytic shellfish toxin concentrations in shellfish tissue ranging from 0 to 5000 ug STX diHCl/100 g.

4. DEFINITIONS

4.1. CRM = Certified reference material

4.2. PSP = Paralytic shellfish poisoning

4.3. C1 = N-sulfocarbamoylgonyautoxin-C1

4.4. C2 = N-sulfocarbamoylgonyautoxin-C2

4.5. C3 = N-sulfocarbamoylgonyautoxin-C3

4.6. C4 = N-sulfocarbamoylgonyautoxin-C4

4.7. dcGTX1 = decarbamoylgonyautoxin-1

4.8. dcGTX2 = decarbamoylgonyautoxin-2

4.9. dcGTX3 = decarbamoylgonyautoxin-3

4.10. dcGTX4 = decarbamoylgonyautoxin-4

4.11. dcSTX = decarbamoylsaxitoxin

4.12. GTX1 = gonyautoxin-1

4.13. GTX2 = gonyautoxin-2

4.14. GTX3 = gonyautoxin-3

4.15. GTX4 = gonyautoxin-4

4.16. GTX5 = gonyautoxin-5

4.17. GTX6 = gonyautoxin-6

4.18. NEO = neosaxitoxin

4.19. STX = saxitoxin

4.20. RCF = Rotor centrifugal force units

5. EQUIPMENT & MATERIALS REQUIRED

5.1. Equipment

5.1.1. Volumetric pipets: 1.0 mL, 2.0mL, 4.0 mL, 10.0 mL and 15.0 mL capacities

5.1.2. Volumetric flasks, various volumes

5.1.3. Analytical & top-load balances

5.1.4. Boiling water bath

5.1.5. Accurate timing device

5.1.6. Sieve, No. 10 mesh

5.1.7. Blender, small food processor or equivalent

5.1.8. 50 mL polypropylene tubes or equivalent

5.1.9. Dispenser capable of delivering 5 mL or equivalent

5.1.10. Centrifuge capable of holding 50 mL polypropylene tubes and of generating ~ 5000 RCF (g's)

5.1.11. Microcentrifuge tubes 1.5-2 mL

5.1.12. Pippettor(s) capable of delivering 20-1000 µL

5.1.13. 13 mm nylon syringe filters (0.2 µm) or equivalent

5.1.13.1. 3 mL disposable syringes if using syringe filters

5.1.14. Microcentrifuge capable of generating ~16000 RCF (g's)

5.1.15. High Recovery autosampler vials and caps

5.1.16. pH meter

5.1.17. Vortex mixer

5.2. Instrumentation:

5.2.1. LC pump system able to generate rapid, reliable binary gradients at flow rates of up to 1.5 mL/min and at pressures of at least 3000 psi

5.2.2. Autosampler system able to communicate with the pumps and data system and provide up to 100 µL injection volumes either in one injection or repeated smaller injections

5.2.3. Column oven able to maintain a column temperature of 50°C

5.2.4. LC fluorescence detector able to achieve the required sensitivity at excitation λ = 330 nm and emission λ = 390 nm

5.2.5. Two post-column pumps able to deliver acid and oxidant at flow rates up to 0.5 mL/min

5.2.6. Knitted reaction coil, 1 mL volume, 5 m x 0.5 mm or equivalent

5.2.7. Post-column reaction oven able to maintain a temperature of 85°C

5.2.8. LC columns

5.2.8.1. GTXs & STXs : Agilent Zorbax Bonus RP, 4.6 x150 mm, 3.5 µm particle size

5.2.8.2. C toxins: Thermo BetaBasic 8, 4.6 x 250 mm, 5 µm particle size

5.3. Reagents

5.3.1. Deionized water (DIW), 18Ω resistance or equivalent

5.3.2. Acetic acid (HOAc), glacial

5.3.2.1. 10% (v/v) HOAc: Pipet 10.0 mL of concentrated HOAc to a 100.0 mL volumetric flask containing 70 mL of DIW, dilute to the mark with DIW and mix well.

5.3.3. DIW (pH 5.00): Acidify DIW to pH 5.00 by dropwise addition of 10% HOAc.

5.3.4. Acetonitrile (MeCN), HPLC grade

5.3.5. Concentrated Hydrochloric acid (HCl), reagent grade

5.3.5.1. 5.0 M HCl: Add 413.2 mL of concentrated HCl to a 1.0 L volumetric flask containing 300 mL of DIW, dilute to the mark with DIW and mix well.

5.3.5.2. 0.1 M HCl: Add 40.0 mL of 5.0 M HCl to a 2.0 L volumetric flask containing 1.5 L of DIW, dilute to the mark with DIW and mix well.

5.3.5.3. 3 mM HCl: Pipet 15.0 mL of 0.1 M HCl to a 500.0 mL volumetric flask containing 300 mL of DIW, dilute to the mark with DIW and mix well.

5.3.6. Ammonium hydroxide (NH_4OH), reagent grade

5.3.6.1. 1% (v/v) NH_4OH: Pipet 1.0 mL of NH_4OH to a 100.0 mL volumetric flask containing 80 mL of DIW, dilute to the mark with DIW and mix well.

5.3.7. 1.0 M Tetrabutyl ammonium dihydrogen phosphate solution

5.3.8. Trichloroacetic acid (TCA), reagent grade

5.3.8.1. 30% (w/v) TCA: Dissolve 15.0 g of TCA in a 50.0 mL volumetric flask, dilute to the mark with DIW and mix well.

5.3.9. Sodium hydroxide (NaOH), reagent grade

5.3.9.1. 5.0 M NaOH: Weigh 200 g of NaOH, dissolve in 1.0 L DIW and mix well.

5.3.10. o-Phosphoric acid (H_3PO_4), reagent grade

5.3.10.1. 0.5 M H_3PO_4: Add 33.9 mL of H_3PO_4 to 800 mL of DIW in a 1.0 L volumetric flask, dilute to the mark with DIW and mix well.

5.3.11. 1-Heptane sulphonate sodium salt monohydrate

5.3.11.1. 0.5 M 1-Heptane sulphonate: Weigh 11.01 g of 1-heptane sulphonate sodium salt monohydrate for every 100.0 mL of DIW. Mix well, and store in fridge as solution degrades rapidly.

5.3.12. Periodic acid (H_5IO_6), reagent grade

5.3.12.1. 0.05 M H_5IO_6: Dissolve 11.4 g of H_5IO_6 in a 1.0 L volumetric flask, dilute to the mark with DIW and mix well.

5.3.13. Nitric acid (HNO_3), reagent grade

5.3.13.1. 0.75 M HNO_3: Add 101.2 mL of concentrated HNO_3 to 1.6 L of DIW. Make to volume (2.0 L), mix well, and then filter through 0.22 µm nylon membrane.

5.3.14. Post-Column Oxidant: Add 400 mL of 0.5 M H_3PO_4 to 1.2 L DIW and stir well. Add 200 mL of 0.05 M H_5IO_6, stir well, and check pH. If pH is approximately 4 then discard and start over. If pH is approximately 1.5 then adjust the pH to 7.8 with 5 M NaOH. Transfer to a 2.0 L volumetric flask, dilute to the mark with DIW and mix well. Filter through 0.45 µm membrane.

5.3.15. GTX & STX mobile phase "A": Add 44.0 mL of 0.5 M heptane sulphonate to 1.8 L DIW and mix well. Add 22.0 mL of 0.5 M H_3PO_4 and mix well. Adjust the pH to 7.1 using concentrated NH_4OH. Transfer to a 2.0 L volumetric flask and dilute to the mark with DIW and mix well. Filter through 0.45 µm membrane.

5.3.16. GTX & STX mobile phase "B": Add 22.0 mL of 0.5 M heptane sulphonate to 800mL DIW and mix well. Add 33.0 mL of 0.5 M H_3PO_4 and mix well. Adjust the pH to 7.1 using concentrated NH_4OH. Add 115 mL of MeCN and mix well. Transfer to a 1.0 L volumetric flask, dilute to the mark with DIW and mix well. Filter through 0.45 µm membrane.

5.3.17. C toxin mobile phase "C": Add 4.0 mL of 1.0 M tetrabutyl ammonium dihydrogen phosphate solution to 1.8 L of DIW using a volumetric pipet. If pH is above 5.8 then adjust the pH to 5.8 by adding 10% (v/v) HOAc dropwise, but if the pH is below 5.8 then adjust the pH to 5.8 by adding 1% NH_4OH dropwise. Transfer to a 2.0 L volumetric flask, dilute to the mark with DIW and mix well. Filter through 0.45 µm membrane.

5.3.18. C toxin mobile phase "D": Add 2.0 mL of 1.0M tetrabutyl ammonium dihydrogen phosphate solution to 900mL DIW using a volumetric pipet. If pH is above 5.8 then adjust the pH to 5.8 by adding 10% (v/v) HOAc dropwise, but if the pH is below 5.8 then adjust the pH to 5.8 by adding 1% NH_4OH dropwise. Add 40mL MeCN and mix well. Transfer to a 1.0L volumetric flask, dilute to the mark with DIW and mix well. Filter through 0.45 µm membrane.

5.4. Standards

5.4.1. Primary Standards- C1, C2, dcGTX2, dcGTX3, dcSTX, GTX1, GTX2, GTX3, GTX4, GTX5, NEO, and STX (National Research Council Institute for Marine Biosciences, Halifax, NS). All standards are obtained from the National Research Council's Certified Reference Material

Program, and have certified values. These standards are then used to make stock and working solutions.

5.4.2. C3 & C4 are NRC in house reference materials that will become commercially available when certification is complete.

5.4.3. Stock Standards-

Stock solutions are approximately 4 fold dilutions of NRC CRM's (Various concentrations based on CRM concentration).

5.4.3.1. Remove CRM ampoules from fridge/freezer and allow to reach room temperature.

5.4.3.2. Weigh an empty 2.0 mL volumetric flask.

5.4.3.3. Open the ampoule of CRM by carefully cracking at the scored line. Transfer as much liquid as possible to the volumetric flask.

5.4.3.4. Weigh the volumetric flask that now contains the CRM and determine the mass of transferred CRM by difference.

5.4.3.5. Dilute to 2.0 mL using 0.003 M HCl for GTX & STX or DIW (pH 5.00) for C toxins and mix well.

5.4.3.6. Weigh the full flask and determine the final volume of the solution.

5.4.3.7. Calculate the concentration based on the CRM documentation.

5.4.4. Working Standards

Working solutions (Various concentrations based on stock concentration). Standard solutions are generally separated into two categories, C toxins and GTXs & STXs. One working standard includes C1, C2, C3 and C4 while another working standard includes dcGTX2, dcGTX3, dcSTX, GTX1, GTX2, GTX3, GTX4, GTX5, NEO and STX.

5.4.4.1. GTX & STX neat mixed working solution

5.4.4.1.1. Weigh an empty 5.0mL volumetric flask after wiping the outside of the flask

5.4.4.1.2. Transfer volume of stock solution from table below to volumetric flask recording weight of flask after each addition.

5.4.4.1.3.

Toxin	**Volume of Stock (u** (Solution from 5.2.2
dcGTX2&	200
GTX2&3	200
dcSTX	200
STX	200
GTX1&4	400
GTX5	400
NEO	400

Dilute to 5.0mL using 0.003 M HCl.

5.4.4.1.4. Weigh full flask to determine final volume of solution.

5.4.4.1.5. Calculate concentration based on dilution factor and stock solution concentrations.

5.4.4.2. C-toxin neat mixed working solution

5.4.4.2.1. Weigh an empty 5.0mL volumetric flask after wiping the outside of the flask.

5.4.4.2.2. Transfer volume of stock solution from table below to volumetric flask recording weight of flask after each addition.

Toxin	**Volume of Stock (uL)** (Solution from 5.2.20)
C1&2	400
C3&4	800

5.4.4.2.3. Dilute to 5.0mL using DIW (pH 5.00).

5.4.4.2.4. Weigh full flask to determine final volume of solution.

5.4.4.2.5. Calculate concentration based on dilution factor and stock solution concentrations.

5.4.4.3. Matrix fortified working standards

5.4.4.3.1. Follow instructions for making up neat working standard (5.4.4.1) using a toxin free, de-proteinated, mussel extract as the diluent.

6. SAFETY PRECAUTIONS

6.1. Follow normal laboratory practices for a safe and healthy working environment.

6.2. Always wear gloves when handling PSP standards and samples.

6.3. Always wear a mask when weighing 1-heptane sulphonate.

6.4. Always work in a fumehood when using NH_4OH.

7. POLICY

7.1. Only Trained and authorized analysts shall perform this analysis.

8. INSTRUCTIONS

8.1. Sampling Procedure

8.1.1. Take a representative sample of the shellfish that require testing, usually consisting of 12-18 market size shellfish. This number should ensure the selection of sound animals suitable for analysis. Ensure the shellfish yield approximately 100 g of meats and shell liquor.

8.1.2. Rinse samples to remove sand, dirt and mud and place in a clean plastic bag.

8.1.3. Mark or tag all samples using waterproof markers for identification purposes. Label the sample in such a way that the identity of the sample can not be lost during shipment.

8.1.4. Ensure that the integrity of the sample is maintained by proper storage. Maintain the state of the sample.

8.1.4.1. Refrigerate samples of shucked or live shellfish immediately after collection by packing in crushed ice and keeping them in ice until examined. The shellfish must not come into direct contact with ice.

8.1.4.2. Keep frozen samples frozen in a freezer or in a carton/cooler with ice packs and ship the sample as quickly as possible to ensure that the sample remains in the frozen state.

8.2. Sample Preparation

8.2.1. Live Bivalve Molluscan Shellfish –

8.2.1.1. Thoroughly clean the outside of the shellfish with running tap water. Open the shell by cutting the adductor muscles without cutting or damaging the viscera. Rinse the inside with tap water to remove sand or other foreign material.

8.2.1.2. Remove tissue of 12-15 animals from the shell, for most shellfish collect the entire shell contents. For scallops, separately collect the digestive gland, adductor muscles, gonad, etc. for analysis as stipulated by regulatory requirements.

8.2.1.3. Collect the tissue to be used for the analyses onto a number 10 sieve. Allow to drain for approximately 5 minutes. Remove any pieces of shell or other foreign matter. Discard draining.

8.2.1.4. Transfer meats to a suitable vessel and blend/grind until homogenous.

8.2.2. Frozen in the shell Bivalve Molluscan Shellfish

8.2.2.1. Allow frozen products to thaw under controlled conditions to prevent decomposition, preferably under refrigeration overnight. Thaw frozen product under controlled conditions to prevent decomposition (preferably refrigerated overnight). Do not drain

8.2.2.2. Remove tissue of 12-15 animals from the shell, for most shellfish collect the entire shell contents. For scallops, separately collect the digestive gland, adductor muscles, gonad, etc. for analysis as stipulated by regulatory requirements.

8.2.2.3. Homogenize as per 8.1.2.4

8.2.3. Refrigerated/Frozen shucked ProductsRefrigerated Shucked Products

8.2.3.1. Refrigerated shucked products, such as clams, mussels, oysters, or scallops, use the sample as provided. – do not drain. Homogenize as per 8.2.1.3

8.2.3.2. Frozen product must be allowed to thaw under controlled conditions to prevent decomposition, preferably under refrigeration overnight. Homogenize as per 8.2.1.4. Use refrigerated shucked products as provided. Do not drain.

8.2.4. Frozen Shucked Product

8.2.4.1. Thaw frozen product under controlled conditions to prevent decomposition (preferably refrigerated overnight). Do not drain

8.2.4.2. Homogenized as per 8.2.1.4

8.2.5. Frozen breaded product

8.2.5.1. If frozen and breaded, thaw, remove breading, and homogenize as per 8.2.1.4

8.3. Extraction

8.3.1. Include the appropriate QA samples with each analytical run (generally a blank sample, spiked sample and a check sample).

8.3.2. Accurately weigh 5 g of homogenized material into 50 mL polypropylene tube and record weight.

8.3.3. Add 5 mL 0.1 M HCl and mix on a vortex mixer.

8.3.4. Check pH and adjust pH to between 2 and 4 using 5M HCl or NaOH if necessary

8.3.5. Cap tubes tightly and place in a boiling water bath for 5 minutes.

8.3.6. Allow tubes to cool to room temperature and check to ensure that the pH is between 2.0 and 4.0. If the pH must be lowered, then add 5 M HCl dropwise while stirring until the pH is <4.0. If the pH is adjusted to below 2, discard sample and start extraction again. If pH must be raised, add 5 M NaOH dropwise while stirring until the pH is between 2.0 and 4.0.

8.3.7. Centrifuge tubes at ~5000 RCF for 5 minutes

8.3.8. Pipette 500.0 µL of □upernatant into a microcentrifuge tube.

8.3.9. Add 25.0 µL of 30% (w/v) TCA and mix using a vortex mixer.

8.3.10. Centrifuge at ~16000 RCF for five minutes.

8.3.11. Add 30.0 µL 1.0 M NaOH and mix using a vortex mixer to neutralize TCA.

8.3.12. Centrifuge at ~16000 RCF for five minutes.

8.3.13. Filter through a 0.2 µm syringe filter into an LC autosampler vial. Divide sample into two LC autosampler vials if GTX & STX and C toxin analyses are being performed on separate instruments.

8.4. LC Conditions

8.4.1. GTX & STX Analysis Conditions

Mobile Phase
A – 11 mM Heptane sulphonate, 5.5 mM H_3PO_4, pH 7.1
B – 11 mm Heptane sulphonate, 16.5 mM H_3PO_4 in 11.5% MeCN, pH 7.1
Column Flow 0.8 mL/minute
Column oven temperature 40°C (see 8.9)
Detector Fluorescence
Excitation λ = 330nm
Emission λ = 390nm

8.4.2. C-Toxins Analysis Conditions

Mobile Phase
C – 2 mM tetrabutyl ammonium phosphate, pH 5.8
D – 2 mM tetrabutyl ammonium phosphate, pH 5.8 in 4% MeCN
Column Flow 0.8 mL/minute
Column oven temperature 15°C

Detector	Fluorescence Excitation λ = 330nm Emission λ = 390nm

8.4.3. Post-Column Reaction Module Conditions

Oxidant Flow Rate	0.4mL/minute
Acid Flow Rate	0.4mL/minute
Reactor Temp.	85°C
Reaction Coil	1mL (5m x 0.5mm)

8.5. Equilibrate the system for at least 20 minutes with 100% solvent A.

8.6. The toxins are separated using the following gradient conditions for the two groups of toxins. These gradient conditions are subject to modification to facilitate proper separation parameters.

Gradient

		Flow Rate (mL/min.)		
Time (min.	% Solvent B	LC	Oxidant	Acid
Gonyautoxin and Saxitoxins				
0	0	0.8	0.4	0.4
7.9	0	0.8	0.4	0.4
8	100	0.8	0.4	0.4
18.5	100	0.8	0.4	0.4
18.6	0	0.8	0.4	0.4
24	0	0.8	0.4	0.4
C-toxins				
0	0	0.8	0.4	0.4
8	0	0.8	0.4	0.4
15	100	0.8	0.4	0.4
16	100	0.8	0.4	0.4
19	0	0.8	0.4	0.4
24	0	0.8	0.4	0.4

8.7. For GTX, STX and C toxins calibrate the instrument with duplicate injections of the matrix fortified working standards.

8.8. Inject aliquots of mixed working solutions (10 µL for the GTX and STX toxins and 5 µL for the C toxins) into the system and separate chromatographically using the gradient conditions shown in 8.6 to ensure system suitability conditions (shown below) are met.

8.9. The step time in the gradient for GTX and STX (see 8.6) and/or column temperature may be altered to facilitate the resolution of GTX3, artefact, and GTX2 peaks. If the artefact peak and GTX2 co-elute, reduce the column temperature to achieve the desired resolution.

Toxins	Conditions
GTXs & STXs	• Artefact peak must be at least 70% baseline resolved between GTX3 and GTX2 • GTX5 must be at least 40% baseline resolved between dcGTX3 and dcGTX2 • dcSTX and STX must be at least 70% baseline resolved • GTX4 retention time must be between 5 and 7 minutes • STX retention time must be between 17 and 23 minutes
C toxins	• C2 must be at least 70% baseline resolved between C1 and C3 • C1 retention time must be between 4 and 7 minutes

8.10. For GTX and STX inject 10 µL of samples (including checks, spikes, blanks and repeats). Peaks are identified by comparison of retention times with recently run standards.

8.11. For C toxins inject 5 µL of samples (including checks, spikes, blanks and repeats). Peaks are identified by comparison of retention times with recently run standards.

8.12. Flush the system regularly to prevent build-up of salts. This should be done at least once a week, and always before long periods of instrument inactivity.

8.12.1. Remove the column from the LC system and flush the LC with DIW or 10% acetonitrile.

8.12.2. Both post-column pumps should be flushed with acid and then with DIW

9. CALCULATIONS

9.1. Using a single point calibration, measure peak areas of the standards

9.2. Measure the peak areas of the sample(s).

9.3. Calculate the contribution of each toxin to the overall toxicity using the following formula:

$$mgSTXeq/kg = \sum \left(uM \left({}^{372.2}/_{1000} \right) * \left({}^{Fvol}/_{Ext.vol} \right) * \left({}^{10}/_{Wt} \right) * \mathrm{Re}Tx \right)$$

Or $$mgSTXeq/kg = \sum \left(uM * \mathrm{Re}Tx * 8.33 \right)$$

Where :
- µM = Concentration in the extract
- Fvol = Final volume of the deproteinzed extract (560µL)
- Ext.vol = Volume of crude extract used (500µL)
- Wt= Weight of sample used
- ReTx= Toxicity of the analyte in relation to Saxitoxin from Table 1

To provide a total toxicity directly comparable to what would be obtained from the MBA when the laboratory uses the FDA STX standard a correction factor of 1.16 must be used with the result from the CFIA-PCOX. This is a result of the FDA STX standard having a nominal value of 100 µg/mL, when in fact that it is determined to be 86 µg/mL when analysed against the certified standard from NRC.

Table 1. Relative Toxicity Values

Toxin	ReTx	Toxin	ReTx
GTX1	0.9940	**NEO**	0.9243
GTX2	0.3592	**STX**	1.0000
GTX3	0.6379	**dcSTX**	0.5131
GTX4	0.7261	**C1**	0.0060
GTX5	0.0644	**C2**	.00963
dcGTX2	0.1538	**C3**	0.0133
dcGTX3	0.3766	**C4**	0.0576

9.4. Add the contributions of all of the individual toxins to obtain the overall toxin concentration for the sample in µg STX equivalents/100g

10. QA/QC CONSIDERATIONS

10.1. Representative chromatograms of the GTX and STX mixed working standards, an unspiked mussel sample and a spiked mussel sample run on the Agilent Zorbax Bonus RP, 4.6 x150 mm, 3.5 µ column are shown in Figure 1. Representative chromatograms of C toxin working standards, an unspiked mussel sample and a spiked mussel sample run on a Thermo BetaBasic 8, 4.6 x 250 mm, 5 µ column are shown in Figure 2.

10.2. Total toxicity spike recoveries (based on 5 determinations at 3 levels, two analysts) range from 94 to 106 %.

10.3. The reproducibility of the method I as determined from incurred material should be between 2 and 6 % at the regulatory limit of 80 ug STX·diHCl/100g.

10.4. LODs and LOQs for GTX, STX and C toxins are shown in Table 2.

Table 2. Estimated Limits of Detection and Quantitation for the individual PST in the validated matrices.

Toxin	Clams		Mussels		Scallops		Oysters	
	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ
GTX4	0.0120	0.036	0.0160	0.048	0.016	0.048	0.026	0.078
GTX1	0.0210	0.063	0.0240	0.072	0.024	0.072	0.037	0.111
dcGTX3	0.0025	0.008	0.0008	0.002	0.018	0.054	0.003	0.008
GTX5	0.0060	0.018	0.0032	0.010	0.007	0.021	0.008	0.024
dcGTX2	0.0070	0.021	0.0021	0.006	0.005	0.014	0.007	0.021
GTX3	0.0025	0.008	0.0012	0.004	0.003	0.008	0.003	0.009
GTX2	0.0310	0.093	0.0220	0.066	0.024	0.072	0.029	0.087
NEO	0.0250	0.075	0.0240	0.007	0.024	0.072	0.026	0.078
dcSTX	0.0096	0.029	0.0077	0.023	0.008	0.023	0.010	0.029
STX	0.0170	0.051	0.0130	0.039	0.013	0.039	0.014	0.042
C-1	0.0004	0.001	0.0002	0.001	0.001	0.003	0.000	0.001
C-2	0.0008	0.002	0.0008	0.002	0.001	0.004	0.009	0.028
Total	0.135	0.404	0.115	0.345	0.143	0.430	0.172	0.515

LOD = 3 xS/N
LOQ= 3 x LOD

10.5. Store GTX & STX CRMs and standards in a refrigerator at 4 ^{o}C when not in use. Stock solutions are stable for two months

10.6. Store C toxin CRMs and standards in a freezer at <-18 ^{o}C when not in use. Stock solutions are stable for two months

10.7. Working standards and matrix fortified standards should be prepared fresh monthly.

10.8. The final extracts may be stored for at least 2 weeks when stored in the refrigerator

10.9. Single point calibration is recommended however depending on the equipment or columns used; a multi-point calibration may be required. In such cases the linearity of the standard curve (r^2) must be greater than 0.95.

10.10. Monitor dcGTX3 for signs of a shoulder on the front of the peak, as this peak will sometimes split. Both the main peak and the front shoulder are dcGTX3.

10.11. The retention times of GTX1 and GTX4 are affected by the matrix, so matrix fortified standards must be used.

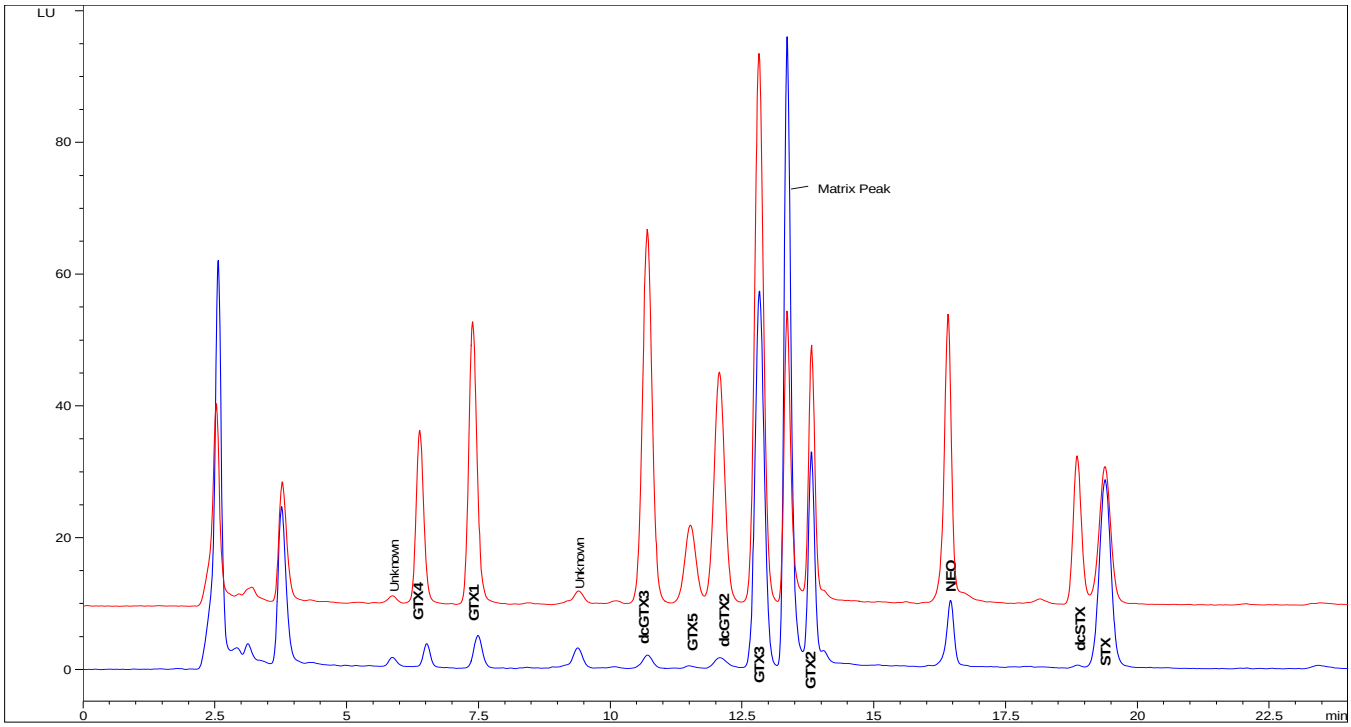


Figure 1. Overlaid chromatographic separation of the Gonyautoxins and Saxitoxins working standard (top) and an incurred mussel tissue (bottom) obtained by the CFIA-PCOX method of analysis.

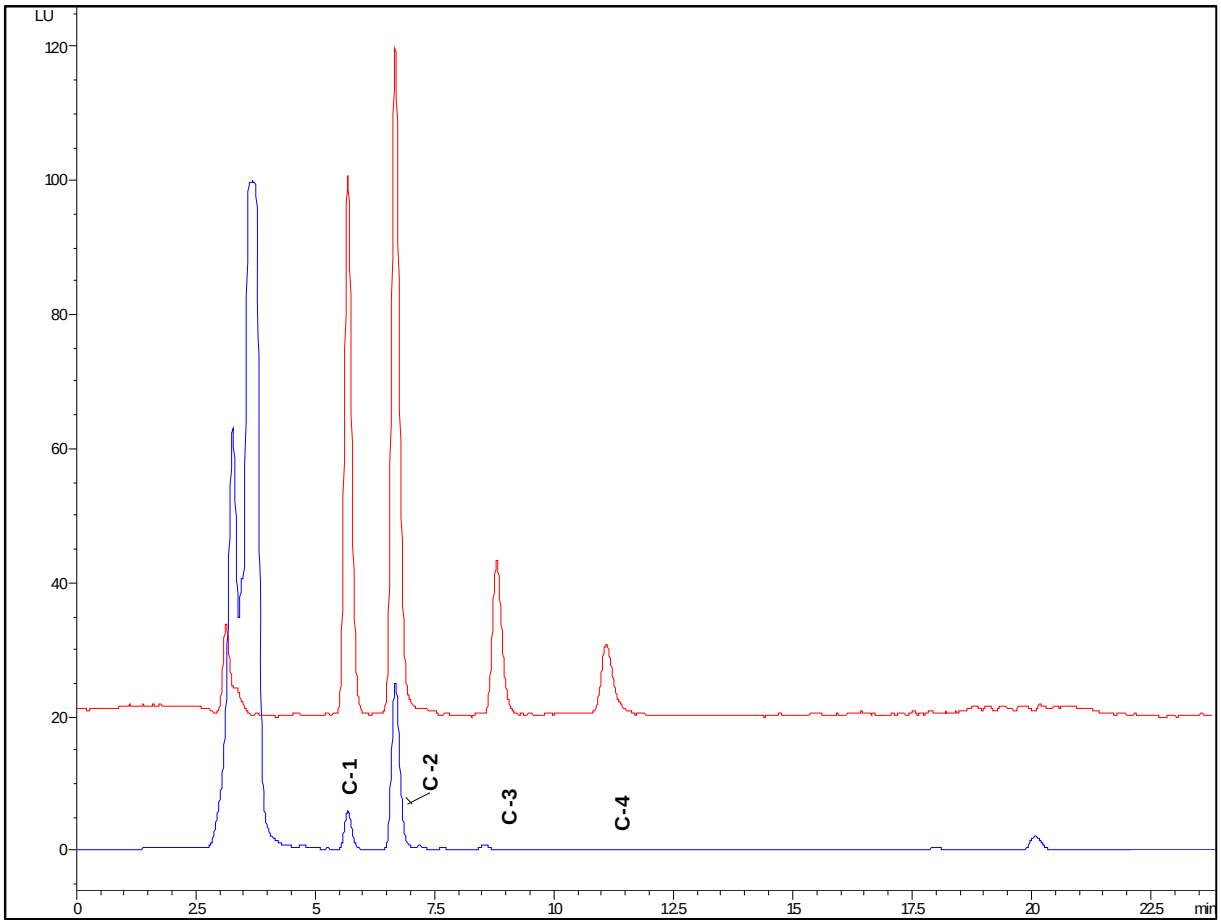


Figure 2. Overlaid chromatographic separation of the N-sulfocarbamoyl-gonyautoxins (C-toxins) working standard (top) and an incurred mussel tissue (bottom) obtained by the CFIA-PCOX method of analysis.

FOOD CHEMICAL CONTAMINANTS

Rapid Postcolumn Methodology for Determination of Paralytic Shellfish Toxins in Shellfish Tissue

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A rapid liquid chromatographic (LC) method with postcolumn oxidation and fluorescence detection (excitation 330 nm, emission 390 nm) for the determination of paralytic shellfish toxins (PSTs) in shellfish tissue has been developed. Extracts prepared for mouse bioassay (MBA) were treated with trichloroacetic acid to precipitate protein, centrifuged, and pH-adjusted for LC analysis. Saxitoxin (STX), neoSTX (NEO), decarbamoylSTX (dcSTX), and the gonyautoxins, GTX1, GTX2, GTX3, GTX4, GTX5, dcGTX2, and dcGTX3, were separated on a polar-linked alkyl reversed-phase column using a step gradient elution; the *N*-sulfocarbamoyl GTXs, C1, C2, C3, and C4, were determined on a C-8 reversed-phase column in the isocratic mode. Relative toxicities were used to determine STX-dihydrochloride salt (diHCl) equivalents (STXeq). Calibration graphs were linear for all toxins studied with STX showing a correlation coefficient of 0.999 and linearity between 0.18 and 5.9 ng STX-diHCl injected (equivalent to 3.9–128 μg STXeq/100 g in tissue). Detection limits for individual toxins ranged from 0.07 μg STXeq/100 g for C1 and C3 to 4.1 μg STXeq/100 g for GTX1. Spike recoveries ranged from 76 to 112% in mussel tissue. The relative standard deviation (RSD) of repeated injections of GTX and STX working standard solutions was <4%. Uncertainty of measurement at a level of 195 μg STXeq/100 g was 9%, and within-laboratory reproducibility expressed as RSD was 4.6% using the same material. Repeatability of a 65 μg STXeq/100 g sample was 3.0% RSD. Seventy-three samples were analyzed by the new postcolumn method and both AOAC Official Methods for PST determination: the MBA ($y = 1.22x + 13.99$, $r^2 = 0.86$) and the precolumn LC oxidation method of Lawrence ($y = 2.06x + 12.21$, $r^2 = 0.82$).

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Testing shellfish for the group of potent neurotoxins responsible for paralytic shellfish poisoning is critical for consumers and for the shellfish industry in general. Paralytic shellfish toxins (PSTs) accumulate in shellfish, and consumption of these shellfish can lead to serious illness and death. Monitoring programs are needed to determine when it is safe to harvest and consume shellfish. The PST group comprises more than 20 different naturally occurring analogs of saxitoxin (STX). The toxins can be subgrouped into 4 categories: the most toxic, carbamate group, which includes STX and neosaxitoxin (NEO); the decarbamoyl group; the deoxy-decarbamoyl group; and the least toxic, *N*-sulfocarbamoyl group. The individual toxin levels of these analogs are usually expressed as STX equivalents (STXeq) so that an overall toxicity of a sample may be calculated (1, 2) when chemical or biological tests other than the mouse bioassay (MBA) are used. The dihydrochloride salt of STX (STX-diHCl) is used as the standard for the MBA; therefore, the regulatory limit is actually 80 μg STX-diHCl equivalents per 100 g of whole tissue. Ensure that the proper units are used when comparing chemical test results to MBA results. All references to STXeq in this paper refer to the diHCl salt.

The MBA has been the regulatory method for over 50 years and is an Official Method of AOAC INTERNATIONAL (3). The MBA method currently serves as the reference method in the European Union (EU) with the EU council directive 91/492/EEC (4) stating that the total PST content must not exceed 80 μg STXeq/100 g tissue. The time from exposure to death is used in the MBA to estimate the amount of toxin present in shellfish, with a detection limit for the method at 40 μg STXeq/100 g. Although the MBA method has proved to be very reliable, there is international pressure to reduce or eliminate testing involving animals (5, 6). The MBA provides little toxin profile information, but has the advantage of reporting the total toxicity of the sample. This method also is subject to considerable variability (7). Alternative methods that could reduce or completely eliminate MBA testing for PSTs in a regulatory environment are becoming very desirable.

A number of different approaches have been investigated to replace the MBA as a regulatory tool, including biological assays (8–11), electrophoresis (12), chemosensors (13), and immunoassays (14, 15). The most common chemical method

Table 1. Relative PST toxicities and concentrations of reference, stock, and working standard solutions

Toxin	Mouse units (MU)/μmole	CRM, μM[a]	Stock standard solution, μM	Working standard solution, μM
GTX4	1803	35	8.3	0.66
GTX1	2468	106	25.2	2.0
dcGTX3	935	32	7.9	0.32
dcGTX2	382	114	28.1	1.1
GTX5	160	65	17.5	1.4
GTX3	1584	39	10.3	0.41
GTX2	892	118	31.0	1.2
NEO	2295	65	16.6	1.3
dcSTX	1274	62	16.1	0.64
STX	2483	65	15.9	0.64
C1	15	114	31.2	2.5
C2	239	35	9.5	0.76
C3	33	34	2.5	0.34
C4	143	27	0.76	0.27

[a] CRM = Certified Reference Material.

uses a combination of liquid chromatography (LC) with either pre- or postcolumn oxidation followed by fluorescence detection (FLD; 16–19). This instrumental technology can screen samples while providing detailed toxin profile information, now that a variety of calibration solutions are available (20). The LC-FLD method of Lawrence et al. has been the subject of a successful interlaboratory study (2) and collaborative study (21) and has been accepted by AOAC as the first analytical alternative to the MBA (22). Although it meets the major safety criteria of equivalency to the MBA, the Lawrence method suffers from several drawbacks when applied in a regulatory environment. The major impediment to widespread use of the Lawrence method is the amount of time required to process samples containing significant amounts of PSTs (23). The Lawrence method also cannot distinguish isomeric toxins that may exhibit significantly different toxicities. This study describes the modification of a postcolumn approach previously reported by Oshima (18) and Thomas et al. (19) to address these shortcomings.

The new postcolumn method performance was compared with the "gold standard" MBA as well as the Lawrence precolumn oxidation method. Fourteen of the most toxic and most commonly occurring PSTs were chosen for the study, including STX; NEO; decarbamoylsaxitoxin (dcSTX); gonyautoxin (GTX)-1,2,3,4,5; decarbamoylgonyautoxin (dcGTX)-2,3; and *N*-sulfocarbamoyl gonyautoxin (C)-1,2,3,4 to ensure that the majority of the toxin profiles could be addressed. This method was evaluated against a number of criteria essential to meeting the needs of a regulatory environment, including the practicality for regulatory work, equivalency of results to the MBA and/or the Lawrence method results, applicability to a variety of toxin profiles, reliability on a daily basis, cost, and ease of use. Instrument and analyst time were also considered as factors. The most important consideration in method acceptance for regulatory use was and continues to be the safety of the consumer. The method was applied to a variety of shellfish matrixes, containing numerous toxin profiles, collected throughout eastern Canada.

METHOD

Apparatus

(**a**) *LC system.*—Agilent 1200 quaternary solvent delivery system, autosampler equipped with 0.1–100 μL variable volume injector, column oven, column-switching valve, and data-handling module (Agilent Technologies, Kirkland, QU, Canada).

(**b**) *Postcolumn reaction system.*—Waters postcolumn reaction module capable of maintaining temperature at 85°C with reagents delivered by Waters Reagent Manager pumps (Waters, Milford, MA).

(**c**) *Reaction coil.*—Supelco knitted teflon tube with total volume of 1.0 mL (Sigma-Aldrich Canada, Oakville, ON, Canada).

(**d**) *Fluorescence detector.*—Agilent 1200 FLD operated at an excitation wavelength of 330 nm and an emission wavelength of 390 nm.

(**e**) *LC columns.*—(*1*) Agilent Zorbax Bonus RP, 4.6 × 150 mm, 3.5 μm; (*2*) Thermo BetaBasic 8, 4.6 × 250 mm, 5 μm (Fisher Scientific, Nepean, ON, Canada).

(**f**) *Centrifuge.*—Eppendorf 5415C equipped with F-45-18-11 rotor; maximum 16 000 × *g*.

Reagents

All solvents and reagents were analytical or LC grade materials. All mobile phase and postcolumn reagents were filtered through a 0.2 μm membrane before use.

(**a**) *Water.*—Glass-distilled or deionized (DIW).

(**b**) *DIW (pH 5.0).*—Acidify DIW to pH 5.0 by dropwise addition of 10% acetic acid (HOAc).

Table 2. Postcolumn LC system suitability conditions

Toxins	Conditions
GTXs and STXs	Artifact peak must be at least 70% baseline-resolved between GTX3 and GTX2
	GTX5 must be at least 40% baseline-resolved between dcGTX3 and dcGTX2
	dcSTX and STX must be at least 70% baseline-resolved
	GTX4 retention time must be between 5 and 7 min
	STX retention time must be between 17 and 23 min
C toxins	C2 must be at least 70% baseline-resolved between C1 and C3
	C1 retention time must be between 4 and 7 min

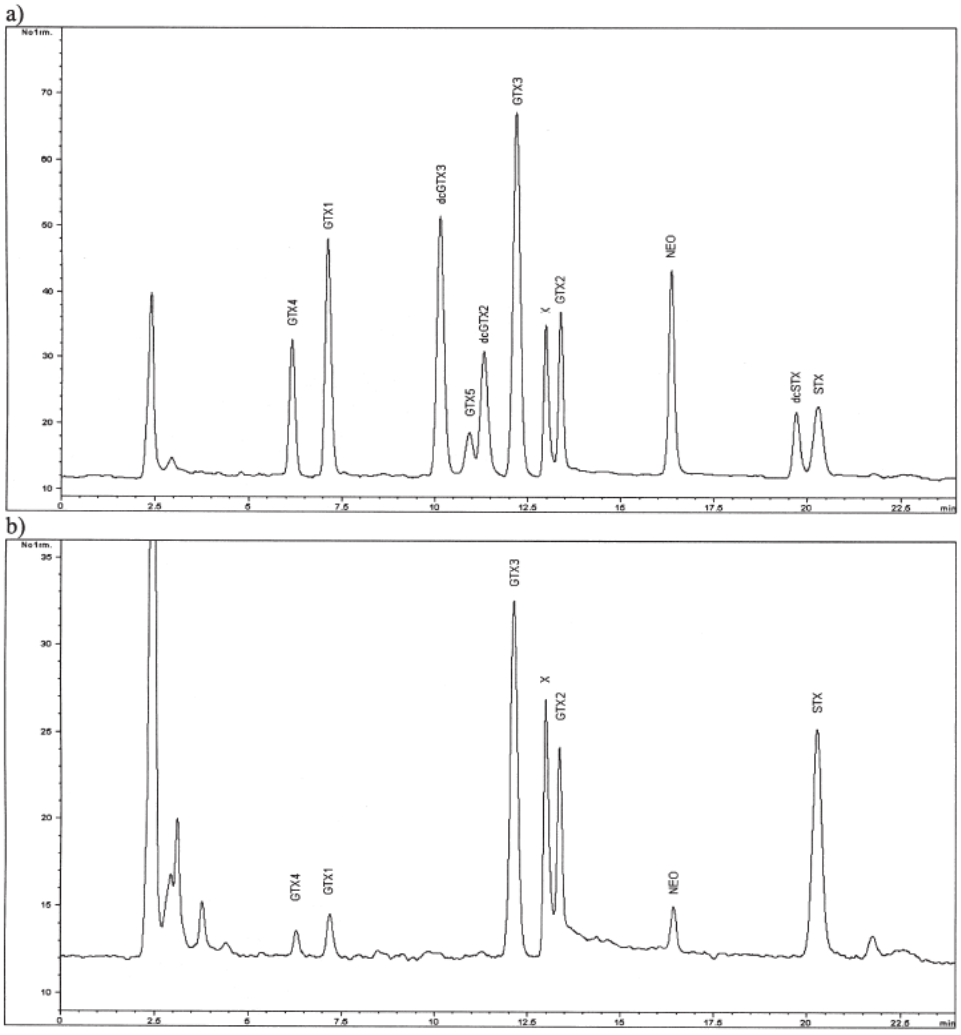

Figure 1. Chromatograms of (a) GTX and STX matrix-matched mixed working solution (10 μL) on an Agilent Zorbax Bonus RP (4.6 × 150 mm, 3.5 μm). Mobile phase: (A) 5.5 mM H_3PO_4, 11 mM heptane sulfonate, pH 7.1. (B) 16.5 mM H_3PO_4, 11 mM heptane sulfonate, pH 7.1 containing 11.5% MeCN. Gradient: 100% mobile phase A for 7.9 min; step to 100% mobile phase B at 8 min; hold for 10.5 min, step to 100% mobile phase A at 18.6 min, 0.8 mL/min. Ox = 5 mM H_5IO_6, 100 mM H_3PO_4, pH 7.8, 0.4 mL/min; H+ = 0.75 M HNO_3, 0.4 mL/min. (b) Mussel sample containing 119 μg STXeq/100 g GTX and STX toxins, conditions as above. In both chromatograms, the artifact peak is labeled "X."

(c) *LC mobile phases (GTXs and STXs).—Solvent A.*—11 mM heptane sulfonate, 5.5 mM phosphoric acid (H_3PO_4) aqueous solution adjusted to pH 7.1 with ammonium hydroxide (NH_4OH). *Solvent B.*—11 mM heptane sulfonate, 16.5 mM H_3PO_4, 11.5% acetonitrile (MeCN) aqueous solution adjusted to pH 7.1 with NH_4OH.

(d) *LC mobile phase (C toxins).*—2 mM tetrabutyl ammonium phosphate aqueous solution adjusted to pH 5.8 using 10% HOAc if too basic or 1% NH_4OH if too acidic. The pH must only be adjusted in one direction, and if the pH is overshot the solution must be remade.

(e) *Postcolumn oxidant.*—100 mM H_3PO_4, 5 mM periodic acid (H_5IO_6) aqueous solution adjusted to pH 7.8 with 5 M sodium hydroxide (NaOH).

(f) *Postcolumn acid.*—0.75 M nitric acid (HNO_3).

(g) *Primary standards.*—National Research Council Canada (NRC) Certified Reference Materials (CRMs) for C1, C2, dcGTX2, dcGTX3, dcSTX, GTX1, GTX2, GTX3, GTX4, GTX5, NEO, and STX; NRC in-house reference materials for C3 and C4 (NRC Institute for Marine Biosciences, Halifax, NS, Canada). These CRMs were used as supplied by the NRC. The lack of a specific salt does not imply the free-base form of

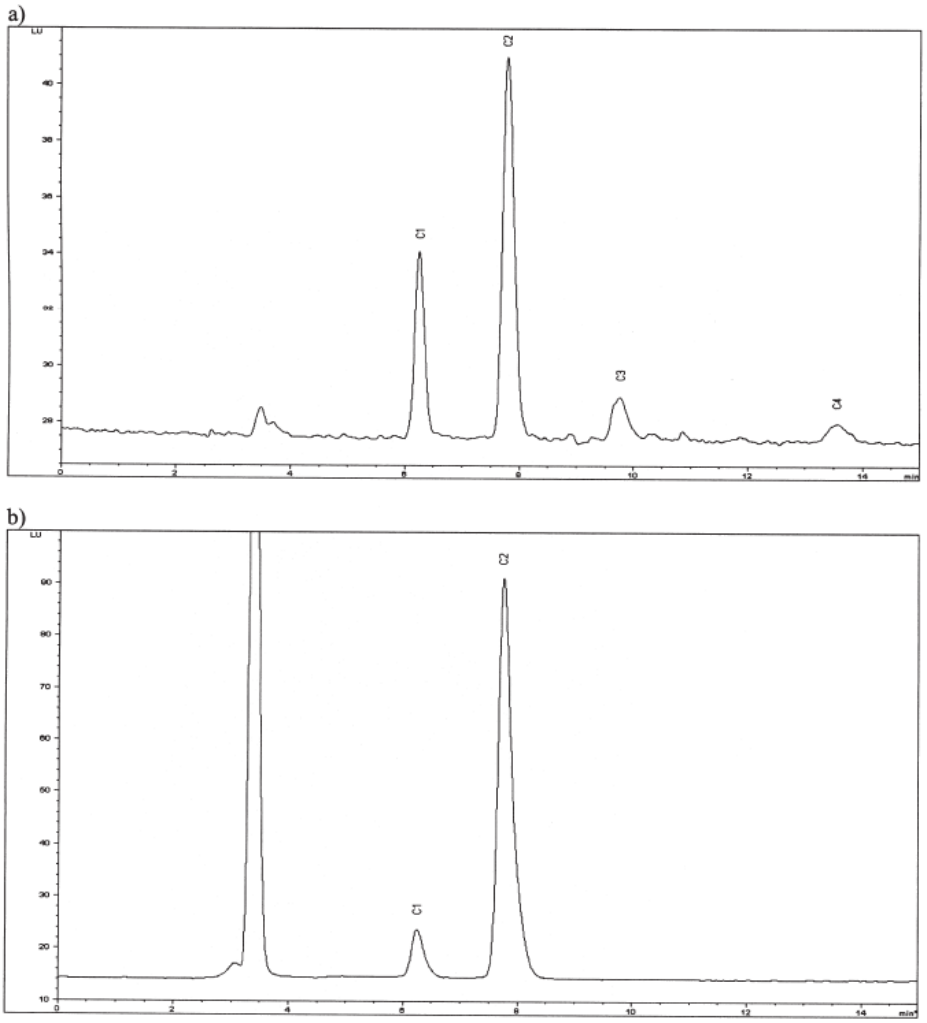

Figure 2. Chromatograms of (a) C toxin mixed working solution (5 μL) on a Thermo BetaBasic 8 (4.6 × 250 mm, 5 μm). Mobile phase (isocratic): 2 mM tetrabutyl ammonium phosphate, pH 5.8, at 0.8 mL/min. Ox = 5 mM H_5IO_6, 100 mM H_3PO_4, pH 7.8, 0.4 mL/min; H+ = 0.75 M HNO_3, 0.4 mL/min. (b) Mussel sample containing 51.4 μg STXeq/100 g C toxins, conditions as above.

toxins in the preceding list, but is simply a list of the toxins used. The NRC has only one form of each toxin available. STX for standardization of MBA was obtained from the U.S. Food and Drug Administration (FDA).

(**h**) *Stock solutions (0.76–31 μM; see Table 1).*—Prepare individual stock standards gravimetrically as per NRC instructions (24). Perform dilutions with 0.003 M HCl for the GTXs and STXs and DIW (pH 5.0) for C toxins.

(**i**) *Neat mixed working solutions (0.269–2.496 μM; see Table 1).*—Prepare 2 solutions, the first containing dcGTX2, dcGTX3, dcSTX, GTX1, GTX2, GTX3, GTX4, GTX5, NEO, and STX, and the second containing C1, C2, C3, and C4 (Table 1). Perform dilutions with 0.003 M HCl for the GTXs and STXs and DIW (pH 5.0) for C toxins.

(**j**) *Matrix-matched mixed working solutions.*—Follow the instructions for the neat mixed working solutions but dilute matrix-matched working solutions using a toxin-free mussel extract as the diluent.

Sampling

Samples of shellstock collected during the summer of 2005 as part of the toxin monitoring program of the Canadian Food Inspection Agency, Dartmouth, NS, Canada, were used in this study. The majority of the samples were collected from coastal regions of New Brunswick, Canada; Nova Scotia, Canada; and Prince Edward Island, Canada but also included offshore and imported products. Samples consisted mainly of mussels (*Mytilus edilus*) and clams (*Mya arenaria*) but included a small number of other species such as scallops and oysters. Samples were shucked and analyzed by MBA on receipt. AOAC MBA extracts were stored at 4°C prior to postcolumn LC analysis, and

Table 3. Method performance statistics for the new postcolumn method and the Lawrence method as applied in the authors' laboratory

Toxins	Lawrence method LOD, μg STXeq/100 g	New postcolumn method LOD, μg STXeq/100 g	New postcolumn method spike recovery, % ± SD[a,b]
GTX4	2.8	1.6	99 ± 13
GTX1	2.8	4.1	112 ± 7
dcGTX3	0.98	0.25	101 ± 8
dcGTX2	0.98	0.67	100 ± 4
GTX5	1.5	0.90	98 ± 5
GTX3	0.80	0.38	102 ± 2
GTX2	0.80	1.5	76 ± 5
NEO	2.8	2.3	106 ± 6
dcSTX	2.0	2.1	102 ± 2
STX	3.0	3.9	100 ± 3
C1	0.002	0.07	100 ± 2
C2	0.002	0.15	95 ± 3
C3	0.05	0.07	NA
C4	0.05	0.41	NA

[a] Average of 5 replicate analyses.

[b] Spiked at approximately 3 × LOD for each toxin.

tissue homogenate was stored at –20°C prior to precolumn LC analysis.

Sample Extraction and Cleanup

Thoroughly clean the outside of the shellfish with fresh water. Shuck the samples onto a No. 10 sieve and drain for 5 min. Homogenize the soft tissue in a standard household blender in preparation for extraction. Prepare a sufficient amount of tissue for MBA, LC-FLD precolumn and postcolumn analyses.

Postcolumn LC-FLD and MBA.—Extract 100 g samples of homogenized shellfish tissue according to the AOAC MBA method (3) using 0.1 M HCl. Store aliquots of the extract in scintillation vials for later injections into mice or for further cleanup and LC postcolumn analysis. Deproteinate samples destined for postcolumn FLD analysis by adding 25 μL 30% (w/v) trichloroacetic acid (TCA) to 500 μL shellfish extract in a microcentrifuge tube. Mix in a Vortex mixer and centrifuge at 16 000 × *g* for 5 min. Add 20 μL 1.0 M NaOH, mix, and centrifuge at 16 000 × *g* for 5 min. Filter through 0.2 μm syringe filter into an autosampler vial in preparation for LC analysis.

Precolumn oxidation LC-FLD.—Extract 5 g homogenized shellfish tissue with 1% HOAc, boil for 5 min, and clean up using C18 solid-phase extraction (SPE) and COOH SPE cartridges according to the method of Lawrence (22) in preparation for LC analysis. Apply the method for "Application of the Method for Routine Analysis" as described by Lawrence (Lawrence screen; 22). If toxins are detected, continue with the full Lawrence method.

LC Postcolumn Determinations

GTX and STX toxins.—Equilibrate the LC system for ≥20 min at a column oven temperature of 40°C with 100% solvent A flowing at 0.8 mL/min. Construct a step gradient as follows: 100% solvent A for 7.9 min; step to 100% solvent B at 8 min; hold for 10.5 min; step to 100% A at 18.6 min; equilibrate for 5.4 min.

C toxins.—Equilibrate the LC system for ≥20 min at a column oven temperature of 20°C with mobile phase flowing at 0.8 mL/min. Operate the system in the isocratic mode.

Postcolumn reaction module.—Oxidant flow rate, 0.4 mL/min; acid flow rate, 0.4 mL/min; reaction oven temperature, 85°C; reaction coil, 5 m × 0.50 mm id.

Inject mixed working solutions (10 μL for GTX and STX toxins and 5 μL for C toxins) to ensure that system suitability conditions (Table 2) are met, and construct a linear regression curve of peak area vs concentration in μM. Inject 10 μL sample extracts, blanks, and spikes for GTX and STX toxins, and 5 μL sample extracts, blanks, and spikes for the C toxins. Calculate the μmoles of STXeq for each toxin in the sample extracts using the linear regression of the calibration graph and the specific relative toxicities of each individual PST (Table 1). For comparison to MBA results, use the following equation to calculate the toxicity in the traditional units of "μg STXeq per 100 g tissue" in the specific case of 0.1 kg tissue being extracted with 0.1 L solvent in a single-step dispersive extraction (final volume = 0.2 L):

Table 4. Percent relative standard deviation (% RSD) of retention time (RT) and instrument response of repeated injections of PST standard solutions determined by this method

Toxin (μg STXeq/100 g)	RT (% RSD)	Peak area (% RSD)
GTX4 (20.69)	0.11[a]	2.2[a]
GTX1 (85.79)	0.16[a]	1.2[a]
dcGTX3 (4.98)	0.23[a]	3.1[a]
dcGTX2 (7.25)	0.18[a]	2.8[a]
GTX5 (3.45)	0.19[a]	2.4[a]
GTX3 (9.37)	0.07[a]	1.5[a]
GTX2 (15.97)	0.03[a]	1.5[a]
NEO (47.79)	0.24[a]	2.8[a]
dcSTX (13.07)	0.45[a]	1.8[a]
STX (25.19)	0.49[a]	1.8[a]
C1 (0.56)	0.46[b]	7.3[b]
C2 (2.72)	0.85[b]	4.5[b]
C3 (0.17)	1.8[b]	15[b]
C4 (0.58)	2.4[b]	11[b]

[a] Average of five 10 μL injections.
[b] Average of five 5 μL injections.

$$\text{Sample toxicity (μg STXeq/100 g)} = \sum_{i=1}^{n} C_i \times T_i \times \frac{0.20}{0.10} \times \frac{372.2}{2483} \times F \times 0.1$$

This is simplified to:

$$\text{Sample toxicity (μg STXeq/100 g)} = \sum_{i=1}^{n} C_i \times T_i \times F \times 0.03$$

where C_i = concentration of each toxin "i" in micromoles per liter (μM); T_i = specific toxicity of each toxin "i" in mouse units per micromole (MU/μmole); F = 1.16 for MBA data calibrated against the FDA STX solution (if the MBA was calibrated against the NRC standard, a value of F = 1.00 would be used); 372.2 = molecular weight of STXdiHCl (g/mole).

This F factor of 1.16 must be applied when comparing data calibrated against the NRC STX CRM with the MBA data, which has been calibrated against the FDA STX standard (100 μg/mL stated concentration). A concentration of 86 μg STX-diHCl/mL is observed for the FDA STX standard when calibrated using the NRC STX CRM (1).

LC Precolumn Determinations

Inject 50 μL cleaned-up extract and the periodate oxidation of the cleaned-up extract onto a Supelcosil LC-18-DB, 4.6 × 15 cm, 5 μm column as described by Lawrence (22). If toxins are detected, inject periodate and/or peroxide oxidations of required fractions according to Lawrence (22). Quantify each toxin by direct comparison to analytical standards. Calculate the amount of PSTs present as μg STXeq/100 g sample using the PST relative toxicity values as described by Lawrence (22) in order to compare to the MBA. Calculate total toxicity by summing the individual toxin contributions. Apply factor of 1.16 as in postcolumn determinations for comparison with MBA data.

MBA Determinations

Inject 17–23 g mice intraperitoneally with 1 mL HCl extract according to the AOAC Official Method **959.08** (3) and record death times. Calculate the amount of PSTs present as μg STXeq/100 g sample using Sommer's Table (3).

Results and Discussion

A new postcolumn method for the determination of PSTs was developed and compared to AOAC Official Methods for PST determination. Oshima's postcolumn method (18) required 3 injections to quantify the 14 toxins included in this study. The number of injections was decreased to 2 by Thomas et al. (19), but the separation of GTXs and STXs took 60 min, and used a trinary mobile phase system. The GTX and STX toxin method was improved by consolidating the trinary mobile phase system into a binary step gradient, which allowed a decreased run time of 24 min. All GTX and STX toxins studied were baseline-resolved with the exception of GTX5, which was 50% baseline-resolved (Figure 1). The C toxins were baseline-resolved and quantified in <15 min (Figure 2) in an isocratic system very similar to that described by Oshima (18). Differences between the new postcolumn method for C toxin determination and Oshima's method (18) include a different cleanup procedure, a different concentration of tetrabutyl ammonium phosphate, a different LC column, and different oxidation conditions. This study included 14 currently available commercial standards. An additional standard, decarbamoylneosaxitoxin (dcNEO), was not included at this time due to co-elution with NEO under the rapid separation system. It is possible to resolve dcNEO and NEO with a 75 min trinary step gradient (19). The oxidation products of dcNEO co-elute with the oxidation products of both dcSTX and STX when the Lawrence method is used (B. Niedzwiadek, Health Canada, Ottawa, ON, Canada, personal communication, 2006). From a regulatory perspective, this was not a major issue in the postcolumn method, as the relative toxicity of dcNEO is less than that of NEO. The worst case scenario would be a slight overestimation of total toxicity, further protecting the consumer. Gonyautoxin-6 (GTX6) was not included in this study due to the lack of standard availability, but elutes

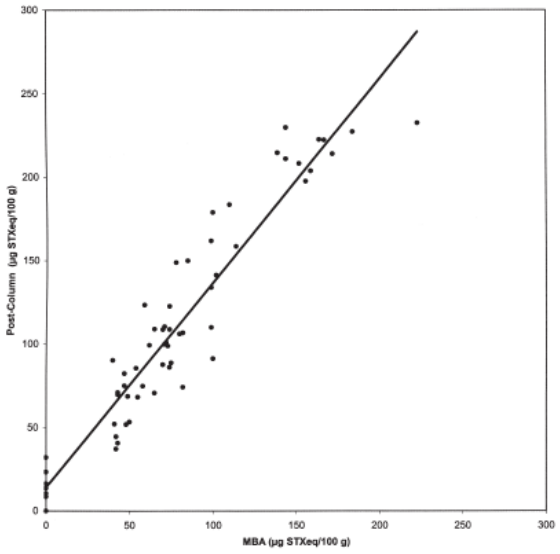

Figure 3. Correlation between results of the MBA method and the new postcolumn method for samples up to 250 µg STXeq/100 g; $y = 1.22x + 13.99$; $r^2 = 0.86$.

immediately before GTX4 under the described chromatographic conditions.

PSTs were extracted using the AOAC MBA method (3) for postcolumn LC analysis; therefore, the toxin profile quantified using the postcolumn method was very similar to that injected into the mouse. Protein remaining in the AOAC MBA extract can be trapped on column frits, leading to the rapid development of backpressure, an attendant decrease in column performance, and possible damage to LC pumping systems. TCA was used to remove protein from the AOAC MBA extract and, in so doing, extended column life. Some concern was expressed that the use of TCA might change the toxin profile even though the pH was returned to its original level quickly. No differences were observed in the toxin profiles following treatment with TCA. However, treatment with TCA increased column life so that approximately 600 samples could be analyzed before significant deterioration of the column was observed. Without TCA treatment, column deterioration is evident after approximately 100 samples have been analyzed.

The LC system performed reliably and was simply shut down at the end of each daily run. No problems were associated with start-up the next day. The postcolumn system (pumps and reaction coil) was flushed once a week with 0.75 M HNO_3 followed by DIW. As a precaution, the column was removed from the LC and the entire fluid path was flushed with 10% MeCN in DIW to prevent line blockage due to the precipitation of buffers. If the system is to be shut down for extended periods, it is recommended that the pumps are not left in the harsh acid or oxidant environment. Following these maintenance procedures, no problems were experienced other than the very minor difficulties that are typically encountered with modern LC pumping systems.

The maximum sample throughput of the new postcolumn method and the Lawrence method was compared because of its importance in a regulatory environment. A single LC system could analyze 31 samples per 24 h period with the postcolumn method, including attendant standards and quality assurance samples. In those situations where the Lawrence screen could be used, approximately 40 samples could be processed in a 24 h period. However, if positive samples are encountered, as is the case in our laboratory where approximately 30% of samples received are positive for PSTs, a combination of the Lawrence screen and full methodologies is required. Using a combination of full and screen methodologies allows only an average of 16 samples to be processed each day. In addition, results from those samples requiring the full method will be delayed up to a further 24 h while the COOH SPE fractions are prepared and oxidized prior to LC analysis. This is a major limitation of the Lawrence methodology in a regulatory environment (23).

Limits of detection (LODs) of the new postcolumn method and the Lawrence method are shown in Table 3. LODs ranged from 0.07 µg STXeq/100 g for C3 to 4.1 µg STXeq/100 g for GTX1 for the postcolumn method. This compared quite favorably with detection limits for the Lawrence method, which ranged from 0.002 µg STXeq/100 g for C1, C2 to 3.0 µg STXeq/100 g for STX as applied in our laboratory. Adequate detection capability for regulatory purposes was supplied by both LC methods. A spiking study near the limit of quantitation for individual toxins demonstrated that the new postcolumn method recovered between 76% (GTX2) and 112% (GTX1) of toxins (Table 3). No spiking data are currently available for C3 or C4 due to the limited supply of standards, but these recoveries are expected to fall within the range of recoveries for other toxins examined.

A calibration graph for STX was linear between 0.18 and 5.9 ng STX injected, which was equivalent to 3.9–128 µg STXeq/100 g in tissue. Calibration graphs for other toxins showed very similar results. The correlation coefficients of the calibration graphs for all toxins ranged from 0.999 to 1.00. Stock and working solutions of GTXs and STXs were stored at 4°C; stock and working solutions for C toxins were stored at ≤–20°C. Standard solutions have been stored for >12 months with no noticeable deterioration.

Working standards were prepared using a mussel tissue extract to assist in the identification of toxins present in the samples, as matrixes caused a slight positive retention time shift for GTX4 and GTX1 in the new postcolumn method. Exact matrix matching of standards was not required for any matrixes studied, including various species of mussels, clams, scallops, and oysters. Matrix-matched standards assisted in resolving interfering peaks, as most samples have an artifact peak (Figure 1, peak X) corresponding to the step gradient solvent front. This artifact peak did not contain any toxins included in this study and was generally well resolved, but over time may co-elute with GTX3 or GTX2. It was found

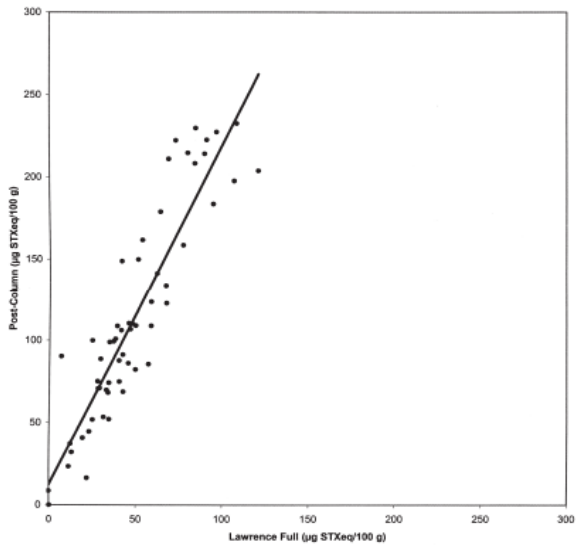

Figure 4. Correlation between results of the full Lawrence method and the new postcolumn method for samples up to 250 µg STXeq/100 g; $y = 2.06x + 12.21$; $r^2 = 0.82$.

that a temperature adjustment of <±5°C easily resolved all 3 peaks with no significant impact on overall run time or separation of other toxins. This adjustment may be effective for up to several weeks, depending on column usage. The first injection each day should contain GTX2, GTX3, and the artifact peak (matrix standard or check sample), and this injection will be used to adjust the column temperature to meet system suitability criteria. The elution conditions (gradient step time, column temperature) must be confirmed each time a new column is used, and after this only small changes to column temperature should be needed. No unresolvable interferences were observed in any of the mussel, clam, oyster, or scallop samples tested. Retention times were stable; the relative standard deviation (RSD) varied from 0.03 to 2.4% (Table 4). Replicate injections of standard and tissue extract solutions indicated good peak response repeatability over the range of concentrations studied with RSDs ranging from 1.2 to a maximum of 15% (Table 4). Quantification was based on peak areas. The method showed good within-laboratory reproducibility; a mussel tissue extract containing 195 µg STXeq/100 g analyzed over 21 days showed an RSD of only 4.6%. The uncertainty of measurement based on precision data for the same mussel tissue extract was 9%. Repeatability RSD of a 65 µg STXeq/100 g mussel tissue analyzed 5 times was 3.0%.

The MBA has a long successful history of preventing consumer illnesses and deaths. Therefore, equivalency to the MBA is essential. More than 50 positive shellfish samples with MBA results between 40 and 223 µg STXeq/100 g were analyzed by MBA, pre- and postcolumn methods. The MBA results were plotted against the postcolumn results in Figure 3; the slope was 1.22 and the correlation coefficient was 0.86. It was expected that the postcolumn results would be slightly higher than the MBA results. It has been reported widely that salt effects lead to an underestimation of the toxicity of shellfish especially with samples near the MBA detection limit (7, 18, 25). The vast majority of samples with MBA results near the regulatory limit show very similar postcolumn results.

The Lawrence method has been approved by AOAC as the first official LC method for PSTs (22). The comparison of MBA and Lawrence screen results exhibited a slope of 0.79 and a correlation coefficient of 0.36. Although the correlation was poor, samples with higher MBA values generally produced higher values in the Lawrence screen method. This points out the necessity of running the full Lawrence method when PSTs are detected if accurate results are to be obtained. The MBA is known to have a large variation (17, 26), due in large part to the fact that it uses a biological system. It was expected that the results from the pre- and postcolumn methods would be quite comparable since neither method uses a biological system. Figure 4 compares the full Lawrence method and the postcolumn method results. A slope of 2.06 indicates that the postcolumn results were approximately 50% higher than the results of the full Lawrence method but the correlation coefficient was good (0.82). Lawrence and Menard (27) initially noted this trend of postcolumn methods producing higher results than precolumn methods. Experiments carried out to determine where toxicity might be lost while using the Lawrence method highlighted 3 stages for potential toxin loss. Standard solutions and positive samples were extracted using the Lawrence method, and monitored at various stages using the new postcolumn LC system. In our laboratory, approximately 7% of the total toxicity was lost during C18 SPE cartridge cleanup, 11% was lost to the pH adjustment after the C18 SPE, and an additional 11% was lost during the COOH SPE cleanup. These losses totaled 29% of overall toxicity, resulting from the full Lawrence cleanup procedure. Correcting for these losses provided a simple solution and provided a corrected slope of 1.4 with the new postcolumn data. There is also an expected difference due to different extractant acids. The HOAc extraction used by the Lawrence method is milder than the HCl extraction used by the AOAC MBA method and is not subject to the Proctor enhancement, which converts *N*-sulfocarbamoyl toxins to the more toxic carbamate forms (28).

Both LC methods were compared in our laboratory to determine the pros and cons of each method in a regulatory environment. The positive aspects of the postcolumn method were easier interpretation of data, separation of all analytes tested, and faster turnaround times for positive samples (31 versus 16 samples/day/LC system assuming a 30% positive rate). The precolumn advantages were excellent chromatographic performance, faster turnaround time when most samples tested negative for PSTs, and no postcolumn system required. One concern with the Lawrence method is the possibility of a single sample accidentally not being

oxidized; a sample would be reported as a false negative if it was not oxidized. Caution must be exercised to ensure that the proper volumes and reagents have been added to each vial before LC injection. Although the postcolumn equipment has a few additional moving parts which may fail in day-to-day operation, postcolumn system failure is very obvious, as all standards, spikes, and control samples would also be affected. The total analysis cost (capital purchases and consumables) for the new postcolumn method was less than that of MBA analysis if capital costs are depreciated over 7 years. The Lawrence screen cost approximately the same as MBA analysis and the full Lawrence method was nearly triple the cost of MBA analysis, due to increased consumable costs (SPE cartridges, filters).

Both the pre- and postcolumn methods have demonstrated that they are viable alternatives to MBA analysis. These LC methods effectively measured the toxin content in shellfish tissue containing a variety of toxin profiles. The main advantages of the new postcolumn method in a regulatory setting were higher throughput and faster turnaround of positive samples. The speed of analysis provided by this method is essential in a regulatory environment where decisions are required on a timely basis.

Future work will concentrate on running the new postcolumn method in parallel with the MBA over one shellfish season to ensure that the method is robust, reliable, and accurate and can be counted upon to protect the health and safety of consumers. Approximately 1000 samples have been analyzed concurrently with no significant problems. Validation data for additional toxins will be generated when standards become available, and alternate extraction methods which may reduce turnaround time will be evaluated.

Acknowledgments

We are grateful to Nancy Peacock, Shelly Hancock, and Myrna Gillis (Canadian Food Inspection Agency, Dartmouth, NS, Canada) for their help with the project, including performing MBA analyses and providing AOAC MBA extracts for LC analysis.

References

(1) Quilliam, M.A. (2004) in *Supplemental Information for PSP CRMs,* NRC Certified Reference Materials Program, National Research Council, Institute for Marine Biosciences, Halifax, NS, Canada, pp 1–5

(2) Lawrence, J.F., Niedzwiadek, B., & Menard, C. (2004) *J. AOAC Int.* **87**, 83–100

(3) *Official Methods of Analysis* (2000) 17th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **959.08**

(4) Council of the European Community (1991) *Off. J. Eur. Commun.* **L268**, 1–14

(5) Balls, M.B., Goldburg, A.M., Fentem, J.H., Broadhead, C.L., Bursch, R.L., Festing, M.F.W., Frazier, J.M., Hendrickson, C.F.M., Jennings, M., van der Kamp, M.D.O., Morton, D.B., Rowan, A.N., Russell, C., Russel, W.M.S., Spielman, H., Stephens, M.L., Stokes, W.S., Straughan, D.W., Yager, J.D., Zurlo, J., & van Zutphen, B.F.M. (1995) *The Report and Recommendation of ECVAM Workshop 11, ATLA* **23**, 883–866

(6) Hess, P., Grune, B., Anderson, D.B., Aune, T., Botana, L.M., Caricato, P., van Egmond, H.P., Halder, M., Hall, S., Lawrence, J.F., Moffat, C., Poletti, R., Richmond, J., Rossini, G.P., Seamer, C., & Vilageliu, J.S. (2006) *The Report and Recommendations of a Joint ECVAM/DG SANCO Workshop (ECVAM Workshop 55), ATLA* **34**, 193–224

(7) Park, D.L., Adams, W.N., Graham, S.L., & Jackson, R.C. (1986) *J. AOAC Int.* **69**, 547–550

(8) Jellett, J.F., Doucette, L.I., & Belland, E.R. (1998) *J. Shellfish Res.* **17**, 1653–1655

(9) Louzao, M.C., Vieytes, M.R., Cabado, A.G., Baptista de Sousa, J.M.V., & Botana, L.M. (2003) *Chem. Res. Toxicol.* **16**, 434–437

(10) David, L.S., & Nicholson, R.A. (2004) *Chemosphere* **55**, 1315–1321

(11) Manger, R.L., Leja, L.S, Lee, S.Y., & Hungerford, J.M. (2003) *J. AOAC Int.* **86**, 540–543

(12) Thibault, P., Pleasance, S., & Laycock, M.V. (1991) *J. Chromatogr.* **542**, 483–501

(13) Gawley, R.E., Pinet, S., Cardona, C.M., Datta, P.K., Ren, T., Guida, W.C., Nydick, J., & LeBlanc, R.M. (2002) *J. Am. Chem. Soc.* **124**, 13448–13453

(14) Usleber, E., Dietrich, R., Burk, C., Schneider, E., & Martlbauer, E. (2001) *J. AOAC Int.* **84**, 1649–1656

(15) Jellett, J.F., Roberts, R.L., Laycock, M.V., Quilliam, M.A., & Barrett, R.E. (2002) *Toxicon* **40**, 1407–1425

(16) Lawrence, J.F., & Niedzwiadek, B. (2001) *J. AOAC Int.* **84**, 1099–1108

(17) Sullivan, J.J., Wekell, M.M., & Kentala, L.L. (1985) *J. Food Sci.* **50**, 26–29

(18) Oshima, Y. (1995) *J. AOAC Int.* **78**, 528–532

(19) Thomas, K., Chung, S., Ku, J., Reeves, K., & Quilliam, M.A. (2006) in *Molluscan Shellfish Safety*, K. Henshilwood, B. Deegan, T. McMahon, C. Cusack, S. Keaveney, J. Silke, M. O'Cinneide, D. Lyons, & P. Hess (Eds), The Marine Institute, Galway, Ireland, pp 132–138

(20) Quilliam, M.A. (2006) in *Molluscan Shellfish Safety*, K. Henshilwood, B. Deegan, T. McMahon, C. Cusack, S. Keaveney, J. Silke, M. O'Cinneide, D. Lyons, & P. Hess (Eds), The Marine Institute, Galway, Ireland, pp 139–144

(21) Lawrence, J.F., Niedzwiadek, B., & Menard, C. (2005) *J. AOAC Int.* **88**, 1714–1732

(22) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **2005.06**

(23) Ben-Gigirey, B., Rodriguez-Velasco, M.L., Villar-Gonzalez, A., & Botana, L.M. (2007) *J. Chromatogr. A* **1140**, 78–87

(24) *Certificate of Analysis* (2006) National Research Council, Institute for Marine Biosciences, Halifax, NS, Canada

(25) Asp, T.N., Larsen, S., & Aune, T. (2004) *Toxicon* **43**, 319–327

(26) Holtrop, G., Petrie, J., McElhiney, J., & Dennison, N. (2006) *Toxicon* **47**, 336–347

(27) Lawrence, J.F., & Menard, C. (1991) *J. AOAC Int.* **74**, 1006–1012

(28) Hall, S., Reichardt, P.B., & Neve, R.A. (1980) *Biochem. Biophys. Res. Commun.* **97**, 649–653

Proposal Subject: Domoic Acid Test Kit

Specific NSSP Guide Reference: Section IV. Guidance Documents, Chapter II. Growing Areas .10 Approved National Shellfish Sanitation Program Laboratory Tests: Microbiological and Biotoxin Analytical Methods.

Text of Proposal/ Requested Action

Mercury Science Inc., in collaboration with the NOAA Center for Coastal Fisheries and Habitat Research has developed a new quantitative immunoassay for the detection of domoic acid. The assay has been commercialized and is currently sold for research use as the Domoic Acid Test Kit (product # DAK-36) (Information online at http://mercuryscience.com/DA).

This product underwent thorough testing by Mercury Science to define the performance characteristics of the assay prior to commercialization. In addition, the product has been independently validated in several labs in a variety of matrices. The results of these internal and external validation studies strongly suggest that the Domoic Acid Test Kit is a rapid, low-cost, and accurate method for analysis of food, water and phytoplankton samples.

At this time, Mercury Science would like to submit a partially complete Method Application to the ISSC Laboratory Methods Review Committee. Please note that the Method Application at this time does not include the completed Single Lab Validation report. The DA analyses to complete Section C. Validation Criteria are currently in progress and will continue throughout the summer. My laboratory has just received funding from the North Pacific Research Board and will be running ISSC Single Laboratory Validation Testing on butter clams (*Saxidomus giganteus*), blue mussels (*Mytilus edulis*), geoducks (*Panopea abrupta*), manila clams (*Venerupis japonica*), oysters (*Crassostrea virginica*) and razor clams (*Siliqua patula*) from Alaska later this summer. The NOAA CCFHR laboratory has similarly received their MERHAB funds last week and will be conducting a parallel Single Laboratory Validation study on butter clams, blue mussels, geoducks, manila clams, oysters, and razor clams from California, Oregon and Washington, oysters from North Carolina and quahogs (*Mercenaria mercenaria*) from Georges Bank, Massachusetts. The goal is to test a broad array of commercial species to ensure that matrix affects do not affect the assay. The results will be made available to the ISSC as they become available.

The work to date includes 1) publishing the complete ELISA methodology and initial validation studies in the December 2008 issue of the Journal of Shellfish Research and 2) completing the first validation series using oysters from North Carolina. The technique was also independently validated by the Quinault tribe in Washington State. They ran the ELISA on razor clam samples gathered by the tribe for a year and sent duplicate samples to the Washington Department of Health HPLC for analyses and have made their results available for inclusion in this preliminary application.

The purpose of this submission is to bring the new method to the attention of the committee in a manner that enables the method to be evaluated in a timely way. I am also seeking the committee's advice and guidance on the validation studies that will be conducted this coming summer by my laboratory and that of Wayne Litaker at NOAA. In the initial study using the oyster tissues I have closely followed the ISSC guidelines, but wanted to ensure that my interpretation was correct. I would therefore request the committee to review the methodology used in the initial oyster validation study to ensure the procedures used meet current requirements and that no additional data need to be gathered. If necessary, the protocol can be altered to meet the committee requirements.
Please find in association with this cover letter a series of materials relevant to the

evaluation of the Domoic Acid Test Kit by the ISSC Laboratory Methods Review Committee.

These items included:

- ISSC Method Application with Section A, Section B**,** and Section D completed (see below).
- A pdf file containing the User Guide for the Domoic Acid Test Kit (DAK-36) that is included in the commercial product. (Also available online at: http://www.mercuryscience.com/DA User Guide 2007A.pdf)
- A pdf file containing a reprint of the research paper entitled " RAPID ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF THE ALGAL TOXIN DOMOIC ACID," published in the December, 2008 issue of Journal for Shellfish Research. This paper describes correlation data comparing the Domoic Acid Test Kit versus HPLC analysis using several sample matrices. (Also available online at: http://mercuryscience.com/LitakerStewartDec2008.pdf)
- An Excel file showing the results of a study done by the Quinault Indian Nation and the Washington Department of Health comparing razor clam analysis performed by the Domoic Acid Test Kit versus HPLC analysis. This independent study used samples collected over a nineteen month period and was planned and performed without any input from Mercury Science or NOAA. (also available online at: http://mercuryscience.com/QINWDOHdata.xls)
- Preliminary tests using oyster spiked materials (see below)

The ELISA method has been used independently in six laboratories and provided results equivalent to those obtained using HPLC, FMOC-HPLC and LC-MS. This is detailed in the Litaker et al. 2008 publication listed above. Based on the correlation studies conducted so far, I request that this method be considered for interim approval by the LMR committee until the remaining validation data can be provided over the next six months. Upon completion of the SLV, consideration for approval of the assay as a Level 4 method will be requested.

Public Health Significance:

The regulatory method for DA detection sanctioned by the Interstate Shellfish Sanitation Conference is a high performance liquid chromatography (HPLC) assay. Though accurate, these analyses are generally run by centralized state facilities with results typically not available for 3 to 14 days after the samples are collected. In more remote communities, many of which depend heavily on subsistence clam harvests, these long delays and the costs of sample analysis are causes for public health concern. The average cost of approximately $100 per sample limits the number of samples that can be analyzed (Harold Rourk, Washington State Department of Health, personal communication). Resource managers in coastal communities have expressed their desire for a cost-effective method for rapid and accurate determination of DA concentrations in shellfish and phytoplankton samples.

Cost Information (if available):

Anticipated cost is $7.00 per duplicate reaction.

Proposed Specific Research Need/Problem to be Addressed:

This research focuses on the development is an accurate, rapid, cost-effective ELISA for use by environmental managers and public health officials to monitor Domoic Acid concentrations in environment samples. The regulatory method for DA detection sanctioned by the Interstate Shellfish Sanitation Conference is a high

performance liquid chromatography (HPLC) assay. Though accurate, these analyses are generally run by centralized state facilities with results typically not available for 3 to 14 days after the samples are collected. In more remote communities, many of which depend heavily on subsistence clam harvests, these long delays and the costs of sample analysis are causes for public health concern. The average cost of approximately $100 per sample limits the number of samples that can be analyzed (Harold Rourk, Washington State Department of Health, personal communication). Resource managers in coastal communities have expressed their desire for a cost-effective method for rapid and accurate determination of DA concentrations in shellfish and phytoplankton samples. The high throughput capacity of the assay also allows for much faster response times when domoic acid events occur. The relatively low cost of the assay means that significantly more sampling is also possible on the same or smaller budget.

How will addressing this research support/improve the mission/role of the ISSC/NSSP/Industry? Support need with literature citations as appropriate.

This Assay will allow better protect public health and provide a rapid response capability when DA outbreaks occurs. It can also be adapted to monitoring phytoplankton samples so that toxic blooms can be identify and tracked. Toxic phytoplankton cells generally appear several weeks before the shellfish become toxic and can be used as an early warning system for when shellfish are likely to become toxic/

More detailed information on the assay and its potential uses is provided in a recently published article:
RAPID ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF THE ALGAL TOXIN DOMOIC ACID, Journal of Shellfish Research, Vol. 27, No. 5, 1301–1310, 2008.
Available online at: http://mercuryscience.com/LitakerStewartDec2008.pdf

Relative Priority Rank in Terms of Resolving Research Need:

Immediate	☐	**Important**	☐
Required	☐	**Other**	☐
Valuable	☐		

Estimated Cost: $7.00 per duplicate sample (~$200.00 for ELISA kit capable of analyzing 36 duplicate samples in 1.5 h)

Proposed Sources of Funding/Support: Grants have been awarded by NPRB and NOAA MERHAB program for the completion of the validation studies.

Time Frame Anticipated: Validation should be completed by January or February 2010.

Action by 2009 Laboratory Methods Review Committee Recommended referral of Proposal 09-105 to the appropriate committee as determined by the Conference Chairman.

Action by 2009 Task Force I Recommended adoption of Laboratory Methods Review Committee recommendation on Proposal 09-105.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force I on Proposal 09-105.

Journal of Shellfish Research, Vol. 27, No. 5, 1301–1310, 2008.

RAPID ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF THE ALGAL TOXIN DOMOIC ACID

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ABSTRACT Domoic acid (DA) is a potent toxin produced by bloom-forming phytoplankton in the genus *Pseudo-nitzschia*, which is responsible for causing amnesic shellfish poisoning (ASP) in humans. ASP symptoms include vomiting, diarrhea, and in more severe cases confusion, loss of memory, disorientation, and even coma or death. This paper describes the development and validation of a rapid, sensitive, enzyme linked immunosorbent assay test kit for detecting DA using a monoclonal antibody. The assay gives equivalent results to those obtained using standard high performance liquid chromatography, fluorenylmethoxycarbonyl high performance liquid chromatography, or liquid chromatography—mass spectrometry methods. It has a linear range from 0.1–3 ppb and was used successfully to measure DA in razor clams, mussels, scallops, and phytoplankton. The assay requires approximately 1.5 h to complete and has a standard 96-well format where each strip of eight wells is removable and can be stored at 4°C until needed. The first two wells of each strip serve as an internal control eliminating the need to run a standard curve. This allows as few as 3 or as many as 36 duplicate samples to be run at a time enabling real-time sample processing and limiting degradation of DA, which can occur during storage. There was minimal cross-reactivity in this assay with glutamine, glutamic acid, kainic acid, epi- or iso-DA. This accurate, rapid, cost-effective, assay offers environmental managers and public health officials an effective tool for monitoring DA concentrations in environment samples.

KEY WORDS: ASP, domoic acid poisoning, ELISA, mussels, scallops, razor clams, test kit

INTRODUCTION

Domoic acid (DA) is a potent toxin produced by bloom-forming phytoplankton in the genus *Pseudo-nitzschia* (Fig. 1). It is a glutamate analog, which acts as a potent excitatory neurotransmitter and causes amnesic shellfish poisoning (ASP) in humans (Quilliam & Wright 1989, Quilliam et al. 1989b, Wright et al. 1989). Symptoms include vomiting, diarrhea, and in more severe cases confusion, loss of memory, disorientation, and even death. As a tricarboxylic acid, fully ionized at seawater pH, DA can behave as a potent trace metal ligand (Rue & Bruland 2001, Wells et al. 2005). DA can bioaccumulate and rapidly transvectors throughout the food chain *via* clams, mussels, crabs, filter feeding fish, and other organisms (Horner & Postel 1993, Scallet et al. 2005, Vigilant & Silver 2007). DA poisoning was first recognized after a lethal event on Prince Edward Island, Canada in 1987 (Wright et al. 1989). Since that time, a number of toxic events have occurred on the United States west coast where DA has been shown to commonly accumulate in the edible parts of razor clams (*Siliqua patula*), mussels (*Mytilus californianus* or *edulis*), and Dungeness crabs (*Cancer magister*) (Wekell et al. 1994, Horner et al. 1997). High levels of DA in razor clams in Oregon and Washington are responsible for beach closures that can last for more than a year. Losses of more than $20 million annually result from these closures caused by lost tourism and reduced recreational and commercial and tribal clam harvests (Adams et al. 2000). DA has also been implicated in the death and illness of brown pelicans (*Pelecanus occidentalis*) and Brandt's cormorants (*Phalacrocorax penicillatus*) (Fritz et al. 1992, Work et al. 1993), California sea lions (*Zalophus californianus*) (Scholin et al. 2000, Trainer et al. 2000, Brodie et al. 2006), sea otters (*Enhydra lutris*) (Kreuder et al. 2003), and possibly whales (Lefebvre et al. 2002).

The regulatory method for DA detection sanctioned by the Interstate Shellfish Sanitation Conference (Quilliam et al. 1989a, Quilliam et al. 1995) is a high performance liquid chromatography (HPLC) assay (Quilliam et al. 1991, Hatfield et al. 1994). Though accurate, these analyses are generally run

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by centralized state facilities with results typically not available for 3–14 days after the samples are collected. In more remote communities, many of which depend heavily on subsistence clam harvests, these long delays and the costs of sample analysis are causes for public health concern. The average cost of approximately $100 per sample limits the number of samples that can be analyzed (Harold Rourk, WA State Department of Health, pers. comm.). Resource managers in coastal communities have expressed their desire for a cost-effective method for rapid and accurate determination of DA concentrations in shellfish and phytoplankton samples. This paper describes the development and optimization of a robust monoclonal antibody based enzyme-linked immunosorbent assay (ELISA) test kit for DA that will meet management needs for rapid detection of DA in environmental samples.

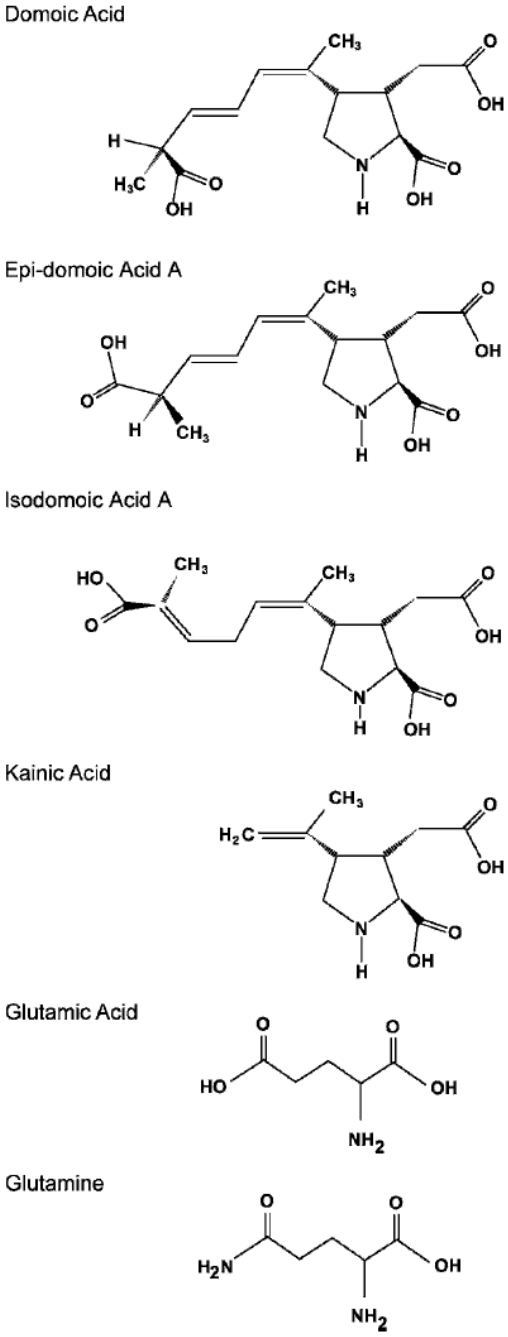

Figure 1. Structure of domoic acid, the isomers epi-domoic acid, isodomoic acid, and two analogues kainic acid and glutamic acid.

MATERIALS AND METHODS

Assay Kit Overview

The DA assay kit was developed jointly by NOAA's National Centers for Coastal Ocean Science, National Ocean Service, and the Northwest Fisheries Science Center, together with an industry partner Mercury Science, Inc., Durham, NC (NOAA/MSI). It was designed as a sequential competitive enzyme linked immunosorbent assay (ELISA) utilizing a high avidity monoclonal antibody (mAb) to DA to ensure assay specificity and consistency across production lots. In the current format, a fixed number of anti-DA mAb binding sites are incubated with dissolved DA in the sample followed by the addition of a DA—horseradish peroxidase (HRP) conjugate. As these binding events occur, the anti-DA mAb molecules are simultaneously captured by antimouse antibodies affixed to the surface of the microtiter plate wells. Subsequent HRP derived color development, readable on standard microplate readers, was inversely proportional to the concentration of DA in the sample matrix. The assay reagents were titrated so that the amount of mAb and the DA–HRP conjugate added produced a maximal absorbance signal of 3 absorbance units when no DA was present. The implementation of this ELISA system required the development and validation of two essential reagents, a high avidity monoclonal antibody to DA and a stable DA-HRP conjugate recognized by the same mAb.

Production of the Anti-Domoic Acid Antibody

Domoic acid (Sigma-Aldrich, St. Louis, MO), was conjugated with bovine serum albumin (BSA) using dicyclohexyl carbodiimide and N-hydroxysuccinimide by a two-step synthetic pathway (Adamczyk et al. 1994). Ten mice were immunized with the DA-BSA immunogen. Serum titers were determined five days after each boost. A fusion was performed on the three mice that showed the greatest response. Hybridoma cell lines and monoclonal antibody production was performed according to the method of Fenderson et al. (1984). The 10 clones with highest affinity mAbs were selected for further growth and their affinity to DA was compared. The most sensitive clone was ultimately selected as the primary mAb for use in the assay development.

DA-HRP Conjugate

Domoic acid (Sigma) was cross-linked to horseradish peroxidase (HRP) using the procedure of Yoon et al. (1993). The reagent was tested for stability and was used to screen for high affinity mAbs after the fusion and for assay development.

Domoic Acid Standards

The DA standards used to calibrate the assay were purchased from the Certified Reference Materials Program at the National Research Council of Canada Institute for Marine Biosciences (Halifax, Nova Scotia, Canada).

Assay Calibration

A series of dose response curves using varying amounts of antibody and DA-HRP were performed to optimize the assay sensitivity. The optimal assay conditions were found to have an effective linear range from approximately 0.1–3.0 ppb. These conditions were used in all the subsequent phases of assay development. The antibody was also tested for cross-reactivity with varying concentrations of kainic acid, glutamine and glutamic acid. These compounds are structurally similar to various portions of DA molecule and have the potential to cross-react with anti-DA mAbs. Glutamine and glutamic acid, in particular, are common in animal tissues, including shellfish.

Calculation of the Parameters Needed to Construct an Internal Domoic Acid Standard for Each Well Strip

Using the optimized DA assay, multiple dose response curves were made using the NRC standards diluted to between 0 and 10 ppb (1–10 ng mL^{-1}) in the assay reaction buffer. The average response derived from each of the individual response curves was calculated and a dose response curve was generated using a four parameter logit-log curve fitting analysis (Ritchie et al. 1981; Fig. 2). Four parameters were derived from this analysis. This first was B_o, the maximal signal, which occurred when no sample DA was present (Fig. 3A). The second was B, the signal produced by a known amount of sample DA. The third was the slope of the logistic transformed data [proportional to the linear portion of the sigmoidal curve describing the relationship between the ln sample DA concentration versus signal (B)]. And the fourth was ED_{50}, the DA concentration at the mid point of the slope curve where half the available anti-DA mAbs in the well are bound to DA-HRP (Fig. 3A). Because the concentration ratio of anti-DA antibody and DA–HRP conjugates are standardized within reagent lots, the kinetics of the reaction were fixed between assay runs (assuming constant temperature), such that the slope and ED_{50} values remain constant. This made it possible to calculate DA concentrations using the four parameter model.

$$\text{DA concentration} = ED_{50}[(B_o/B) - 1]^{-\text{slope}}$$

Because the slope and ED_{50} are constants, all that was needed to calculate the DA concentrations was an accurate B_o and the B estimates from individual samples. In the assay, the mean value for B_o for each strip of wells was determined by adding sample dilution buffer lacking DA to the first two wells in that strip. Duplicate aliquots from each of three extracted samples diluted with sample buffer were then added to the six remaining wells to obtain the B values. Duplicates were run to ensure assay replicability. It should be noted that B_o (the maximal value with no DA added) can have noticeable variation between assays depending on differences in temperature and development time as shown in Figure 2A. However, when the B values for each strip are divided by B_o, the kinetics of the curve become normalized (i.e., replicable between strips and between runs) (see Fig. 2B). In this way the average B_o values serves as an internal standard that can be used in place of a standard curve provided the variation in the B_o is not above or below certain limits, which are specified in the calculation software described later.

Domoic Acid ELISA Test Kit Procedure

The 96-well assay tray used in the assay contained 12 strips. Each strip of 8 wells could be removed and stored until it was needed. The first two wells of each strip were used as a control (no DA added). The remaining six wells were used to analyze three samples in duplicate. This format provided the flexibility of running anywhere from 3–36 duplicate samples at a time. For unknown sample analysis, extracts were diluted to a final concentration ranging from 0.3–3 to ppb using the sample buffer (phosphate salt solution, pH 7.8, containing casein). For

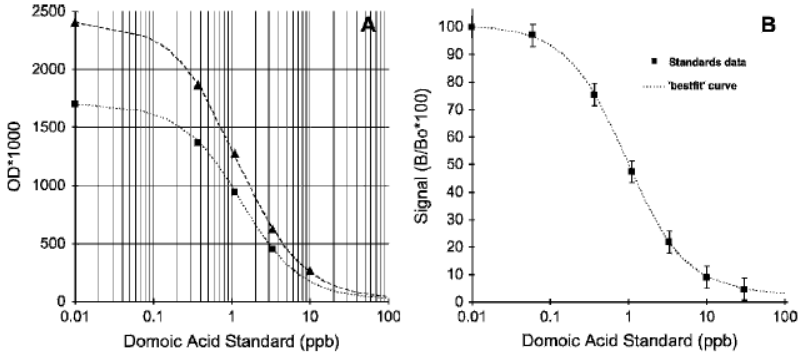

Figure 2. (A) Representative dose response curves for domoic acid analyzed on different days. It should be noted that B_o (the average of the maximal 450 nm absorbance values from the first two wells of a strip to which no DA is added) can vary noticeably between assays depending on differences in ambient temperature and development time. (B) The mean and SD in signal from eight normalized domoic acid dose response curves carried out over the course of several weeks. These data were specifically normalized by dividing each of the resultant absorbance values by B_o. The result of this normalization process, given that the concentrations of antidomoic acid antibody and HRP-domoic acid conjugate are fixed, is that the resultant curves are replicable between rows and between assays done on different days. The black squares and error bars indicate the mean value at each given domoic acid concentration ± 1 SD.

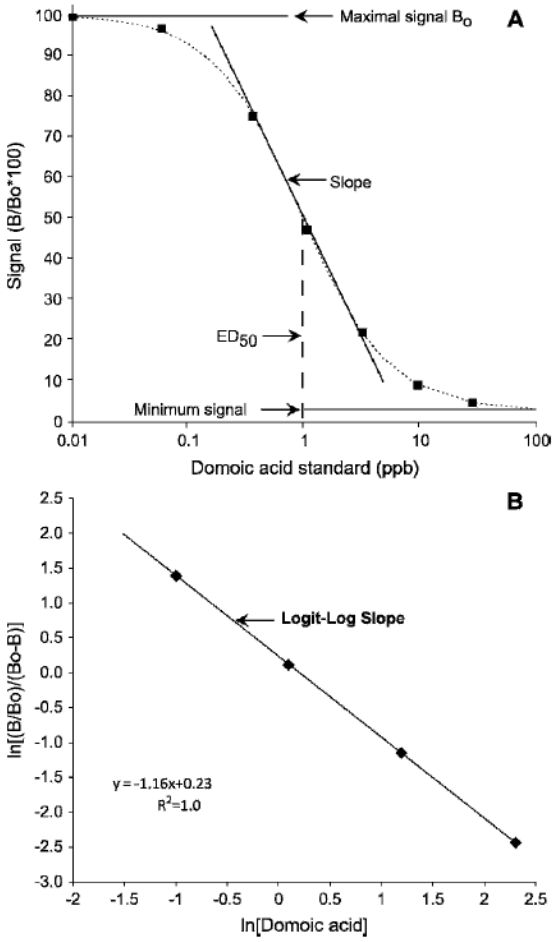

Figure 3. (A) DA concentrations versus the corresponding ELISA absorbance values, which were normalized by dividing by maximal (B_o) absorbance value. (B) Log-logit transform of the data shown in Fig. 3A. From this analysis it was possible to calculate the parameters needed to accurately calculate domoic acid concentrations using the ELISA assay. These parameters include B_o, the maximal absorbance value at 450 nm obtained from the first two wells of a strip to which no free domoic acid is added and B, the 450 nm absorbance value for a given sample, slope of the logit-log transformed data, which were proportional to the linear portion of the sigmoidal curve describing the relationship between the ln DA concentration versus signal (B), and ED_{50}, the mid point of the slope curve where half the available anti-DA mAbs are bound to DA.

clam tissues containing DA, sample dilutions of 1:50 and 1:1000 were typically used. Preliminary tests with razor clam extracts showed that a 25-fold dilution in sample dilution buffer eliminated matrix effects in ELISA analysis.

The assay was initiated by adding 50 µL of the anti-DA antibody to each well using a multi channel pipettor. Next, 50 µL of the control solution (sample buffer without DA) was added to the first two wells in each row. Duplicate 50 µL aliquots from the diluted DA extracts were then added to the remaining wells in each strip and the plate incubated at room temperature for 30 min on an orbital shaker set to vigorously mix the solution in each well (PerkinElmer Waltham, MA 1296–004 DELFIA Plateshake set on high). Vigorous mixing is key to obtaining replicable results from one run to the next. In this step, the bulk of the native DA will bind to available mAbs in proportion to the DA concentration. At the end of the incubation, 50 µL of DA HRP conjugate was added to each well and the plate incubated a second time for 30 min at room temperature on an orbital shaker. The DA-HRP will then bind to remaining available mAb sites. After the incubation, the plate was washed three times with wash solution [Tris-HCl buffered salt solution (pH 7.8) containing Tween 20 and sodium azide as a preservative] using a commercial plate washer, making certain the fluid was completely aspirated from all the wells. Alternatively, these washes can be done manually by adding wash solution to wells using a multichannel pipettor and then flicking all fluid from the wells. The manual method may result in slightly higher variability. Next, 100 µL of K-Blue TMB substrate (5.5′-tetramethylbenzidine, Neogen Corporation, Lexington, KY) was added to each well. The plate was placed on an orbital shaker for no more than 5 min, or until adequate color development was observed. Color development was terminated by adding 100 µL stop solution (1N hydrochloric acid) to each well. The absorbance in each well was measured at 450 nm using a Thermo Ascent MultiSkan plate reader (Thermo Scientific, Waltham, MA). The DA concentrations were determined using the sample (B) and control (B_o) absorbances, the original tissue weights, and the volume of 20% or 50% methanol used to extract each sample. The actual calculations were made using a Microsoft Excel work sheet (Microsoft Corporation, Redmond, WA), which incorporates the constants for the four parameter model described above. This worksheet can be downloaded from Stewart (2008). Processing time for this assay was ~1.5 h.

Routine Tissue Extraction

In the case of razor clams and scallops, pooled samples of 10–12 individual shellfish were cleaned, and ground to a smooth and uniform homogenate in a commercial blender (Waring model HGBSS56, Torrington, CT). Clams were pooled because previous studies of DA in razor clams from the Washington coast indicated that the coefficient of variation for DA between clams in a population exceeded 100% (Wekell et al. 2002). If the homogenate appeared to be forming a gel caused by unusually high lipid content, an equal weight of water was added and the dilution noted. Approximately 2 g of homogenized tissue were added to a tared 50 mL conical tube and the weight recorded to the nearest 0.01 g. Next, 18 mL of 50% methanol were added and the samples mixed at high speed on a vortex mixer for 2 min. Once the extraction was completed the tubes were spun in a table top centrifuge for 20 min at 10,000× g or until a tight pellet and clear supernatant were obtained. If the samples did not clear despite the spinning at high speed, the supernatant was poured into a syringe, then passed through a 0.45 µm Millex HA syringe filter (Millipore, Billerica, MA) to remove proteins and other compounds that can form micelles, whereas soluble DA remained in the filtrate. At this point the homogenate was ready for analysis by ELISA and HPLC. If necessary, the sample was stored at 4°C for up to 24 h in an explosion proof refrigerator prior to analysis.

Phytoplankton Extraction

Approximately 0.1–1.0 L of cultured cells or sea water samples were filtered onto a GF/F filter, which was immediately frozen at –80°C until the filter could be processed. For processing, the filter was placed in a 5 mL conical BD Falcon Tube (Becton Dickinson, Franklin Lakes, NJ) and 3 mL of 20% methanol were added. The samples were then sonicated using a Thermo Fisher Scientific Model 100 Sonic Dismembrator with a 1/8 inch probe (model 15-338-80, Fisher Scientific, Waltham, MA) until the filter was completely homogenized. Care was taken to prevent the probe from rupturing the tube. The sonicator probe was cleaned very carefully with 20% methanol between samples to prevent cross-contamination. Next the homogenate was centrifuged at 3000 *g* for 10 min. The supernatant was then passed through a disposable Whatman GD/X 0.2 µm syringe filter (Florham Park, NJ) into a 5 mL tube. At this point the sample was split for analysis using both the ELISA and HPLC assays.

HPLC Validation of DA concentration from Razor Clam Tissues

HPLC is the accepted standard method for measuring DA and is the basis of the current official method for regulatory action in the U.S. (AOAC Official Method 991.26). The lower detection level for the standard assay is ~0.5 ppm. This technique was used to validate the DA concentration in the razor clams in this study. Briefly, 10–15 mL of the clarified supernatant prepared as described above was transferred into a 25 mL disposable plastic syringe and filtered through 0.45 micron HA Millipore filter (Bedford, MA) into a labeled scintillation vial. Salt clean-up was done with solid phase extraction columns (Hatfield et al. 1994). Strong anion exchange (SAX) solid phase extraction (SPE) cartridges (Whatman, Florham Park, NJ) were conditioned by washing successively with 6 mL of methanol, 6 mL of deionized water, and 6 mL of 50% methanol. The SPE clean up also removes tryptophan, which is a major source of false positives in HPLC-UV detection of DA because it coelutes with DA. Each sample was then drawn through a conditioned SAX SPE cartridge at a rate of 1 drop per second using a vacuum manifold. Flow was stopped when the meniscus was just above the top of the column. The columns were washed with 5 mL of 0.1 M NaCl in 10% aqueous acetonitrile (10% acetonitrile: 90% deionized water). The columns were immediately moved to a new row in the vacuum manifold and the DA eluted from the SPE cartridge using 5 mL of 0.5 M NaCl in aqueous 10% acetonitrile (10:90, acetonitrile:deionized water) and collected in 5 mL graduated centrifuge tubes. Flow was stopped when eluant reached 4.9 mL in the graduated centrifuge tube. The graduated centrifuge tube was removed from the manifold and the actual volume recorded. The graduated centrifuge tubes were capped and the eluant immediately mixed by shaking the tube vigorously 5–10 times. Tissues from the other invertebrate species examined (Table 1) were processed similarly, except that the extracts were filtered through Nanospec MF GHP 0.45 µm centrifugal filters (Pall, Ann Arbor, MI) instead of SPE columns before HPLC analysis. Eluted samples were transferred to HPLC analysis vials. The HPLC conditions were as follows: Vydac TP210 column (Grace, Deerfield, IL), 2.1 by 250 mm, 40°C, elution of DA in 10% acetonitrile containing 0.1% trifluoroacetic acid (TFA). Twenty µl of each sample were injected into the column and eluted isocratically at 0.3 mL per min. The retention time for the DA peak was about 6–8 min depending on the column. Canadian NRC DACS standards at concentration of 1 ppm in 10% acetonitrile solution were run simultaneously (Hardstaff et al. 1990).

HPLC Detection of Domoic Acid in Phytoplankton Using Fluorenylmethoxycarbonyl (FMOC) Derivatization

A more sensitive fluorescent fluorenylmethoxycarbonyl chloride (FMOC) derivatization method (Pocklington et al. 1990) was used to determine particulate DA concentrations in phytoplankton samples, which typically contained less DA than shellfish tissues. The samples were processed on a Hewlett-Packard 1090 HPLC using a Vydac 201TP, 5 µm, 25 cm column, HP 1046A fluorescence detector, and column heater set to 40°C with the following modification. In our analysis, solvents A (HPLC Water with 0.1% v/v TFA) and B (acetonitrile with 0.1% v/v TFA) were pumped at 0.2 mL/min and the linear gradient elution was changed allowing for increased separation and resolution of the domoic acid peak. The initial gradient went from 70% A and 30% B at time of injection to

TABLE 1.

Intertidal invertebrates sampled from several locations around Monterey Bay in November 2006. HPLC-UV analysis detected significant levels of compounds comigrating with iso- and epi-domoic acid standards. These crude methanolic extracts were used to challenge the NOAA and Biosense ELISAs. The goal was to establish the extent to which the ELISA assays are confounded by the presence of coeluting compounds called as the domoic acid isomers epi- and iso-domoic acid by HPLC-UV assay. Nondetect samples are represented as 0 values.

Organism	Combined epi and iso-DA by HPLC (ppb)	DA Concentration by NOAA ELISA (ppb)	% Total DA Detected by NOAA ELISA	DA Concentration by Biosennse ELISA (ppb)	% Total DA Detected by Biosense ELISA
Chthamalus fissus/dalli	281.7	0.00	0.00	0.02	0.01
Chthamalus fissus/dalli	1,137.1	15.41	1.36	1.53	0.13
Littorina scutulata	198.7	10.57	5.32	3.02	1.52
Littorina scutulata	682.0	15.98	2.34	1.02	0.15
Littorina scutulata	119.5	0.00	0.00	0.17	0.14
Lottia digitalis	236.7	0.00	0.00	0.10	0.04
Lottia digitalis	477.9	13.91	2.91	0.09	0.02
Lottia digitalis	390.6	10.31	2.64	0.78	0.20

60% A and 40% B over 0–10 min, then held constant for 10 min; adjusted to 0% A and 100% B from 20–30 min, held isocratic for 2 min; adjusted from 0% A and 100% B to 70% A and 30% B over 2 min, and then held constant at these (initial) conditions until the end of the run at 45 min. Dihydrokainic acid was used as an internal standard, as described by Pocklington et al. (1990).

A subset of phytoplankton samples was validated to confirm the presence of DA (by mass) using liquid chromatography-mass spectrometry (LC-MS) on a ThermoFinnigan Quantum Discovery Max TSQ ESI Mass Spectrometer coupled to a HP 1100 series binary pump HPLC, following the general protocol of Quilliam et al. (1989a). Samples for LC-MS were prepared as for HPLC, but were then dried down under vacuum and redissolved in 100% methanol prior to injection. The HPLC conditions for the reverse phase were programmed for a linear gradient elution of 10:90% acetonitrile:deionized water (both containing 0.1% formic acid) up to 0:100% water:acetonitrile over 30 min.

Testing Cross-Reactivity of the ELISA Against Glutamine, Kainic Acid and Putative Isomers Epi-DA and Iso-DA

Domoic acid is structurally similar to glutamine, glutamic acid and kainic acid, all of which can potentially co-occur with DA in sample extracts (Fig. 1). To test for potential cross-reactivity with these compounds, the NOAA/MSI ELISA kit was run using concentrations of glutamine, glutamic acid and kainic acid ranging from 10 ppb to 5 ppm. The ED_{50} for each compound was calculated and then divided by ED_{50} for DA and multiplied by 100 to determine percent cross-reactivity (Table 2). A majority of DA in razor clams and phytoplankton is in the form shown at the top of Figure 1. However, samples sometimes contain a larger quantity of compounds closely eluting with DA on standard HPLC runs that have been identified as the DA conformers epi- and iso-DA (Wright et al. 1990, Kotaki et al. 2005). To determine if the mAb used in this assay could detect these DA isomers, and the extent of interference by such coeluting compounds present in crude extracts of intertidal barnacle, limpet, and snail samples, crude methanolic extracts of these tissues were assays using HPLC-UV and both the NOAA/MSI and Biosense (Biosense Laboratories, Bergen, Norway) ELISA methods. These intertidal invertebrate extracts exhibited high levels of the putative epi-DA and iso-DA isomers as called by comigration on HPLC chromatograms. These compounds are generally near detection limits in razor clams, crabs, and to a lesser extent in mussels, and therefore these extracts provided novel matrices for evaluating the accuracy of NOAA/MSI ELISA.

TABLE 2.

Cross-reactivity of the NOAA/MSI ELISA with kainic acid. glutamine, and glutamic acid.

Analyte	% Reactivity in the Domoic Acid Assay
Domoic acid	100
Kainic acid	0.3
Glutamine	<0.1
Glutamic acid	<0.1

Data Analyses

Analytical results for DA concentrations determined from razor clams, mussels, scallops and phytoplankton cells determined by HPLC, FMOC-HPLC, LC-MS and the NOAA/MSI ELISA were compared using linear regression analysis (Sokal & Rohlf 1995). The performance of the NOAA/MSI and Biosense ELISA kits was also compared using a subset of the phytoplankton samples. This comparison involved simultaneously analyzing phytoplankton extracts using the two kits and comparing the results with those obtained using FMOC-HPLC. All samples were run within a 24 h period to prevent differential degradation of DA, which may occur in some samples. Data were compared using linear regression analysis.

RESULTS AND DISCUSSION

The NOAA/MSI ELISA accurately measured NRC standard DA concentrations (Fig. 4) and gave equivalent results for razor clam (Fig. 5), mussel (Fig. 6), scallop (Fig. 7), and phytoplankton extracts (Fig. 8) as obtained when using HPLC, FMOC-HPLC, or LC-MS methods. When the variability in the NOAA/MSI ELISA and FMOC-HPLC method were compared using replicate phytoplankton extracts they were found to be comparable (Fig. 9). The primary advantage of the NOAA/MSI ELISA over HPLC methods, besides a significantly lower cost per sample was much higher throughput. As many as 36 samples can be completed in <1.5 h after tissue extraction.

The NOAA/MSI format was also flexible. An internal control was incorporated into each strip, which eliminated the necessity of running a standard curve each time the assay was performed. Any unused strips could be removed and stored in a desiccator pouch at 4°C for at least six months without compromising assay performance. This allowed as few as 3 samples to be run in real time thereby avoiding the degradation of DA that can occur during storage, particularly once the samples have been extracted (Smith et al. 2006). For example, when phytoplankton samples were run within 24 h using the Biosense ELISA kit, which has been validated by an international collaborative study, and is officially approved by the AOAC International for regulatory detection of DA in shellfish,

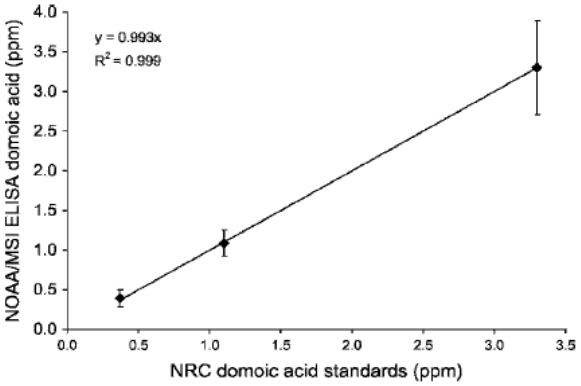

Figure 4. Relationship between various concentrations of National Research Council of Canada (NRC) domoic acid standards and the resultant NOAA/Mercury Science (NOAA/MSI) ELISA values determined using 10 different plates.

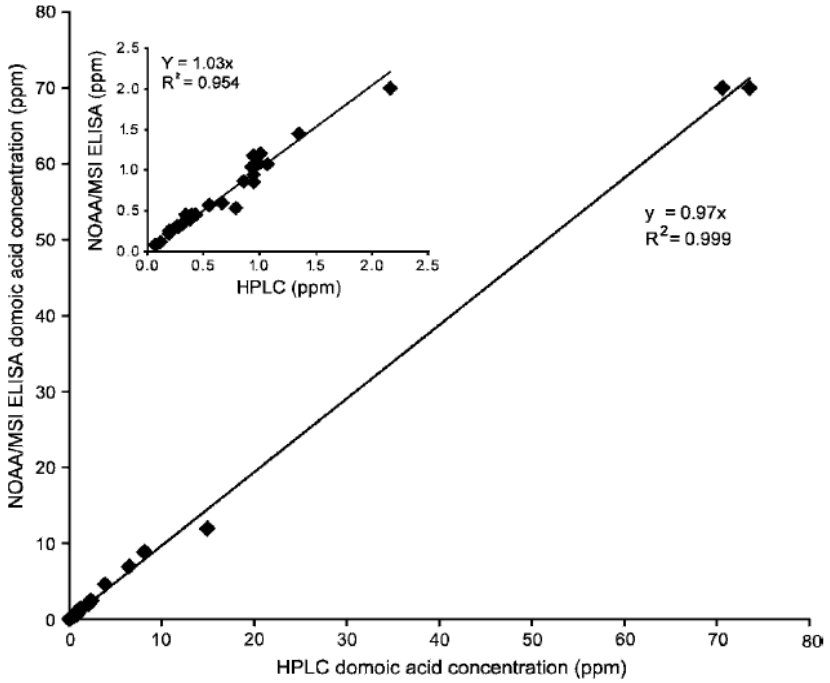

Figure 5. Domoic acid concentrations in razor clam tissues determined from replicate tissue extracts analyzed using HPLC and NOAA/Mercury Science (NOAA/MSI) ELISA. The inset shows an expanded version of the regression analysis for sample containing less than 2.5 ppm domoic acid.

and the NOAA/MSI ELISA kit, equivalent results were obtained (Fig. 10, $r^2 = 0.97$). In contrast, when samples were run two weeks apart the correlation dropped to $r^2 = 0.79$, indicating DA degradation.

The ability to efficiently run a small number of samples in real time was not incorporated into other DA ELISA formats. For example, the Biosense DA ELISA kit includes reagents for only two standard curves (product insert), therefore, only two batches of samples can be run per kit. This means that when small numbers of samples are being collected, they may have to be stored until a sufficient number of samples have been accumulated to maximize the number of samples per kit. This could lead to sample degradation and a critical delay in reporting when samples surpass the regulatory limit of 20 ppm.

Another advantage of the NOAA/MSI assay is that it could be run in either a quantitative or screening mode when assaying shellfish tissues. For quantitative analysis, several dilutions were assayed simultaneously to obtain an accurate DA concentration.

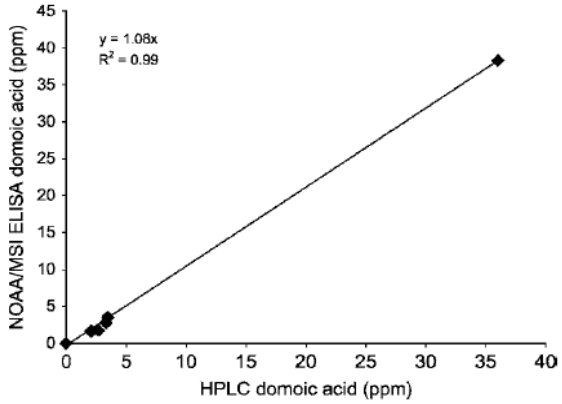

Figure 6. Domoic acid concentrations in mussel tissues determined using HPLC and the NOAA/Mercury Science (NOAA/MSI) ELISA. Aliquots from each sample were run simultaneously.

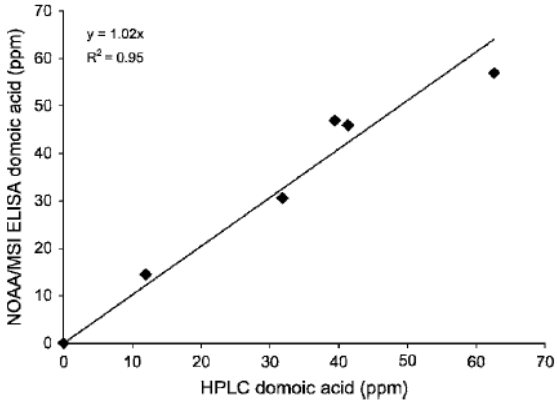

Figure 7. Concentration of domoic acid in scallop tissues extracted from the scallop (*Pecten maximus*) using the standard NOAA/Mercury Science (NOAA/MSI) protocol.

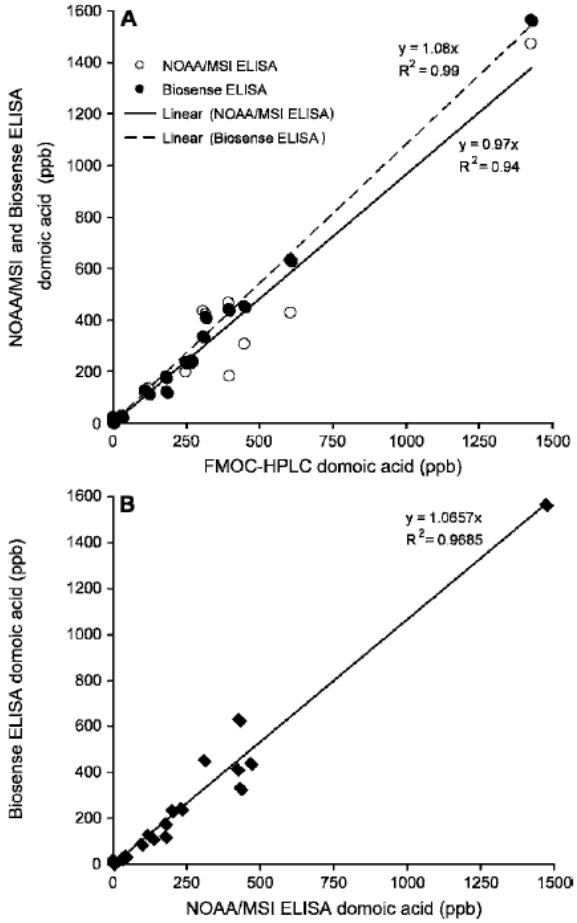

Figure 8. (A) Domoic acid concentrations measured from split phytoplankton sample extracts, which were measured within 24 h by FMOC-HPLC and either the NOAA/Mercury Science (NOAA/MSI) or Biosense ELISAs. (B) Comparison of domoic acid concentrations measured in split samples by either HPLC or ELISA.

Alternatively, to rapidly screen for DA concentrations of concern, the sample extracts were diluted 1:1,000 before running the assay. Taking into account the 1:10 dilution that occurred during the extraction process, the 1:1,000 dilution reduced samples in the 20 ppm DA range to ~2 ppb in the diluted sample. This concentration was within the linear range of the assay (0.1–3 ppb). Tissue samples with 5–10 fold less DA, and far below levels of concern, would show no detectible DA at this dilution. Tissues containing initial DA concentrations >30 ppm would be off scale and indicate a significant DA concentration requiring action. Any samples from this rapid screening that were of concern could then be diluted and run again to obtain an accurate concentration. The NOAA/MSI ELISA test kit also comes with a simple Excel spreadsheet, which allowed the toxin concentrations to be quickly and easily calculated in either a quantitative or rapid screening mode. All that had to be entered was the B_o (no DA added) and sample absorbance data from each strip, the weight of the extracted tissue samples, and the extraction volumes.

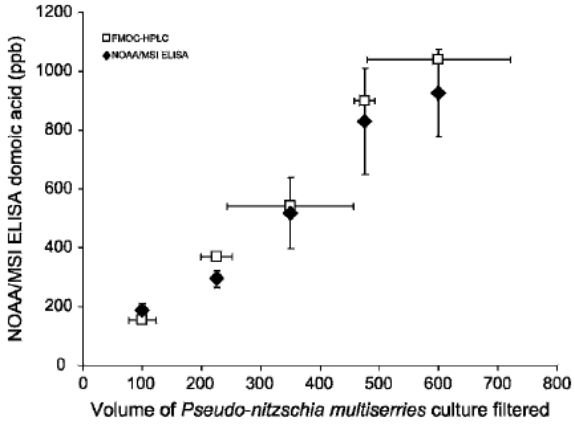

Figure 9. Comparison of the variability between phytoplankton extracts measured using FMOC-HPLC (□) and the NOAA/Mercury Science ELISA (♦). Error bars indicate ±1 SD. Because the error bars largely overlap, for clarity, the standard deviation for the FMOC-HPLC is plotted in the horizontal direction and the NOAA ELISA in the vertical direction.

The NOAA/MSI and Biosense ELISA kits were tested against crude methanolic extracts of several intertidal invertebrates, which HPLC identified as containing >100 ppb levels of epi-DA and iso-DA. These compounds are reported to be less toxic DA congeners based on receptor binding assays (Sawant et al. 2007). Results from both ELISA kits revealed the presence of only trace amounts of DA equivalents in the extracts. The NOAA/MSI ELISA cross-reactivity with these compounds ranged from 0% to 5.3% and the Biosense ELISA cross-reactivity from 0.01% to 1.5% (Table 1) indicating that the ELISA assays are relatively insensitive to cogener interference. It should also be noted that the regulatory methods for assessing human safety are currently based on measuring DA alone, not the combination of DA, iso-DA and epi-DA. These results indicated that both the NOAA/MSI and Biosense

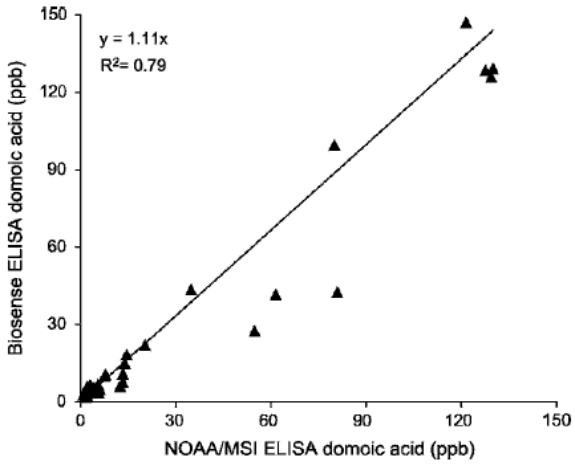

Figure 10. NOAA/MSI ELISA versus Biosense ELISA for phytoplankton samples when the two assays were run several weeks apart showing the increased variability, caused by differential degradation or absorption rates, when samples are not measured within the same 24 h period.

ELISA methods provide DA values comparable to the HPLC values currently used as a basis for regulatory decisions.

Measuring low concentrations of DA in real time is particularly important because the presence or absence of DA contamination is frequently patchy and associated with variable onshore transport of toxic phytoplankton blooms (e.g., Trainer et al. 2002). Depending on prevailing winds and currents, one harvest area can become highly contaminated over a short period whereas adjacent regions remain uncontaminated (Trainer et al. 2000). These differentially affected regions frequently include areas where significant commercial and recreational clam harvests occur. This variability complicates monitoring programs designed to protect human health. The current standard practice involves shipping shellfish samples to a centralized facility for HPLC analyses, introducing delays between 3–14 days from the date of sample collection to reporting results. This turnaround time is too slow to adequately protect subsistence shellfish harvesters who rely on clams consumed within a day or two of harvest. The cost of HPLC analysis is also relatively high per sample and requires a substantially higher capital investment compared with the NOAA/MSI ELISA method. Having an economical technique for better assessing the degree of contamination locally, and in real time, is of great value for local resource managers and public health officials.

The ability to detect DA in phytoplankton using the NOAA/MSI kits would further benefit environmental monitoring programs designed to detect the early onset of toxic *Pseudo-nitzschia* blooms. It is known that increases in the *Pseudo-nitzschia* capable of producing DA often precedes the contamination of shellfish and other filter feeders by a week or two (Trainer & Suddleson 2005). A combination of cell counts and direct toxicity measurements should provide timely predictions for marine resource managers and public health officials. The kit is now commercially available with MSI authorized to market, manufacture and distribute the 96-well plate format test kits. We anticipate completing the necessary validation procedures to qualify the 96 well plate format for regulatory use by public health officials. We are also developing a field test kit that can be used to detect DA levels in shellfish tissues above or below 20 ppm within 10 min after extraction. The test will require no laboratory equipment other than a homogenizer and can be used directly in the field by non-technical personnel, including shellfish harvesters and members of citizen monitoring groups and local volunteers.

In summary, the NOAA/MSI ELISA test kit provides an accurate, flexible and cost effective method for measuring DA in clam, mussel and scallop tissues, as well as in phytoplankton samples. The assay yields concentrations for DA that are indistinguishable from those obtained by HPLC. With further validation, the NOAA/MSI ELISA kit is expected to be approved as a regulatory method for making decisions concerning public health. The short assay (1.5-h) processing time, and relatively low cost, compared with HPLC analysis, mean that the ELISA can be used in more remote locations by environmental managers and public health officials to provide near real-time monitoring capacities.

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LITERATURE CITED

Adamczyk, M., A. Buko, Y. Y. Chen, J. R. Fishpaugh, J. C. Gebler & D. D. Johnson. 1994. Characterization of protein-hapten conjugates. 1. Matrix-assisted laser desorption ionization mass spectrometry of immune BSA-hapten conjugates and comparison with other characterization methods. *Bioconjug. Chem.* 5:631–635.

Adams, N. G., M. Lesoing & V. L. Trainer. 2000. Environmental conditions associated with domoic acid in razor clams on the Washington coast. *J. Shellfish Res.* 19:1007–1015.

Brodie, E. C., F. M. D. Gulland, D. J. Greig, M. Hunter, J. Jaakola, J. St. Leger, T. A. Leighfield & F. M. Van Dolah. 2006. Domoic acid causes reproductive failure in California sea lions (*Salophus californianus*). *Mar. Mamm. Sci.* 22:700–707.

Fenderson, B. A., D. A. O'Brien, C. F. Millette & E. M. Eddy. 1984. Stage specific expression of three cell surface carbohydrate antigens during murine spermatogenesis detected with monoclonal antibodies. *Dev. Biol.* 103:117–131.

Fritz, L., M. A. Quilliam, J. L. C. Wright, A. M. Beale & T. M. Work. 1992. An outbreak of domoic acid poisoning attributed to the pennate diatom *Pseudonitzschia australis*. *J. Phycol.* 28:439–442.

Hardstaff, W. R., W. D. Jamieson, J. E. Milley, M. A. Quilliam & P. G. Sim. 1990. Reference materials for domoic acid, a marine neurotoxin. *Fresenius J. Anal. Chem.* 338:520–525.

Hatfield, C. L., J. C. Wekell, E. J. Gauglitz, Jr. & H. J. Barnett. 1994. Salt clean-up procedure for the determination of domoic acid by HPLC. *Nat. Toxins* 2:206–211.

Horner, R. A. & J. R. Postel. 1993. Toxic diatoms in western Washington waters (U.S. west coast). *Hydrobiologia* 269/270:197–205.

Horner, R. A., D. L. Garrison & F. G. Plumley. 1997. Harmful algal blooms and red tide problems on the United States west coast. *Limnol. Oceanogr.* 42:1076–1088.

Kotaki, Y., E. F. Furio, M. Satake, N. Lundholm, T. Katayama, K. Koike, V. P. Fulgueras, F. A. Bajarias, Y. Takata, K. Kobayashi, S. Sato, Y. Fukuyo & M. Kodama. 2005. Production of isodomoic acids A and B as major toxin components of a pennate diatom *Nitzschia navis-varingica*. *Toxicon* 46:946–953.

Kreuder, C., M. A. Miller, D. A. Jessup, L. J. Lowenstine, M. D. Harris, J. A. Ames, T. E. Carpenter, P. A. Conrad & J. A. K. Mazet. 2003. Patterns of mortality in the southern sea otter (*Enhydra Lutris nereis*) from 1998–2001. *J. Wildl. Dis.* 39:495–509.

Lefebvre, K. A., S. Bargu, T. Kieckhefer & M. W. Silver. 2002. From sanddabs to blue whales: the pervasiveness of domoic acid. *Toxicon* 40:971–977.

Pocklington, R., J. E. Milley, S. S. Bates, C. J. Bird, A. S. W. de Freitas & M. A. Quilliam. 1990. Trace determination of domoic acid in seawater and phytoplankton by high performance liquid chromatography of the fluorenylmethoxycarbonyl (FMOC) derivative. *Int. J. Environ. Anal. Chem.* 38:351–368.

Quilliam, M. A. & J. L. C. Wright. 1989. The amnesic shellfish poisoning mystery. *Anal. Chem.* 61:1053A–1060A.

Quilliam, M. A., M. Xie & W. R. Hardstaff. 1991. A rapid extraction and clean-up procedure for the determination of domoic acid in tissue samples. Canadian National Research Council, Technical Report no. 64, NRCC 33001. Ottawa, Canada. 52 pp.

Quilliam, M. A., M. Xie & W. R. Hardstaff. 1995. Rapid extraction and cleanup for liquid chromatographic determination of domoic acid in unsalted seafood. *J. AOAC Int.* 78:543–554.

Quilliam, M. A., P. G. Sim, A. W. McCulloch & A. G. McInnes. 1989a. High-performance liquid chromatography of domoic acid, a marine neurotoxin, with application to shellfish and plankton. *Int. J. Environ. Anal. Chem.* 36:139–154. Int.

Quilliam, M. A., B. A. Thomson, G. J. Scott & K. W. Siu. 1989b. Ion-spray mass spectrometry of marine neurotoxins. *Rapid Commun. Mass Spectrom.* 3:145–150.

Ritchie, D. G., J. M. Nickerson & G. M. Fuler. 1981. Two simple programs for the analysis of data from enzyme-linked immunosorbent assays on a programmable desktop calculator. *Anal. Biochem.* 110:281–290.

Rue, E. & K. Bruland. 2001. Domoic acid binds iron and copper: A possible role for the toxin produced by the marine diatom *Pseudo-nitzschia*. *Mar. Chem.* 76:127–134.

Sawant, P. M., B. A. Wearea, P. T. Holland, A. I. Selwood, K. L. King, C. M. Mikulski, G. J. Doucette, D. O. Mountfort & D. S. Kerra. 2007. Isodomoic acids A and C exhibit low KA receptor affinity and reduced *in vitro* potency relative to domoic acid in region CA1 of rat hippocampus. *Toxicon* 50:627–638.

Scallet, A. C., L. C. Schmued & J. N. Johannessen. 2005. Neuro-histochemical biomarkers of the marine neurotoxicant, domoic acid. *Neurotoxicol. Teratol.* 27:745–752.

Scholin, C. A., F. Gulland, G. J. Doucette, S. Benson, M. Busman, F. P. Chavez, J. Cordaro, R. DeLong, A. DeVogelaere, J. Harvey, M. Haulena, K. Lefebvre, T. Lipscomb, S. Loscutoff, L. J. Lowenstine, R. Marin, III P. E. Miller, W. A. McLellan, P. D. R. Moeller, C. L. Powell, T. Rowles, P. Silvagni, M. Silver, T. Spraker, V. L. Trainer & F. M. Van Dolah. 2000. Mortality of sea lions along the central California coast linked to a toxic diatom bloom. *Nature* 403:80–84.

Smith, E. A., E. P. Papapanagiotou, N. A. Brown, L. A. Stobo, S. Gallacher & A. M. Shanks. 2006. Effect of storage on amnesic shellfish poisoning (ASP) toxins in king scallops (*Pecten maximus*). *Harmful Algae* 5:9–19.

Sokal, R. R. & F. J. Rohlf. 1995. Biometry: The principles and practice of statistics in biological research. 3rd edition. New York: W. H. Freeman and Co. 887 pp.

Stewart, T. N. 2008. Template for calculating DA concentrations using the NOAA/MSI ELISA. http://www.mercuryscience.com/DA.html. Last accessed 4/22/2008.

Trainer, V. L. & M. Suddleson. 2005. Monitoring approaches for early warning of domoic acid events in Washington State. *Oceanography* 18:228–237.

Trainer, V. L., B. M. Hickey & R. A. Horner. 2002. Biological and physical dynamics of domoic acid production off the Washington USA coast. *Limnol. Oceanogr.* 47:1438–1446.

Trainer, V. L., N. G. Adams, B. D. Bill, C. M. Stehr, J. C. Wekell, P. Moeller, M. Busman & D. Woodruff. 2000. Domoic acid production near california coastal upwelling zones, June 1998. *Limnol. Oceanogr.* 45:1818–1833.

Vigilant, V. L. & M. W. Silver. 2007. Domoic acid in benthic flatfish on the continental shelf of Monterey Bay, California, USA. *Mar. Biol.* 151:2053–2062.

Wekell, J. C., V. L. Trainer, D. Ayres & D. Simons. 2002. A study of spatial variability of domoic acid in razor clams: recommendations for resource management on the Washington coast. *Harmful Algae* 3:1–9.

Wekell, J. C., E. J. Gauglitz, Jr., H. J. Barnett, C. L. Hatfield, D. Simons & D. Ayres. 1994. Occurrence of domoic acid in Washington state razor clams (*Siliqua patula*) during 1991–1993. *Nat. Toxins* 2:197–205.

Wells, M. L., C. G. Trick, W. P. Cochlan, M. P. Hughes & V. L. Trainer. 2005. Domoic acid: The synergy of iron, copper, and the toxicity of diatoms. *Limnol. Oceanogr.* 50:1908–1917.

Work, T. M., B. Barr, A. M. Beale, L. Fritz, M. A. Quilliam & J. L. C. Wright. 1993. Epidemiology of DA poisoning in brown pelicans (*Pelecanus occidentalis*) and Brandt's cormorants (*Phalacrocarax penicillatus*) in California. *J. Zoo Wildl. Med.* 24:54–62.

Wright, J. L. C., M. Falk, A. G. McInnes & J. A. Walter. 1990. Identification of isodomoic acid D and two new geometrical isomers of domoic acid in toxic mussels. *Can. J. Chem.* 68:22–25.

Wright, J. L. C., R. K. Boyd, A. S. W. de Freitas, M. Falk, R. A. Foxall, W. D. Jamieson, M. V. Laycock, A. W. McCulloch, A. G. McInnes, P. Odense, V. P. Pathak, M. A. Quilliam, M. A. Ragan, P. G. Sim, P. Thibault, J. A. Walter, D. J. A. Gilgan, D. J. A. Richard & D. Dewar. 1989. Identification of domoic acid, a neuroexcitatory amino acid, in toxic mussels from eastern Prince Edward Island. *Can. J. Chem.* 67:481–490.

Yoon, D. Y., M. J. Choi, I. S. Choe, T. W. Chung & S. M. Byun. 1993. Influence of the conjugation site on the specificity of monoclonal antibodies to progesterone and on the performance of direct enzyme immunoassay. *Biochem. Mol. Biol. Int.* 31:553–563.

Doc. 2007A

Domoic Acid Screening Test Kit

Colorimetric Immunoassay
for the detection of
Domoic Acid
in environmental samples

Instructions and User Guide

FOR SCIENTIFIC RESEARCH USE

Manufactured by
Mercury Science Inc.
Tel: (866) 861-5836

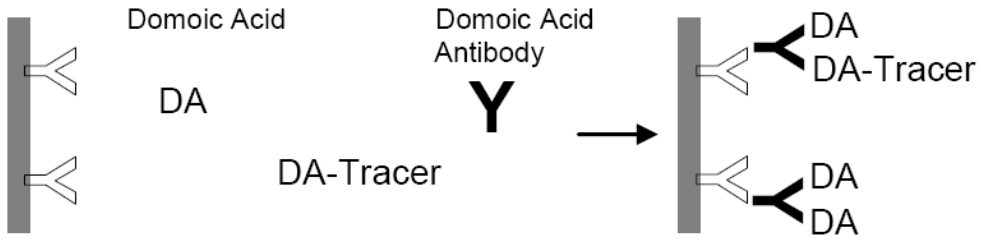
Domoic Acid Screening Test Kit

For Scientific Research Use Only.
This product is not to be used for In Vitro or In Vivo Diagnosis.

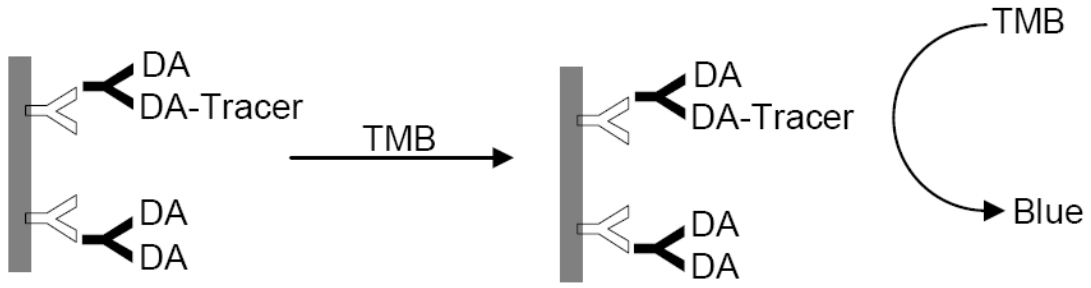
PRINCIPLES OF THE ASSAY
This product contains an antibody (Ab) that binds Domoic Acid and has been developed for the semi-quantitative detection of Domoic Acid in sample extracts. The signal of samples and a control are compared to determine the amount of Domoic Acid present.

The Domoic Acid assay is a solid phase colorimetric immunoassay, based on competition between Domoic Acid and enzyme-labelled Domoic Acid (DA-Tracer) for anti-Domoic Acid antibody. Samples containing Domoic Acid inhibit the binding of the DA-Tracer to the antibody molecules. Both the Ab-Domoic Acid and Ab-DA-Tracer complexes are captured on the surface of the microtiter plate wells.

Following a wash step, the addition of an enzyme substrate (TMB) forms a color proportional to the amount of DA-Tracer in the well. The amount of color measured is inversely proportional to the concentration of Domoic Acid in the sample.



Solid phase
anti-mouse IgG



TEST KIT CONTENTS Each Domoic Acid test kit contains reagents for testing a maximum of 36 samples in duplicate.
The expiry date of the test kit is stated on the outer label.
Store the kit between 2°C and 8°C.

Reagents Store the reagents between 2°C and 8°C when not in use.

Component	Quantity
Control Solution	1 vial 2 mL
The control is a phosphate-buffered salt solution with casein. Contains sodium azide as a preservative.	
Sample Dilution Buffer	1 bottle 50 mL
Ready-to-use phosphate buffered (pH 7.8) salt solution with casein. Contains sodium azide as a preservative.	
Domoic Acid- Tracer	1 vial 7.5 mL
The tracer is in a MOPS-buffered solution containing bovine protein as a stabilizer and methylisothiazolone, bromonitrodioxane, and Proclin 300 as preservatives.	
Domoic Acid Antibody	1 vial 7.5 mL
The antibody is in phosphate-buffered salt solution with casein. Contains sodium azide as a preservative.	
Wash Concentrate	1 bottle 40 mL
A 25-fold concentration of Tris-HCl buffered (pH 7.8) salt solution with Tween 20. Contains sodium azide as a preservative. Prepare for use by mixing entire contents with 960 mL of distilled water and placing in platewasher WASH Bottle.	
Substrate Solution	1 bottle 15 mL
Tetramethylbenzidine and H_2O_2	Keep away from direct sunlight.
Stop Solution	1 bottle 15 mL
1 N Hydrochloric Acid	
Anti-Mouse IgG Microtitration Strips	1 plate (12 x 8 wells)

WARNINGS AND PRECAUTIONS
For research use only. Handle all samples as potentially hazardous.
Disposal of all waste should be in accordance with local regulations.

SCREENING ASSAY PROCEDURE

Perform each determination in duplicate for the Control and unknowns. All sample extracts should be filtered prior to analysis. All reagents and samples should be brought to room temperature prior to use. Use only the number of strips needed. Keep unused strips stored in their aluminum foil pouch with the included desiccant until needed.

1. Pipet 50 uL of the diluted Domoic Acid Antibody solution into each well.

2. Pipet 50 uL of each Control or sample into a well using the sequence shown in the table below. **Always use wells A and B on each strip as Controls.** Always perform duplicate analyses of samples. Three samples can be tested per strip. The example below shows the testing of eight samples.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Con-trol	Con-trol	Con-trol									
B	Con-trol	Con-trol	Con-trol									
C	1st Unk	4th Unk	7th Unk									
D	1st Unk	4th Unk	7th Unk									
E	2nd Unk	5th Unk	8th Unk									
F	2nd Unk	5th Unk	8th Unk									
G	3rd Unk	6th Unk										
H	3rd Unk	6th Unk										

3. Shake the wells for 30 minutes.

4. Pipet 50 uL of the Domoic Acid Tracer solution into each well.

5. Shake the wells for 30 minutes.

6. Wash the strips 3 times on the platewasher. Tap the strips upside-down firmly on a paper towel to blot away any excess wash solution that may remain in the wells.

7. Add 100 uL of Substrate Solution to each well. Shake the plate for five minutes.

8. Add 100 uL of Stop Solution to each well. Shake the plate briefly.

9. Measure the absorbance in each well. Note: If Control absorbance is greater than 3.0 AU, remove 50 uL from ALL WELLS and measure absorbance.

10. The data can be analyzed using the Excel worksheet available at the following link:

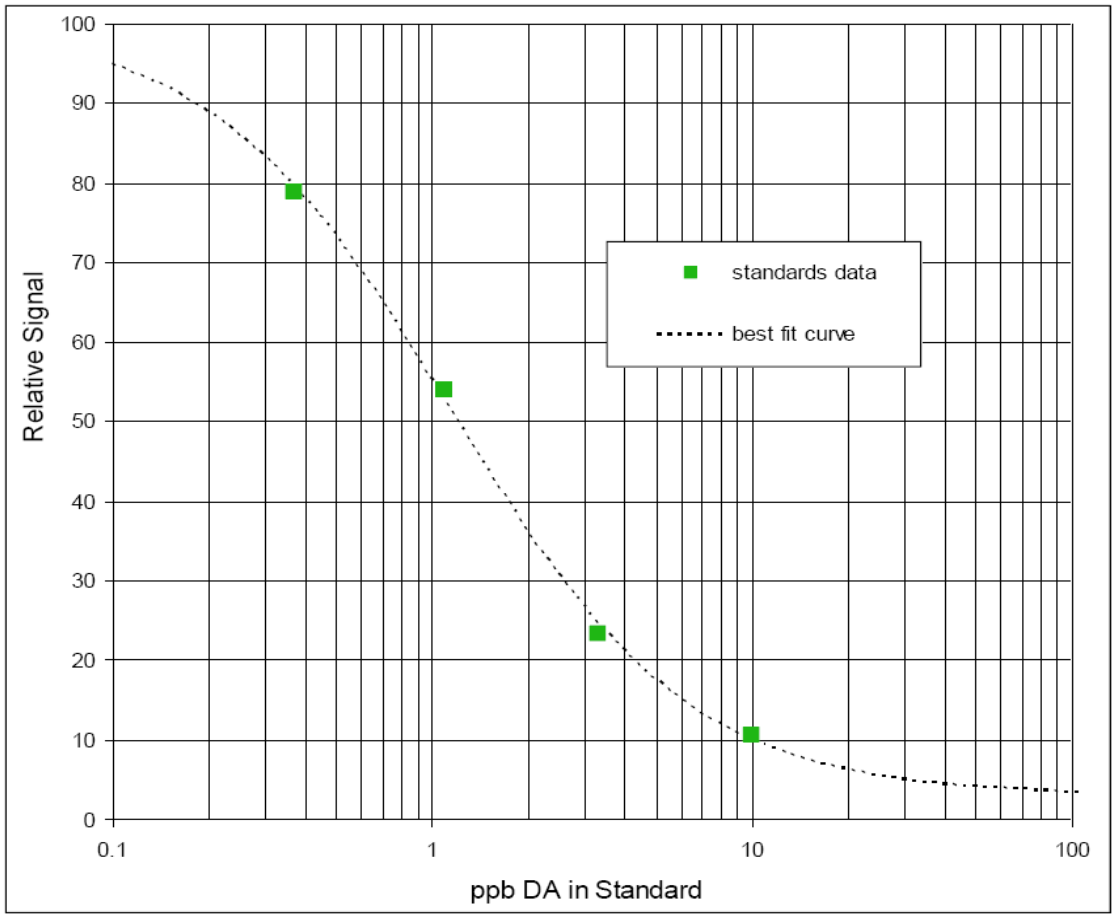
http://mercuryscience.com/Domoic Acid Quantitation 8Well Strip.xls

PERFORMANCE CHARACTERISTICS

Reproducibility

Inter-Assay Standard Curve

The average values and standard deviation of 5 separate standard curves is shown below.



Intra-assay Signal Precision

Analysis of 12 replicates for five different samples

	A	B	C	D	E
Signal (% of Control)	99.5	76.5	47.5	23.5	10.4
Standard Deviation	1.4	1.2	2.0	2.3	1.1
% Coeff. Var.	1.4	1.6	4.2	9.8	10.9

Intra-assay Concentration Precision

Analysis of 3 different samples measured in 6 separate quantitative assays.

	A	B	C
Average Conc. (ppb)	0.56	1.54	3.66
Standard Deviation (ppb)	0.01	0.13	0.19
% Coeff. Var.	2.1	8.6	5.3

PERFORMANCE CHARACTERISTICS (Cont.)

Detection Limit

The detection limit is defined as the minimum concentration of Domoic Acid that can be distinguished from a blank standard with 95% confidence. A detection limit of 0.1 ppb Domoic Acid in extraction buffer has been demonstrated with this assay.

Cross Reactivity

This assay is specific for the detection of domoic acid. The ability of the assay to detect structurally related compounds is shown in the following table.

Analyte	% Reactivity
Domoic Acid	100
Kainic Acid	0.3
Glutamic Acid	less than 0.1
Glutamine	less than 0.1

PROCEDURAL NOTES

Please read all instructions thoroughly before using this kit. Do not mix reagents from kits having different lot numbers. Do not use kits after the expiration date printed on the kit label.

Reagents should be at room temperature when used.

During washing steps, check that each well is completely filled during wash solution additions. After washing is complete, invert the wells and tap them gently against a paper towel to remove excess liquid.

The platewasher should be rinsed with distilled water at the end of each day of use to prevent clogging of the dispensing and aspirating ports. Prime the platewasher with wash solution before the first wash each day.

Care must be taken during each step to prevent contamination of reagents and equipment Do not use the same pipet tip in two different reagents.

For Technical Assistance, contact Mercury Science Inc: (866) 861-5836.

Additional Information

MATERIALS REQUIRED BUT NOT SUPPLIED WITH THE KIT

The Domoic Acid test kit is part of a complete system of immunodiagnostic reagents and instrumentation. The system requires the following equipment.

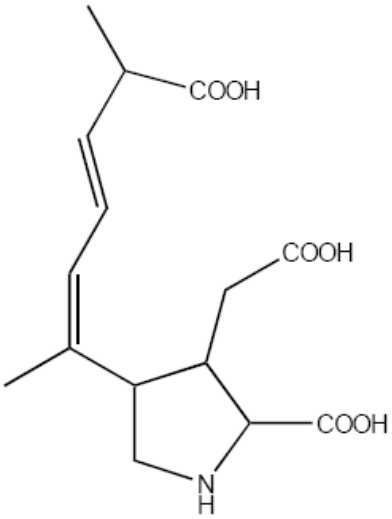
1. Microtiterplate Reader able to measure Absorbance at 450 nm
2. Platewasher
3. Plate Shaker
4. 8 Channel pipet
5. Pipetmen (P10, P200 and P1000)

Other Notes:

- Perform each Control and Sample in duplicate wells.
- All sample extracts should be filtered prior to analysis.
- All reagents and samples should be brought to room temperature prior to use.
- Use only the number of strips needed.
- Keep unused strips stored in their aluminum foil pouch with the included desiccant until needed.
- If Control absorbance is greater than 3.0 AU, remove 100 uL from ALL WELLS and repeat absorbance measurement.

An Excel worksheet has been developed to analyze results and quantitate the amount of domoic acid in extracts. Send your request for the "Domoic Acid Quantitation Worksheet - DAK-36" to: info@mercuryscience.com

Structure of Domoic Acid

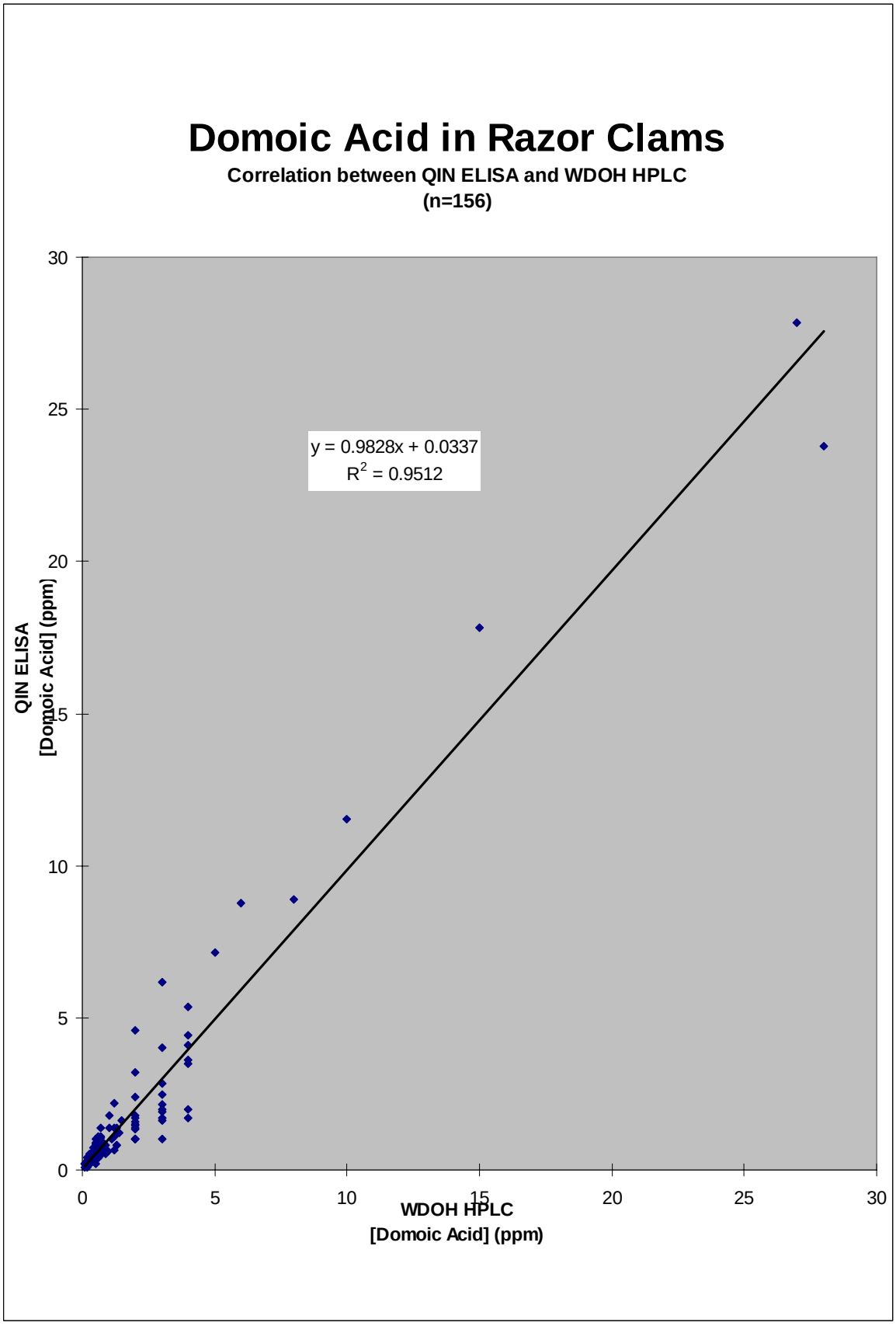


Domoic Acid Test Kit

Summary Protocol Sheet

Add Antibody	50 uL
Add Control and Samples	50 uL
Incubate	Shake for 30 minutes
Add Tracer	50 uL
Incubate	Shake for 30 minutes
Wash	"3 WASHES" program
TMB	Add 100 uL, shake for 5 minutes
Stop	Add 100uL
Measure	Absorbance at 450 nm

Note: If Control absorbance is greater than 3.0 AU, remove 100 uL from ALL WELLS and repeat absorbance measurement.


Domoic Acid in Razor Clams
Correlation between QIN ELISA and WDOH HPLC
(n=156)
y = 0.9828x + 0.0337
R2 = 0.9512
QIN ELISA
[Domoic Acid] (ppm)
WDOH HPLC
[Domoic Acid] (ppm)
0
5
10
15
20
25
30

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

(http://www.issc.org/client_resources/lmr%20documents/i.%20issc%20lab%20method%20application%20checklist.pdf)

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

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Methods Review (LMR) Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

Name of the New Method	DOMOIC ACID RAPID ENZYME-LINKED IMMUNOSORBENT ASSAY – 96 Well Format
Name of the Method Developer	Mercury Science Inc. and the National Oceanic and Atmospheric Administration
Developer Contact Information	Attn: Tom Stewart 4802 Glendarion Dr. Durham, NC 27713 Phone: (866) 861-5836

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.	Y	Faster, more affordable DA analysis
2. What is the intended purpose of the method?	Y	Monitoring shellfish and water samples for DA
3. Is there an acknowledged need for this method in the NSSP?	Y	Faster analysis decreases public health risks
4. What type of method? i.e. chemical, molecular, culture, etc.	Y	Enzyme-Linked Immunosorbent Assay (ELISA)
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title	Y	DOMOIC ACID RAPID ENZYME-LINKED IMMUNOSORBENT ASSAY – 96 Well Format
Method Scope	Y	For the analysis of food, phytoplankton, and water
References	Y	Peer Reviewed Publication, Independent Correlation Study
Principle	Y	Competitive ELISA
Any Proprietary Aspects	Y	Unique Antibody and Enzyme Conjugate
Equipment Required	Y	Equipment is listed for this method
Reagents Required	Y	Reagents are listed for this method
Sample Collection, Preservation and	Y	Requirements are described for this method

Storage Requirements		
Safety Requirements	Y	Normal Good Lab Practices
Clear and Easy to Follow Step-by-Step Procedure	Y	See User Guide supplied with DA Test kit.
Quality Control Steps Specific for this Method	Y	Described below
C. Validation Criteria		
1. Accuracy / Trueness		SLV Testing in Progress – see preliminary results using oysters
2. Measurement Uncertainty		SLV Testing in Progress– see preliminary results using oysters
3. Precision Characteristics (repeatability and reproducibility)		SLV Testing in Progress– see preliminary results using oysters
4. Recovery		SLV Testing in Progress– see preliminary results using oysters
5. Specificity		SLV Testing in Progress
6. Working and Linear Ranges		See publication Dec 2008 issue Journal Shellfish Research - 0.3 to 3 ppb
7. Limit of Detection		Linear range
8. Limit of Quantitation / Sensitivity		SLV Testing in Progress
9. Ruggedness		SLV Testing in Progress
10. Matrix Effects		SLV Testing in Progress
11. Comparability (if intended as a substitute for an established method accepted by the NSSP)		Results from one independent study are included
D. Other Information		
1. Cost of the Method	Y	$200 per 36 duplicate samples
2. Special Technical Skills Required to Perform the Method	Y	Some ELISA experience or training required
3. Special Equipment Required and Associated Cost	Y	See list
4. Abbreviations and Acronyms Defined	Y	See list
5. Details of Turn Around Times (time involved to complete the method)	Y	90 minutes
6. Provide Brief Overview of the Quality Systems Used in the Lab	Y	See attached
Submitters Signature 	Date: June 18, 2009	
Submission of Validation Data and Draft Method to Committee	Date:	
Reviewing Members	Date:	
Accepted	Date:	
Recommendations for Further Work	Date:	

II. DEFINITIONS

1. **Accuracy/Trueness** - Closeness of agreement between a test result and the accepted reference value.
2. **Analyte/measurand** - The specific organism or chemical substance sought or determined in a sample.
3. **Blank** - Sample material containing no detectable level of the analyte or measurand of interest that is subjected to the analytical process and monitors contamination during analysis.
4. **Comparability** – The acceptability of a new or modified method as a substitute for an established method in the NSSP. Comparability must be demonstrated for each substrate or tissue type by season and geographic area if applicable.
5. **Fit for purpose** – The analytical method is appropriate to the purpose for which the results are likely to be used.
6. **HORRAT value** – HORRAT values give a measure of the acceptability of the precision characteristics of a method.[4]
7. **Limit of Detection** – the minimum concentration at which the analyte or measurand can be identified. Limit of detection is matrix and analyte/measurand dependent.[4]
8. **Limit of Quantitation/Sensitivity** – the minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.
9. **Linear Range** – the range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.
10. **Measurement Uncertainty** – A single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects. **.** **Matrix** – The component or substrate of a test sample.
12. **Method Validation** – The process of verifying that a method is fit for purpose.[1]
13. **Precision** – the closeness of agreement between independent test results obtained under stipulated conditions.[1, 2] There are two components of precision:
    a. **Repeatability** – the measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.
    b. **Reproducibility** – the measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.
14. **Quality System** - The laboratory's quality system is the process by which the laboratory conducts its activities so as to provide data of known and documented quality with which to demonstrate regulatory compliance and for other decision–making purposes. This system includes a process by which appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.
15. **Recovery –** The fraction or percentage of an analyte or measurand recovered following sample analysis.
16. **Ruggedness** – the ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]
17. **Specificity** – the ability of a method to measure only what it is intended to measure.[1]
18. **Working Range –** the range of analyte or measurand concentration over which the method is applied.

REFERENCES:

7. Eurachem Guide, 1998. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. LGC Ltd. Teddington, Middlesex, United Kingdom.
8. IUPAC Technical Report, 2002. Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, Pure Appl. Chem., Vol. 74, (5): 835-855.
9. Joint FAO/IAEA Expert Consultation, 1999. Guidelines for Single-Laboratory Validation of Anilytical Methods for Trace-Level Concentrations of Organic Chemicals.
10. MAF Food Assurance Authority, 2002. A Guide for the Validation and Approval of New Marine Biotoxin Test Methods. Wellington, New Zealand.
11. National Environmental Laboratory Accreditation. , 2003. Standards. June 5.
12. EPA. 2004. EPA Microbiological Alternate Procedure Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Methods: Guidance. U.S. Environmental Protection Agency (EPA), Office of Water Engineering and Analysis Division, 1200 Pennsylvania Avenue, NW, (4303T), Washington, DC 20460. April.

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

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

Information: Applicants shall attach all procedures, with materials, methods, calibrations and interpretations of data with the request for review and potential approval by the ISSC. The ISSC also recommends that submitters include peer-reviewed articles of the procedure (or similar procedures from which the submitting procedure has been derived) published in technical journals with their submittals. Methods submitted to the ISSC LMR committee for acceptance will require, at a minimum, 6 months for review from the date of submission.

Note: The applicant should provide all information and data identified above as well as the following material, if applicable:

Justification for New Method

• Name of the New Method.

DOMOIC ACID RAPID ENZYME-LINKED IMMUNOSORBENT ASSAY – 96 Well Format
(Marketed by Mercury Science Inc. as Product # DAK-36 Domoic Acid Test Kit.)

• Specify the Type of Method (e.g., Chemical, Molecular, or Culture).

Enzyme linked immunosorbent assay (ELISA) using an anti-domoic acid monoclonal antibody

• Name of Method Developer.

The DA assay kit was developed jointly by NOAA's National Centers for Coastal Ocean Science, National Ocean Service, and the Northwest Fisheries Science Center, together with an industry partner Mercury Science, Inc., Durham, North Carolina

• Developer Contact Information [e.g., Address and Phone Number(s)].

Mercury Science Inc.
Attn: Tom Stewart
4802 Glendarion Dr.
Durham, NC 27713
Phone: (866) 861-5836

• Date of Submission.

June 18, 2009

• Purpose and Intended Use of the Method.

The method is an accurate, rapid, cost-effective tool for use by environmental managers and public health officials to monitor Domoic Acid concentrations in environment samples.

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

The regulatory method for DA detection sanctioned by the Interstate Shellfish Sanitation Conference is a high performance liquid chromatography (HPLC) assay. Though accurate, these analyses are generally run by centralized state facilities with results typically not available for 3 to 14 days after the samples are collected. In more remote communities, many of which depend heavily on subsistence clam harvests, these long delays and the costs of sample analysis are causes for public health concern. The average cost of approximately $100 per sample limits the number of samples that can be analyzed (Harold Rourk, Washington State Department of Health, personal communication). Resource managers in coastal communities have expressed their desire for a cost-effective method for rapid and accurate determination of DA concentrations in shellfish and phytoplankton samples. The high throughput capacity of the assay also allows for much faster response times when domoic acid events occur. The relatively low cost of the assay means that significantly more sampling is also possible on the same or smaller budget.

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

This ELISA is sensitive to organic solvents such as methanol. Sample extracts that contain methanol can be diluted with Sample Dilution Buffer (provided in the kit) to reduce methanol concentrations to less than 1%.

• Other Comments.

The implementation of this ELISA system required the development and validation of two essential reagents, a high avidity monoclonal antibody to DA and a stable DA-HRP conjugate recognized by the same monoclonal antibody.

Method Documentation

• Method Title.

Domoic Acid Rapid Enzyme-Linked ImmunoSorbent Assay (ELISA) – 96 Well Format

• Method Scope.

The method is a sequential competitive enzyme linked immunosorbent assay (ELISA) utilizing a high avidity monoclonal antibody (mAb) to DA to ensure assay specificity and consistency across production lots. The assay is specific for Domoic Acid and can be used for the analysis of tissue extracts, phytoplankton samples, and water samples.

• References.

RAPID ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF THE ALGAL TOXIN DOMOIC ACID, Journal of Shellfish Research, Vol. 27, No. 5, 1301–1310, 2008.
Available online at: http://mercuryscience.com/LitakerStewartDec2008.pdf

User Guide Available Online at: http://www.mercuryscience.com/DA User Guide 2007A.pdf

• Principle.

A fixed number of anti-DA mAb binding sites are incubated with dissolved DA in the sample followed by the addition of a DA – horseradish peroxidase (HRP) conjugate. As these binding events occur, the anti-DA mAb molecules are simultaneously captured by anti-mouse antibodies affixed to the surface of the microtiter plate wells. Following a wash step, subsequent HRP derived color development, readable on standard microplate readers, was inversely proportional to the concentration of DA in the sample matrix. The assay reagents were titrated so that the amount of mAb and the DA–HRP conjugate added produced a maximal absorbance signal of approximately 2.5 absorbance units when no DA was present.

• Analytes/Measurands.

Domoic Acid

• Proprietary Aspects.

The assay uses a unique monoclonal antibody and enzyme conjugate developed by Mercury Science Inc.

• Equipment.

Microtiterplate orbital shaker
Automated microtiterplate washer
Multichannel pipette
Pipetman (P20, P200, P1000) or equivalent
Microtiterplate reader (capable of reading at 450nm)

• Reagents.

1. anti-DA antibody
2. DA-HRP conjugate
3. Assay Buffer
4. Control Solution
5. Wash solution
6. TMB substrate
7. Stop solution

• Media.

Tissue samples are extracted using a solvent of Methanol:Water (50:50, v:v)
Extracts are diluted into an aqueous sample buffer prior to analysis by the ELISA.

Water samples are filtered and buffered prior to analysis by the ELISA.

Phytoplankton samples are ruptured by appropriate methods in aqueous sample buffer prior to analysis by the ELISA.

• Matrix or Matrices of Interest.

Butter clam (*Saxidomus giganteus*), blue mussel (*Mytilus edulis*), geoduck (*Panopea abrupta*), manila clam (*Venerupis japonica*), oyster (*Crassostrea virginica*), quahog (*Mercenaria mercenaria*) and razor clam (*Siliqua patula*) tissues, as well as phytoplankton and water samples

• Sample Collection, Preservation, Preparation, Storage, Cleanup, etc.

Shellfish preparation: In the case of shellfish, pooled samples of 10-12 individuals are cleaned, and ground to a smooth and uniform homogenate in a commercial blender. Approximately 2 g of homogenized tissue are added to a tared 50 mL conical tube and the weight recorded to the nearest 0.01g. Next, 18 mL of 50% methanol are added and the samples mixed at high speed on a vortex mixer for 2 min. Once the extraction is complete, the tubes are spun in a table top centrifuge for 20 min at 10,000xg or until a tight pellet and clear supernatant are obtained. If the samples do not clear despite the spinning at high speed, the supernatant is passed through a 0.45 µm syringe filter. The extract is then diluted 1:100 or 1:1000 into Sample Dilution Buffer and is ready for analysis by ELISA. If necessary, the sample may be stored at 4ºC for up to 24 h in a refrigerator prior to analysis.

Phytoplankton preparation: Approximately 0.1 to 1.0 L of cultured cells or sea water samples are filtered onto a GF/F filter which can be immediately frozen at -80^{o}C until the filter can be processed or processed immediately. For processing, filters are placed in a 5mL conical tube and 3 mL of 20% methanol are added. The samples are sonicated until the filter is completely homogenized. Care is needed to prevent the probe from rupturing the tube. The sonicator probe is cleaned carefully with 20% methanol between samples to prevent cross-contamination. Next the homogenate is centrifuged at 3000xg for 10 minutes. The supernatant is passed through a 0.2 µm syringe filter. The extract is then diluted into Sample Dilution Buffer and is ready for analysis by ELISA.

Storage of test kit: Any unused strips can be removed and stored in a desiccator pouch at 4^{o}C for at least six months without compromising assay performance

• Safety Requirements.

General Good Laboratory Practices should be followed at all times.
Safety Glasses should be worn at all times.
The Stop solution in the assay contains 1 M hydrochloric acid. Care must be taken to avoid skin or eye contact with the Stop solution.

• Other Information (Cost of the Method, Special Technical Skills Required to Perform the Method, Special Equipment Required and Associated Cost, Abbreviations and Acronyms Defined and Details of Turn Around Times [Time Involved to Complete the Method]).

Cost of the Method: The DAK 36 Domoic Acid Test Kit costs $200 and contains sufficient assay reagents to perform 36 sample analyses (less than $6 per sample)

Special Technical Skills Required to Perform the Method: It is recommended that users have prior experience performing ELISA assays or receive training from Mercury Science Inc.

Special Equipment Required and Associated Cost (estimated):

- Microtiterplate orbital shaker $500
- Automated microtiterplate washer $5,000
- Multichannel pipette $700
- Pipetmen (P20, P200, P1000) (or equivalent) $1,500
- Microtiterplate reader (capable of reading at 450nm) $6,500

This equipment is commonly available in most state laboratories.

Abbreviations and Acronyms Defined:

ELISA – Enzyme-Linked Immunosorbent Assay
HRP – Horseradish Peroxidase
TMB – Tetramethylbenzidine
DA – Domoic Acid
mAb – monoclonal Antibody

Details of Turn Around Times: As many as 36 sample extracts can be analyzed in <1.5 hours.

• Test Procedures, (Be Specific and Provide Easy-to-Follow Step-by-Step Procedures and indicate critical steps.).

The 96 well assay tray used in the assay contains 12 strips. Each strip of 8 wells can be removed and stored until it is needed. The first two wells of each strip are used as a control (no DA added). The remaining six wells are used to analyze 3 samples in duplicate. This format provided the flexibility of running anywhere from 3 to 36 duplicate samples at a time.

1. For unknown sample analysis, extracts are diluted to a final concentration ranging from 0.3 to 3 to ppb using the Sample Dilution Buffer [phosphate salt solution, pH 7.8, containing casein]. For clam tissues containing DA, sample dilutions of 1:100 and 1:1000 are typically used. (Preliminary tests with razor clam extracts showed that a 25-fold dilution in sample dilution buffer eliminates matrix effects in ELISA analysis.)
2. The immunoassay is started by adding 50 µl of the anti-DA antibody reagent to each well using a multi-channel pipette.
3. Next, 50 µl of the Control solution (sample buffer without DA) is added to the first two wells in each strip.
4. Duplicate 50 ul aliquots from the diluted DA extracts are then added to the remaining wells in each strip and the plate is shaken at room temperature for 30 minutes on an orbital shaker set to vigorously mix the solution in each well. **Vigorous mixing is key to reaching equilibrium in the allotted time and obtaining replicable results from one run to the next.** In this step, DA in the sample binds to available mAb in proportion to [DA].
5. At the end of the incubation, 50 µl of DA HRP conjugate is added to each well and the plate is shaken a second time for 30 min at room temperature on an orbital shaker. The DA-HRP will bind to available mAb sites.
6. Following Step 5, the plate is washed three times with wash solution [Tris-HCl buffered salt solution (pH 7.8) containing Tween 20 and sodium azide as a preservative] using a commercial plate washer, making certain the fluid is completely aspirated from all the wells. *Alternatively, these washes can be done manually by adding wash solution to wells using a multichannel pipettor and then flicking all fluid from the wells*. The manual method may result in slightly higher variability.
7. Next, 100 µL of SureBlue TMB substrate (5,5'-tetramethylbenzidine, kpl.com) is added to each well.
8. The plate is placed on an orbital shaker for no more than 5 minutes, or until adequate color development is observed.

9. Color development is terminated by adding 100 µL stop solution (1N hydrochloric acid) to each well.
10. The absorbance in each well is measured at 450 nm using a plate reader.
11. The DA concentrations are determined using the sample (B) and control (B_o) absorbances, the original tissue weights, and the volume of 20% or 50% methanol used to extract each sample. The actual calculations are made using a Microsoft Excel work sheet that incorporates the constants for a four parameter model (DA concentration $=ED_{50}(B_o/B\ -1)^{-slope}$). This worksheet can be downloaded from:

http://www.mercuryscience.com/Domoic%20Acid%20Quantitation%208Well%20Strip.xls

Processing time for this assay is approximately 1.5 hours.

• Quality Control (Provide Specific Steps.).

Bo signals should be greater than 1.5 AU and less than 3.0 AU. When Bo values are greater than 3.0, the user can remove 50 ul of the yellow solution from ALL wells on that strip and re-read the signal.

Duplicate signals should be within 10% of their average value. For example: Two duplicate wells having AU values of 1.500 and 1.600 are acceptable because the difference between the values and their average (1.550) is less than 10%. If two duplicate wells have AU values of 1.000 and 1.400, this result is invalid and should be retested because the variation between the values is too great because: (1.200 - 1.000)/1.000 = 20%

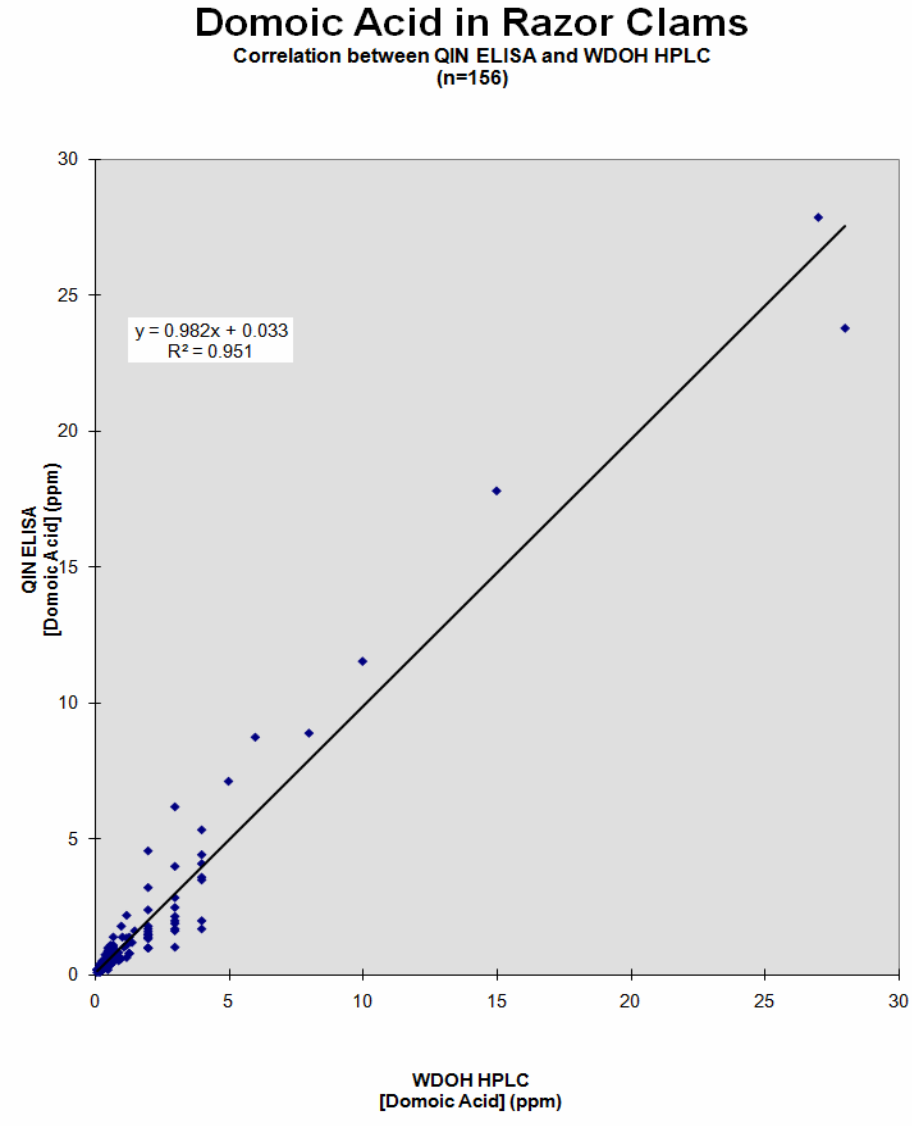
Domoic Acid standard solutions can be run as needed to QC the accuracy of the assay. QC protocols can be developed on a case-by-case basis with assistance provided by Mercury Science Inc.

• Validation Criteria (Include Accuracy / Trueness, Measurement Uncertainty, Precision [Repeatability and Reproducibility], Recovery, Specificity, Working and Linear Ranges, Limit of Detection, Limit of Quantitation / Sensitivity, Ruggedness, Matrix Effects and Comparability (if intended as a substitute for an established method accepted by the NSSP).

A preliminary validation study using oyster tissue has been completed and provided to the committee for feedback. Oysters were selected because they were locally available and could be run prior to the submission date. These data should be considered preliminary. In addition, an informal validation study was conducted by the Quinault Tribe and the Washington Department of Health and included below. The remaining validation studies are will be done in the latter part of the summer and fall 2009. Results will be provided to the LRM committee as they become available.

During internal validation studies at Mercury Science, the assay was found to have an effective quantitative range from approximately 0.3 to 3.0 ppb using domoic acid standard solutions.

- Comparability: The graph below shows the results of a year-long study done by the Quinault Indian Nation (QIN) and the Washington Department of Health (WDOH) comparing razor clam analysis performed by the Domoic Acid Test Kit versus HPLC analysis. One hundred fifty six samples were compared. This independent study was planned and performed without any input from Mercury Science or NOAA.



Additional correlation studies are reported in the following research paper:

RAPID ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF THE ALGAL TOXIN DOMOIC ACID, Journal of Shellfish Research, Vol. 27, No. 5, 1301–1310, 2008.
Available online at: http://mercuryscience.com/LitakerStewartDec2008.pdf

- Data and Statistical Analyses Performed for Each Validation Criterion Tested (Be Specific and Provide Clear Easy-to-Follow Step-by-Step Procedures.). Preliminary study presented for feedback from the committee

- Calculations and Formulas Used for Each Validation Criterion Tested. Testing in Progress

- Results for Each Validation Criterion Tested. Testing in Progress

- Discussion of Each Validation Criterion Tested. Testing in Progress

- Summary of Results. Testing in Progress

Additional Requirement
If a laboratory method is found acceptable for use in the National Shellfish Sanitation Program and adopted by the Interstate Shellfish Sanitation Conference, the method submitter will draft a laboratory checklist that can be used to evaluate laboratories performing their procedure. The checklist will be submitted to the ISSC and reviewed by the Laboratory Quality Assurance Committee for Conference approval.

(For guidance: refer to the checklists in the National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish 2003, Guidance Documents, Chapter II – Growing Areas, .11 Evaluation of Laboratories by State Laboratory Evaluation Officers Including Laboratory Evaluation Checklists.)

VII. SLV Documents for Marine Biotoxin and Non-MPN Based Microbiological Methods (http://www.issc.org/lmrforms.aspx)

VII. #1 Marine Biotoxin and Non-MPN Based Microbiological Methods SOP – Accuracy/Trueness & Measurement Uncertainty

VALIDATION CRITERIA

Accuracy/Trueness is the closeness of agreement between test results and the accepted reference value. To determine method accuracy/trueness, the concentration of the targeted analyte/measurand/organism of interest as measured by the analytical method under study is compared to a reference concentration.

Measurement uncertainty is a single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.

Procedure: This procedure is applicable for use with either growing waters or shellfish tissues. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take two (2) aliquots of either the homogenate or growing water sample appropriately sized for your work and spike one(1) of the two (2) aliquots with a suitable known concentration of the target analyte/measurand/organism of interest. Do not spike the second aliquot. This is the sample blank. For microbiological methods determine the concentration of the target organism of interest used to spike each sample by plating on/in appropriate agar. Process both aliquots of sample as usual to determine the method concentration for the target analyte/measurand/organism of interest. For growing waters do twenty (20) samples collected from a variety of growing areas. For shellfish do twenty (20) samples for each shellfish tissue type of interest collected from a variety of growing areas, the same growing area harvested on different days or from different process lots. **Use a variety of concentrations spanning the range of concentrations of importance in the application of the method to spike sample homogenates or growing water samples**. Both the low and high level spike concentrations must yield determinate values when analyzed by the method under study.

Data:

Working Range _The working range is 0.3 to 3.0 ppb and samples are diluted into the effective range so the working range is 0 to over 100 ppm

Sample Type _Shellfish Tissue__

Agar used to determine spike concentration ___Not applicable__

Organism used for spiking Oyster (*Crassostrea virginica*)

Sample Spike conc/plate count Sample blank conc Spiked sample conc from analysis

The regulatory limit for DA is 20 ppm in shellfish tissue and the dynamic range of the assay was tested using oyster tissues spiked with 2.3 to 35.5 ppm domoic acid. The standard spikes of domoic acid were calibrated using the Canadian NRC standards. The following procedure was used.

Extraction:

1. Live oysters were shucked on 3/30 and 3/31/2009 and homogenized 12 at a time in a blender and stored in 50mL tubes in -80C freezer
2. Samples thawed just prior to use
3. 2 g oyster weighed out in 50mL tube and exact weigh recorded to nearest mg
4. 18mL 50% MeOH added to tube
5. DA added to the homogenate so that the final concentrations in 20 mL were 0.25, .5, 1, 2, 4 ppm. This is equivalent to 2.5,5,10,20 or 40ppm in 2g oyster that is subsequently extracted into the total 20 ml volume.
6. Each tube vortexed for 1 min

ELISA

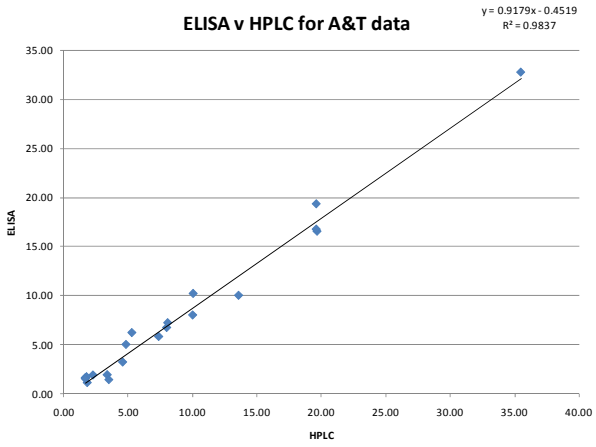
1. ~1.4mL from each tube were transferred into a 2mL microfuge tube
2. Samples in microfuge tubes centrifuged at 14,000 rpm for 5 min
3. Aliquots of the resulting supernatant were diluted with ELISA kit sample dilution buffer with a 2 step dilution series so each extract contained ~2ppb
4. Diluted extracts processed on ELISA following kit instructions

HPLC was used to determine initial spike concentration using the following procedure:

1. Spiked 50mL tubes centrifuged at 3000rpm for 20 min
2. Supernatant filtered with 25mm GF/F filter first, and then filtered with .45um syringe tip filter with 30mL syringe
3. SPE tubes pre-conditioned with 6mL MilliQ water, then 3mL 100% MeOH, then 50% MeOH
4. 5mL of extract though SPE tube, 1 drop per second
5. Washed with 5mL .1M NaCl
6. Eluted/ collected with 5 mL .5M NaCL in 15mL tube
7. ~1mL pipetted with 9 inch glass Pasteur pipette into clear HPLC vial
8. Run through HPLC- 20uL injection, .3mL/min, 15 min/sample….
9. Area and time of peak recorded
10. The DA concentration in each oyster extract was estimated using the previously determined standard curve where peak area =15.704 x DA concentration, R^2=0.9977.

Results

Sample #	Sample Spike conc (HPLC)	Sample blank conc	Spiked sample conc. from analysis (ELISA)
1	5.32	0.00	6.20
2	10.07	0.00	10.18
3	19.69	0.00	16.53
4	35.50	0.00	32.74
5	8.02	0.00	6.72
6	2.30	0.00	1.88
7	4.60	0.00	3.20
8	1.70	0.00	1.60
9	8.10	0.00	7.20
10	1.80	0.00	1.70
11	3.40	0.00	1.90
12	7.40	0.00	5.80
13	13.60	0.00	10.00
14	19.63	0.00	16.74
15	1.85	0.00	1.10
16	3.53	0.00	1.40
17	4.86	0.00	4.99
18	1.70	0.00	1.50
19	10.03	0.00	7.99
20	19.63	0.00	19.32
Average	9.14	0.00	7.93



The results of this preliminary study showed an excellent correlation between the HPLC and the ELISA assay, but with a slope of 0.92 instead of 1.0. This means the ELISA assay consistently underestimated the HPLC validated DA concentrations by ~10%. Preliminary tests using other shellfish tissues have shown a slope of approximately 1.0 (Litaker et al. 2008). I will do additional tests to determine whether or not the lower slope is due to matrix effects unique to oysters.

A consequence of this underestimation is that some of the statistical analyses below will show a significant difference between the spike concentration and the ELISA results. Given that this is the first time I have run through the calibration assay procedures I would request that the committee to wait for additional data before making any judgments concerning the robustness of the assay. Instead, I would like to use the preliminary oyster data to get the committee's feedback on whether I have adequately completed the necessary statistical analyses correctly and to obtain further clarifications concerning several of the analyses. The feedback will then be used for finalizing the subsequent analyses done in my laboratory and by the NOAA CCFHR laboratory.

For shellfish samples, repeat for each tissue type of interest.

DATA HANDLING

Accuracy/Trueness

The accuracy/trueness of a method consists of two distinct components, the portion due to the method itself regardless of the laboratory performing it and the portion contributed by the laboratory's performance. In a single laboratory method validation, it is impossible to distinguish the contribution of each to the overall accuracy/trueness of the method. Consequently, what is being estimated is the accuracy/trueness of the method as implemented by the laboratory performing the analysis. Good accuracy/trueness suggests the appropriateness of the method and the laboratory's performance of it for the intended work. Poor accuracy/trueness on the other hand indicates the potential unsuitability of the method and/or the laboratory's performance of it for the intended work.

Accuracy /trueness will be determined by calculating the closeness of agreement between the test results and either a known reference value or a reference value obtained by plate count for microbiological methods.

Measurement uncertainty

Measurement uncertainty can be determined by subtracting the results for each spiked sample from the reference value for the sample and calculating the 95% confidence interval of these differences. The confidence interval of these differences represents the range in values within which the true measurement uncertainty lies. A narrow range in values indicates that the method as implemented by the laboratory produces reliable results.

Use the log transformed data for both the plate count and the microbial results obtained from the spiked samples. If necessary use the sample blank (converted to logs for microbiological methods) to correct the spiked sample

for matrix effects and calculate the two-sided, 95% confidence interval for the difference in concentrations between the reference and the spiked samples. This range in counts represents the measurement uncertainty of the method as implemented by the laboratory.

Data Summary:
Calculated % accuracy/trueness ___86.84____

Again, the reason for the lower than expected accuracy is the fact that the slope of the relationship was 0.92 between the ELISA and HPLC for this first set of oyster samples.

Sample #	Sample Spike conc (HPLC)	Spiked sample conc. from analysis (ELISA)	Difference (ppm)
1	5.32	6.2	-0.88
2	10.07	10.18	-0.11
3	19.69	16.53	3.16
4	35.5	32.74	2.76
5	8.02	6.72	1.3
6	2.3	1.88	0.42
7	4.6	3.2	1.4
8	1.7	1.6	0.1
9	8.1	7.2	0.9
10	1.8	1.7	0.1
11	3.4	1.9	1.5
12	7.4	5.8	1.6
13	13.6	10	3.6
14	19.63	16.74	2.89
15	1.85	1.1	0.75
16	3.53	1.4	2.13
17	4.86	4.99	-0.13
18	1.7	1.5	0.2
19	10.03	7.99	2.04
20	19.63	19.32	0.31
Average	9.14	7.93	1.21
stdev			1.21832223
95% confidence interval			0.53393371

Calculated measurement uncertainty __0.5 ppm___

VII. #2 Marine Biotoxin and Non-MPN Based Microbiological Methods SOP – Ruggedness

VALIDATION CRITERIA

Ruggedness is the ability of a particular method to withstand relatively minor changes in analytical technique, reagents or environmental factors likely to arise in different test environments.

Procedure: This procedure is applicable for use with either growing waters or shellfish tissue. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish type of interest use a minimum of 10 – 12 animals. For each sample take two (2) aliquots of either the growing water sample or shellfish homogenate appropriately sized for your work. Spike both aliquots with a suitable concentration of the target analyte/measurand/organism of interest. Process both aliquots of the sample as usual to determine method concentration for the target analyte/measurand/organism of interest. For the second aliquot of each sample, however, use a different batch or lot of culture media and/or test reagents as appropriate to process this aliquot. For growing waters, do ten (10) samples collected from a variety of growing waters. For shellfish do ten (10) samples for each shellfish tissue type of interest collected from a variety of growing areas, the same growing area harvested on different days or from different process lots. Use the same two batches or lots of culture media and/or test reagents to process each sample such that "batch or lot 1" is used to process the first aliquot of each sample and "batch or lot 2" is used to process the second aliquot of each sample. Use a range of concentrations which spans the range of the method's intended application to spike the sample aliquots. However both aliquots of the same sample must be spiked with the same concentration of the target analyte/measurand/organism of interest. Process samples over a period of several days.

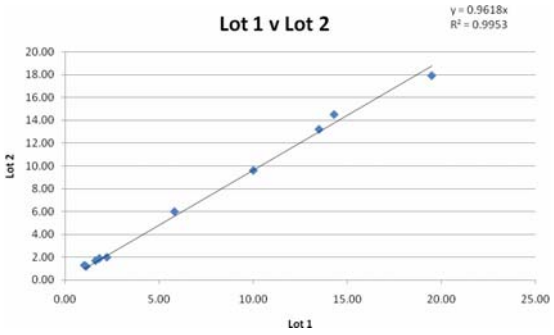
Data:

Sample type ___Oyster tissue_____
Sample Conc "Batch or Lot 1" Conc "Batch or Lot 2"
Media and/or Reagents Media and/or Reagents

Procedure:
Samples were spiked and extracted as listed in section VII. #1 Marine Biotoxin and Non-MPN Based Microbiological Methods SOP – Accuracy/Trueness & Measurement Uncertainty. After the sample was diluted in the 2-step dilution series, the sample was processed on two different ELISA kits with different lot numbers. Samples were processed between 5/19/09 and 5/27/09.

Sample #	Lot 1	Lot 2
1	1.60	1.70
2	13.50	13.20
3	2.20	2.00
4	14.30	14.50
5	1.80	1.90
6	5.80	6.00
7	10.00	9.60
8	19.50	17.90
9	1.10	1.20
10	1.00	1.30

The R^2 between the results for the two batches was 0.995 and the slope was y=0.96



For shellfish samples, repeat for each tissue type of interest.

DATA HANDLING

Ruggedness

In the day to day operations of the laboratory there will be changes in the batches/lots of culture media and/or test reagents used to process samples. Environmental factors are also likely to change over time. None of these factors, however, should adversely impact test results if the method as implemented is sufficiently rugged to be used routinely for regulatory monitoring.

Procedure: To determine whether the method as implemented is sufficiently rugged to withstand the types of changes anticipated to occur in routine use, a two-sided t-test at a significance level (α) of .05 will be used on the data to ascertain if results obtained using different culture media and/or test reagent batches/lots under slightly varying environmental conditions are significantly affected by such minor changes. Either a paired t-test or Welch's t-test will be used depending upon the shape of the distribution produced by the data for each batch/lot and their respective variances. Use log transformed data for the results obtained from microbiological methods. The appropriate t-test to be used for the analysis is determined in the following manner.

1. Test the symmetry of the distribution of results from both batch/lot 1 and batch/lot 2.
2. Calculate the variance of both batch/lot 1 and batch/lot 2 data.
3. Values for the test of symmetry for either batch/lot 1 or batch/lot 2 outside the range of -2 to +2 indicate a significant degree of skewness in the distribution.
4. A ratio of the larger of the variances of either batch/lot 1 or batch/lot 2 to the smaller of the variances of either batch/lot 1 or batch/lot 2 >2 indicates a lack of homogeneity of variance.
5. Use either the paired t-test or Welch's t-test for the analysis based on the following considerations.
    - If the distributions of the data from batch/lot 1 and batch/lot 2 are symmetric (within the range of -2 to +2) and there is homogeneity of variance, use a paired t-test for the analysis.
    - If the distributions of the data from batch/lot 1 and batch/lot 2 are symmetric (within the range of -2 to +2) but there is a lack of homogeneity of variance in the data, use Welch's t-test for the analysis.
    - If the distribution of the data from batch/lot 1 and batch/lot 2 are skewed (outside the range of -2 to +2) and the skewness for both groups is either positive for both or negative for both and there is homogeneity of variance in the data, use the paired t-test for the analysis.
    - If the distributions of the data from batch/lot 1 and batch/lot 2 are skewed and the skewness for both groups is either positive for both or negative for both but the data lacks homogeneity of variance, use Welch's t-test to analyze the data.

Paired T-test results – assumption that the variances are equal

Sample #	Lot 1	Lot 2
1	1.6	1.7
2	13.5	13.2
3	2.2	2
4	14.3	14.5
5	1.8	1.9
6	5.8	6
7	10	9.6
8	19.5	17.9
9	1.1	1.2
10	1	1.3
mean	7.08	6.93
stdev	6.7677	6.3808
t		0.0504
df		18
Significantly different		no

Welch's t-test

The t-value assuming unequal variance was 0.9599.
DF = 18
Two-tailed probability 0.3498, NS

Data Summary:
Value for the test of symmetry of the distribution of batch/lot 1 data _Not determined__
Value for the test of symmetry of the distribution of batch/lot 2 data _Not determined__
Variance of batch/lot 1 data _6.767701_____
Variance of batch/lot 2 data __6.380883_____
Ratio of the larger to the smaller of the variances of batch/lot 1 and batch/lot 2 _1.0606__
Is there a significant difference between batch/lot 1 samples and batch/lot 2 samples ____N__

Neither the paired or Welch's t-test estimates showed a significant difference between batches

VII. #3 Marine Biotoxin and Non-MPN Based Microbiological Methods SOP – Precision & Recovery

VALIDATION CRITERIA
Precision is the closeness of agreement between independent test results obtained under stipulated conditions.
Recovery is the fraction or percentage of an analyte/measurand/organism of interest recovered following sample analysis.
Procedure: This procedure is applicable for use with either growing waters or shellfish tissue. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take four (4) aliquots of either the shellfish homogenate or growing water sample appropriately sized for the work. Spike one of the four aliquots with a low (but determinable by the method under study) concentration of the target analyte/measurand/organism of interest. Spike the second aliquot of the growing water sample or shellfish homogenate with a medium

concentration of the target analyte/measurand/organism of interest. Spike the third aliquot of the growing water sample or shellfish homogenate with a high (but determinable by the method under study) concentration of the target analyte/measurand/organism of interest. Do not spike the fourth aliquot of the growing water sample or shellfish homogenate. This is the sample blank. Spiking levels must cover the range in concentrations important to the application of the method (working range). For microbiological methods determine the concentration of the target organism of interest used to spike each aliquot by plating in/on appropriate agar. Process each aliquot including the sample blank as usual to determine the method concentration for the target analyte/measurand/organism of interest. Do two (2) replicates for each of the three (3) spiked aliquots. Replicate analysis is unnecessary for the sample blank. Do only one sample blank per sample. For growing waters, do ten (10) samples collected from a variety of growing areas. For shellfish, do ten (10) samples for each shellfish tissue type of interest collected from a variety of growing areas, the same growing area harvested on different days or from different process lots. Use the same spiking levels for each of the ten (10) samples analyzed in this exercise (i.e. 10^1, 10^3 and 10^5).

Data:

Working Range _The working range is 0.3 to 3.0 ppb and samples are diluted into the effective range so the working range is 0 to over 100 ppm
Sample Type _Shellfish Tissue__
Agar used to determine spike concentration ___Not applicable__
Organism used for spiking Oyster (*Crassostrea virginica*)

Procedure: Samples were spiked and extracted as listed in section VII. #1 Marine Biotoxin and Non-MPN Based Microbiological Methods SOP – Accuracy/Trueness & Measurand Uncertainty. Each sample was spiked with a low, medium and high concentration of approximately 2.5, 20, and 40ppm in the tissue sample. HPLC was used to determine actual spike concentration.

Sample Spike conc/Plate count/Conc of blank Conc in spiked sample from analysis

	Aliquot 1	Aliquot 2			Aliquot 3			Aliquot 4		
Sample #	Blank	L spike	L_a	L_b	M spike	M_a	M_b	H spike	H_a	H_b
1	0.00	2.60	3.00	2.50	20.14	20.50	19.40	39.93	33.70	38.50
2	0.00	2.71	2.85	2.96	19.10	19.17	19.90	39.28	31.66	33.55
3	0.00	2.26	2.11	2.19	19.64	23.42	22.29	39.84	29.32	30.24
4	0.00	2.50	1.48	1.86	19.21	16.09	16.57	35.50	32.74	30.30
5	0.00	2.62	2.08	1.87	19.11	14.01	15.92	36.56	30.95	30.84
6	0.00	2.45	2.00	2.70	15.89	17.11	13.72	34.97	26.14	27.82
7	0.00	1.99	2.06	2.31	16.42	13.00	12.36	35.32	25.44	27.08
8	0.00	1.70	1.60	1.70	14.77	13.50	13.16	27.30	19.50	19.40
9	0.00	2.14	1.80	1.70	14.60	12.50	12.40	29.48	27.40	27.70
10	0.00	1.80	1.70	1.80	14.84	12.90	12.20	30.49	26.80	30.60

~~1L 1L~~ ~~a~~
~~1L~~ ~~b~~
~~1M 1M~~ ~~a~~
~~1M~~ ~~b~~
~~1H 1H~~ ~~a~~
~~1H~~ ~~b~~
~~1B~~
~~2L 2L~~ ~~a~~
~~2 L~~ ~~b~~
~~2M 2M~~ ~~a~~
~~2M~~ ~~b~~
~~2H 2H~~ ~~a~~
~~2H~~ ~~b~~
~~2B~~
~~“ “~~
~~“ “~~
~~“ “~~
~~“ “~~
~~10L 10L~~ ~~a~~
~~10L~~ ~~b~~
~~10M 10M~~ ~~a~~
~~10M~~ ~~b~~
~~10H 10H~~ ~~a~~
~~10H~~ ~~b~~
~~10B~~

L, M and H refer to low, medium and high concentrations respectively. L_a, L_b, M_a, M_b, H_a and H_b refer to the replicate determinations of the sample aliquots spiked with low (L), medium (M) and high (H) concentrations of the target analyte/measurand/organism of interest. B refers to the sample blank.

For shellfish samples, repeat for each tissue type of interest.

DATA HANDLING

Precision

To determine the precision of the method as implemented by the laboratory over the range in concentrations important to the intended application of the method, the data is manipulated in the following manner:

1. Convert the plate counts and spiked sample results for the microbiological methods to logs.
2. If necessary, use the sample blank (converted to logs for the microbiological methods) to correct the results from the spiked samples for matrix effects.
3. Perform a nested or hierarchical analysis of variance (ANOVA) on the corrected spiked sample data using the following variance components.

		Low					**Medium**				**High**				
		L spike	La	(La)^2	Lb	(Lb)^2	M spike	Ma	(Ma)^2	Mb	(Mb)^2	H spike	Ha	(Ha)^2	Hb	(Hb)^2
		2.6	3	9	2.5	6.25	20.14	20.5	420.25	19.4	376.36	39.93	33.7	1135.69	38.5	1482.25
		2.71	2.85	8.1225	2.96	8.7616	19.1	19.17	367.489	19.9	396.01	39.28	31.66	1002.36	33.55	1125.6
		2.26	2.11	4.4521	2.19	4.7961	19.64	23.42	548.496	22.29	496.844	39.84	29.32	859.662	30.24	914.458
		2.5	1.48	2.1904	1.86	3.4596	19.21	16.09	258.888	16.57	274.565	35.5	32.74	1071.91	30.3	918.09
		2.62	2.08	4.3264	1.87	3.4969	19.11	14.01	196.28	15.92	253.446	36.56	30.95	957.903	30.84	951.106
		2.45	2	4	2.7	7.29	15.89	17.11	292.752	13.72	188.238	34.97	26.14	683.3	27.82	773.952
		1.99	2.06	4.2436	2.31	5.3361	16.42	13	169	12.36	152.77	35.32	25.44	647.194	27.08	733.326
		1.7	1.6	2.56	1.7	2.89	14.77	13.5	182.25	13.16	173.186	27.3	19.5	380.25	19.4	376.36
		2.14	1.8	3.24	1.7	2.89	14.6	12.5	156.25	12.4	153.76	29.48	27.4	750.76	27.7	767.29
		1.8	1.7	2.89	1.8	3.24	14.84	12.9	166.41	12.2	148.84	30.49	26.8	718.24	30.6	936.36
Subgroup sample number	n(I, j, l)		10		10			10		10			10		10	
Subgroup sum	Sum (i, j, l)		20.68		21.59			162.2		157.92			283.65		296.03	Sum
Subgroup variance	[(Sum (i, j, l))^2]/n(I, j, l)		42.77		46.61			2630.88		2493.87			8045.73		8763.38	22023.24
Group sample number	n(i)		20					20					20			60
Group sum	Group sum		42.27					320.12					579.68			942.07

Group mean	Xhat (i)	2.17	16.46	30.95	
Group variance	[(Xhat (i))^2]/n(i)	89.3376	5123.84	16801.4	22014.62

C	14791.59808
Total SS	7859.977618
Among all subgroups SS	7231.65
error SS	628.33
Groups SS	7223.025403
Subgroups SS	8.62
Total DF	59
Groups DF	2
Among all subgroups DF	5
Subgroups DF	3
Error DF	54

Source of Variation	SS	DF	MS
Total	7859.98	59	
Among all subgroups	7231.65	5	
Groups	7223.03	2	3611.52
Subgroups	8.62	3	2.87
Error	628.33	54	11.64

Ho: There is no significant difference among the replicates (a,b) in affecting domoic acid concentration.
HA: There is a significant difference among replicates (a,b) in affecting domoic acid concentration.

$F = 2.87/11.64 = 0.25$ $F_{0.05(1),3,54} = 2.79$ $F < F_{0.05(1),3,54}$ Do not reject Ho.

The replicates are NOT significantly different

Ho: There is no difference in Domoic Acid concentration among the three concentrations (L, M, H).
HA: The three concentrations (L, M, H) are significantly different.

$F = 3611.52/2.87 = 1258.37$ $F_{0.05(1),2,3} = 9.55$ $F > F_{0.05(1),2,3}$ Reject H0

The concentrations are significantly different.

Source of variation Degrees of freedom Sum of Squares Mean Square
Samples 9
Concentrations in samples 20
Determinations within concentrations 30
Total 59

4. Calculate the variance ratio (F) at the 95% confidence interval for the variance components, concentrations in samples/determinations within concentrations. If the variance ratio is significant this indicates that the precision of the method as implemented by the laboratory is not consistent over the range in concentrations important to the intended application.

Per the ISSC instructions, I used F = Concentrations in samples/determinations within concentrations = 2.87/3611.52 = 0.00079

$F_{0.05(1),2,3} = 9.55$ F <<< $F_{0.05(1),2,3}$ Accept H0.

So, there is no significant difference in precision among each of the three concentrations (L,M,H)

If the variance ratio is not significant, calculate the coefficient of variation of the spiked sample data by:

1. Calculating the average concentration of the analyte/measurand/organism of interest in the spiked samples. For microbiological methods log transformed data is used for this calculation.

   Avg. concentration of Domoic acid in the spiked samples

   Low 2.17
   Med 16.46
   High 34.867

2. Calculate the standard deviation of the spiked sample data by taking the square root of the nested ANOVA variance component, **Total**.

   Standard deviation of spiked sample data

	SD
Low	0.43
Med	3.25
High	5.23

3. Divide the standard deviation of the spiked sample data by the average concentration of the analyte/measurand/organism of interest calculated for the spiked samples. For microbiological methods log transformed data is used for this calculation; and

   Low 0.20
   Med 0.20
   High 0.17

4. Multiply the quotient above by 100. This is the coefficient of variation of the method over the range of concentrations of importance in the application of the method as implemented by the laboratory.

   Low 20
   Med 20
   High 17

Recovery

The recovery of the target analyte/measurand/organisms of interest must be consistently good over the range of concentrations of importance to the application of the method under study to be of benefit in the intended work. To determine whether recovery by the method as implemented by the laboratory is consistent over the range in concentrations important to the application of the method, the data is manipulated in the following manner:

1. Convert plate count and spiked sample results for the microbiological methods to logs.
2. If necessary, use the sample blank (converted to logs for microbiological methods) to correct the results from the spiked samples for matrix effects.
3. For each sample determine the average of the replicates at each concentration such that there is only one value, the average of the two replicates at each concentration tested.
4. For each sample subtract the average for the replicates from its associated spike concentration/plate count value.

Sample	Spike	Average ELISA	Spike-ELISA
8L	1.7	1.65	0.05
10L	1.8	1.75	0.05
7L	1.99	2.18	-0.19
9L	2.14	1.75	0.39
3L	2.26	2.15	0.11
6L	2.45	2.35	0.1
4L	2.5	1.67	0.83
1L	2.6	2.75	-0.15
5L	2.62	1.97	0.65
2L	2.71	2.91	-0.2
9M	14.6	12.45	2.15
8M	14.77	13.33	1.44
10M	14.84	12.55	2.29
6M	15.89	15.41	0.47
7M	16.42	12.68	3.74
2M	19.1	19.53	-0.43
5M	19.11	14.96	4.15
4M	19.21	16.33	2.88
3M	19.64	22.86	-3.22
1M	20.14	19.95	0.19
8H	27.3	19.45	7.85
9H	29.48	27.55	1.93
10H	30.49	28.7	1.79
6H	34.97	26.98	7.99
7H	35.32	26.26	9.05
4H	35.5	31.52	3.98
5H	36.56	30.9	5.67
2H	39.28	32.61	6.68

5. Perform a one way analysis of variance (ANOVA) on the data formatted by sample concentration with the following variance components:

Source of variation Degrees of freedom Sum of Squares Mean Square
Concentration 2
Error 27
Total 29

Source of Variation	Sum of Squares	d.f.	Mean Squares	F
Between	181.9	2	90.93	20.22
Error	121.4	27	4.496	
Total	303.2	29		

Group A (low): Number of items= 10
Mean = 0.16400
95% confidence interval for Mean: -1.212 thru 1.540
Standard Deviation = 0.353
High = 0.8300 Low = -0.2000
Median = 7.5000E-02
Average Absolute Deviation from Median = 0.252

Group B (medium): Number of items= 10
Mean = 1.3660
95% confidence interval for Mean: -9.8640E-03 thru 2.742
Standard Deviation = 2.20
High = 4.150 Low = -3.220
Median = 1.795
Average Absolute Deviation from Median = 1.68

Group C (high): Number of items= 10
Mean = 5.8830
95% confidence interval for Mean: 4.507 thru 7.259
Standard Deviation = 2.92
High = 10.06 Low = 1.790
Median = 6.175
Average Absolute Deviation from Median = 2.44

The probability of this result, assuming the null hypothesis, is less than 0.0001. The highest spikes had greater variability. Those in regulatory range (Low and Medium) were less variable.

6. Calculate the variance ratio (F) at the 95% confidence interval for the mean square for concentration divided by the mean square for error. If the variance ratio or F test is significant at the 95% confidence interval, perform Tukey's Honestly Significant Difference (HSD) to compare recovery by concentration. A significant F test suggests that recovery of the method as implemented by the laboratory is not consistent over the range in concentrations important to the application of the method and may not be suitable for the work intended.

   F= 90.93/4.496 = 20.22
   Numerator degrees of freedom = 2
   Denominator degrees of freedom = 27
   Probability Value: 0.000004

   This confirms greater variability in recovery at the higher spike concentrations

If the variance ratio or F test is not significant at the 95% confidence interval, conclude that the recovery is consistent over the range in concentrations important to the application of the method and calculate the overall percent recovery of the method as implemented by the laboratory.

To determine the percent recovery of the method as implemented by the laboratory, the data is manipulated in the following manner:

1. Use log transformed data for microbiological methods.
2. If necessary use the sample blank (converted to logs for microbiological methods) to correct the results from the spiked samples for matrix effects.
3. Calculate the average spike concentration/plate count by summing over concentrations and dividing by 30.

   18.17

4. Calculate the average concentration of analyte/measurand/organism of interest in the spiked samples from the analysis by summing over concentrations and replicates and dividing by 60.

   15.7

5. Divide the average concentration of analyte/measurand/organism of interest from the analysis of the spiked samples by the average concentration from the spike/plate counts then multiply by 100. This is the percent recovery of the method as implemented by the laboratory.

86.4%

Data Summary:

- Is the variance ratio at the 95% confidence interval for the variance components, concentrations in samples/determinations within concentrations significant? **Y**
- If the variability of the method as implemented by the laboratory is consistent over the range in concentrations important to its intended applications, what is the coefficient of variation? **NA/_____%**
- Is the one way analysis of variance to determine the consistency of recovery of the method under study significant? **Y**
- At what concentrations is the one way analysis of variance significant? **NA/___?_____**
- What is the overall percent recovery of the MPN based method under study? **NA/__86.4___%**

VII. #4 Marine Biotoxin and Non-MPN Based Microbiological Methods SOP – Specificity

VALIDATION CRITERIA

Specificity is the ability of the method to measure only what it is intended to measure. To determine method specificity samples containing suspected interferences (interfering organisms/compounds/toxins) are analyzed in the presence of the analyte/measurand/targeted organism of interest.

Procedure: This procedure is applicable for use with either growing waters or shellfish tissue. Make every effort to use samples free of the targeted analyte/measurand/organism of interest. For each shellfish tissue type of interest use a minimum of 10-12 animals per sample. For each sample take three (3) aliquots of either the shellfish homogenate or growing water sample appropriately sized for the work and spike two (2) of the three (3) with a low but determinate level (by the method under study) of the targeted analyte/measurand/ organism of interest. Take one of these two (2) aliquots and also spike it with a moderate to high level of a suspected interfering organism/compound/toxin if not naturally incurred. Do not spike the third aliquot. This is the sample blank. Process each aliquot, the sample blank, the aliquot spiked with the targeted analyte/measurand/organism of interest and the aliquot spiked with the targeted analyte/measurand/organism of interest in the presence of the suspected interfering organism/compound/toxin as usual to determine the method concentration for the targeted analyte/measurand/organism of interest. Do five (5) replicates for each aliquot excluding the sample blank. Do one sample blank per analysis. Repeat this process for all suspected interfering organisms/compounds/toxins.

Data:

Glutamine and Glutamic are structurally related to domoic acid and present in shellfish tissues. Hence they represent potentially important competitors. These compounds were therefore tested to determine if high concentrations would interfere with the DA ELISA.

Name of suspected interfering organism/compound/toxin #1 _____ Glutamine _____
Sample type ____Shellfish Tissue _____
Sample blank concentration for the targeted analyte/measurand/organism of interest __0.0__
Concentration of aliquot spiked with targeted analyte/measurand/ with targeted analyte/measured: see below
Organism of interest organism: oyster

Procedure:

1. 2000 ppm solutions of Glutamine and Glutamic acid were made by mixing 26.7mg Glutamine in 13.35mL dH_2O and 26.8 mg Glutamic Acid in 13.4 mL dH2O
2. 2 g thawed oyster sample weighed into 50 mL tube
3. 17mL 50% MeOH added to tube
4. 3.34 µL 90% 1670ppm DA added to make 2.5ppm DA spike
5. Sample vortexed
6. Sample split into two 15mL tubes
7. 500 µL 50% MeOH added to DA-only tube
8. For tube spiked with interfering compound, 250mL 50% MeOH added + 250 µL 2000ppm Gulatime/Glutamic Acid for an ~55ppm spike in shellfish tissue
9. Samples then processed by ELISA and HPLC as described previously.

Replicate	Conc. of spike	Conc. of Spike Glutamine
1	1.70	1.70
2	1.60	1.70
3	1.70	1.60
4	1.90	2.10
5	1.70	2.20
Avg	1.72	1.86
mean	1.7	1.9
Standard deviation	0.1	0.2
SIavg	0.925	

Name of suspected interfering organism/compound/toxin #2 _____ Glutamic Acid _____
Sample type ____Shellfish Tissue _____
Sample blank concentration for the targeted analyte/measurand/organisn of interest __0.0__
Concentration of aliquot spiked with targeted analyte/measurand/ with targeted analyte/measured: see below
Organism of interest organism: oyster

Replicate	Conc of spike	Conc of Spike Glutamic Acid
1	1.90	1.80
2	1.60	1.80
3	1.50	1.40
4	1.30	1.50
5	1.90	1.50
Avg	1.64	1.60
Standard deviation	0.2	0.2
SIavg	1.025	

Repeat for each suspected interfering organism tested.

DATA HANDLING

The **Specificity index** will be used to test the specificity of the method in the presence of suspected interfering organisms/compounds/toxins. The **Specificity index (SI)** is calculated as indicated below:

Specificity index (SI) = Sample spiked with target of interest only
Sample spiked with both target and suspected interferences

All microbiological count data must be converted to logs before analysis. Samples spiked with both the targeted analyte/measurand/organism of interest and the targeted anaalyte/measurand/organism of interest in the presence of a suspected interfering organism/compound/toxin may have to be corrected for matrix effects before determining the Specificity index (SI). The sample blank accompanying the analysis is used for this purpose. Any corrections that may be necessary to microbiological data for matrix effects are done using log transformed data.

The Specificity index should equal one (1) in the absence of interferences. To test the significance of a Specificity index other than one (1) for any suspected interfering organism/compound/toxin, a two-sided t-test is used. For each suspected interfering organism/compound/toxin calculate the average **Specificity Index (SI)** for the 5 replicatesanalyzed for each sample by obtaining the average concentration for both the aliquot containing the targeted analyte/measurand/organism of interest only and the aliquot containing the targeted analyte/measurand/organism of interest in the presence of suspected interfering organisms/compounds/toxins and using the formula below.

SI_{avg} = Avg concentration of sample spiked with target of interest only
Avg concentration of sample spiked with both target and suspected interferences

Perform a two-sided t-test at the .05 significance level to determine if the average Specificity index (SI) obtained from the 5 replicates of each analysis differs from one (1).

Repeat for all interfering organisms/compounds/toxins tested.

Data Summary:

Interfering organism/compound/toxin #1 _____Glutamine_____ SI_{avg} 0.925_____

Significant difference from 1 _____

Interfering organism/compound/toxin #2 ____Glutamic Acid_____ SI_{avg} 1.025____

Significant difference from 1 _____

Glutamine Two tailed T-test 95% confidence level

T=2.0
DF=8
Confidence Level 91.95%
Not Significant

Glutamic Acid

T=0.3162
DF=8
Confidence Level 24.01%
Not Significant

VII. #5 Marine Biotoxin and Non-MPN Based Microbiological Methods SOP – **Linear Range, Limit of Detection, Limit of Quantitation/Sensitivity**

VALIDATION CRITERIA

Linear Range is the range within the working range where the results are proportional to the concentration of the analyte/measurand/organism of interest present in the sample.

Limit of Detection is the minimum concentration at which the analyte/measurand/organism of interest can be identified.

Limit of Quantitation/Sensitivity is the minimum concentration of the analyte/measurand/organism of interest that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.

Procedure: This procedure is applicable for use with either growing waters or shellfish tissue. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take at least six (6) aliquots of either the growing water sample or shellfish homogenate appropriately sized for your work and spike five (5) of the six (6) aliquots with five (5) different concentrations (i.e. 10^a, 10^b …10^n) of the target analyte/measurand/organism of interest spanning 50 – 150% of the working range/range of interest for the method under study. Do not spike the sixth or last aliquot of each sample. This is the sample blank. For microbiological methods determine the concentration of the target analyte/measurand/organism of interest used to spike each aliquot of each sample by plating in/on appropriate agar. Do not use aliquots of the same master solution/culture to spike all the samples in this exercise. A separate master solution /culture should be used for each sample. Process each aliquot including the sample blank as usual to determine method concentration for the target analyte/measurand/organism of interest. Do three (3) replicates for each aliquot excluding the sample blank. Do only one blank per sample. For growing waters do ten (10) samples collected from a variety of growing areas. For shellfish do ten (10) samples for each shellfish tissue type of interest collected from a variety of growing areas, the same growing area harvested on different days or from different process lots. Use the same spiking levels for each of the ten (10) samples analyzed (10^a, 10^b …10^n).

This is a section where I could use guidance by the committee. The assay has a wide dynamic range because samples are diluted into the 0.3 to 3 ppb linear range of the assay. It is this aspect of the assay which makes it difficult to implement the instructions provided above. The actual linear range was determined as by diluting the standards to various levels and testing the assay multiple times. This was a necessary step in developing the critical parameters needed by the data analysis software provided with the kit to back calculate DA values from the B and Bo values (see article published in the December 2008 issue of the Journal of Shellfish Research for details). I need to know if the data presented in the published article are sufficient to meet the committee's requirements for determining the linear range and limits of detection. If not, please recommend what procedure should be followed considering that the samples must be diluted. This is similarly true for determining the dynamic range of the assay.

Data: Testing in progress

Sample type _____
Working range/Range of interest _____
Range in spiking levels used _____
Agar used to determine spike concentration _____
Organism used for spiking _____
Aliquot 0* 1 2 3 4 5
Sample 1
Spike conc./plate count
Response, replicate 1
Response, replicate 2
Response, replicate 3
Aliquot 0* 1 2 3 4 5
Sample 2
Spike conc./plate count
Response, replicate 1

Response, replicate 2
Response, replicate 3
Sample 3
Spike conc./plate count
Response, replicate 1
Response, replicate 2
Response, replicate 3
Sample 4
Spike conc./plate count
Response, replicate 1
Response, replicate 2
Response, replicate 3
Sample 5
Spike conc./plate count
Response, replicate 1
Response, replicate 2
Response, replicate 3
Sample 6
Spike conc./plate count
Response, replicate 1
Response, replicate 2
Response, replicate 3
Sample 7
Spike conc./plate count
Response, replicate 1
Response, replicate 2
Response, replicate 3
Sample 8
Spike conc./plate count
Response, replicate 1
Response, replicate 2
Response, replicate 3
Aliquot 0* 1 2 3 4 5
Sample 9
Spike conc./plate count
Response, replicate 1
Response, replicate 2
Response, replicate 3
Sample 10
Spike conc./plate count
Response, replicate 1
Response, replicate 2
Response, replicate 3
*** Unspiked sample blank**

Response is the signal data (absorbance, florescence, Ct value), colonies, plaques, etc resulting from the analysis.
For shellfish samples repeat for each tissue type of interest**.**

DATA HANDLING

Linear Range

To determine the range within the working range where the results are proportional to the concentration of the target analyte/measurand/organism of interest present, the data is manipulated in the following manner.

1. Convert the plate counts and spiked sample results for the microbiological methods to logs.
2. If necessary, use the sample blank (converted to logs for microbiological methods) to correct the results from the spiked samples for matrix effects.
3. Divide the response obtained for each replicate tested by the concentration of the spiked analyte/measurand/organism of interest which gave rise to it. Use log values for the microbiological data.
4. Plot the data obtained above on the y-axis against the log of the concentration of the spiked analyte/measurand/organism of interest which gave rise to the respective data point on the x-axis. Connect the points. This is the relative response line.
5. Calculate the mean of the values obtained (in step 3) when the response for each replicate tested is divided by the concentration of the spiked analyte/measurand/organism of interest which gave rise to it.
6. Plot this value on the y-axis of the graph obtained in step 4 at each log concentrations of the analyte/measurand/organism of interest spiked into the samples. Connect the points to form a horizontal line. This constitutes the line of constant response
7. Multiply the value obtained in step 5 by 0.95 and 1.05.
8. Plot these values on the y-axis of the graph obtained in steps 4 and 6 at each log concentration of the analyte/measurand /organism of interest spiked into the samples. Connect the points to form two horizontal lines which bracket the line of constant response.
9. The method is linear up to the point where the relative response line (obtained in step 4) intersects either of the lines obtained above.
10. The linear range of the method as implemented by the laboratory is comprised of the range in concentrations obtained by taking the antilogs of the concentrations of the spiked analyte/measurand/organism of interest bracketed within the horizontal lines of the plot obtained in step 8 above.

Limit of Detection and Limit of Quantitation/Sensitivity

To determine the minimum concentration at which the analyte/measurand/organism of interest can be identified and subsequently quantified with an acceptable level of precision and accuracy under the conditions of the test, the data is manipulated in the following manner.

1. Calculate the coefficient of variation or relative standard deviation for each concentration of analyte/measurand/organisn of interest spiked into the samples. Use the log transformed data for manipulating microbiological results.
2. Plot the coefficient of variation/relative standard deviation on the y-axis for each concentration of analyte/measurand/organism of interest spiked into the samples and plotted on the x-axis. Use log transformed concentration values for the microbiological data.
3. Fit the curve and determine from the graph the concentration of analyte/measurand/organism of interest which gave rise to a coefficient of variation/relative standard deviation of 10%. This is the limit of quantitation/sensitivity of the method as implemented by the laboratory.
4. Divide the value for the limit of quantitation/sensitivity obtained from step 3 above by 3.3 or determine the concentration of analyte/measurand/organism of interest that gave rise to a coefficient of variation/relative standard deviation of 33%. This value is the limit of detection of the method as implemented by the laboratory.

For single laboratory validation, the concepts of "blank + 3σ" and "blank + 10σ" generally suffice for determining the limit of detection and the limit of quantitation/sensitivity. Since the blank is in theory zero (0), then the limit of detection and the limit of quantitation /sensitivity become 3σ and 10σ respectively. An absolute standard deviation of 3 and 10 equates to a coefficient of variation/relative standard deviation of 33% and 10% respectively. Accordingly the limit of detection and the limit of quantitation/sensitivity become the concentration of analyte/measurand/organism of interest which give rise to these values.

Data Summary:

Linear range of the method as implemented _____

The limit of detection of the method as implemented _____

The limit of quantitation/sensitivity of the method as implemented _____

IX. SLV Documents for New or Modified Methods as Alternatives to NSSP Methods http://www.issc.org/client_resources/lmr%20documents/ix%20%20_1%20new%20or%20modified%20methods%20as%20alternatives.pdf

IX. #1 SOP for the Single Laboratory Validation of New or Modified Analytical Methods Intended as Alternatives to Officially Recognized NSSP Methods – Comparing Methods

VALIDATION CRITERIA

Comparability is the acceptability of a new or modified analytical method as a substitute for an established method in the NSSP. To be acceptable the new or modified method must not produce a significant difference in results when compared to the officially recognized method. Comparability must be demonstrated for each substrate or tissue type of interest by season and geographic area if applicable.

Comparison of Methods:

New or modified methods demonstrating comparability to officially recognized methods must not produce significantly different results when compared

Procedure to compare the new or modified method to the officially recognized method: This procedure is applicable for use with either growing waters or shellfish tissue. For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take two (2) aliquots and analyze one by the officially recognized method and the other by the alternative method. Actual samples are preferable; but, in cases where the occurrence of the analyte/measurand/organism of interest is intermittent (such as marine biotoxins), spiked samples can be used. Samples having a variety of concentrations which span the range of the method's intended application should be used in the comparison. Analyze a minimum of thirty (30) paired samples for each season from a variety of growing areas for a total of at least 120 samples over the period of a year for naturally incurred samples. For spiked samples analyze a minimum of ten (10) samples for each season from a variety of growing areas for a total of at least 40 samples over the period of a year.

Data:

Sample type ____Shellfish tissue- oyster_____

Date Sample/Station # Conc. Recognized method Conc. Alternative Method

Data still being gathered to answer this question.

~~1~~
~~2~~
~~3~~
~~4~~
~~5~~
~~6~~
~~7~~
~~8~~
~~9~~
~~10~~
~~n~~
~~n is the last sample in the comparison~~

For shellfish samples, repeat for each tissue type of interest

Data handling to compare the new or modified method to the officially recognized

Two methods of analysis are considered to be comparable when no significant difference can be demonstrated in their results. To determine whether comparability in methods exists, a two-sided t-test at a significance level (α) of .05 will be used to test the data. Either a paired t-test or Welch's t-test will be used depending upon the shape of the distributions produced by the data for each method and their respective variances. Use log transformed data for the results obtained from microbiological methods. The appropriate t-test to be used for the analysis is determined in the following manner.

1. Test the symmetry for the distribution of results from both the officially recognized analytical method and the proposed alternative analytical method.
2. Calculate the variance of the data for both the officially recognized analytical method and the proposed alternative analytical method.
3. Values for the test of symmetry for either method outside the range of -2 to +2 indicate a significant degree of skewness in the distribution.
4. A ratio of the larger of the variances of either method to the smaller of the variances of either method >2 indicates a lack of homogeneity of variance.
5. Use either the paired t-test or Welch's t-test for the analysis of the data based on the following considerations.
    - If the distribution of the data from the officially recognized analytical method and the proposed alternative analytical method are symmetric (within the range of -2 to +2) and there is homogeneity of variance use a paired t-test for the data analysis.
    - If the distributions of the data for both analytical methods are symmetric (within the range -2 to +2) but there is a lack of homogeneity of variance in the data, use Welch's t-test for the analysis of the data.
    - If the distributions of the data from the officially recognized and proposed alternative analytical methods are skewed (outside the range -2 to +2) and the skewness for both methods is either positive for both or negative for both and there is homogeneity of variance in the data, use the paired t-test for the analysis of the data.
    - If the distributions of the data from the officially recognized and the proposed alternative analytical methods are skewed and the skewness for both analytical methods is either positive or negative for both but the data lacks homogeneity of variance, use Welch's t-test to analyze the data.

Data summary for the comparison of the new or modified method to the officially recognized method:
Value for the test of symmetry for the distribution of the data generated by the officially recognized method _____
Value for the test of symmetry for the distribution of the data generated by the proposed alternative method _____
Variance of the data generated from the officially recognized analytical method _____
Variance of the data generated from the proposed alternative analytical method _____
Ratio of the larger to the smaller of the variances generated by the officially recognized and proposed analytical methods _____
Is there a significant difference between the analytical methods Y/N

Proposal Subject: Brevetoxin (NSP) ELISA Kit

Specific NSSP Guide Reference: Section IV. Guidance Documents, Chapter II Growing Areas, .10 Approved National Shellfish Sanitation Program Laboratory Tests: Microbiological and Biotoxin Analytical Methods

Section II. Model Ordinance Chapter III. Laboratory @.02 Methods C. Biotoxin

Text of Proposal/ Requested Action See attached ISSC Method Application

Faster and easier to perform methods are needed to satisfy the needs of the regulatory community and shellfish industry. The proposed ELISA method is a fast and easy to perform method with ready to use reagents i.e. analyst only needs to extract shellfish sample or dilute water sample before analysis. The proposed ELISA also provides a quantitative and/or semi-quantitative screening for shellfish extracts and/or water samples. This assay is part of Abraxis platform for marine toxin testing and complements the company's other offering for PSP, DSP, and ASP testing. The proposed ELISA can be used on-site (boat, dock) or established analytical laboratories.

Public Health Significance:

Cost Information (if available): As low as $15 per sample.

Action by 2009 Laboratory Methods Review Committee Recommended no action on Proposal 09-106. Rationale: Insufficient data.

Action by 2009 Task Force I Recommended adoption of Laboratory Methods Review Committee recommendation on Proposal 09-106.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force I on Proposal 09-106.

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

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Methods Review (LMR) Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

Name of the New Method	Brevetoxin (NSP) ELISA Kit	
Name of the Method Developer	Abraxis LLC	
Developer Contact Information	Fernando Rubio 54 Steamwhistle Drive Warminster, PA 18974 Phone: (215) 357-3911 FAX: (215) 357-5232	
Checklist	**Y/N**	**Submitter Comments**
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.		Shellfish are filter feeders that pump large quantities of water through their bodies when actively feeding. During this process, shellfish can concentrate toxigenic micro-algae and other substances from the water column when they are present. The ability of shellfish to concentrate chemical pollutants from water can lead to accumulation of these toxins to levels that constitute a public health hazard. Red tides containing Brevetoxin have caused mortality events in fish, and sea mammals. In humans, Brevetoxin (NSP) poisoning causes a combination of gastro-intestinal and neurological symptoms. Some of the currently available methods used for the detection and monitoring of brevetoxin in water and shellfish are not conducive for the quick on-site or real time, dockside or ship board monitoring of this toxin. For example: 1) the mouse bioassay is labor intensive, requires the use and destruction of many vertebrate animals, analyses is only performed in a few laboratories with a low turn around time, 2) a research ELISA has been developed by the University of North Carolina, however, this assay requires the user to coat plates with antibodies before analysis, a process that takes at least two days to complete before an analytical result is obtained. Therefore, faster and easier to perform methods are needed to satisfy the needs of the regulatory community and shellfish industry. The proposed ELISA method is a fast and easy to perform method with ready to use reagents i.e. analyst only needs to extract shellfish sample or dilute water sample before analysis. The proposed ELISA also provides a quantitative and/or semi-quantitative screening for shellfish extracts and/or water samples.

		This assay is part of Abraxis platform for marine toxin testing and complements the company's other offering for PSP, DSP, and ASP testing.
2. What is the intended purpose of the method?		The fast analysis of Brevetoxin (NSP) in shellfish extracts and/or water quality monitoring. The proposed ELISA can be used on-site (boat, dock) or established analytical laboratories.
3. Is there an acknowledged need for this method in the NSSP?		Yes. NSSP Guidance Documents, Chapter II Constitution by-laws and procedures of the Interstate Shellfish Sanitation Conference. Procedure XVI. Procedure for acceptance and approval of analytical methods for the NSSP. And: National Shellfish Sanitation Program 2003 Model Ordinance III. Laboratory @ 02 Methods C. Biotoxin. Methods for the analysis of shellfish and shellfish harvest waters shall be: 1) The current APHA method used in bioassay for *Karemia breve* toxin.
4. What type of method? i.e. chemical, molecular, culture, etc.		Immunochemical Method.
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title		Abraxis ELISA Kit for the Screening of Brevetoxin in Shellfish Extract and/or Harvest Waters.
Method Scope		A Method for the screening out negative brevetoxin samples in shellfish regulatory labs, to determine if shellfish are safe to harvest and/or distribute. A method for water classification for brevetoxin around harvest areas and to screen for toxic phytoplankton in seawater to provide early warning.
References		Maucher, J.M., Briggs, L.R, Podmore, C., Ramsdell, J.S. (2007) Optimization of blood collection card method/ELISA for monitoring exposure of bottlenose dolphin to brevetoxin-producing red tides. *Environmental Science & Technology*, 41: 563-567. Inter-lab study data performed by several labs including Ag Research in New Zealand, Cawthron Institute in New Zealand and NOAA is available upon request.
Principle		**The test is a direct competitive ELISA based on the recognition of Brevetoxin by specific antibodies. Brevetoxin, when present in a sample, and a Brevetoxin enzyme-conjugate compete for the binding sites of sheep anti-brevetoxin antibodies that have been immobilized in the wells of a microtiter plate. After a washing step and addition of the substrate solution, a color signal is produced. The intensity of the blue color is inversely proportional to the concentration of Brevetoxin present in the sample. The color reaction is stopped after a specified time and the color is evaluated using an ELISA reader. The concentrations of the samples are determined by interpolation using the**

		standard curve constructed with each run.
Any Proprietary Aspects		Immunoreagents and sample diluent.
Equipment Required		Pipettes and plate reader. Blender for shellfish extraction.
Reagents Required		Reagents provided in the ELISA kit. In addition methanol is needed.
Sample Collection, Preservation and Storage Requirements		Water samples need to be collected in glass vials and preserved according to users guide (attached). Diluted shellfish extracts should be stored in glass vials. All dilution should be done using provided sample diluent. If not analyzed promptly, samples should be stored refrigerated for up 2 days or frozen if longer periods are required.
Safety Requirements		As with any laboratory procedure, gloves and goggles should be used during the processing and analysis of samples.
Clear and Easy to Follow Step-by-Step Procedure		User's guide and an easy to follow flow chart are provided with each kit (attached).
Quality Control Steps Specific for this Method		As with any analytical procedure laboratory controls (positive and negative) are recommended.
C. Validation Criteria		
1. Accuracy / Trueness		Data ran by AgResearch, New Zealand is provided as an attachment,
2. Measurement Uncertainty		@ 0.042 ng/mL in water SD 0.002 CV 4.8% @ 0.210 ng/mL in water SD 0.010 CV 4.8% @ 0.443 ng/mL in water SD 0.064 CV 14.5%
3. Precision Characteristics (repeatability and reproducibility)		< 15%
4. Recovery		Average water recovery 86%, shellfish extract recovery 104%
5. Specificity		PbTx-3 100% Deoxy PbTx-2 133% PbTx-5 127% PbTx-2 102% PbTx-9 83% PbTx-6 13% PbTx-1 5%
6. Working and Linear Ranges		0.01-2 ng/mL water or 0.5-100 ng/gm or in shellfish extract or higher depending on dilution.
7. Limit of Detection		0.05 ng/mL
8. Limit of Quantitation / Sensitivity		0.01 ng/mL in water; 4.5 ng/gm in shellfish extract
9. Ruggedness		Since and analytical curve is run with each assay and the samples are compared to the standard curve, the proposed ELISA is rugged.
10. Matrix Effects		If used according to instructions (dilutions), none detected

11. Comparability (if intended as a substitute for an established method accepted by the NSSP)		Method is intended as a screening method to complement other accepted NSPP methods: i.e. mouse bioassay.
D. Other Information		
1. Cost of the Method		As low as $15 per sample
2. Special Technical Skills Required to Perform the Method		Some technical skills are required. Familiarity with laboratory setting is adequate. Kit Manufacturer's on-site training is available.
3. Special Equipment Required and Associated Cost		As low as $1,800. Strip reader and pipette
4. Abbreviations and Acronyms Defined		ELISA: Enzyme linked immuno sorbent assay NSP: neurotoxic shellfish poisoning
5. Details of Turn Around Times (time involved to complete the method)		40 samples can be run in duplicate in approximately 2 hours. Shellfish sample extraction requires approximately 15 minutes
6. Provide Brief Overview of the Quality Systems Used in the Lab		The ELISA kits are manufactured following GMP and GLP procedures.

Submitters Signature	Date:
Submission of Validation Data and Draft Method to Committee	Date:
Reviewing Members	Date:
Accepted	Date:
Recommendations for Further Work	Date:
Comments:	

DEFINITIONS

1. Accuracy/Trueness - Closeness of agreement between a test result and the accepted reference value.
2. Analyte/measurand - The specific organism or chemical substance sought or determined in a sample.
3. Blank - Sample material containing no detectable level of the analyte or measurand of interest that is subjected to the analytical process and monitors contamination during analysis.
4. Comparability - The acceptability of a new or modified method as a substitute for an established method in the NSSP. Comparability must be demonstrated for each substrate or tissue type by season and geographic area if applicable.
5. Fit for purpose - The analytical method is appropriate to the purpose for which the results are likely to be used.
6. HORRAT value - HORRAT values give a measure of the acceptability of the precision characteristics of a method.[4]
7. Limit of Detection - the minimum concentration at which the analyte or measurand can be identified. Limit of detection is matrix and analyte/measurand dependent.[4]
8. Limit of Quantitation/Sensitivity - the minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.
9. Linear Range - the range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.
10. Measurement Uncertainty - A single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.
11. Matrix - The component or substrate of a test sample.
12. Method Validation - The process of verifying that a method is fit for purpose.[1]
13. Precision - the closeness of agreement between independent test results obtained under stipulated conditions.[1, 2] There are two components of precision:
    a. Repeatability - the measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.
    b. Reproducibility - the measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.
14. Quality System - The laboratory's quality system is the process by which the laboratory conducts its activities so as to provide data of known and documented quality with which to demonstrate regulatory compliance and for other decision-making purposes. This system includes a process by which appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.
15. Recovery - The fraction or percentage of an analyte or measurand recovered following sample analysis.
16. Ruggedness - the ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]
17. Specificity - the ability of a method to measure only what it is intended to measure.[1]
18. Working Range - the range of analyte or measurand concentration over which the method is applied.

REFERENCES:

1. Eurachem Guide, 1998. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. LGC Ltd. Teddington, Middlesex, United Kingdom.

2. IUPAC Technical Report, 2002. Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, Pure Appl. Chem., Vol. 74, (5): 835-855.
3. Joint FAO/IAEA Expert Consultation, 1999. Guidelines for Single-Laboratory Validation of Anilytical Methods for Trace-Level Concentrations of Organic Chemicals.
4. MAF Food Assurance Authority, 2002. A Guide for the Validation and Approval of New Marine Biotoxin Test Methods. Wellington, New Zealand.
5. National Environmental Laboratory Accreditation. , 2003. Standards. June 5.
6. EPA. 2004. EPA Microbiological Alternate Procedure Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Methods: Guidance. U.S. Environmental Protection Agency (EPA), Office of Water Engineering and Analysis Division, 1200 Pennsylvania Avenue, NW, (4303T), Washington, DC 20460. April.

ABRAXIS

Testing for Assay Ruggedness

The performance of the brevetoxin ELISA was tested when small changes in the operating conditions were made similar to those likely to arise in different laboratories. This was undertaken not only to demonstrate that the assay is resistant to small departures from the conditions specified in the protocol but also to identify conditions requiring critical control and to set limits for steps in the protocol.

Based on a fractional factorial design (Table 1), as described by Wernimont *et al.* (1996), seven different experimental factors were selected. Two levels for each factor in eight assay runs were used in the study, i.e., under optimal and sub-optimal conditions. The effect of the sub-optimal condition was determined as the percentage difference between assay parameters (I_{20}, I_{50}, I_{80}) and sample concentrations measured using the optimal and sub-optimal assay conditions (Table 2). The study was carried out on eight plates each with a standard curve for the analysis of three shellfish extracts at two dilutions that fell within the working range of the standard curve. Contaminated shellfish extracts were used as samples for the test because contaminated dolphin blood extracts were not available. Each standard or dilution of shellfish extract was analysed in duplicate.

Table 1. Test design

Factor Value	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8
A or a	A	A	A	A	a	a	a	a
B or b	B	B	b	b	B	B	b	b
C or c	C	c	C	c	C	C	C	c
D or d	D	D	d	d	d	d	D	D
E or e	E	e	E	e	e	E	e	E
F or f	F	f	f	F	F	f	f	F
G or g	G	g	g	G	g	G	G	g
Observed result:	s	t	u	v	w	x	y	z

Summary:
Percentage differences in the quantification of samples determined using both optimal and sub-optimal conditions were, except for one point (16%), below 15%. This indicates that overall the assay is robust in terms of incubation times, temperature and plate shaking during TMB development.

The ruggedness test has, however, identified the incubation *temperature* during the competitive binding step with sample or standard, as a critical factor in effecting assay parameters (I_{20}, I_{50}, I_{80}) and to a lesser extent the incubation *time* for this step.

Reference:
Wernimont, G.T. (1996) Use of statistics to develop and evaluate analytical methods, Fifth Edition. AOAC International, Gaitersburg, MD, USA.

Table 2. Ruggedness test for the brevetoxin ELISA

	Experimental factor	**Optimal** or sub-optimal condition	Differences determined using optimal and sub-optimal conditions (%)					
			I_{20}	I_{50}	I_{80}	Sample A	Sample B	Sample C
1	Fridge temp. during capture Ab binding step	A: **4°C**						
		a: 10°C	+14	+7	0	+3	+3	0
2	Temp during specific Ab binding step	B: **22°C**						
		b: 30°C	-5	-2	+1	+1	-3	0
3	Incubation time with sample or standard	C: **1 hr**						
		c: 1hr 15min	**+24**	**+16**	+10	+4	+11	**+16**
4	Incubation temp with sample or standard	D: **22°C**						
		d: 30°C	**+43**	**+43**	**+43**	+3	+5	+8
5	Temp. for TMB development	E: **22°C**						
		e: 4°C	+1	+3	+4	+2	+5	-2
6	Time for TMB development	F: **15 min**						
		f: 20 min	+1	-1	+1	+1	+6	-1
7	Shaking for TMB development	G: **Yes**						
		g: No	-11	-3	+6	-5	-6	-4

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Abraxis Comments: Although a difference is seen in curve parameters when the assay is incubated at different temperatures (this is to be expected with any immunoasay), due to the fact that a standard curve is run with each batch of samples, the result obtained on those unknown samples should not change.

ABRAXIS

Within-run and Between-run Variability Studies
Brevetoxin ELISA

1. Within-run Variability

Five methanolic extracts of Green-lipped mussels (*Perna canaliculus*) were analyse on ten different days to determine within-run variability for the ELISA. Four replicate of each sample were analysed and the extracts were stored at -20°C between assays.

The within-run variability (RSD_R, i.e., residual standard deviation repeatability) was calculated for each concentration measurement and an overall mean RSD_R was calculated for each run. Finally, an overall mean for the 10 days was calculated. The within-day repeatability for all analyses of the extracts, ranged from 4.0 to 11.6% with an overall mean RSDr value of 6.6%.

Table 1. Within-run variability for the analysis of shellfish extracts

# of runs	Replicates (n)	Sample ID	Mean PbTx-3 equivalents (ng/mL)	SD	RSD_R (%)	Mean RSD_R (%)	Overal RSD_R (%)
1	4	1	1967	75	4		
	4	2	1307	72	6		
	4	3	1217	61	5		
	4	4	180	12	7		
	4	5	25	1	5		
						5.2	
2	4	1	2145	142	7		
	4	2	2019	191	10		
	4	3	1324	86	7		
	4	4	211	33	16		
	4	5	25	2	7		
						9.0	
3	4	1	1813	136	20		
	4	2	1368	117	9		
	4	3	1251	51	4		
	4	4	159	30	19		
	4	5	23	2	7		
						11.6	
4	4	1	1897	106	6		
	4	2	1639	49	3		
	4	3	1441	39	3		
	4	4	244	14	6		
	4	5	27	1	3		
						4.0	

Table 1. Contd.

# of runs	Replicates (n)	Sample ID	Mean PbTx-3 equivalents (ng/mL)	SD	RSD_R (%)	Mean RSD_R (%)	Overall RSD_R (%)
5	4	1	2153	95	4		
	4	2	1608	66	4		
	4	3	1368	66	5		
	4	4	226	14	6		
	4	5	28	1	3		
						4.6	
6	4	1	2686	112	4		
	4	2	2224	75	3		
	4	3	1934	32	2		
	4	4	294	18	6		
	4	5	23	3	13		
						5.7	
7	4	1	2573	81	3		
	4	2	1997	99	5		
	4	3	2030	120	6		
	4	4	326	25	8		
	4	5	38	3	8		
						5.9	
8	4	1	2462	201	8		
	4	2	1816	124	7		
	4	3	1757	171	10		
	4	4	311	32	10		
	4	5	36	3	8		
						8.7	
9	4	1	2277	145	6		
	4	2	1599	93	6		
	4	3	1518	106	7		
	4	4	261	18	7		
	4	5	29	1	3		
						5.9	
10	4	1	2199	132	6		
	4	2	1624	103	6		
	4	3	1496	48	3		
	4	4	222	10	5		
	4	5	35	1	3		
						4.6	**6.6**

2. Between-run Variability

The data in Table 1 was combined to calculate the between-day variability for each sample analysed on ten different occasions over a three month period.

Table 2. Between-run variability for the analysis of shellfish extracts

# of runs	Replicates (n)	Sample ID	Overall mean (ng/mL)	SD	RSD_R (%)	Overall RSD_R (%)
10	40	1	2217	288	**13.0**	
10	40	2	1720	292	**17.0**	
10	40	3	1534	283	**18.4**	
10	40	4	243	55	**22.6**	
10	40	5	29	6	**19.1**	**18.0**

The between-day variability (RSD_R, i.e., residual standard deviation reproducibility) for the analysis of brevetoxin concentrations in shellfish ranging from 26 to 1500 ng/mL, was below 18.0 %.

Agresearch

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New Zealand

Importance of Brevetoxin Determination

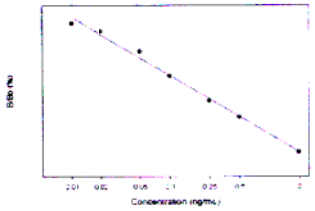
Neurotoxic shellfish poisoning (NSP) is caused by polyether toxins known as Brevetoxins. Brevetoxins (PbTxs) are produced by the dinoflagellate *Karenia brevis*, which causes harmful algal blooms (HABs) know as red tides. The Brevetoxins are toxic to fish, marine mammals, birds and humans, but not to shellfish. Contamination of shellfish with Brevetoxin has been associated with the presence of harmful algal blooms in various parts of the world.

Mortality events attributed to HABs have been documented for fish, manatee, dolphins, and seabirds. In man, NSP causes dose-dependent symptoms of diarrhea, nausea, and vomiting, chills, sweats, reversal of temperature, hypotension, arrhythmias, numbness, tingling, bronchoconstriction, paralysis, seizures, and coma.

The Brevetoxin ELISA allows the determination of 40 samples in duplicate determination. Only a few milliliters of sample are required. The test can be performed in less than 2 hours.

Performance Data

Test sensitivity: The limit of detection for Brevetoxin is calculated as: Xn +/- 3SD (n=20) and is equal to 0.005 ng/ml in water and 22.5 ng/gm in diluted shellfish (when using a dilution factor of 450). The concentration of residue necessary to cause 50% inhibition (50% B/B_0) is approximately 0.16 ng/mL. Determinations closer to the middle of the calibration range of the test yield the most accurate results.



Test reproducibility: Coefficients of variation (CVs) for standards: <10%, CVs for samples: <15%.

Selectivity: This ELISA recognizes Brevetoxin and other NSP toxins to varying degrees:

Cross-reactivities:

PbTx-3	100%
Deoxy PbTx-2	133%
PbTx-5	127%
PbTx-2	102%
PbTx-9	83%
PbTx-6	13%
PbTx-1	5%

No cross-reactivity was shown with any of the following common PSP shellfish toxins: saxitoxin, neosaxitoxin, dc-STX, gonyautoxins-1/4, gonyautoxins-2/3, B-2; B-1; C-1/2 and domoic acid.

Samples: Salt Water and shellfish samples (after recommended dilution) were tested for matrix effects in the ELISA. No matrix effects were determined.

General Limited Warranty: Abraxis LLC warrants the products manufactured by the Company, against defects and workmanship when used in accordance with the applicable instructions for a period not to extend beyond the product's printed expiration date. **Abraxis makes no other warranty, expressed or implied. There is no warranty of merchantability or fitness for a particular purpose**

For ordering or technical assistance contact

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R031808

Brevetoxin (NSP) ELISA, Microtiter Plate

ABRAXIS

Enzyme-Linked Immunosorbent Assay for the Determination of Brevetoxin (NSP) in Water and Contaminated Samples

Product No. 520026

1. General Description

The Brevetoxin ELISA is an immunoassay for the quantitative and sensitive detection of Brevetoxin. Brevetoxin is one of the toxins associated with neurotoxic shellfish poisoning (NSP). This test is suitable for the quantitative and/or qualitative detection of Brevetoxin in water samples as well as shellfish samples. For shellfish samples a sample preparation is required. If necessary, positive samples can be confirmed by HPLC, GC/MS, or other conventional methods.

2. Safety Instructions

The standard solutions in this test kit contain small amounts of Brevetoxin (PbTx-3). In addition, the substrate solution contains tetramethylbenzidine and the stop solution contains diluted sulfuric acid. Avoid contact of stopping solution with skin and mucous membranes. If these reagents come in contact with the skin, wash with water.

3. Storage and Stability

The Brevetoxin ELISA should to be stored in the refrigerator (4–8°C). The solutions must be allowed to reach room temperature (20-25°C) before use. Reagents may be used until the expiration date on the box.

4. Test Principle

The test is a direct competitive ELISA based on the recognition of Brevetoxin by specific antibodies. Brevetoxin, when present in a sample, and a Brevetoxin enzyme-conjugate compete for the binding sites of sheep anti-brevetoxin antibodies that have been immobilized in the wells of a microtiter plate. After a washing step and addition of the substrate solution, a color signal is produced. The intensity of the blue color is inversely proportional to the concentration of Brevetoxin present in the sample. The color reaction is stopped after a specified time and the color is evaluated using an ELISA reader. The concentrations of the samples are determined by interpolation using the standard curve constructed with each run.

5. Limitations of the Brevetoxin ELISA, Possible Test Interference

Numerous organic and inorganic compounds commonly found in samples have been tested and found not to interfere with this test. However, due to the high variability of compounds that might be found in samples, test interferences caused by matrix effects can not be completely excluded. Mistakes in handling the test can also cause errors. Possible sources for such errors can be:

Inadequate storage conditions of the test kit, incorrect pipetting sequence or inaccurate volumes of the reagents, too long or too short incubation times during the immune and/or substrate reaction, extreme temperatures during the test performance (lower than 10°C or higher than 30°C).

The Abraxis Brevetoxin ELISA kit provides screening results. As with any analytical technique (GC, HPLC, etc.) positive samples requiring some action should be confirmed by an alternative method.

Working Instructions

A. Materials Provided

1. Microtiter plate coated with sheep anti-Brevetoxin
2. Standards PbTx-3 (8): 0, 0.010, 0.025, 0.05, 0.1, 0.25, 0.5, 2.0 ng/mL
3. Brevetoxin-HRP Conjugate, 6 mL
4. Sample Diluent (1X), 2 X 30 mL. Use to dilute samples
5. Wash Solution (5X) Concentrate, 100 mL
6. Color Solution (TMB), 12 mL
7. Stop Solution, 2 X 6 mL
8. Sea Water Pre-treatment Solution, 25 mL

B. Test Preparation

Micro-pipetting equipment and pipette tips for pipetting the standards and the samples are necessary. We recommend using a multi-channel pipette or a stepping pipette for adding the conjugate, the substrate solution and the stop solution in order to equalize the incubations periods of the standard solutions and the samples on the entire microtiter plate. Please use only the reagents and standards from one package lot in one test, as they have been adjusted in combination.

1. Adjust the microtiter plate and the reagents to room temperature before use.
2. Remove the number of microtiter plate strips required from the foil bag. The remaining strips are stored in the foil bag and zip-locked closed. Store the remaining kit in the refrigerator (4-8°C).
3. The standard solutions, enzyme conjugate, substrate and stop solution are ready to use and do not require any further dilutions.
4. Dilute the Wash Buffer at a ratio of 1:5. If using the entire bottle (100 mL), add to 400 mL of deionized or distilled water.
5. The stop solution should be handled with care as it contains diluted H_2SO_4.

C. Assay Procedure

1. Add 50 µL of the **standard solutions or the samples** (water) or **sample extracts** (shellfish) into the wells of the test strips according to the working scheme given. We recommend using duplicates or triplicates.
2. Add 50 µL of **enzyme conjugate** solution to the individual wells successively using a multi-channel pipette or a stepping pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a rapid circular motion on the benchtop for about 30 seconds. Be careful not to spill contents. Incubate for 60 minutes.
3. After incubation, remove the covering and vigorously shake the contents of these wells into a sink. Wash the strips three times using the 1X washing buffer solution. Use at least a volume of 250 µL of washing buffer for each well and each washing step. Remaining buffer in the wells should be removed by patting the plate dry on a stack of paper towels.
4. Add 100 µL of **substrate solution** to the wells. The strips are incubated for 30 min at room temperature. Protect the strips from direct sunlight.
5. Add 100 µL of **stop solution** to the wells in the same sequence as for the substrate solution.
6. Read the absorbance at 450 nm using a microplate ELISA photometer within 15 minutes after the addition of the stopping solution.

D. Evaluation

The evaluation of the ELISA can be performed using commercial ELISA evaluation programs (4-Parameter (preferred) or Logit/Log). For manual evaluation, calculate the mean absorbance value for each of the standards. Calculate the %B/B_0 for each standard by dividing the mean absorbance value for each standard by the Zero Standard (Standard 0) mean absorbance. Construct a standard curve by plotting the %B/B_0 for each standard on the vertical linear (y) axis versus the corresponding Brevetoxin concentration on the horizontal logarithmic (x) axis on graph paper. %B/B_0 for samples will then yield levels in ppb of Brevetoxin by interpolation using the standard curve. Samples showing lower concentrations of Brevetoxin compared to standard 1 (0.01 ng/mL) are considered as negative. Samples showing a higher concentration than standard 7 (2.0 ng/mL) must be diluted further to obtain more accurate results.

E. Additional Materials (not delivered with the test kit)

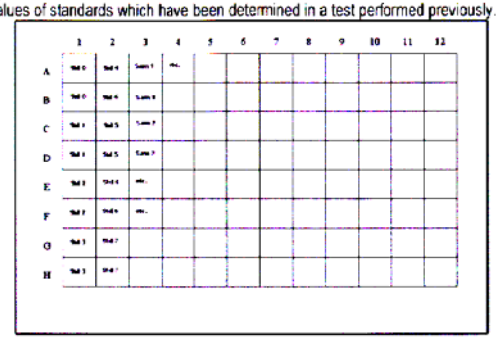
1. Micro-pipettes with disposable plastic tips (10-200 and 200-1000 µL)
2. Multi-channel pipette (10-250 µL) or stepper pipette with plastic tips (10-250 µL)
3. Microtiter plate washer (optional)
4. Microtiter plate reader (wave length 450 nm)
5. Shaker for microtiter plates (optional)

F. Working Scheme

The microtiter plate consists of 12 strips of 8 wells which can be used individually for the test. The standards must be run with each test. Never use the values of standards which have been determined in a test performed previously.

Std 0-Std 7: Standards
0; 0.010; 0.025; 0.05; 0.1; 0.25
0.5; 2.0 (ng/mL) or ppb

Sam1, Sam2, etc.: Samples



G. Preparation of Samples

I. Mussels

NOTE: For regulatory purposes, a 100g sample needs to be used. Extraction solution volume needs to be adjusted accordingly.

1. Mussels are removed from their shells, washed with deionized water, thoroughly dried and homogenized (Waring blender, Polytron or equivalent).
2. A 1.0 gm portion of the homogenized mussels is then placed in a 40 mL glass vial.
3. Add 9.0 mL of a methanol/deionized water solution (9:1 v/v).
4. Vial is capped and hand shaken vigorously for 2 minutes.
5. Centrifuge mixture for 10 minutes at 3000 g. Collect the supernatant.
6. Remove 20 uL of collected extract and dilute to 1.0 mL with Sample Diluent (equals a 1:50 dilution).
7. Analyze diluted extracts as samples (Assay Procedure step 1)

The Brevetoxin concentration contained in the samples is determined by multiplying the concentration of the diluted extract by a factor of 450. Highly contaminated samples outside the range of the curve should be diluted further and re-analyzed.

II. Sea Water

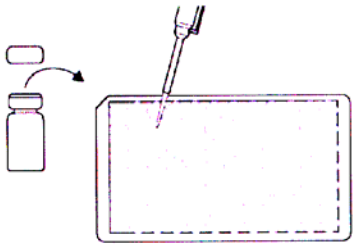
1. Collect 2 mL of sea water sample in a glass container.
2. To prevent loss of Brevetoxin to the glass surface, immediately add 0.5 mL of Sea Water Pretreatment Solution, mix by hand.
3. Analyze preserved sample as samples (Assay Procedure step 1)

The Brevetoxin concentration contained in the sea water sample is determined by multiplying the concentration of the diluted sample by a factor of 1.25. Highly contaminated samples outside the range of the curve should be diluted in Sample Diluent (PN 205226), and re-analyzed. Additional Sample Diluent or Sea Water-Pretreatment Solution (PN 205227) can be purchased from Abraxis.

Brevetoxin (NSP) Plate, Detailed ELISA Procedure

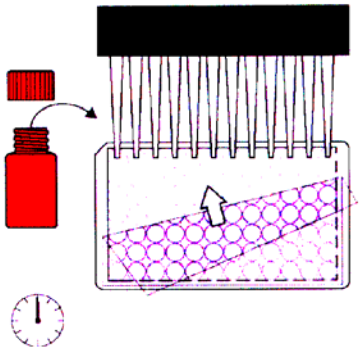
1. Addition of Standards, Samples

Add 50 uL of the standard solutions or samples into the wells of the test strips according to the working scheme given. We recommend using duplicates or triplicates.



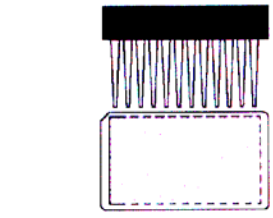
2. Addition of Enyzme Conjugate

Add 50 uL of the enzyme conjugate to the individual wells successively using a multi- channel pipette or a stepping pipette. Cover and mix for 30 seconds by rotating on benchtop. Incubate for 60 minutes at room temperature.

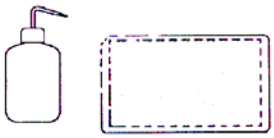


3. Washing of Plates

After incubation, remove the covering and vigorously shake the contents of the wells into a sink. Wash the strips three times with a multi-channel pipette or wash bottle using the diluted 1X washing buffer solution. Please use at least a volume of 250 uL of washing buffer for each well and each washing step. Remaining buffer in the wells should be removed by patting the plate dry on a stack of paper towels.

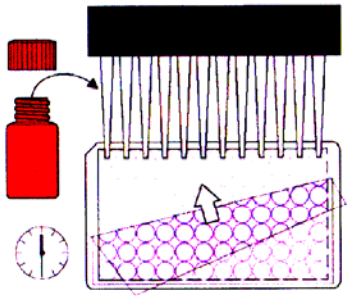


OR



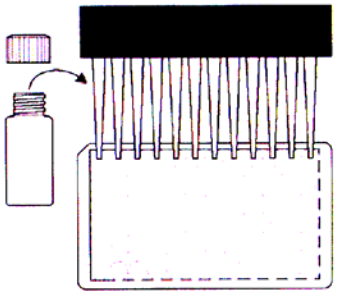
4. Addition of Substrate/Color Solution

Add 100 uL of substrate/color solution to the individual wells successively using a multi-channel pipette or a stepping pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a rapid circular motion on the benchtop. Be careful not to spill contents. Incubate the strips for 30 min at room temperature.



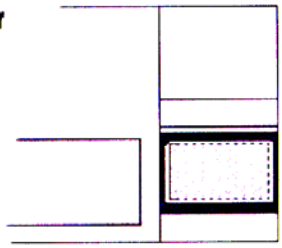
5. Addition of Stopping Solution

Add 100 uL of stop solution to the wells in the same sequence as for the substrate solution using a multi- channel pipette or a stepping pipette.



6. Measurement of Color

Read the absorbance at 450 nm using a microplate ELISA reader. Calculate results.



For Ordering or Technical Assistance Contact:
ABRAXIS, LLC
54 Steamwhistle Drive, Warminster, PA 18974
Phone: 215-357-3911 Fax: 215-357-5232
www.abraxiskits.com

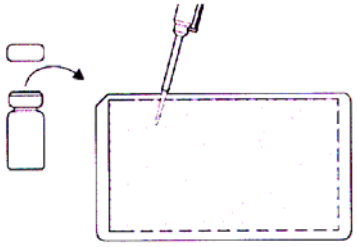
ABRAXIS

Brevetoxin (NSP) Plate Kit Part # 520026

Brevetoxin (NSP) Plate, Concise ELISA Procedure

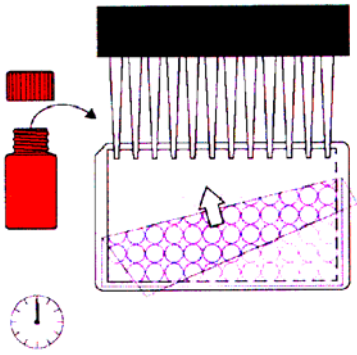
1. Addition of Standards, Samples

Add 50 uL of standard solutions or samples.



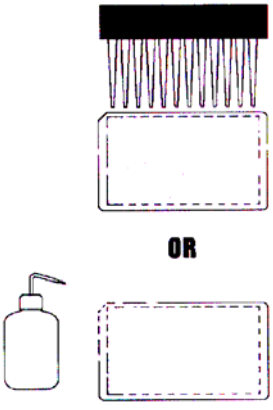
2. Addition of Enyzme Conjugate

Add 50 uL of enzyme conjugate. Cover and mix for 30 seconds by rotating on benchtop. Incubate for 60 minutes at room temperature.



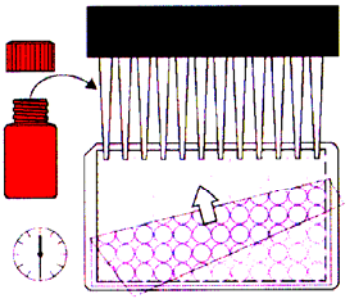
3. Washing of Plates

Wash the plates three times with 250 uL of diluted 1X washing buffer.



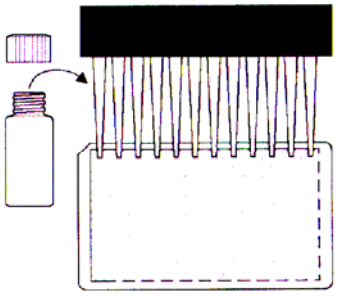
4. Addition of Substrate/Color Solution

Add 100 uL of substrate/color solution. Incubate 30 minutes at room temperature and away from direct sunlight.



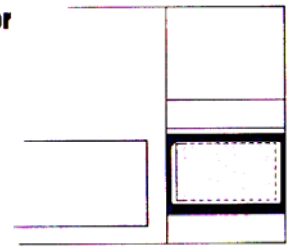
5. Addition of Stopping Solution

Add 100 uL of stop solution.



6. Measurement of Color

Measure color at 450 nm. Calculate results.



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Brevetoxin (NSP) Plate Kit Part # 520026

Proposal Subject: Saxitoxin (PSP) ELISA Kit

Specific NSSP Guide Reference: Section IV. Guidance Documents, Chapter II Growing Areas, .10 Approved National Shellfish Sanitation Program Laboratory Tests: Microbiological and Biotoxin Analytical Methods

Section II. Model Ordinance Chapter III. Laboratory @.02 Methods C. Biotoxin

Text of Proposal/ Requested Action See attached ISSC Method Application

Faster, easier, and/or more reliable methods are needed to satisfy the needs of the regulatory community and shellfish industry. The proposed ELISA method is a fast and easy to perform method with ready to use reagents i.e. analyst only needs to extract shellfish sample or dilute water sample before analysis. The proposed ELISA also provides a quantitative and/or semi-quantitative screening for shellfish extracts and/or water samples. This assay is part of Abraxis platform for marine toxin testing and complements the company's other offering for NSP, DSP, and ASP testing. The proposed ELISA can be used on-site (boat, dock) or established analytical laboratories.

Public Health Significance:

Cost Information (if available): As low as $15 per sample.

Action by 2009 Laboratory Methods Review Committee Recommended no action on Proposal 09-107. Rationale: Insufficient data.

Action by 2009 Task Force I Recommended adoption of Laboratory Methods Review Committee recommendation on Proposal 09-107.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force I on Proposal 09-107.

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

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Methods Review (LMR) Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

Name of the New Method	Saxitoxin (PSP) ELISA Kit	
Name of the Method Developer	Abraxis LLC	
Developer Contact Information	Fernando Rubio 54 Steamwhistle Drive Warminster, PA 18974 Phone: (215) 357-3911 FAX: (215) 357-5232	
Checklist	**Y/N**	**Submitter Comments**
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.		Shellfish are filter feeders that pump large quantities of water through their bodies when actively feeding. During this process, shellfish can concentrate toxigenic micro-algae and other substances from the water column when they are present. The ability of shellfish to concentrate chemical pollutants from water can lead to accumulation of these toxins to levels that constitute a public health hazard. Dinoflagellates producing Saxitoxin have caused mortality events in fish, and sea mammals. In humans, Saxitoxin (PSP) poisoning causes neurological symptoms that can lead to respiratory paralysis and even death. Some of the currently available methods used for the detection and monitoring of saxitoxin in water and shellfish are not conducive for the quick on-site or real time, dockside or ship board monitoring of this toxin. For example: 1) the mouse bioassay is labor intensive, requires the use and destruction of many vertebrate animals, analyses is only performed in a few laboratories with a low turn around time, 2) a lateral flow ELISA developed by Jellet Rapid Testing Ltd., however, this assay seems to produce a high degree of false positives. Therefore, faster, easier and/or more reliable methods are needed to satisfy the needs of the regulatory community and shellfish industry. The proposed ELISA method is a fast and easy to perform method with ready to use reagents i.e. analyst only needs to extract shellfish sample or dilute water sample before analysis. The proposed ELISA also provides a quantitative and/or semi-quantitative screening for shellfish extracts and/or water samples.

		This assay is part of Abraxis platform for marine toxin testing and complements the company's other offering for NSP, DSP, and ASP testing.
2. What is the intended purpose of the method?		The fast analysis of Saxitoxin (PSP) in shellfish extracts and/or water quality monitoring. The proposed ELISA can be used on-site (boat, dock) or established analytical laboratories.
3. Is there an acknowledged need for this method in the NSSP?		Yes. NSSP Guidance Documents, Chapter II Constitution by-laws and procedures of the Interstate Shellfish Sanitation Conference. Procedure XVI. Procedure for acceptance and approval of analytical methods for the NSSP. And: National Shellfish Sanitation Program 2003 Model Ordinance III. Laboratory @ 02 Methods C. Biotoxin. Methods for the analysis of shellfish and shellfish harvest waters shall be: 1) The current AOAC and APHA methods used in bioassay for paralytic shellfish poisoning toxins
4. What type of method? i.e. chemical, molecular, culture, etc.		Immunochemical Method.
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title		Abraxis ELISA Kit for the Screening of Saxitoxin in Shellfish Extract and/or Harvest Waters.
Method Scope		A Method for the screening out negative saxitoxin samples in shellfish regulatory labs, to determine if shellfish are safe to harvest and or distribute. A method for water classification for saxitoxin around harvest areas and to screen for toxic phytoplankton in seawater to provide early warning. A method that provides multiple simultaneous results (depending on chosen cut-off values). This can be easily done because the assay is run with multiple STX concentrations.
References		Etheridge, S., Deeds, J, Easy, D., Laycok, M., Caulfield, C., Deardorff, D., Church, J., PSP & TTX Kits: Regulatory Perspectives. Satellite Workshop to the Gordon Conference on Mycotoxins and Phycotoxins 2007, Maine, USA, E. Hignutt, S.W. Longan, Environmental Health Laboratory, State of Alaska, Anchorage, AK; Comparison of HILIC/Tandem Mass Spectrometry, Abraxis ELISA and Mouse Bioassay for Determination of PSP in Shellfish. To be presented at the 2008 AOAC Annual Meeting, Dallas, Texas.
Principle		**The test is a direct competitive ELISA based on the recognition of Saxitoxin by specific antibodies. Saxitoxin, when present in a sample and a saxitoxin-**

		enzyme-conjugate compete for the binding sites of rabbit anti-saxitoxin antibodies in solution. The saxitoxin antibodies are then bound by a second antibody (sheep anti-rabbit) immobilized on the plate. After a washing step and addition of the substrate solution, a color signal is produced. The intensity of the blue color is inversely proportional to the concentration of the Saxitoxin present in the sample. The color reaction is stopped after a specified time and the color is evaluated using an ELISA reader. The concentrations of the samples are determined by interpolation using the standard curve constructed with each run.
Any Proprietary Aspects		Immunoreagents and sample diluent.
Equipment Required		Pipettes and plate reader. Blender for shellfish extraction.
Reagents Required		Reagents provided in the ELISA kit. In addition diluted hydrochloric acid or vinegar and rubbing alcohol (depending on extraction procedure chosen by analyst).
Sample Collection, Preservation and Storage Requirements		Water samples need to be collected in glass vials and preserved according to users guide (attached). Diluted shellfish extracts should be stored in glass vials. All dilution should be done using provided sample diluent. If not analyzed promptly, samples should be stored refrigerated for up 2 days or frozen if longer periods are required.
Safety Requirements		As with any laboratory procedure, gloves and goggles should be used during the processing and analysis of samples.
Clear and Easy to Follow Step-by-Step Procedure		User's guide and an easy to follow flow chart are provided with each kit (attached).
Quality Control Steps Specific for this Method		As with any analytical procedure laboratory controls (positive and negative) are recommended.
C. Validation Criteria		
1. Accuracy / Trueness		Provided as an attachment.
2. Measurement Uncertainty		@ 0.046 ng/mL in water SD 0.004 CV 8.7% @ 0.087 ng/mL in water SD 0.004 CV 4.6% @ 0.227 ng/mL in water SD 0.008 CV 3.5%
3. Precision Characteristics (repeatability and reproducibility)		< 15%
4. Recovery		Average water recovery 112%; shellfish extract 96%.
5. Specificity		Saxitoxin (STX) 100% (per definition) Decarbamoyl STX 29% GTX 2 & 3 23% GTX-5B 23% Sulfo GTX 1 & 2 2.0% Decarbamoyl GTX 2 & 3 1.4% Neosaxitoxin 1.3% Decarbamoyl Neo STX 0.6% GTX 1 & 4 <0.2%
6. Working and Linear Ranges		0.02-0.4 ng/mL water or 20-400 ng/gm in shellfish extract or higher depending on dilution.
7. Limit of Detection		0.015 ng/mL
8. Limit of Quantitation / Sensitivity		0.02 ng/mL in water; 20 ng/gm in shellfish extract
9. Ruggedness		Since and analytical curve is run with each assay and the samples are compared to the standard curve, the proposed ELISA is rugged.
10. Matrix Effects		If used according to instructions (dilutions), none detected

11. Comparability (if intended as a substitute for an established method accepted by the NSSP)		Method is intended as a screening method to complement other accepted NSPP methods: i.e. mouse bioassay. Some comparison data is provided as an attachment.
D. Other Information		
1. Cost of the Method		As low as $15 per sample
2. Special Technical Skills Required to Perform the Method		Some technical skills are required. Familiarity with laboratory setting is adequate. Kit Manufacturer's on-site training is available.
3. Special Equipment Required and Associated Cost		As low as $1,800. Strip reader and pipette
4. Abbreviations and Acronyms Defined		ELISA: Enzyme linked immuno sorbent assay PSP: paralytic shellfish poisoning
5. Details of Turn Around Times (time involved to complete the method)		40 samples can be run in duplicate in approximately 2 hours. Shellfish sample extraction requires approximately 15 minutes
6. Provide Brief Overview of the Quality Systems Used in the Lab		The ELISA kits are manufactured following GMP and GLP procedures.

Submitters Signature	Date:
Submission of Validation Data and Draft Method to Committee	Date:
Reviewing Members	Date:
Accepted	Date:
Recommendations for Further Work	Date:
Comments:	

DEFINITIONS

1. Accuracy/Trueness - Closeness of agreement between a test result and the accepted reference value.
2. Analyte/measurand - The specific organism or chemical substance sought or determined in a sample.
3. Blank - Sample material containing no detectable level of the analyte or measurand of interest that is subjected to the analytical process and monitors contamination during analysis.
4. Comparability - The acceptability of a new or modified method as a substitute for an established method in the NSSP. Comparability must be demonstrated for each substrate or tissue type by season and geographic area if applicable.
5. Fit for purpose - The analytical method is appropriate to the purpose for which the results are likely to be used.
6. HORRAT value - HORRAT values give a measure of the acceptability of the precision characteristics of a method.[4]
7. Limit of Detection - the minimum concentration at which the analyte or measurand can be identified. Limit of detection is matrix and analyte/measurand dependent.[4]
8. Limit of Quantitation/Sensitivity - the minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.
9. Linear Range - the range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.
10. Measurement Uncertainty - A single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.
11. Matrix - The component or substrate of a test sample.
12. Method Validation - The process of verifying that a method is fit for purpose.[1]
13. Precision - the closeness of agreement between independent test results obtained under stipulated conditions.[1, 2] There are two components of precision:
    a. Repeatability - the measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.
    b. Reproducibility - the measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.
14. Quality System - The laboratory's quality system is the process by which the laboratory conducts its activities so as to provide data of known and documented quality with which to demonstrate regulatory compliance and for other decision-making purposes. This system includes a process by which appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.
15. Recovery - The fraction or percentage of an analyte or measurand recovered following sample analysis.
16. Ruggedness - the ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]
17. Specificity - the ability of a method to measure only what it is intended to measure.[1]
18. Working Range - the range of analyte or measurand concentration over which the method is applied.

REFERENCES:

7. Eurachem Guide, 1998. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. LGC Ltd. Teddington, Middlesex, United Kingdom.
8. IUPAC Technical Report, 2002. Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, Pure Appl. Chem., Vol. 74, (5): 835-855.
9. Joint FAO/IAEA Expert Consultation, 1999. Guidelines for Single-Laboratory Validation of Anilytical Methods for Trace-Level Concentrations of Organic Chemicals.
10. MAF Food Assurance Authority, 2002. A Guide for the Validation and Approval of New Marine Biotoxin Test Methods. Wellington, New Zealand.
11. National Environmental Laboratory Accreditation. , 2003. Standards. June 5.
12. EPA. 2004. EPA Microbiological Alternate Procedure Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Methods: Guidance. U.S. Environmental Protection Agency (EPA), Office of Water Engineering and Analysis Division, 1200 Pennsylvania Avenue, NW, (4303T), Washington, DC 20460. April.

ACCURACY OF PSP ELISA METHOD

PSP Analysis of Shellfish

Dockside Samples Analysis by: Jellet, Abraxis, Mouse Bioassay

Coordinates	SeaWatch #	Depth	Species	MBA result	Lab #	Jellett Result	Abraxis Result* (@40 ug/100g)	Abraxis Result ** (@80 ug/100g)
41 35 80 68 23 33	1	73	SC	40 ug/100g	22	Pos	Pos	Neg
41 34 77 68 23 43	2	105	SC	41	23	Pos	Pos	Neg
41 33 56 68 22 57	3	123	SC	45	24	Pos	Pos	Neg
41 32 65 68 21 19	4	110	SC	39	25	Pos	Pos	Neg
41 35 13 67 58 05	16	117	SC	<39	26	Pos	Neg	Neg
41 08 54 68 33 74	20	98	SC	<40	27	Pos	Neg	Neg
41 37 84 68 10 79	23	86	SC	<41	28	Pos	Neg	Neg
41 36 46 68 09 38	24	91	SC	<39	29	Pos	Neg	Neg
41 35 58 68 09 38	25	80	SC	<39	30	Pos	Neg	Neg
41 47 02 67 45 90	29	102	SC	74	31	Pos	Pos	Pos
41 46 85 67 47 23	30	106	SC	79	32	Pos	Pos	Pos

Abraxis cut-off for positive = can be chosen at 40 or 80 ug/100g simultenously.
other multiple cut-off values can also be chosen.

Data provided by Wallace and Associates

Saxitoxin in Freshwater
Sample Preparation

1. Intended Use
For the detection of Saxitoxin in freshwater samples: groundwater, surface water, drinking water, effluent.

2. Materials Required (Not Provided)
Pipettes capable of delivering 100 and 900µL
Glass sample collection vials with Teflon lined caps

3. Notes and Precautions
Immediately upon collection, freshwater samples should be preserved with 10X Concentrated Sample Diluent to prevent adsorptive loss of Saxitoxin from the sample. This step is necessary for freshwater samples only. Saltwater samples do not require additional reagents for preservation due to their naturally occurring salts.

4. Procedure
Add 100µL of 10X Concentrated Sample Diluent per 900µL of Sample. Cap container and invert several times to thoroughly mix.

The sample is now ready to analyze according to the procedure described in the Abraxis Saxitoxin Kit package insert.

5. Evaluation of Results
Results obtained with freshwater samples which have been preserved with 10X Concentrated Sample Diluent as described above must be multiplied by a factor of 1.1 to account for the initial dilution of samples with 10X Diluent.

6. Performance Data
Recovery
Four (4) freshwater samples were spiked with various levels of Saxitoxin, preserved as described above, and then assayed using the Saxitoxin Assay. The following results were obtained:

	Recovery				
Amount of Saxitoxin Added (ppb)	Mean (ppb)	48 Hours Mean (ppb)	1 Week Mean (ppb)	S.D. (ppb)	%
0.04	0.046	0.046	0.050	0.002	117.9
0.08	0.087	0.085	0.086	0.001	107.5
0.2	0.227	0.217	0.217	0.006	110.1
Average					111.8

7. Assistance

For ordering or technical assistance contact:
Abraxis LLC
54 Steamwhistle Drive
Warminster, PA 18974
Tel.: (215) 357-3911
Fax: (215) 357-5232
Email: info@abraxiskits.com
Web: www.abraxiskits.com

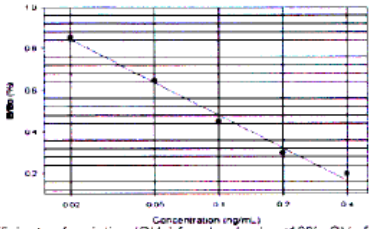
082708

Importance of the Saxitoxin Determination
Saxitoxin is one of the "paralytic shellfish poisons" (PSP), produced by several marine dinoflagellates and fresh water algae. Contamination of shellfish with saxitoxin has been associated with harmful algal blooms throughout the world.

In man, PSP causes dose-dependent perioral numbness or tingling sensations and progressive muscular paralysis, which can result in death through respiratory arrest. The maximum tolerance levels established by the EU and FDA are 40-80 ug per 100 ug edible portion of fresh, frozen, or tinned shellfish.

The Saxitoxin ELISA allows the determination of 42 samples in duplicate determination. Only a few milliliters of sample are required. The test can be performed in about 1 hour.

Performance Data
Test sensitivity: The detection limit for Saxitoxin is 0.015 ng/mL (mean of 6 blank determinations minus 3 SD). The middle of the test (50% B/B_0) is at approximately 0.09 ng/mL. Determinations close to the middle of the tests gives the most accurate results.



Test reproducibility: Coefficients of variation (CVs) for standards: <10%, CVs for samples: <15%.

Selectivity: This ELISA recognizes Saxitoxin and other PSP toxins with varying degrees.

Cross-reactivities:		
	Saxitoxin (STX)	100% (per definition)
	Decarbamoyl STX	29%
	GTX 2 & 3	23%
	GTX-5B	23%
	Sulfo GTX 1 & 2	2.0%
	Decarbamoyl GTX 2 & 3	1.4%
	Neosaxitoxin	1.3%
	Decarbamoyl Neo STX	0.6%
	GTX 1 & 4	<0.2%

Cross-reactivities with other classes of algal toxins have not been observed.

Samples: Drinking water, ground water, and surface water were tested for matrix effects in the ELISA. No matrix effects were determined.

General Limited Warranty: Abraxis LLC warrants the products manufactured by the Company, against defects and workmanship when used in n accordance with the applicable instructions for a period not to extend beyond the product's printed expiration date. **Abraxis makes no other warranty, expressed or implied. There is no warranty of merchantability or fitness for a particular purpose**

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WEB: WWW.abraxiskits.com

R082508

Saxitoxin (PSP) ELISA, Microtiter Plate

Enzyme-Linked Immunosorbent Assay for the Determination of Saxitoxin (PSP) in Water and Contaminated Samples

Product No. 52255B

1. General Description

The Saxitoxin ELISA is an immunoassay for the quantitative and sensitive detection of Saxitoxin. Saxitoxin is one of the toxins associated with paralytic shellfish poisoning (PSP). This test is suitable for the quantitative and/or qualitative detection of Saxitoxin in water samples as well as other contaminated samples. For shellfish samples a sample preparation is required. If necessary, positive samples can be confirmed by HPLC, GC/MS, or other conventional methods.

2. Safety Instructions

The standard solutions in the test kit contain small amounts of Saxitoxin. In addition the substrate solution contains tetramethylbenzidine and the stop solution contains diluted sulfuric acid. Avoid contact of stopping solution with skin and mucous membranes. If these reagents come in contact with the skin, wash with water.

3. Storage and Stability

The Saxitoxin ELISA should to be stored in the refrigerator (4–8°C). The solutions have to be allowed to reach room temperature (20-25°C) before use. Reagents may be used until the expiration date on the box.

4. Test Principle

The test is a direct competitive ELISA based on the recognition of Saxitoxin by specific antibodies. Saxitoxin, when present in a sample and a saxitoxin-enzyme-conjugate compete for the binding sites of rabbit anti-saxitoxin antibodies in solution. The saxitoxin antibodies are then bound by a second antibody (sheep anti-rabbit) immobilized on the plate. After a washing step and addition of the substrate solution, a color signal is produced. The intensity of the blue color is inversely proportional to the concentration of the Saxitoxin present in the sample. The color reaction is stopped after a specified time and the color is evaluated using an ELISA reader. The concentrations of the samples are determined by interpolation using the standard curve constructed with each run.

5. Limitations of the Saxitoxin ELISA, Possible Test Interference

Numerous organic and inorganic compounds commonly found in water samples have been tested and found not to interfere with this test. However, due to the high variability of compounds that might be found in water samples, test interferences caused by matrix effects can't be completely excluded. Mistakes in handling the test also can cause errors. Possible sources for such errors can be:

Inadequate storage conditions of the test kit, wrong pipetting sequence or inaccurate volumes of the reagents, too long or too short incubation times during the immune and/or substrate reaction, extreme temperatures during the test performance (lower than 10°C or higher than 30°C).

The Abraxis Saxitoxin ELISA kit provides screening results. As with any analytical technique (GC, HPLC, etc.) positive samples requiring some action should be confirmed by an alternative method.

Working Instructions

A. Materials Provided

1. Microtiter plate coated with a second antibody (sheep anti-rabbit).
2. Standards (6): 0, 0.02, 0.05, 0.1, 0.2, 0.4 ng/mL
3. Antibody Solution (rabbit anti-saxitoxin), 6 mL
4. Saxitoxin-HRP Conjugate, 6 mL
5. Sample Diluent Concentrate (10X), 2 X 25 mL. Use to dilute samples
6. Wash Solution (5X) Concentrate, 100 mL
7. Color Solution (TMB), 12 mL
8. Stop Solution, 12 mL

B. Test Preparation

Micro-pipetting equipment and pipette tips for pipetting the standards and the samples are necessary. We recommend using a multi-channel pipette or a stepping pipette for adding the antibody, the substrate solution and the stop solution in order to equalize the incubations periods of the standard solutions and the samples on the entire microtiter plate. Please use only the reagents and standards from one package lot in one test, as they have been adjusted in combination.

1. Adjust the microtiter plate and the reagents to room temperature before use.
2. Remove the number of microliter plate strips required from the foil bag. The remaining strips are stored in the foil bag and zip-locked closed. Store the remaining kit in the refrigerator (4-8°C).
3. The standard solutions, enzyme conjugate, substrate and stop solution are ready to use and do not require any further dilutions.
4. Dilute the Wash Buffer at a ratio of 1:5. If using the entire bottle (100 mL) then add to 400 mL of deionized or distilled water. Dilute the Sample Diluent at a ratio of 1:10 with deionized water.
5. The stop solution has to be handled with care as it contains diluted H_2SO_4.
6. Freshwater samples must be preserved immediately upon collection to prevent loss of saxitoxin from the samples. Please refer to the Saxitoxin in Freshwater Sample Preparation Bulletin for details.

C. Assay Procedure

1. Add 50 µL of the **standard solutions or the samples** (water) or sample extracts (shellfish) into the wells of the test strips according to the working scheme given. We recommend using duplicates or triplicates.
2. Add 50 µL of **enzyme conjugate** solution to the individual wells successively using a multi- channel pipette or a stepping pipette.
3. Add 50 µL of **antibody solution** to the individual wells successively using a multi- channel pipette or a stepping pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a rapid circular motion on the benchtop for about 30 seconds. Be careful not to spill contents.
4. Incubate the strips for 30 min at room temperature.
5. Wash the strips four times using the washing buffer solution. Please use at least a volume of 300 µL of washing buffer for each well and each washing step. Remaining buffer in the wells should be removed by patting the plate dry on a stack of paper towels.
6. Add 100 µL of **substrate solution** to the wells. The strips are incubated for 30 min at room temperature. Protect the strips from direct sunlight.
7. Add 100 µL of **stop solution** to the wells in the same sequence as for the substrate solution.
8. Read the absorbance at 450 nm using a microplate ELISA photometer within 15 minutes after the addition of the stopping solution.

D. Evaluation

The evaluation of the ELISA can be performed using commercial ELISA evaluation programs (4-Parameter (preferred) or Logit/Log). For a manual evaluation calculate the mean absorbance value for each of the standards. Calculate the $\%B/B_0$ for each standard by dividing the mean absorbance value for each standard by the Zero Standard (Standard 0) mean absorbance. Construct a standard curve by plotting the $\%B/B_0$ for each standard on a vertical linear (y) axis versus the corresponding Saxitoxin concentration on horizontal logarithmic (x) axis on graph paper. $\%B/B_0$ for samples will then yield levels in ppb of Saxitoxin by interpolation using the standard curve.

The concentrations of the samples are determined using this standard curve. Samples showing lower concentration of Saxitoxin compared to standard 1 (0.02 ng/mL) are considered as negative. Samples showing a higher concentration than standard 6 (0.4 ng/mL) must be diluted further to obtain more accurate results.

E. Additional Materials (not delivered with the test kit)

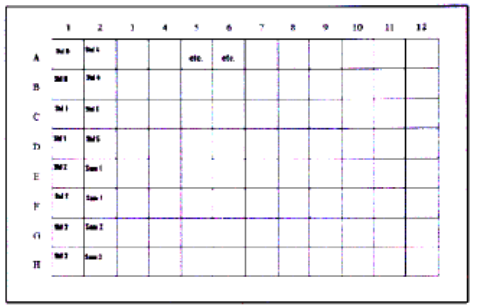
1. Micro-pipettes with disposable plastic tips (10-200, and 200-1000 µL)
2. Multi-channel pipette (10-300 µL) or stepper pipette with plastic tips (10-300 µL)
3. Microtiter plate washer
4. Microtiter plate reader (wave length 450 nm)
5. Shaker for microliter plates (optional)

F. Working Scheme

The microtiter plate consists of 12 strips of 8 wells, which can be used individually for the test. The standards have to be run with each test. Never use the values of standards which have been determined in a test performed previously.

Std 0-Std 5: Standards
0; 0.02; 0.05; 0.10; 0.20; 0.40 ppb

Sam1, Sam2, etc.: Samples



G. Preparation of Sample (Mussels)

NOTE: If for regulatory purposes a 100 g sample is needed, extraction solution volume should be adjusted accordingly.

1. Mussels are removed from their shells, washed with deionized water and homogenized.
2. Mix 10 gm of homogenized mussels with 10 mL of 0.1M HCl and boil for 5 minutes while stirring.
3. Allow to cool and centrifuge for 10 minutes at approximately 3500 g.
4. Adjust pH to < pH 4.0 with 5 N HCl.
5. Remove 10 uL and dilute to 10 mL with Sample Dilution Buffer (1:1,000 dilution).
6. Run in the assay as sample (Assay Procedure step 1).

The STX concentration in the samples is determined by multiplying the concentration of the diluted extract by a factor of 2,000. Highly contaminated samples (outside the range of the curve), should be diluted further and re-analyzed, we recommend further dilutions of 1:10 with sample dilution buffer. The dilution factor will then be 20,000. Samples with low contamination of STX or samples that contain STX congeners with low cross-reactivity (see chart) can be detected in the assay by diluting samples 1:250 before analysis. The assay has low cross-reactivity against GTX 1 & 4, therefore food samples containing these congeners at low concentrations might be underestimated by this ELISA.

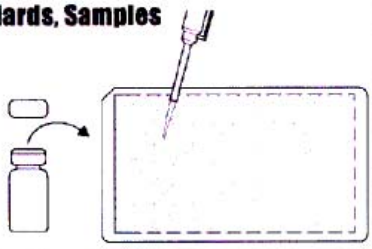
H. Alternative Sample Preparation

1. Mussels are removed from their shells, washed with deionized water, dried and homogenized using a Polytron or equivalent.
2. A 1.0 gm portion is then mixed with 6 mL methanol/DI water (80/20) using a Polytron or equivalent.
3. Centrifuge mixture for 10 minutes at 3000 g. Collect supernatant.
4. Add 2 mL methanol/deionized water (80/20) to the mussel tissue residue. Re-centrifuge the mixture for 10 minutes. Add supernatant to first portion.
5. Bring the volume of the collected supernatant to 10 mL with methanol deionized water (80/20). Filter extract through a 0.45 um filter (Millex HV, Millipore).
6. Remove 10 uL and dilute to 1.0 mL with sample Dilution Buffer (1:100 dilution), then analyze as samples (Assay Procedure, step 1). The STX concentration in the samples is determined by multiplying the concentration of the diluted extract by a factor of 1,000.

Saxitoxin (PSP) Plate, Detailed ELISA Procedure

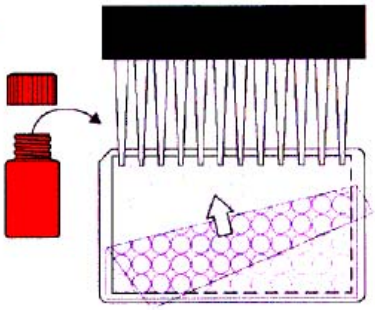
1. Addition of Standards, Samples

Add 50 uL of the standard solutions, control or samples into the wells of the test strips according to the working scheme given. We recommend using duplicates or triplicates.



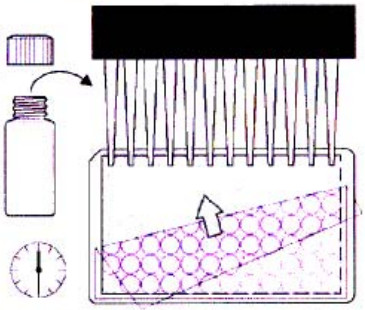
2. Addition of Enyzme Conjugate

Add 50 uL of the enzyme conjugate to the individual wells successively using a multi- channel pipette or a stepping pipette.



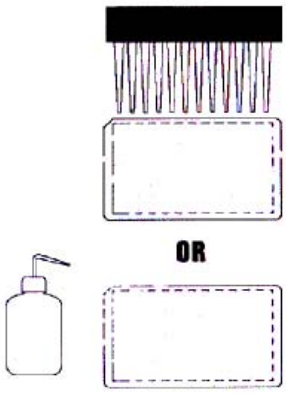
3. Addition of Antibody Solution

Add 50 uL of the Saxitoxin anitbody solution to the individual wells successively using a multi-channel pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a rapid circular motion on the benchtop. Be careful not to spill contents. Incubate the strips for 30 min at room temperature.



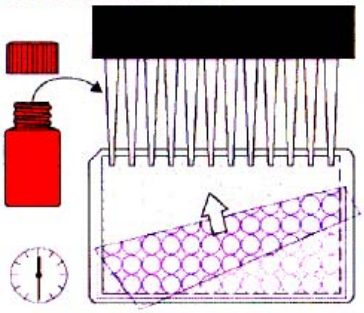
4. Washing of Plates

After incubation, remove the covering and vigorously shake the contents of the wells into a sink. Wash the strips three times with a multi-channel pipette or wash bottle using the diluted 1X washing buffer solution. Please use at least a volume of 300 uL of washing buffer for each well and each washing step. Remaining buffer in the wells should be removed by patting the plate dry on a stack of paper towels. Repeat steps an additional three times.



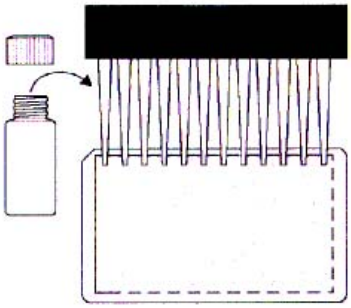
5. Addition of Substrate/Color Solution

Add 100 uL of substrate/color solution to the individual wells successively using a multi-channel pipette or a stepping pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a rapid circular motion on the benchtop. Be careful not to spill contents. Incubate the strips for 30 min at room temperature.



6. Addition of Stopping Solution

Add 100 uL of stop solution to the wells in the same sequence as for the substrate solution using a multi- channel pipette or a stepping pipette.



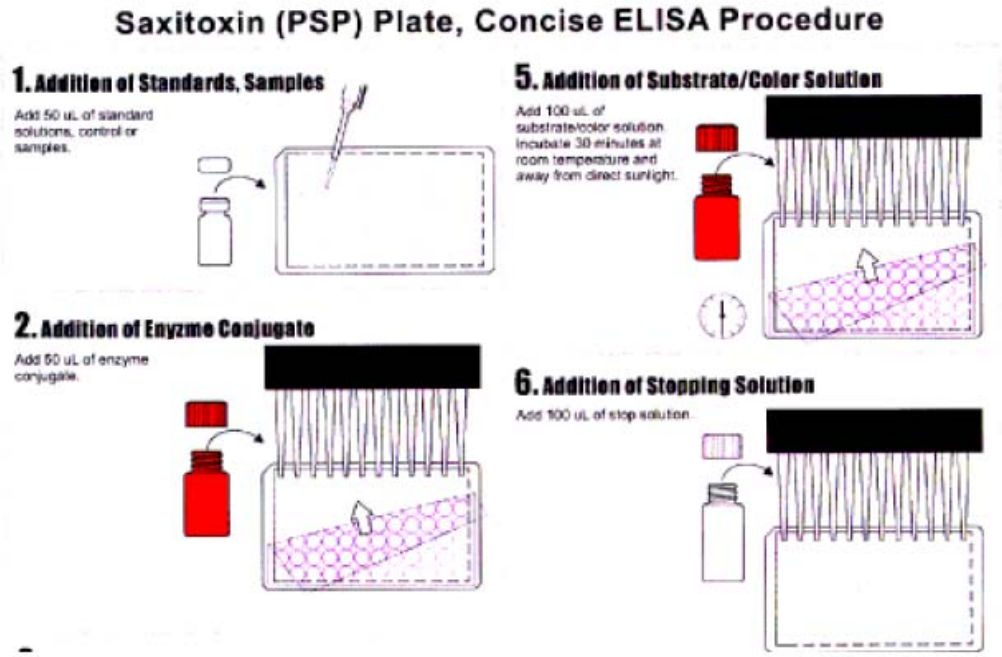
7. Measurement of Color

Read the absorbance at 450 nm using a microplate ELISA reader. Calculate results.

For Ordering or Technical Assistance Contact:
ABRAXIS, LLC
54 Steamwhistle Drive, Warminster, PA 18974
Phone: 215-357-3911 Fax: 215-357-5232
www.abraxiskits.com

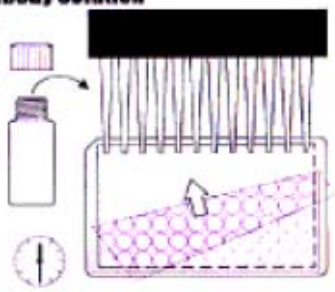
ABRAXIS

Saxitoxin (PSP) Plate Kit Part # 52255B


Saxitoxin (PSP) Plate, Concise ELISA Procedure
1. Addition of Standards, Samples
Add 50 uL of standard solutions, control or samples.
2. Addition of Enyzme Conjugate
Add 50 uL of enzyme conjugate.
5. Addition of Substrate/Color Solution
Add 100 uL of substrate/color solution. Incubate 30 minutes at room temperature and away from direct sunlight.
6. Addition of Stopping Solution
Add 100 uL of stop solution.

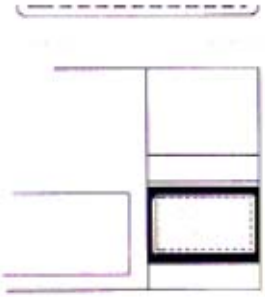
3. Addition of Antibody Solution

Add 50 uL of the antibody solution. Cover and mix for 30 seconds by rotating on benchtop. Incubate for 30 minutes at room temperature



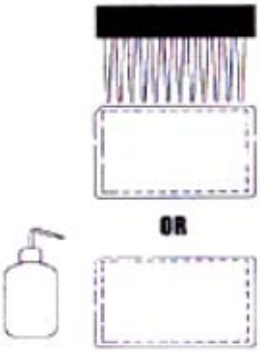
7. Measurement of Color

Measure color at 450 nm.
Calculate results.



4. Washing of Plates

Wash the plates four times with 300 uL of diluted 1X washing buffer.



For Ordering or Technical Assistance Contact:
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54 Steamwhistle Drive, Warminster, PA 18974
Phone: 215-357-3911 Fax: 215-357-5232
www.abraxiskits.com

ABRAXIS

Saxitoxin (PSP) Plate Kit Part # 522558

Proposal Subject: *Vibrio vulnificus* Risk Management Plan

Specific NSSP Guide Reference: 1999 NSSP Guide Model Ordinance
Chapter II. Risk Assessment and Risk Management

Text of Proposal/ Requested Action Modify 1999 Model Ordinance Chapter II. by adding new Section @. 04:

Chapter II. Risk Assessment and Risk Management.

@. 04 *Vibrio vulnificus* Risk Management

Risk Management Plan

(1) For states having 2 or more etiologically confirmed shellfish-borne *Vibrio vulnificus* illnesses traced to the consumption of commercially harvested raw or undercooked oysters that originated from the waters of that state, the Authority shall develop and implement a *Vibrio vulnificus* risk management plan. Etiologically confirmed means those cases in which laboratory evidence of a specific agent is obtained and specified criteria are met.

(2) The plan may include the following elements and shall define the administrative procedures and resources necessary to accomplish (i.e., establish and maintain) them;

(a) Education/Consumer intervention;

(b) Pre-harvest controls to reduce *Vibrio vulnificus* levels in oyster shellstock; and

(c) Post-harvest controls to reduce *Vibrio vulnificus* levels in oyster shellstock.

(3) The plan shall include controls and interventions that are designed to reduce the rate of etiologically confirmed shellfish-borne *Vibrio vulnificus* septicemia illnesses reported in core states from the consumption of commercially harvested raw or undercooked oysters by 40 percent by the end of 2005 and by 60 percent by 2007. The rate of illness shall be calculated as the number of illnesses divided by the production of oysters from the states bordering the Gulf of Mexico, based on National Marine Fisheries Service landing data. Core states shall be Florida, Texas, California, Louisiana, Georgia, South Carolina, and Alabama. The baseline data for measuring illness reduction shall be the reported illnesses in the core states for the period 1996 to 1999, inclusive, as compiled by the Southeast Regional Office of the U.S. Food and Drug Administration. The data used for measuring goal attainment shall begin with 2001 data. See §B. (1) below.

(4) At a minimum, the plan shall include the following controls and interventions:

(a) Education/Consumer intervention - Implementing ~~of those~~ portions of the ISSC Education/Consumer Intervention Plan that are relevant to the state;

(b) Pre-harvest Controls - Based on the results of the annual FDA state shellfish program evaluation, assuring that all certified dealers comply with the time/temperature requirements contained in VIII.03, IX.05, XI.01A. (3), XII.01A. (3), XIII.01A. (3), and XIV.01A. (3). *[Ed. note: see proposed language for XI.01A. (3),*

XII.01A. (3), XIII.01A. (3), and XIV.01A. (3) in Issue 00-208.]

(c) Post-harvest Controls

(i) Providing assistance, as necessary, for the further study of dockside icing to investigate its effects on shelf-life and variations in the effectiveness of the method as a result of seasonal and regional differences;

(ii) Implementing dockside icing requirements if the study results are favorable and illness reduction targets are not met as described in §(5) below;

(iii) Supporting, as necessary, the commercialization of existing post-harvest technologies and the development of new technologies;

(iv) Providing incentives to add refrigeration capacity to harvest vessels; and

(v) Selecting and preparing for the implementation of one or more of the controls contained in II. @. 04A. (6), in case such implementation becomes necessary, as described in that paragraph.

(5) If the illness reduction goal contained in II. @. 04A. (3) is less than 25 percent by the end of Year 4 (2004); the goal must be reassessed through a thorough review of the more intensive epidemiological investigations of illnesses for years 2001-2004.

[Submitter's note: The details of this more intensive epidemiological investigation are being discussed by the Vibrio Management Committee (VMC). Final recommendations will be made available following the VMC meeting on June 13 and 14.]

(6) Affected states must implement one or more of the following control strategies on January 1, 2008, if the illness reductions fail to meet the requirements of §(5) above.

[*Submitter's note: The Committee is discussing multiple options for appropriate control strategies. They include:*

(a) Labeling oysters when water temperatures reach a certain level (65□ Fahrenheit is being discussed);

(b) Requiring post-harvest treatment when water temperatures exceed a certain level (65□ Fahrenheit is being discussed);

(c) Closing growing areas when water temperatures exceed a certain level (65□ Fahrenheit is being discussed);

(d) Labeling shellfish, "For shucking and cooking only" based on Vibrio vulnificus levels in meats;

(e) Requiring post-harvest treatment based on levels of Vibrio vulnificus in meats at harvest;

(f) Closing growing areas based on Vibrio vulnificus levels in meats at harvest;

(g) Labeling oysters "For shucking and cooking only" during certain months;

(h) Requiring post-harvest treatment during certain months;

(i) Closing certain shellfish growing areas during certain months.

Submitter's note: Final recommendations will be made available following the VMC meeting on June 13 and 14.]

Epidemiological Plan

(1) Core states referenced in §A. above will administer a survey to

determine the Vibrio vulnificus disease reporting practices in each state for the period 1996-1999. The development and implementation plan for the survey will be initiated through the ISSC with participation of state public health officers, epidemiologists and others as determined. Continued surveillance will be necessary to indicate changes to reporting practices during 2000-2007. This is fundamental to establishing the illness baseline as described in §A. (3) above and in tracking future illness report data.

(2) Beginning in calendar year 2001, a new shellfish-borne Vibrio vulnificus disease investigation team will rapidly investigate any case of etiologically confirmed shellfish-borne Vibrio vulnificus septicemia illnesses in core states. This team will gather customary epidemiological information as well as the level of awareness of risk in those who have suffered etiologically confirmed shellfish-borne Vibrio vulnificus septicemia illnesses. The ISSC will assist in initiating this team.

Public Health Significance: This plan is aimed at reducing exposure to *Vibrio vulnificus*, especially in at-risk populations. These controls, by potentially decreasing exposure, can in turn potentially reduce oyster-borne *Vibrio vulnificus* septicemia illnesses.

Cost Information (if available): Unknown

Action by 2000 Vibrio Management Committee Recommended adoption of 00-201 as substituted by the Vibrio Management Committee (VMC).

Text of Proposal:
Modify Model Ordinance Chapter II. by adding Section @. 04:

***@*. 04 *Vibrio vulnificus* Risk Management**

(A) For states having 2 or more etiologically confirmed shellfish-borne *Vibrio vulnificus* illnesses since 1995 traced to the consumption of commercially harvested raw or undercooked oysters that originated from the waters of that state (Source State), the Authority shall develop and implement *a Vibrio vulnificus* risk management plan.

(B) The plan shall define the administrative procedures and resources necessary to accomplish (i.e. establish and maintain) involvement by the state in a collective illness reduction program. The goal of the program will be to reduce the rate of etiologically confirmed shellfish-borne *Vibrio vulnificus* septicemia illnesses reported in core states (Florida, Texas, California, Louisiana, Georgia, South Carolina, and Alabama) from the consumption of commercially harvested raw or undercooked oysters by 40 percent, collectively, by the end of 2005 and by 60 percent, collectively, by the end of 2007. The rate of illness shall be calculated as the number of illnesses adjusted for population and rate of reporting divided by the production of oysters from the states bordering the Gulf of Mexico, based on National Marine Fisheries Service landing data verified by Silver Spring, Maryland, headquarters. The goal may be reevaluated prior to the year 2005 and adjusted in the event that new science, data or information becomes available.

(C) The plan shall also include identification and preparation for

implementation of one or more of the following controls, or equivalent controls, which shall be implemented should the 60 percent illness reduction goal not be achieved by 2007. This portion of the plan shall be completed no later than December 2006. The temperature and month-of the-year parameters identified in the following controls may be adjusted as needed to achieve the established illness reduction goal.

(1) Labeling all oysters, "For shucking by a certified dealer," when the Average Monthly Maximum Water Temperature exceeds 75°F;

(2) Subjecting all oysters to an Authority-approved post-harvest treatment that reduces the *Vibrio vulnificus* levels to 3MPN/g or less," when the Average Monthly Maximum Water Temperature exceeds 75°F;

(3) Closing shellfish growing areas when the Average Monthly Maximum Water Temperature exceeds 75°F;

(4) Labeling all oysters, "For shucking by a certified dealer," during the months of May through September, inclusive;

(5) Subjecting all oysters to a post-harvest treatment that is both approved by the Authority and reduces the *Vibrio vulnificus* levels to 3MPN/g or less during the months of May through September, inclusive;

(6) Closing shellfish growing areas during the months of May through September, inclusive.

Modify the NSSP Guide for Control of Molluscan Shellfish by adding the following Guidance Document (numbering to be determined at time of publication of the next revision).

***Vibrio vulnificus* Management Guidance Document**

***Vibrio vulnificus* Management**

The voting delegates at the 1999 Annual Meeting in New Orleans created the Vibrio Management Committee (VMC). At the 2000 annual meeting the voting delegates will be asked to adopt the VMC's recommendation of reducing the rate of etiologically confirmed shellfish-borne *Vibrio vulnificus* septicemia. The goal is to reduce those illnesses reported in core states (Florida, Texas, California, Louisiana, Georgia, South Carolina, and Alabama) from the consumption of commercially harvested raw or undercooked oysters by 40 percent by the end of 2005 and by 60 percent by the end of 2007. The Core States are the states that have consistently reported *Vv* cases since 1995. The rate of illness shall be calculated as the number of illnesses adjusted for population and rate of reporting divided by the production of oysters from the states bordering the Gulf of Mexico, based on National Marine Fisheries Service landing data verified by Silver Spring, Maryland, headquarters. This adjustment will be performed in consultation with statisticians and epidemiologists from core states and federal agencies. The baseline data and all future data for measuring illness reduction shall be the reported illnesses in the core states for the period 1996 to 1999, inclusive, as compiled by the Southeast Regional Office of the U.S. Food and Drug Administration. The data used for measuring goal attainment shall begin with 2001 data. The formula for calculating for the rate of illness is as follows:

(number of cases) x (CDC adjustment factor)

population

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production

The VMC members will include, at a minimum, industry and state shellfish control authority representatives from *Vibrio vulnificus* Illness Source and Core States, FDA, NOAA, EPA, CDC, state epidemiologists; as well as industry and shellfish control representatives from other regions. *Vibrio vulnificus* Illness Source States are those states reporting 2 or more etiologically confirmed shellfish-borne *Vibrio vulnificus* illnesses since 1995 traced to the consumption of commercially harvested raw or undercooked oysters that originated from the waters of that state. Core states are Florida, Texas, California, Louisiana, Georgia, South Carolina and Alabama. Etiologically confirmed means those cases in which laboratory evidence of a specific agent is obtained and specified criteria are met.

The VMC will meet at least annually to develop and approve work plans and review progress. The first plan will be in place for a one-year period, followed by three biennial plans. The first work plan and progress review period will be from January 2001 to December 31, 2001. The next work plan period will be from January 1, 2002 to December 31, 2003, January 1, 2004 to December 31, 2005; then January 1, 2006 to December 31, 2007.

Work plans will include goals, tasks, performance measures and assessment methods to track and achieve progress towards the illness reduction goals. The work plans will be developed by the VMC and approved by the VMC membership. The chair of the VMC will deliver a written annual progress report, including a summary of the previous year's progress made in the education program, to the ISSC March executive board meeting. The report shall be made available to the general membership. The biennial work plan structure, outlined below, provides adaptive management and assures consistent progress towards the illness reduction goals.

Work plans developed by the VMC shall include the following elements and shall define the administrative procedures and resources necessary for accomplishment (i.e. establishment and maintenance):

(a) An ISSC Consumer Education Program targeted toward individuals who consume raw oysters and whose health condition(s) increase their risk for *Vibrio vulnificus* infection. The Education Program's objectives will be 1) to increase the target audience's awareness that eating raw oysters can be life-threatening to them, and; 2) to change the at-risk group's oyster-eating behavior, i.e., to reduce or stop eating raw oysters. The ISSC Education Committee and the *Vibrio vulnificus* Education Subcommittee will assist in the development and oversight for this program.

(i) The Consumer Education Program will focus educational efforts in the Core States. The Education Program will make educational materials available to states upon request.

(ii) Educational approaches will emphasize partnerships with

health and advocacy organizations, and include dissemination of printed materials, posting materials on the Internet, broadcast of television spots, press releases, and other measures deemed effective such as the USDA Physician Notification Program.

(iii) Periodic administration of Behavior Risk Factor State Surveys (BRFSS) and other survey assessments at the state level shall be explored as a means of assessing the effectiveness of educational interventions.

(b) Administration of a survey to determine the current *Vibrio vulnificus* disease reporting and education in each state;

(c) Creation of a shellfish-borne *Vibrio vulnificus* disease investigation team that will be available to assist in collection of epidemiological information associated with confirmed shellfish-borne *Vibrio vulnificus* septicemia illness. This team will assist in gathering customary epidemiological information as well as the level of awareness of risk in those who have suffered etiologically confirmed shellfish-borne *Vibrio vulnificus* septicemia illnesses. A small ISSC team with recognized epidemiological officers will assist in rapid investigation of any case. This team will work cooperatively with existing local, state and federal disease investigation programs.

(d) Industry-implemented post-harvest controls to reduce *Vibrio vulnificus* levels in oyster shellstock which may include: time-temperature, post harvest treatment (i.e. hydrostatic pressure, cool pasteurization, IQF, and irradiation--pending approval), rapid chilling and other emerging technologies.

(e) To encourage implementation of post harvest controls the Conference will pursue options such as SBA low interest loans; revolving loans; cost sharing; demonstration projects; state-industry partnerships; FDA label incentives; PHT specific growing area classifications; targeted time/temperature assessment by FDA during annual shellfish program evaluations; assistance, as necessary, for the further study and possible implementation of dockside icing to investigate its effects on shelf life and variations in the effectiveness of the method as a result of seasonal and regional differences and incentives to add refrigeration capacity to harvest vessels. The goal will be to provide incentives necessary to post-harvest treat 20 percent of all oysters intended for the raw, half-shell market during the months of May through September harvested from a source state by the end of the third year (December 31, 2003). The assessment will include the capacity of all operational plants and the capacity of plants under construction. Should the 20 percent goal not be accomplished, the VMC will pursue additional incentives to achieve the goals.

(f) **A VMC compilation and review of the data on rates of illness will be made available to the ISSC at the ISSC Biennial meeting following the year in which the data was gathered. In the event that the data is not available at the time of the meeting, the VMC shall meet and review the data when it becomes available and issue a compilation report, which will be made available to the entire ISSC membership. In the event there is no Biennial meeting scheduled for a certain year, the VMC shall meet and review the data when it becomes available and issue a compilation report which will be made available to the entire conference.**

(g) A VMC evaluation of the effectiveness of reduction efforts will be conducted at the end of the fifth year (December 31, 2005). The evaluation will determine whether the 40 percent, 5-year illness reduction goal or education/consumer intervention or post harvest controls performance measures set forth in prior work plans have been achieved. Should the VMC evaluation indicate the 40 percent, 5 year goal has not been accomplished, the committee will identify additional harvest controls in the 2006 - 2007 work plan to assure achievement of the 60 percent illness reduction goal by the close of the seventh year. In addition, the VMC will evaluate the requirements in Section 04.C. with the possibility of changing the controls to achieve remaining illness reduction goals.

PUBLIC HEALTH SIGNIFICANCE: **The purpose of the National Shellfish Sanitation Program is to promote and improve the sanitation of shellfish (oysters, clams, mussels and scallops) moving in interstate commerce through federal/state cooperation and uniformity of State Shellfish Programs. This includes protection of the public health by reducing the prevalence of food borne hazards. Complete elimination of illness is difficult to attain but public health programs should be designed to provide the greatest level of public health protection possible. The vision of public health officials must focus on maximizing protection with the most practical public health measures available. This plan is designed to assure a significant reduction in *Vibrio vulnificus* septicemia illnesses through a combination of consumer education, processing incentives and, if necessary, mandatory harvesting or processing controls.**

COST INFORMATION: Unknown.

In addition the Committee recommended:

(1) Issue 00-201 become effective October 1, 2000; and the requirement for the *Vibrio vulnificus* Management Plans specified in Section .04A. be developed by these states by April 1, 2001;
(2) Establish a new VMC technical subcommittee that would come up with a list of research and market-related questions and needs relative to the design of a PHT incentive program; and
(3) Ensure that the VMC establishes and performs all necessary evaluations of goals, tasks, performance measures, assessment measures and data collection elements contained in the new Model Ordinance Section @. 04 *Vibrio vulnificus* Risk Management, and in the *Vibrio vulnificus* Management Guidance Document.

Action by 2000 Task Force II

Recommended adoption of Issue 00-201 as substituted by the Vibrio Management Committee (VMC) and further amended as follows:

TEXT OF PROPOSAL:

Modify Model Ordinance Chapter II. By adding Section @. 04:

@. 04 *Vibrio vulnificus* Risk Management **for Oysters**.

(A) For states having 2 or more etiologically confirmed shellfish-borne *Vibrio vulnificus* illnesses since 1995 traced to the consumption of commercially harvested raw or undercooked oysters that originated from the waters of that state (Source State), the Authority shall develop and implement *a Vibrio vulnificus* risk management plan.

(B) The plan shall define the administrative procedures and resources necessary to accomplish (i.e. establish and maintain) involvement by the state in a collective illness reduction program. **The Plan shall include, at a minimum, the ISSC Consumer Education Program targeted toward individuals who consume raw oysters and whose health condition(s) increase their risk for *Vibrio vulnificus* illnesses**. The goal of the Vibrio Risk Management Plan will be to reduce the rate of etiologically confirmed shellfish-borne *Vibrio vulnificus* septicemia illnesses, reported in core states, ~~which may include (Florida, Texas, California, Louisiana, Georgia, South Carolina, and Alabama) to be determined by the VMC after a thorough review of statistical and epidemiological information~~ from the consumption of commercially harvested raw or undercooked oysters by 40 percent, collectively, by the end of 2005 and by 60 percent, collectively, by the end of 2007. **The core states include Florida, Texas, California, Louisiana, Georgia, South Carolina, and Alabama. The list of core states may be adjusted if after a thorough review, epidemiological and statistical data demonstrates that it would be appropriate.** The rate of illness shall be calculated as the number of illnesses adjusted for population and rate of reporting divided by the production of oysters from the states bordering the Gulf of Mexico, based on National Marine Fisheries Service landing data verified by Silver Spring, Maryland, headquarters. The goal may be reevaluated prior to the year 2005 and adjusted in the event that new science, data or information becomes available.

(C) The plan shall also include identification and preparation for implementation of one or more of the following controls, or equivalent controls, which shall be implemented should the 60 percent ~~illness~~ **rate of illness** reduction goal not be achieved by 2007. This portion of the plan shall be completed no later than December 2006. The temperature and month-of the-year parameters identified in the following controls may be adjusted as needed to achieve the established illness reduction goal.

(1) Labeling all oysters, "For shucking by a certified dealer," when the Average Monthly Maximum Water Temperature exceeds 75°F;

(2) Subjecting all oysters **intended for the raw, half-shell market** to an Authority-approved post-harvest treatment that reduces the *Vibrio vulnificus* levels to 3MPN/g or less," when the Average Monthly Maximum Water Temperature exceeds 75°F;

(3) Closing shellfish growing areas **for the purpose of harvest of**

oysters intended for the raw, half-shell market when the Average Monthly Maximum Water Temperature exceeds 75°F;

(4) Labeling all oysters, "For shucking by a certified dealer," during the months of May through September, inclusive;

(5) Subjecting all oysters **intended for the raw, half-shell market** to a post-harvest treatment that is both approved by the Authority and reduces the *Vibrio vulnificus* levels to 3MPN/g or less during the months of May through September, inclusive;

(6) Closing shellfish growing areas **for the purpose of harvesting oysters intended for the raw, half-shell market** during the months of May through September, inclusive.

Modify the NSSP Guide for the Control of Molluscan Shellfish by adding the following Guidance Document (numbering to be determined at time of publication of the next revision.)

***Vibrio vulnificus* Management Guidance Document**

***Vibrio vulnificus* Management**

The voting delegates at the 1999 Annual Meeting in New Orleans created the Vibrio Management Committee (VMC). At the 2000 annual meeting the voting delegates will be asked to adopt the VMC's recommendation of reducing the rate of etiologically confirmed shellfish-borne *Vibrio vulnificus* septicemia. The goal is to reduce ~~those~~ **the rate of** illness reported in core states ~~from~~ **due to** the consumption of commercially harvested raw or undercooked oysters by 40 percent by the end of 2005 and by 60 percent by the end of 2007. The Core States are the states that have consistently reported *Vibrio vulnificus* cases since 1995. **The list of core states may be adjusted if after a thorough review, epidemiological and statistical data demonstrates that it would be appropriate.** The rate of illness shall be calculated as the number of illnesses adjusted for population and rate of reporting divided by the production of oysters from the states bordering the Gulf of Mexico, based on National Marine Fisheries Service landing data verified by Silver Spring, Maryland, headquarters. This adjustment will be performed in consultation with statisticians and epidemiologists from core states and federal agencies. The baseline data and all future data for measuring illness reduction shall be the reported illnesses in the core states for the period 1996 to 1999, inclusive, as compiled by the Southeast Regional Office of the U.S. Food and Drug Administration. The data used for measuring goal attainment shall begin with 2001 data. The formula for calculating the rate of illness is as follows:

$$\frac{\text{(number of cases) x (}\sout{\text{CDC}}\ \textbf{illness reporting}\ \text{adjustment factor)}}{\text{population}}$$

$$\frac{\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad}{\text{production}}$$

The VMC members will include, at a minimum, **balanced representation from** industry and state shellfish control **authorities** from *Vibrio vulnificus* Illness Source and Core States, FDA, NOAA, EPA, CDC, state epidemiologists; as well as industry and shellfish control representatives from other regions. *Vibrio vulnificus* Illness Source States are those states reporting 2 or more etiologically confirmed shellfish-borne *Vibrio vulnificus* illnesses since 1995 traced to the consumption of commercially harvested raw or undercooked oysters that originated from the waters of that state. Core states are Florida,

Texas, California, Louisiana, Georgia, South Carolina and Alabama **or those states determined to be appropriate after a thorough review of epidemiological and statistical data.** Etiologically confirmed means those cases in which laboratory evidence of a specific agent is obtained and specified criteria are met.

Recognizing the increasing importance and roles for the VMC, the Committee leadership will be expanded and structured in a similar manner as stated in the ISSC By-Laws for Task Forces (reference: ISSC By-Law, Article I Task Forces). The VMC Chair shall alternately be selected from a state shellfish control authority and from industry. The Board Chairman, with approval of the Board, shall appoint a VMC Chair and Vice-Chair. If the VMC Chair represents a state shellfish control authority, the Vice-Chair shall be an industry representative. At the end of the VMC Chair's term of office, the Vice Chair will become Chairman and a new Vice Chair will be appointed who represents the same segment of the Conference as the outgoing VMC Chair. A VMC Chair and Vice Chair should be appointed before October 1, 2000 in order to be consistent with plans for annual VMC meetings and with the effective date of *Vibrio vulnificus* Risk Management Plans. Likewise, the term of office should be for (2) years.

The VMC will meet at least annually to develop and approve work plans and review progress. The first plan will be in place for a one-year period, followed by three biennial plans. The first work plan and progress review period will be from January 2001 to December 31, 2001. The next work plan period will be from January 1, 2002 to December 31, 2003, January 1, 2004 to December 31, 2005**;** then January 1, 2006 to December 31, 2007.

Work plans will include goals, tasks, performance measures and assessment methods to track and achieve progress towards the illness reduction goals. The work plans will be developed by the VMC and approved by the VMC membership. The chair of the VMC will deliver **a written annual progress report, including a summary of the previous year's progress made in the education program, to the ISSC March executive board meeting. The report shall be made available to the general membership. The biennial work plan structure, outlined below,** provides adaptive management and assures consistent progress towards the illness reduction goals.

Work plans developed by the VMC shall include the following elements and shall define the administrative procedures and resources necessary for accomplishment (i.e. establishment and maintenance):

(a) An ISSC Consumer Education Program targeted toward individuals who consume raw oysters and whose health condition(s) increase their risk for *Vibrio vulnificus* infection. The Education Program's objectives will be 1) to increase the target audience's awareness that eating raw, untreated oysters can be life-threatening to them, and; 2) to change the at-risk group's oyster-eating behavior, i.e., to reduce or stop eating raw, untreated oysters. The ISSC Education Committee and the *Vibrio vulnificus* Education Subcommittee will assist in the development and oversight for this program.

(i) The Consumer Education Program will focus educational efforts in the Core States. The Education Program will make educational materials available to states upon request.

(ii) Educational approaches will emphasize partnerships with health and advocacy organizations, and include dissemination of printed materials, posting materials on the Internet, broadcast of television spots, press releases, and other measures deemed effective such as the USDA Physician Notification Program.

(iii) Periodic administration of Behavior Risk Factor State Surveys (BRFSS) and other survey assessments at the state level shall be explored as a means of assessing the effectiveness of educational interventions.

(b) Administration of a survey to determine the current *Vibrio vulnificus* disease reporting and education in each state.

(c) Creation of a shellfish-borne *Vibrio vulnificus* disease investigation team that will be available to assist in collection of epidemiological information associated with confirmed shellfish-borne *Vibrio vulnificus* septicemia illness. This team will assist in gathering customary epidemiological information as well as the level of awareness of risk in those who have suffered etiologically confirmed shellfish-borne *Vibrio vulnificus* septicemia illnesses. A small ISSC team with recognized epidemiological officers will assist in rapid investigation of any case. This team will work cooperatively with existing local, state and federal disease investigation programs.

(d) Industry-implemented post-harvest controls to reduce *Vibrio vulnificus* levels in oyster shellstock which may include: time-temperature, post harvest treatment (i.e. hydrostatic pressure, cool pasteurization, IQF, and irradiation--pending approval), rapid chilling and other emerging technologies.

(e) To encourage implementation of post harvest controls the Conference will pursue options such as ~~SBA low interest loans; revolving loans; cost sharing; demonstration projects; state industry partnerships;~~ **market development;** FDA label incentives; PHT specific growing area classifications; targeted time/temperature assessment by FDA during annual shellfish program evaluations; assistance, as necessary, for the further study and possible implementation of dockside icing to investigate its effects on shelf life and variations in the effectiveness of the method as a result of seasonal and regional differences and incentives to add refrigeration capacity to harvest vessels. The goal will be to provide incentives necessary to post-harvest treat 20 percent of all oysters intended for the raw, half-shell market during the months of May through September harvested from a source state by the end of the third year (December 31, 2003). The assessment will include the capacity of all operational plants and the capacity of plants under construction. Should the 20 percent goal not be accomplished, ~~the VMC will pursue additional incentives to achieve the goals.~~ **the VMC will investigate and report their findings as to why the goal was not reached.**

(f) The VMC will develop a list of issues relating to public health, various technologies; including Post-harvest treatments; marketability; shelf -life and similar matters that lend themselves to investigation. The VMC will work with FDA, NOAA, CDC, EPA, the shellfish industry and other entities as appropriate to obtain or facilitate the investigation of the issues listed and take the results into account as it develops plans or recommended Issues for the ISSC.

~~(f)~~**(g)** A VMC compilation and review of the data on rates of illness will be made available to the ISSC at the ISSC Biennial meeting following the year in which the data was gathered. In the event that the data is not available at the time of the meeting, the VMC shall meet and review the data when it becomes available and issue a compilation report, which will be made available to the entire ISSC membership. In the event there is no Biennial meeting scheduled for a certain year, the VMC shall meet and review the data when it becomes available and issue a compilation report which will be made available to the entire conference.

~~(g)~~**(h)** A VMC evaluation of the effectiveness of reduction efforts will be conducted at the end of the fifth year (December 31, 2005). The evaluation will determine whether the 40 percent, 5-year ~~illness reduction~~ goal to **reduce the rate of illness** or education/consumer intervention or post harvest controls performance measures set forth in prior work plans have been achieved. Should the VMC evaluation indicate the 40 percent, 5 year goal has not been accomplished, the committee will identify additional harvest controls in the 2006 - 2007 work plan to assure achievement of the 60 percent ~~illness~~ reduction **in the rate of illness** goal by the close of the seventh year. In addition, the VMC will evaluate the requirements in Section 04.C. with the possibility of changing the controls to achieve remaining illness reduction goals.

PUBLIC HEALTH SIGNIFICANCE: The purpose of the NSSP is to promote and improve the sanitation of shellfish (oysters, clams, mussels and scallops) moving in interstate commerce through federal/state cooperation and uniformity of State Shellfish Programs. This includes protection of the public health by reducing the prevalence of food borne hazards. Complete elimination of illness is difficult to attain but public health programs should be designed to provide the greatest level of public health protection possible. The vision of public health officials must focus on maximizing protection with the most practical public health measures available. This plan is designed to assure a significant reduction in *Vibrio vulnificus* septicemia illnesses through a combination of consumer education, processing incentives and, if necessary, mandatory harvesting or processing controls.

COST INFORMATION: Unknown.
The Task Force further recommended adoption of the 2000 Vibrio Management Committee recommendations # 1, 2, and 3.

Action by 2000 General Assembly The 2000 General Assembly referred Issue 00-201 to appropriate committee as determined by the Conference Chairman.

Action by USFDA

Concurred with Conference action.

Action by 2001 Vibrio vulnificus Subcommittee

Recommended adoption of Issue 00-201 as amended and presented in the 2001 Issue packet:

TEXT OF PROPOSAL:

Modify Model Ordinance Chapter II. By adding Section @. 04:

@. 04 *Vibrio vulnificus* Risk Management for Oysters.

(A) For states having 2 or more etiologically confirmed shellfish-borne *Vibrio vulnificus* illnesses since 1995 traced to the consumption of commercially harvested raw or undercooked oysters that originated from the waters of that state (Source State), the Authority shall develop and implement *a Vibrio vulnificus* risk management plan.

(B) The Source State's *Vibrio vulnificus* management plan shall define the administrative procedures and resources necessary to accomplish (i.e. establish and maintain) involvement by the state in a collective illness reduction program. The Plan shall include, at a minimum, the ISSC Consumer Education Program targeted toward individuals who consume raw oysters and whose health condition(s) increase their risk for *Vibrio vulnificus* illnesses. The goal of the *Vibrio vulnificus* Risk Management Plan will be to reduce the rate of etiologically confirmed shellfish-borne *Vibrio vulnificus* septicemia illnesses reported collectively by ~~core reporting states, collectively~~ California, Florida, Louisiana, Texas, from the consumption of commercially harvested raw or undercooked oysters by 40 percent, ~~collectively, by the end of~~ for years 2005 and 200~~5~~6 (average) and by 60 percent for years 2007 and ~~collectively, by the end of~~ 200~~7~~8 (average) ~~from the current rate of 0.306/million~~ from the average illness rate for the years 1995 - 1999 of 0.306/million~~. The core reporting states include Florida, Texas, California, and Louisiana.~~ The list of ~~core reporting~~ states (California, Florida, Louisiana, Texas) used to calculate rate reduction may be adjusted if after a thorough review, epidemiological and statistical data demonstrates that it would be appropriate. The illness rate shall be calculated as the number of illnesses per unit of population. The goal may be reevaluated prior to the year 200~~5~~6 and adjusted in the event that new science, data or information becomes available.

(C) The Source States' *Vibrio vulnificus* management plan shall also include identification and preparation for implementation of one or more of the following controls, or equivalent controls, which shall be implemented should the 60 percent rate of illness reduction goal not be achieved collectively by 200~~7~~8. The control measures identified in the plan shall be appropriate to the state and reflect that state's contribution to the number of Vv illnesses and the controls that have been implemented by each state. This portion of the plan shall be completed no later than December 200~~6~~7. The temperature and month-of the-year parameters identified in the following controls may be adjusted by the ISSC Executive Board as recommended by the Vibrio Management Committee (VMC) on a state by state basis, as needed to achieve the established illness reduction goal. The adjustment to the State's plan can take into account the illness rate reduction that has occurred since the last review of the plan.

(1) Labeling all oysters, "For shucking by a certified dealer," when the Average Monthly Maximum Water Temperature exceeds 75°F;
(2) Subjecting all oysters intended for the raw, half-shell market to an Authority-approved post-harvest treatment that reduces the *Vibrio vulnificus* levels to 3MPN/g or less," when the Average Monthly Maximum Water Temperature exceeds 75°F;
(3) Closing shellfish growing areas for the purpose of harvest of oysters intended for the raw, half-shell market when the Average Monthly Maximum Water Temperature exceeds 75°F;
(4) Labeling all oysters, "For shucking by a certified dealer," during the months of May through September, inclusive;
(5) Subjecting all oysters intended for the raw, half-shell market to a post-harvest treatment that is both approved by the Authority and reduces the *Vibrio vulnificus* levels to 3MPN/g or less during the months of May through September, inclusive;
(6) Closing shellfish growing areas for the purpose of harvesting oysters intended for the raw, half-shell market during the months of May through September, inclusive.

Modify the NSSP Guide for the Control of Molluscan Shellfish by adding the following Guidance Document (numbering to be determined at time of publication of the next revision.)

***Vibrio vulnificus* Management Guidance Document**

***Vibrio vulnificus* Management**

The voting delegates at the 1999 Annual Meeting in New Orleans created the Vibrio Management Committee (VMC). Subsequently, *Vibrio vulnificus* and *Vibrio parahaemolyticus* subcommittees have been charged to develop appropriate illness control measures for these two pathogens. The VMC provides guidance and oversight to the subcommittees. Subcommittee recommendations are reviewed by the VMC before submittal to Task Forces. At the 2001 annual meeting, Task Forces will review the VMC's recommendation of reducing the rate of etiologically confirmed shellfish-borne *Vibrio vulnificus* septicemia with the intention to submit the recommendation to the voting delegates. The goal is to reduce the rate of illness reported in ~~core reporting states~~ California, Florida, Louisiana and Texas due to the consumption of commercially harvested raw or undercooked oysters by 40 percent by the end of 200~~5~~6 and by 60 percent by the end of 200~~7~~8. ~~The Core Reporting States are Louisiana, California, Florida, and Texas. The list of core reporting.~~ The list of states may be adjusted if after a thorough review, epidemiological and statistical data demonstrates that it would be appropriate. The rate of illness shall be calculated as the number of illnesses adjusted for population. This adjustment will be performed in consultation with statisticians and epidemiologists from ~~core reporting states~~ California, Florida, Louisiana and Texas and Federal agencies. The baseline data and all future data for measuring illness reduction shall be the reported illnesses in the ~~core reporting states~~ California, Florida, Louisiana and Texas for the period 1995 to 1999, inclusive, as compiled by the Southeast Regional Office of the U.S. Food and Drug Administration. The data used for measuring goal attainment shall begin with 200~~1~~2 data. For the purpose of maintaining an accurate count of the number of illnesses report by each state (California, Florida, Louisiana and Texas) ~~Core Reporting State~~, the following will apply:

(a) Illness cases counted are those reported by ~~Core Reporting States~~

California, Florida, Louisiana and Texas;
(b) Each illness case is recorded under the state that reports it;
(c) Each case is not counted more than once; and
(d) In the event more than one report per case is filed, the case is recorded under the state of diagnosis.

The formula for calculating the rate of illness is as follows:

$$\frac{\text{(number of cases)}}{\text{population}}$$

The ~~VMC~~ Vv subcommittee members will include, at a minimum, balanced representation from industry and state shellfish control authorities from *Vibrio vulnificus* Illness Source States ~~and Core Reporting States~~ California, Florida, Louisiana and Texas, FDA, NOAA, EPA, CDC, state epidemiologists; as well as industry and shellfish control representatives from other regions. *Vibrio vulnificus* Illness Source States are those states reporting two (2) or more etiologically confirmed shellfish-borne *Vibrio vulnificus* illnesses since 1995 traced to the consumption of commercially harvested raw or undercooked oysters that originated from the waters of that state. ~~Core reporting states are Florida, Texas, California, and Louisiana, or those states determined to be appropriate after a thorough review of epidemiological and statistical data.~~ Etiologically confirmed means those cases in which laboratory evidence of a specific agent is obtained and specified criteria are met.

Recognizing the increasing importance and roles for the, the Committee leadership will be expanded and structured in a similar manner as stated in the ISSC By-Laws for Task Forces (reference: ISSC By-Law, Article I Task Forces). The VMC Chair shall alternately be selected from a state shellfish control authority and from industry. The Board Chairman, with approval of the Board, shall appoint a VMC Chair and Vice-Chair. If the VMC Chair represents a state shellfish control authority, the Vice-Chair shall be an industry representative. At the end of the VMC Chair's term of office, the Vice Chair will become Chairman and a new Vice Chair will be appointed who represents the same segment of the Conference as the outgoing VMC Chair. A VMC Chair and Vice Chair should be appointed before October 1, 200~~0~~1 in order to be consistent with plans for annual VMC meetings and with the effective date of *Vibrio vulnificus* Risk Management Plans. Likewise, the term of office ~~should~~ shall be for (2) years.

The VMC will meet at least annually to develop and approve annual VMC work plans for *Vibrio vulnificus* illness reduction and review progress. ~~The first plan will be in place for a one-year period, followed by three biennial plans.~~ A series of work plans, each covering a one-year period shall be adopted. The first work plan and progress review period will ~~be from January 2001 to December 31, 2001.~~ cover a seventeen-month period from August 1, 2001 to December 31, 2003 followed subsequently by annual work plans. ~~The next work plan period will be from January 1, 2002 to December 31, 2003, January 1, 2004 to December 31, 2005; then January 1, 2006 to December 31, 2007.~~

Work plans will include goals, tasks, performance measures and assessment methods to track and achieve progress towards the illness reduction goals. The work plans will be developed by the VMC and approved by the VMC membership. The chair of the VMC will deliver a written annual progress report, including a summary of the previous year's progress made in the education program, to the ISSC March executive board meeting. The report shall be made available to the general membership. The ~~biennial~~ annual work plan structure, outlined below, provides adaptive management and assures consistent progress

towards the illness reduction goals. If annual assessment of progress towards achieving the illness rate reduction goals show inadequate progress the VMC shall incorporate actions into current and subsequent work plans to assure success in achieving those goals. In addition, if annual review shows inadequate progress the VMC will develop issues for deliberation at the 2005 biennial meeting to consider actions such as:

- increased educational efforts,
- limited harvest restriction,
- reduction in time from harvest to refrigeration,
- phased-in post-harvest treatment requirements, or
- other equivalent controls.

Work plans developed by the VMC shall include the following elements and shall define the administrative procedures and resources necessary for accomplishment (i.e. establishment and maintenance):

(a) An ISSC Consumer Education Program targeted toward individuals who consume raw oysters and whose health condition(s) increase their risk for *Vibrio vulnificus* infection. The Education Program's objectives will be 1) to increase the target audience's awareness that eating raw, untreated oysters can be life-threatening to them, and; 2) to change the at-risk group's oyster-eating behavior, i.e., to reduce or stop eating raw, untreated oysters. The ISSC Vibrio Management Committee and the *Vibrio vulnificus* Education Subcommittee will ~~assist~~ evaluate Year 2001 survey results ~~will be~~ and compare~~d to~~ them with the Year 2003 or 2004 survey results to ~~demonstrate that determine~~ the effectiveness in meeting the two objectives of the *Vv* education effort: (1) Show 40% increase in awareness of risk from Vv; and (2) Show 15% increase in at-risk consumers no longer eating raw oysters while minimizing impacts to non-at-risk consumer raw oyster consumption. ~~in the development and oversight for this program~~.

(i) The Consumer Education Program will focus educational efforts ~~in the Core Reporting States~~ California, Florida, Louisiana and Texas. The Education Program will make educational materials available to additional states upon request.

(ii) Educational approaches will emphasize partnerships with health and advocacy organizations, and include dissemination of printed materials, posting materials on the Internet, broadcast of television spots, press releases, and other measures deemed effective such as the USDA Physician Notification Program.

(iii) Survey assessments at the state level shall be used as a means of assessing the baseline knowledge and effectiveness of educational interventions.

(b) Administration of a survey to determine the current *Vibrio vulnificus* disease reporting and education in each state;

(c) Creation of a ~~A committee~~ working group ~~will be created~~ to work cooperatively with local, state, and federal agencies and ~~program~~ programs to assist in the collection of environmental and epidemiological data to further expand on the current information available. A coordinator may be utilized to facilitate the activities of this ~~subcommittee~~ working group to develop standardized collection of environmental and epidemiological information from harvest to consumer.

(d) Industry-implemented post-harvest controls to reduce *Vibrio vulnificus* levels in oyster

shellstock which may include: time-temperature, post harvest treatment (i.e. hydrostatic pressure, cool pasteurization, IQF, and irradiation--pending approval), rapid chilling and other emerging technologies.

(e) Pursuit of ISSC options ~~To encourage implementation of post harvest controls the Conference will pursue options~~ such as industry education and communication; FDA label incentives; PHT specific growing area classifications; targeted time/temperature assessment by FDA during annual shellfish program evaluations; assistance, as necessary, for the further study and possible implementation of dockside icing to investigate its effects on shelf life and variations in the effectiveness of the method as a result of seasonal and regional differences and incentives to add refrigeration capacity to harvest vessels. The goal will be to provide incentives necessary to post-harvest treat 20 percent of all oysters intended for the raw, half-shell market during the months of May through September harvested from a ~~source state~~ Source State by the end of the third year (December 31, 200~~3~~4. The assessment will include the capacity of all operational plants and the capacity of plants under construction. Should the 20 percent goal not be accomplished, the VMC will investigate and report their findings as to why the goal was not reached.

(f) Development by the VMC of ~~The VMC will develop~~ a list of issues relating to public health, various technologies~~;~~ including Post-harvest treatments; marketability; shelf -life and similar matters that lend themselves to investigation. The VMC will work with FDA, NOAA, CDC, EPA, the shellfish industry and other entities as appropriate to obtain or facilitate the investigation of the issues listed and take the results into account as it develops plans or recommended Issues for the ISSC.

(g) Provision for a ~~A~~ VMC compilation and review of the data on rates of illness which will be made available to the ISSC at the ISSC Biennial meeting following the year in which the data was gathered. In the event that the data is not available at the time of the meeting, the VMC shall meet and review the data when it becomes available and issue a compilation report, which will be made available to the entire ISSC membership. In the event there is no Biennial meeting scheduled for a certain year, the VMC shall meet and review the data when it becomes available and issue a compilation report which will be made available to the entire ~~conference~~ membership.

(h) Provision for a ~~A~~ VMC evaluation of the effectiveness of reduction efforts which will be conducted at the end of the fifth year (December 31, 200~~5~~6). The evaluation will determine whether the 40 percent, 5-year goal to reduce the rate of illness or education/consumer intervention or post harvest controls performance measures set forth in prior work plans have been achieved. Should the VMC evaluation indicate the 40 percent, 5 year goal has not been accomplished, the committee will identify additional harvest controls in the 200~~6~~7 - 200~~7~~8 work plan to assure achievement of the 60 percent reduction in the rate of illness goal by the close of the seventh year. In addition, the VMC will evaluate the requirements in Section 04.C. with the possibility of changing the controls to achieve remaining illness reduction goals.

(i) Should a disagreement arise between FDA and the Authority on the equivalency of a control as described in .04c, the Vv Subcommittee will be requested to provide guidance.

PUBLIC HEALTH SIGNIFICANCE: The purpose of the National Shellfish Sanitation

Program is to promote and improve the sanitation of shellfish (oysters, clams, mussels and scallops) moving in interstate commerce through federal/state cooperation and uniformity of State Shellfish Programs. This includes protection of the public health by reducing the prevalence of food borne hazards. Complete elimination of illness is difficult to attain but public health programs should be designed to provide the greatest level of public health protection possible. The vision of public health officials must focus on maximizing protection with the most practical public health measures available. This plan is designed to assure a significant reduction in *Vibrio vulnificus* septicemia illnesses through a combination of consumer education, processing incentives and, if necessary, mandatory harvesting or processing controls.

COST INFORMATION: Unknown.

Action by 2001 Vibrio vulnificus Subcommittee

Recommended the following changes to Issue 00-201 at the July 22, 2001 subcommittee meeting:

TEXT OF PROPOSAL:

Modify Model Ordinance Chapter II. By adding Section @. 04:

@. 04 *Vibrio vulnificus* Risk Management for Oysters.

(A) For states having 2 or more etiologically confirmed shellfish-borne *Vibrio vulnificus* illnesses since 1995 traced to the consumption of commercially harvested raw or undercooked oysters that originated from the waters of that state (Source State), the Authority shall develop and implement *a Vibrio vulnificus* management plan.

(B) The Source State's *Vibrio vulnificus* management plan shall define the administrative procedures and resources necessary to accomplish (i.e. establish and maintain) involvement by the state in a collective illness reduction program. ~~The Plan shall include, at a minimum, the ISSC Consumer Education Program targeted toward individuals who consume raw oysters and whose health condition(s) increase their risk for *Vibrio vulnificus* illnesses~~. The goal of the *Vibrio* <u>*vulnificus*</u> Management Plan will be to reduce the rate of etiologically confirmed shellfish-borne *Vibrio vulnificus* septicemia illnesses reported collectively by <u>California, Florida, Louisiana, Texas</u>, from the consumption of commercially harvested raw or undercooked oysters by 40 percent, for years 2005 and 2006 (average) and by 60 percent for years 2007 and 2008 (average) from the average illness rate for the years 1995 - 1999 of 0.306/million. The list of states (California, Florida, Louisiana, Texas) used to calculate rate reduction may be adjusted if after a thorough review, epidemiological and statistical data demonstrates that it would be appropriate. The illness rate shall be calculated as the number of illnesses per unit of population. The goal may be reevaluated prior to the year 2006 and adjusted in the event that new science, data or information becomes available.

(C) The Source States' *Vibrio vulnificus* management plan **<u>shall include, at a minimum:</u>**

<u>(1) The ISSC Consumer Education Program targeted toward individuals who consume raw oysters and whose health condition(s) increase their risk for *Vibrio vulnificus* illnesses;</u>

<u>(2) A process to collected standardized information for each *Vibrio vulnificus* illness: including underlying medical conditions; knowledge of disease status; prior counseling on avoidance of high risk foods, including</u>

raw oysters; existence of consumer advisories at point of purchase or consumption; and, if possible, whether consumer was aware and understood the advisories;

(3) A standardized process for tracking products implicated in *Vibrio vulnificus* illnesses;

(4) Identification and preparation for achieving a goal of post-harvest treatment capacity of 25 percent of all oysters intended for the raw, half-shell market during the months of May through September harvested from a Source State by the end of the third year (December 31, 2004). The percentage of post harvest treatment will include the capacity of all operational plants and the capacity of plants under construction;

(5) Identification and preparation for implementation of required post harvest treatment capacity of 50% of all oysters intended for the raw, half-shell market during the months of May through September, harvested from a Source State, which shall be implemented should the 40 percent illness reduction goal not be achieved by December 31, 2006. The percentage of post harvest treatment will include the capacity of all operational plants and the capacity of plants under construction. In the alternative, the state may utilize the control measures, or equivalent control measures, listed in .04, (C), (6) (a), (b), (c), and (d) below for such periods of time which, in combination with post harvest treatment, will provide equivalent outcomes. This portion of the plan shall be completed no later than December 31, 2005; and

(6) Identification and preparation for implementation of one or more of the following controls, or equivalent controls, which shall be implemented should the 60 percent rate of illness reduction goal not be achieved collectively by 2008. The control measures identified in the plan shall be appropriate to the state and reflect that state's contribution to the number of Vv illnesses and the controls that have been implemented by each state. This portion of the plan shall be completed no later than December 2007. The temperature and month-of the-year parameters identified in the following controls may be adjusted by the ISSC Executive Board as recommended by the Vibrio Management Committee (VMC) on a state by state basis, as needed to achieve the established illness reduction goal. The adjustment to the State's plan can take into account the illness rate reduction that has occurred since the last review of the plan.

(a) Labeling all oysters, "For shucking by a certified dealer," when the Average Monthly Maximum Water Temperature exceeds 75°F;

(b) Subjecting all oysters intended for the raw, half-shell market to an Authority-approved post-harvest treatment that reduces the *Vibrio vulnificus* levels to 3MPN/g or less," when the Average Monthly Maximum Water Temperature exceeds 75°F;

(c) Closing shellfish growing areas for the purpose of harvest of oysters intended for the raw, half-shell market when the Average Monthly Maximum Water Temperature exceeds 75°F;

(d) Labeling all oysters, "For shucking by a certified dealer," during the months of May through September, inclusive;

(e) Subjecting all oysters intended for the raw, half-shell market to a post-harvest treatment that is both approved by the Authority and reduces the *Vibrio vulnificus* levels to 3MPN/g or less during the months of May through September, inclusive;

(f) Closing shellfish growing areas for the purpose of harvesting oysters

intended for the raw, half-shell market during the months of May through September, inclusive.

Modify the NSSP Guide for the Control of Molluscan Shellfish by adding the following Guidance Document (numbering to be determined at time of publication of the next revision.)

***Vibrio vulnificus* Management Guidance Document**

***Vibrio vulnificus* Management**

The voting delegates at the 1999 Annual Meeting in New Orleans created the Vibrio Management Committee (VMC). Subsequently, *Vibrio vulnificus* and *Vibrio parahaemolyticus* subcommittees have been charged to develop appropriate illness control measures for these two pathogens. The VMC provides guidance and oversight to the subcommittees. Subcommittee recommendations are reviewed by the VMC before submittal to Task Forces. At the 2001 annual meeting, Task Forces will review the VMC's recommendation of reducing the rate of etiologically confirmed shellfish-borne *Vibrio vulnificus* septicemia with the intention to submit the recommendation to the voting delegates. The goal is to reduce the rate of illness reported in California, Florida, Louisiana and Texas due to the consumption of commercially harvested raw or undercooked oysters ~~by 40 percent by the end of 2006 and by 60 percent by the end of 2008.~~ **by 40 percent, for years 2005 and 2006 (average) and by 60 percent for years 2007 and 2008 (average) from the average illness rate for the years 1995 - 1999 of 0.306/million.** The list of states may be adjusted if after a thorough review, epidemiological and statistical data demonstrates that it would be appropriate. The rate of illness shall be calculated as the number of illnesses adjusted for population. This adjustment will be performed in consultation with statisticians and epidemiologists from California, Florida, Louisiana and Texas and Federal agencies. The baseline data and all future data for measuring illness reduction shall be the reported illnesses in the California, Florida, Louisiana and Texas for the period 1995 to 1999, inclusive, as compiled by the Southeast Regional Office of the U.S. Food and Drug Administration. The data used for measuring goal attainment shall begin with 2002 data. For the purpose of maintaining an accurate count of the number of illnesses report by each state (California, Florida, Louisiana and Texas), the following will apply:

(a) Illness cases counted are those reported by California, Florida, Louisiana and Texas;
(b) Each illness case is recorded under the state that reports it;
(c) Each case is not counted more than once; and
(d) In the event more than one report per case is filed, the case is recorded under the state of diagnosis.

The formula for calculating the rate of illness is as follows:

$$\frac{\text{number of cases}}{\text{population}}$$

The V.v. subcommittee members will include, at a minimum, balanced representation from industry and state shellfish control authorities from *Vibrio vulnificus* Illness Source States California, Florida, Louisiana and Texas, FDA, NOAA, EPA, CDC, state epidemiologists; as well as industry and shellfish control representatives from other

regions. *Vibrio vulnificus* Illness Source States are those states reporting two (2) or more etiologically confirmed shellfish-borne *Vibrio vulnificus* illnesses since 1995 traced to the consumption of commercially harvested raw or undercooked oysters that originated from the waters of that state. Etiologically confirmed means those cases in which laboratory evidence of a specific agent is obtained and specified criteria are met.

Recognizing the increasing importance and roles for the, the Committee leadership will be expanded and structured in a similar manner as stated in the ISSC By-Laws for Task Forces (reference: ISSC By-Law, Article I Task Forces). The VMC Chair shall alternately be selected from a state shellfish control authority and from industry. The Board Chairman, with approval of the Board, shall appoint a VMC Chair and Vice-Chair. If the VMC Chair represents a state shellfish control authority, the Vice-Chair shall be an industry representative. At the end of the VMC Chair's term of office, the Vice Chair will become Chairman and a new Vice Chair will be appointed who represents the same segment of the Conference as the outgoing VMC Chair. A VMC Chair and Vice Chair should be appointed before October 1, 2001 in order to be consistent with plans for annual VMC meetings and with the effective date of *Vibrio vulnificus* Risk Management Plans. Likewise, the term of office shall be for (2) years.

The VMC will meet at least annually to develop and approve annual VMC work plans for *Vibrio vulnificus* illness reduction and review progress. A series of work plans, each covering a one-year period shall be adopted. The first work plan and progress review period will cover a seventeen-month period from August 1, 2001 to December 31, 2003 followed subsequently by annual work plans. Work plans will include goals, tasks, performance measures and assessment methods to track and achieve progress towards the illness reduction goals. The work plans will be developed by the VMC and approved by the VMC membership. The chair of the VMC will deliver a written annual progress report, including a summary of the previous year's progress made in the education program, to the ISSC March executive board meeting. The report shall be made available to the general membership. The annual_work plan structure, outlined below, provides adaptive management and assures consistent progress towards the illness reduction goals. If annual assessment of progress towards achieving the illness rate reduction goals show inadequate progress the VMC shall incorporate actions into current and subsequent work plans to assure success in achieving those goals. In addition, if annual review shows inadequate progress the VMC will develop issues for deliberation at the 2005 biennial meeting to consider actions such as:

- increased educational efforts,
- limited harvest restriction,
- reduction in time from harvest to refrigeration,
- phased-in post-harvest treatment requirements, or
- other equivalent controls.

Work plans developed by the VMC shall include the following elements and shall define the administrative procedures and resources necessary for accomplishment (i.e. establishment and maintenance):

(a) An ISSC Consumer Education Program targeted toward individuals who consume raw oysters and whose health condition(s) increase their risk for *Vibrio vulnificus* infection. The Education Program's objectives will be 1) to increase the target audience's awareness that eating raw, untreated oysters can be life-threatening to them, and; 2) to change the at-risk group's oyster-eating

behavior, i.e., to reduce or stop eating raw, untreated oysters. The ISSC Vibrio Management Committee and the *Vibrio vulnificus* Education Subcommittee will evaluate Year 2001 survey results and compare them with the Year 2003 or 2004 survey results determine the effectiveness in meeting the two objectives of the *Vv* education effort: (1) Show 40% increase in awareness of risk from Vv; and (2) Show 15% increase in at-risk consumers no longer eating raw oysters while minimizing impacts to non-at-risk consumer raw oyster consumption.

(i) The Consumer Education Program will focus educational efforts **in** California, Florida, Louisiana and Texas. The Education Program will make educational materials available to additional states upon request.

(ii) Educational approaches will emphasize partnerships with health and advocacy organizations, and include dissemination of printed materials, posting materials on the Internet, broadcast of television spots, press releases, and other measures deemed effective such as the USDA Physician Notification Program.

(iii) Survey assessments at the state level shall be used as a means of assessing the baseline knowledge and effectiveness of educational interventions.

(b) Administration of a survey to determine the current *Vibrio vulnificus* disease reporting and education in each state.

(c) Creation of a working group to work cooperatively with local, state, and federal agencies and programs to assist in the collection of environmental and epidemiological data to further expand on the current information available. A coordinator may be utilized to facilitate the activities of this working group to develop standardized collection of environmental and epidemiological information from harvest to consumer.

(d) Industry-implemented post-harvest controls to reduce *Vibrio vulnificus* levels in oyster shellstock which may include: time-temperature, post harvest treatment (i.e. hydrostatic pressure, cool pasteurization, IQF, and irradiation--pending approval), rapid chilling and other emerging technologies.

(e) Pursuit of ISSC options such as industry education and communication; FDA label incentives; PHT specific growing area classifications; targeted time/temperature assessment by FDA during annual shellfish program evaluations; assistance, as necessary, for the further study and possible implementation of dockside icing to investigate its effects on shelf life and variations in the effectiveness of the method as a result of seasonal and regional differences and incentives to add refrigeration capacity to harvest vessels. The goal will be to provide incentives necessary to post-harvest treat ~~20~~ **25** percent of all oysters intended for the raw, half-shell market during the months of May through September harvested from a Source State by the end of the third year (December 31, 2004). The assessment will include the capacity of all operational plants and the capacity of plants under construction. Should the ~~20~~ **25** percent goal not be accomplished, the VMC will investigate and report their findings as to why the goal was not reached.

(f) Development by the VMC of a list of issues relating to public health, various technologies~~,~~ including Post-harvest treatments; marketability; shelf -life and similar matters that lend themselves to investigation. The VMC will work with FDA, NOAA, CDC, EPA, the shellfish industry and other entities as appropriate to obtain or facilitate the investigation of the issues listed and take the results into account as it develops plans or recommended Issues for the ISSC.

(g) Provision for a VMC compilation and review of the data on rates of illness, which will be made available to the ISSC at the ISSC Biennial meeting following the year in which the data was gathered. In the event that the data is not available at the time of the meeting, the VMC shall meet and review the data when it becomes available and issue a compilation report, which will be made available to the entire ISSC membership. In the event there is no Biennial meeting scheduled for a certain year, the VMC shall meet and review the data when it becomes available and issue a compilation report which will be made available to the entire membership.

Provision for a VMC evaluation of the effectiveness of reduction efforts, which will be conducted at the end of the fifth year (December 31, 2006). The evaluation will determine whether the 40 percent, 5-year goal to reduce the rate of illness or education/consumer intervention or post harvest controls performance measures set forth in prior work plans have been achieved. Should the VMC evaluation indicate the 40 percent, 5 year goal has not been accomplished, the committee will identify additional harvest controls in the 2007 - 2008 work plan to assure achievement of the 60 percent reduction in the rate of illness goal by the close of the seventh year. In addition, the VMC will evaluate the requirements in Section 04.C. with the possibility of changing the controls to achieve remaining illness reduction goals.

Should a disagreement arise between FDA and the Authority on the equivalency of a control as described in .04~~c~~**(C)**, the V.v. Subcommittee will be requested to provide guidance.

The *Vibrio vulnificus* Subcommittee further recommended the following:

1) **Request the Executive Board request FDA to meet with the Irradiation petition submitter to establish a timetable under which FDA will review the petition.**

2) **Request the Executive Board request FDA and the state of California seek additional funding to increase the education of at-risk consumers in California, particularly in southern California,**

3) **Recommended that the Chairman appoint a committee to develop further guidance language for implementation of .04 (C) (1)-(5).**

4) **Recommended adoption of an effective date of October 1, 2001, and further recommended an expedited review by FDA.**

Action by 2001 Vibrio Management Committee

Recommended adoption of the *V. vulnificus* Subcommittee Report recommendations.

Action by 2001 Task Force II

Recommended adoption of 2001 Vibrio Management Committee Report recommendations.

The Task Force further recommended the Executive Board Chairman appoint an appropriate committee which shall develop a threshold for adoption of *Vibrio vulnificus* management plans (.04)(A), and for development of an exit strategy for source states.

Action by 2001 General Assembly

Adopted recommendation of 2001 Task Force II.

Action by USFDA

Concurred with Conference action.

This issue was referred back to the ISSC V*ibrio vulnificus* Subcommittee following its marginal defeat at the 2000 ISSC. While FDA was disappointed that the 2000 Conference voted to refer Issue 00-201 back to committee, we believe the dedicated efforts of the *Vibrio vulnificus* Subcommittee over the ensuing year resulted in ISSC adoption of a stronger and more workable plan to reduce *Vibrio vulnificus* illnesses associated with raw shellfish consumption. Issue 00-201 was designed to reduce *Vibrio vulnificus* septicemia illnesses through post harvest treatment (PHT) processing, consumer education, and, if necessary, mandatory harvesting and/or processing controls. FDA looks forward to working with states as they develop and implement *Vibrio vulnificus* management plans. We also look forward to our continued participation on the ISSC *Vibrio* Management Committee (VMC*), Vibrio vulnificus* Subcommittee, and *Vibrio vulnificus* Education Subcommittee to implement measures (including data collection, data analysis, and development of annual work plans by the VMC) set forth in the "*Vibrio vulnificus* Management Guidance Document" which was adopted as part of Issue 00-201.

During review of Issue 00-201, FDA noted that adopted in the third sentence of Chapter II. @. 04(C)(5) did not include alternatives (e) and (f) of 04(C)(6) should the 40% illness reduction goal not be achieved. It is our understanding that alternatives (e) and (f), which appear to have been inadvertently omitted, will be considered at the January meeting of the ISSC Executive Board for inclusion as alternatives in 04(C)(5).

Action by 2003 Vibrio vulnificus Subcommittee

Recommended that the baseline illness reduction rate of 1995 – 99 of 0.306 per million be modified in Chapter II @ 04 B to 0.303 per million to reflect the elimination of 1 case from the database.

Action by 2003 Vibrio Management Committee

Recommended adoption of Vv Subcommittee recommendation on Proposal 00-201.

Action By 2003 Task Force II

Recommended adoption of Vibrio Management Committee recommendation on Proposal 00-201.

Action By 2003 General Assembly

Adopted recommendations of 2003 Task Force II.

Action By USFDA	Concurred with Conference Action.
Action by 2005 *Vv* Subcommittee	Recommended the Vibrio Management Committee communicate to the Executive Board that the Conference has made significant progress toward achieving the 40% illness reduction goal as reflected in the 2004 rates compared to the baseline in the core states. Additionally, FDA has found all states required to implement Vv Management Plan are in compliance with the Model Ordinance. It should be noted that this is not an indication for a reduction in current efforts.
Action by 2005 Vibrio Management Committee	Recommended adoption of the Vv Subcommittee recommendation on Proposal 00-201. Additionally, the VMC adopted the following motion: In the three (3) Gulf Core States the illness rate reduction was 32% from their baseline. In all four Core States the reduction was 47%. Likely factors that contributed to the illness reduction include increased voluntary post harvest processing, education of at-risk individuals and California's action to ban non-post harvest processed oysters. It is recommended that the Conference continue to pursue additional methods to measure success or failure of the Risk Management Plan in both the Core States and nationally.
Action by 2005 Task Force II	Recommended adoption of the Vibrio Management Committee recommendations on Proposal 00-201.
Action by 2005 General Assembly	Adopted recommendation of 2005 Task Force II.
Action by USFDA	With reservation, FDA concurs with action taken on Proposal 00-201. Although FDA recognizes that a 47% reduction in *Vibrio vulnificus (Vv)* illnesses has been achieved in the Core reporting states, the Agency believes that this reduction is primarily the result of California's ban on non-post harvest processed Gulf oysters. At the 2005 Conference FDA proposed that California be removed from the list of Core states and that one or more additional states with consistent *Vv* illness reporting records be substituted. The *Vv* Subcommittee did not concur with FDA's recommendation and retained California as a Core state for measuring the success of the *Vv* Action Plan. FDA maintains the position that California should be removed as a Core reporting state and that illness reduction rates that include California provide a false indication of success relative to the *Vv* Action Plan illness reduction goals. FDA requests that the ISSC Executive Board direct the Vibrio Management Committee (VMC), during its March meeting, to reconsider the decision of the *Vv* Subcommittee to retain California as a Core reporting state.
Action by 2007 Vibrio Mgmt Committee	Recommended that the Vibrio Management Committee continue to monitor the activities of Proposal 00-201.
Action by 2007 Task Force II	Recommended adoption of the Vibrio Management Committee recommendation on Proposal 00-201.
Action by 2007 General Assembly	Adopted recommendation of 2007 Task Force II.

Action by USFDA

December 20, 2007

Concurred with Conference action with the following comments and recommendations for ISSC consideration.

At the 2007 Biennial Meeting, Dr. Alvin Rainosek advised the Conference that current efforts under the *Vibrio vulnificus* Management Plan are not likely to achieve the ISSC's 60% illness reduction goal by the end of 2008. FDA strongly encourages source states and the shellfish industry to begin preparing for the implementation of controls outlined in NSSP Model Ordinance Chapter II @ .04 and intended to ensure a 60% illness reduction in years subsequent to 2008. FDA anticipates that source states will be prepared to implement these controls at the conclusion of 2008 should the 60% reduction goal not be met. FDA also anticipates that implementation of those controls, should they be needed, will achieve a 60% illness reduction by the end of 2009 as determined by the average number of illnesses for the years 2008 and 2009 combined.

Action by VMC October 2009

1. a. Recommended that FDA submit a proposal for deliberation by a Special ISSC conference to be held in 2010.

   b. In the interim, it is requested that FDA, in coordination with ISSC fund a robust economic impact and consumer acceptance analysis to inform the ISSC deliberations on the proposal. An impacts analysis guidance committee will be appointed to guide and make recommendations on the components of the impacts analysis study.

2. Recommended that a workgroup be established to develop criteria for an economic analysis. The workgroup will use the criteria for an economic impact analysis for rulemaking as a guide. The study should include a taste acceptance component. The workgroup should include, but not be limited to, at least one industry member and one regulatory member from the east, west and gulf coasts.

3. Recommended that May 1, 2011, be set as date for implementation of Model Ordinance Section II @ .04, *Vibrio* Management Plan for Oysters.

4. Recommended that the *Vibrio* Management Committee meet at the Spring 2010 meeting of the Executive Board.

5. Recommended that the findings of the *Vibrio* vulnificus Illness Review Subcommittee be accepted. The Subcommittee found that 17 cases in 2007 met the criteria and 13 cases in 2008 met the criteria. After adjusting for population changes, the illness rate reduction was calculated to be 35.2% from the baseline period.

Action by 2009 Task Force II

Recommended adoption of *Vibrio* Management Committee Recommendation No. 1.a. and b. on Proposal 00-201.

Recommended adoption of *Vibrio* Management Committee Recommendation No. 2. on Proposal 00-201 with instruction to add a consumer representative to the work group.

Recommended adoption of *Vibrio* Management Committee Recommendation No. 3 on Proposal 00-201.

Action by 2009 Task Force II (continued)

Recommended adoption of *Vibrio* Management Committee Recommendation No. 4 on Proposal 00-201.

Recommended adoption of *Vibrio* Management Committee Recommendation No. 5 on Proposal 00-201.

Action by 2009 General Assembly

Voted no action on Proposal 00-201 Recommendation 1.a.

Adopted recommendation of 2009 Task Force II on Proposal 00-201 Recommendation 1.b.

Adopted recommendation of 2009 Task Force II on Proposal 00-201 Recommendation 2.

Voted no action on Proposal 00-201 Recommendation 3. The previous implementation date of May 1, 2010 remains in effect.

Adopted recommendation of 2009 Task Force II on Proposal 00-201 Recommendation 4.

Adopted recommendation of 2009 Task Force II on Proposal 00-201 Recommendation 5.

Adopted a motion that the Vibrio Management Committee, at its fall 2010 meeting, evaluate the effects of the Vibrio Management Plans implemented May 1, 2010, and make recommendations to the Executive Board.

Adopted a motion that the Executive Board write a letter to FDA stating that the unilateral actions taken to regulate *Vv* under the Seafood HACCP Regulations are not consistent with the MOU between the ISSC and FDA.

Proposal Subject: Identification of Wet Stored Shellstock

Specific NSSP Guide Reference: NSSP Guide Section II Model Ordinance
Chapter X. General Requirements for Dealers
@ .05 Shellstock Identification B. Tags (2)

Text of Proposal/ Requested Action

.05 B. (2) The dealers tag…
- (a) The dealer's name…
- (b) The dealer's certification…
- (c) The original shellstock …
- (d) The date of harvest…
- (e) If depurated …
- (f) The most precise…
- (g) **When the shellstock has been transported from the original area and wet stored in another approved growing area within the same state for at least two weeks, the dealer will:**
  - **(i) use the date shellstock was harvested from the last growing area as the harvest date;**
  - **(ii) identify the last growing area as the harvest location.**
- ~~(g)~~ **(h)** When the shellstock has been transported across state lines…
- ~~(h)~~ **(i)** The type and quantity …
- ~~(i)~~ **(j)** The following statement…
- ~~(j)~~ **(k)** All shellstock intended…

Public Health Significance: There is no guidance in the Model Ordinance on tagging shellstock that is moved from one growing area to another within the same state. After 2 weeks in a growing area, the shellstock would have the characteristics of the new growing area and the product should be tagged appropriately. This will facilitate product recall and trace backs in the event of human illnesses.

Cost Information (if available): None

Action by 2003 Task Force II Recommended referral of Proposal 03-204 to the appropriate committee as determined by the Conference Chairman.

Action by 2003 General Assembly Adopted recommendation of 2003 Task Force II.

Action by USFDA Concurred with Conference Action.

Action by 2005 Post Harvest Processing Committee Recommended adoption of Proposal 03-204 with the following change to (g):

(i) use the date shellstock was harvested from the ~~last~~ most recent growing area as the harvest date;
(ii) identify the ~~last~~ most recent growing area as the harvest location.

Action by 2005 Task Force II Recommended referral of Proposal 03-205 to appropriate committee as determined by the Conference Chairman.

Action by 2005 General Assembly	Adopted recommendation of 2005 Task Force II.
Action by USFDA	Concurred with Conference action.
Action by 2007 Traceability/PHP Committees	Recommended no action on Proposal 03-204. Rationale – No scientific information has been provided to support the suggestion that shellstock harvested and wet stored for a specified period of time in a site other than the original harvest site takes on the characteristics of the wet storage area.
Action by 2007 Task Force II	Recommended referral of Proposal 03-204 back to the Post Harvest Processing Committee with direction to address confusion over whether activity is wet storage, relay, or transplanting under aquaculture and to secure whatever science is available relative to length of time in growing area to take on new characteristics of that growing area.
Action by 2007 General Assembly	Adopted recommendation of 2007 Task Force II.
Action by USFDA	December 20, 2007 Concurred with Conference action.
Action by 2009 Post Harvesting Committee	Recommended no action on Proposal 03-204. Rationale: Two weeks is insufficient for shellfish to take on characteristics of new growing area. In addition, in 2007 changes were made to labeling of wet stored shellstock that addressed this and adoption of this proposal would be redundant. Further recommended that FDA investigate the similarities and differences between US and other countries' systems for labeling wet stored shellfish and request FDA evaluate whether the differences between the systems place US firms at a competitive disadvantage. Ask that FDA report back to the conference and include time lines for changes to approach.
Action by 2009 Task Force II	Recommended adoption of Post Harvest Processing Committee recommendation on Proposal 03-204.
Action by 2009 General Assembly	Adopted recommendation of 2009 Task Force II on Proposal 03-204.

Proposal Subject: Requirements to Conduct Product Recall

Specific NSSP Guide Reference: NSSP Guide Section II. Model Ordinance
Chapter II. Risk Assessment and Risk Management
@.02 Presence of Human Pathogens in Shellfish Meats

Text of Proposal/ Requested Action

B. Growing Area Investigation
- (1) The Authority shall…
- (2) The Authority shall …
- (3) When the Authority determines that the growing area is not properly classified **or that the growing area may be the source of the pathogens** the Authority shall take immediate action to:
  - (a) Change the existing classification to the correct classifications; or
  - (b) Close the growing area until the correct classification can be determined~~.~~**; and**
  - **(c) Promptly initiate recall procedures consistent with the Recall Enforcement Policy Title 21 of Code of Federal Regulations Part 7.**
- **(4) When the Authority determines that illegal harvesting is taking place, the Authority shall promptly initiate recall procedures consistent with the Recall Enforcement Policy Title 21 Code of Federal Regulations Part 7 for all shellfish that may be falsely represented.**

C. Distribution and Processing
- (1) The Authority shall …
- (2) The Authority shall …
- (3) When the Authority determines that a problem exists in the distribution or processing of the shellfish, the Authority shall take immediate steps to correct the problem **and promptly initiate recall procedures consistent with the Recall Enforcement Policy Title 21 of Code of Federal Regulation Part 7**.

Public Health Significance: The Model Ordinance is not clear regarding the disposition of shellfish that have been harvested and then found positive for the presence of human pathogens. Failure to initiate recall procedures when human pathogens are known to be present in shellfish meats is inconsistent with the conservative public health approach of the NSSP and jeopardizes consumer health. Furthermore, while the Model Ordinance addresses a finding of no illegal harvesting (@.02 B. (2)), it is silent regarding what happens when illegal harvesting is determined. Adoption of the proposed language clarifies what action is to be taken when human pathogens are found present in shellfish meats.

Cost Information (if available): N/A

Action by 2007 Task Force II Recommended referral of Proposal 07-200 to an appropriate committee as determined by the Conference Chairman.

Action by 2007 General Assembly Adopted recommendation of 2007 Task Force II.

Action by USFDA December 20, 2007
Concurred with Conference action.

Action by 2009 Product Recall Committee

Recommended adoption of Proposal 07-200 as amended by the Committee.

B. Growing Area Investigation

(1) The Authority shall…

(2) The Authority shall …

(3) When the Authority determines that the growing area is not properly classified **~~or that the growing area may be the source of the pathogens~~** the Authority shall take immediate action to:

(a) Change the existing classification to the correct classifications; or

(b) Close the growing area until the correct classification can be determined; and

(c) Promptly initiate recall procedures consistent with the Recall Enforcement Policy Title 21 of Code of Federal Regulations Part 7.

(4) When the Authority determines that the growing area may be the source of pathogens the Authority shall promptly initiate recall procedures consistent with the Recall Enforcement Policy title 21 of Code of Federal Regulations Part 7 if the pathogens exceed tolerance levels.

~~(4~~(5) When the Authority determines that illegal harvesting is taking place, the Authority shall promptly initiate recall procedures consistent with the Recall Enforcement Policy Title 21 Code of Federal Regulations Part 7 for all shellfish that may be falsely represented.

C. Distribution and Processing

(1) The Authority shall …

(2) The Authority shall …

(3) When the Authority determines that a problem exists in the distribution or processing of the shellfish, the Authority shall take immediate steps to correct the problem and promptly initiate recall procedures consistent with the Recall Enforcement Policy Title 21 of Code of Federal Regulation Part 7.

Action by 2009 Task Force II

Recommended adoption of Product Recall Committee recommendation on Proposal 07-200.

Action by 2009 General Assembly

Adopted recommendation of 2009 Task Force II on Proposal 07-200.

Proposal Subject: Incorporating In-shell Product Concept into Model Ordinance Definitions

Specific NSSP Guide Reference: NSSP Guide Section II. Model Ordinance
B. Definitions of Terms

Text of Proposal/ Requested Action Change current definitions and add new definitions as listed below and renumber Section II. Model Ordinance B. Definitions of Terms section appropriately.

(16) Commingle or Commingling means the act of combining different lots of ~~shellstock or shucked~~ shellfish.

(90) Reshipper (RS) means a person who purchases ~~shucked~~ shellfish ~~or shellstock~~ from dealers and sells the product without repacking or relabeling to other dealers, wholesalers, or retailers.

(102) Shellstock Shipper (SS) means a dealer who grows, harvests, buys, or repacks and sells shellstock. They are not authorized to shuck shellfish nor to repack shucked shellfish. A shellstock shipper may also **buy, repack, and sell in-shell product as well as** ship shucked shellfish.

NEW **In-shell product packing means the process of placing in-shell product into containers for introduction into commerce.**

NEW **Lot of in-shell product means a single type of container of in-shell product of no more than one day's harvest from a single defined growing area.**

NEW **Repacking in-shell product means the practice of removing in-shell product from containers and placing it into other containers.**

Public Health Significance: This proposal is one of several that are part of an effort to incorporate the concept of in-shell product throughout the Model Ordinance.

Cost Information (if available): No cost.

Action by 2009 Task Force II Recommended adoption of Proposal 09-200 as submitted.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force II on Proposal 09-200.

Proposal Subject:	Post Harvest Handling Definition
Specific NSSP Guide Reference:	NSSP Guide Section II. Model Ordinance B. Definitions of Terms
Text of Proposal/ Requested Action	Add a new definition for Post Harvest Handling as follows and renumber Definitions Section appropriately. **Post Harvest Handling means any handling technique which has been established by a certified dealer and/or licensed harvester using the Hazard Analysis Critical Control Point guidelines that have been proven to result in a low historical risk of incidence of illnesses to consumers from naturally occurring bacteria as determined by the SSCA.**
Public Health Significance:	The use of Post-Harvest Handling techniques by certified dealers and licensed harvesters are proven to provide consumers of raw molluscan shellfish with a low incidence of illnesses caused by naturally occurring bacteria using HACCP controls
Cost Information (if available):	Less than the cost of closing oyster harvest areas, requiring oysters be shucked when shucking oysters is not profitable or requiring post-harvest processing of oysters.
Action by 2009 Task Force II	Recommended referral of Proposal 09-201 to an appropriate committee as determined by the Conference Chairman.
Action by 2009 General Assembly	Adopted recommendation of 2009 Task Force II on Proposal 09-201.

Proposal Subject: Prohibit Commingling of In-shell Product.

Specific NSSP Guide Reference: NSSP Guide Section II Model Ordinance
Chapter I. Shellfish Sanitation Program
@.01 Administration

Text of Proposal/ Requested Action

G. Commingling.

(1) Except for any shellstock included in the Authority's commingling plan, the Authority shall not permit the commingling of shellstock.

(2) If the Authority permits shellstock commingling, the Authority shall develop a commingling management plan. The plan shall:

(a) Minimize the commingling dates of harvest and growing areas;
(b) Define a primary dealer;
(c) Limit the practice of commingling to primary dealers;
(d) Limit commingling to shellstock harvested from specific growing areas within the State as identified by the Authority and purchased directly from harvesters; and
(e) Define how the commingled shellstock will be identified.

(3). The Authority shall not permit the commingling of in-shell product.

Public Health Significance: There is currently no restriction on commingling of in-shell product in the Model Ordinance. The proposed change provides such a restriction.

Cost Information (if available): None.

Action by 2009 Task Force II Recommended adoption of Proposal 09-202 as amended.

G. Commingling.

(1) Except for any ~~shellstock~~ shellfish included in the Authority's commingling plan, the Authority shall not permit the commingling of ~~shellstock~~ shellfish.

(2) If the Authority permits ~~shellstock~~ shellfish product commingling, the Authority shall develop a commingling management plan. The plan shall:

(a) Minimize the commingling dates of harvest and growing areas;
(b) Define a primary dealer;
(c) Limit the practice of commingling to primary dealers;
(d) Limit commingling to ~~shellstock~~ shellfish harvested from specific growing areas within the State as identified by the Authority and purchased directly from harvesters; and
(e) Define how the commingled ~~shellstock~~ shellfish will be identified.

~~(3). The Authority shall not permit the commingling of in-shell product.~~

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force II on Proposal 09-202.

Proposal Subject: Continuing Education Requirement for Certified Shellfish Dealers

Specific NSSP Guide Reference: NSSP Guide Section II. Model Ordinance
Chapter I. Shellfish Sanitation Program
@.02 Dealer Certification A. General

Text of Proposal/ Requested Action

(2) Certification shall be given only to persons who meet the established requirements established for certification.

a. All persons prior to applying for plant certification shall complete 3 hours annually of continuing education hours to maintain certification by the Authority and listing the ICSSL. Continuing Education hours could include attendance at ISSC meetings attendance at regional shellfish sanitation conferences, attendance at regional shellfish association meetings, or any other conference or meeting approved by the Authority.

Public Health Significance: This requirement will better inform certified dealers of new guidelines set forth in the NSSP.

Cost Information (if available): The cost would include registration fee and certification certificate for dealer to attend continuing education course.

Action by 2009 Task Force II: Recommended referral of Proposal 09-203 to an appropriate committee as determined by the Conference Chairman.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force II on Proposal 09-203.

Proposal Subject: Addition to the Requirements for the Authority During a Suspected Oyster Related Outbreak of ***Norovirus***

Specific NSSP Guide Reference: NSSP Guide Section II. Model Ordinance
Chapter II. Risk Assessment and Risk Management
@ .01Outbreaks of Shellfish-Related Illness

Text of Proposal/ Requested Action

A. When shellfish are implicated in an illness outbreak involving two (2) or more persons not from the same household (or one or more persons in the case of paralytic shellfish poisoning [PSP]**, and in the case of *Norovirus* being reported from more than one retail outlet or location of consumption)**, the Authority shall determine whether an epidemiological association exists between the illness and the shellfish consumption by reviewing:

(1) Each consumer's food history;
(2) Shellfish handling practices by the consumer and/or retailer;
(3) Whether the disease has the potential or is known to be transmitted by shellfish; and
(4) Whether the symptoms and incubation period of the illnesses are consistent with the suspected etiologic agent.

Public Health Significance:

The basis for this addition is to allow the authority time to determine if the suspected oyster-related *Norovirus* outbreak is due to growing area problems or problems associated with the location where the oysters were served. Due to the nature of *Norovirus,* it would be expected that if the suspected outbreak were growing area related, illnesses would be seen at more than one location. With the known prevalence of *Norovirus* throughout society and the ease with which it can be spread by human to human and human to food contact, it is difficult to determine the actual cause within 24 hours when faced with illness reported from a single location.

The Centers for Disease Control and Prevention (CDC) estimates that *Norovirus* causes 23 million cases of acute gastroenteritis annually, making *Norovirus* the leading cause of gastroenteritis in the United States (CDC, 2006; Fankhauser, et al., 2002, Mead, et al., 1999).

Of viruses, only the common cold is reported more often than viral gastroenteritis (*Norovirus)* (Benson & Merano, 1998).

According to the CDC:

Food and drinks can very easily become contaminated with *Norovirus* because the virus is so small and because it probably takes fewer than 100 *Norovirus* particles to make a person sick. Food can be contaminated either by direct contact with contaminated hands or work surfaces that are contaminated with stool or vomit, or by tiny droplets from nearby vomit that can travel through air to land on food. Although the virus cannot multiply outside of human bodies, once on food or in water, it can cause illness.

People working with food who are sick with *Norovirus* gastroenteritis are a particular risk to others, because they handle the food and drink many other people will consume. Since the virus is so small, a sick food handler can easily – without meaning to – contaminate the food he or she is handling. Many of those eating the contaminated food may become ill, causing an outbreak.

Outbreaks of *Norovirus* gastroenteritis have taken place in restaurants, cruise ships, nursing homes, hospitals, schools, banquet halls, summer camps, and family dinners – in other words, places where often people have consumed water and/or food prepared or handled by others. It is estimated that as many as half of all food-related outbreaks of illness may be caused by *Norovirus*. In many of these cases, sick food handlers were thought to be implicated.

Cost Information (if available): Not Available.

Action by 2009 Task Force II: Recommended no action on Proposal 09-204.

Rationale: Inconsistent with public health requirements of the Program.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force II on Proposal 09-204.

Proposal Subject: Addition to the Requirements for the Authority During a Suspected Oyster Related Outbreak of *Norovirus*

Specific NSSP Guide Reference: NSSP Guide Section II. Model Ordinance
Chapter II Risk Assessment and Risk Management
@.01 Outbreaks of Shellfish-Related Illness

Text of Proposal/ Requested Action

A. When shellfish are implicated in an illness outbreak involving two (2) or more persons not from the same household (or one or more persons in the case of paralytic shellfish poisoning [PSP]) the Authority shall determine whether an epidemiological association exists between the illness and the shellfish consumption by reviewing:

(1) Each consumer's food history;
(2) Shellfish handling practices by the consumer and/or retailer;
(3) Whether the disease has the potential or is known to be transmitted by shellfish; and
(4) Whether the symptoms and incubation period of the illnesses are consistent with the suspected etiologic agent.

NOTE: For additional guidance refer to the International Association of Milk, Food, and Environmental Sanitarians' *Procedures to Investigate Food Borne Illness*.

B. When the Authority has determined an epidemiological association between an illness outbreak and shellfish consumption, the Authority shall conduct an investigation of the illness outbreak within 24 hours to determine whether the illness is growing area related or is the result of post-harvest contamination or mishandling. **In the case of a suspected N*orovirus* outbreak, the investigation shall begin when an epidemiological association between illnesses and the consumption of shellfish is confirmed through sampling; or, if an epidemiological association is determined by linking illnesses from more than one location with the consumption of shellfish.**

Public Health Significance: The basis for this addition is to allow the authority time to determine if the suspected oyster-related *Norovirus* outbreak is due to growing area problems or problems associated with the location where the oysters were served. Due to the nature of *Norovirus*, it would be expected that if the suspected outbreak were growing area related, illnesses would be seen at more than one location. With the known prevalence of *Norovirus* throughout society and the ease with which it can be spread by human to human and human to food contact, it is difficult to determine the actual cause within 24 hours when faced with illness reported from a single location.

The Centers for Disease Control and Prevention (CDC) estimates that *Norovirus* causes 23 million cases of acute gastroenteritis annually, making *Norovirus* the leading cause of gastroenteritis in the United States (CDC, 2006; Fankhauser, et al., 2002, Mead, et al., 1999).

Of viruses, only the common cold is reported more often than viral gastroenteritis (***Norovirus***) (Benson & Merano, 1998).

According to the CDC:

Food and drinks can very easily become contaminated with *Norovirus* because the virus is so small and because it probably takes fewer than 100 *Norovirus* particles to make a person sick. Food can be contaminated either by direct contact with contaminated hands or work surfaces that are contaminated with stool or vomit, or by tiny droplets from nearby vomit that can travel through air to land on food. Although the virus cannot multiply outside of human bodies, once on food or in water, it can cause illness.

People working with food who are sick with *Norovirus* gastroenteritis are a particular risk to others, because they handle the food and drink many other people will consume. Since the virus is so small, a sick food handler can easily – without meaning to – contaminate the food he or she is handling. Many of those eating the contaminated food may become ill, causing an outbreak.

Outbreaks of *Norovirus* gastroenteritis have taken place in restaurants, cruise ships, nursing homes, hospitals, schools, banquet halls, summer camps, and family dinners – in other words, places where often people have consumed water and/or food prepared or handled by others. It is estimated that as many as half of all food-related outbreaks of illness may be caused by *Norovirus.* In many of these cases, sick food handlers were thought to be implicated.

Cost Information (if available): Not Available.

Action by 2009 Task Force II: Recommended adoption of Proposal 09-205 as amended.

B. When the Authority has determined an epidemiological association between an illness outbreak and shellfish consumption, the Authority shall conduct an investigation of the illness outbreak within 24 hours to determine whether the illness is growing area related or is the result of post-harvest contamination or mishandling. ~~In the case of a suspected N*orovirus* outbreak, the investigation shall begin when an epidemiological association between illnesses and the consumption of shellfish is confirmed through sampling; or, if an epidemiological association is determined by linking illnesses from more than one location with the consumption of shellfish.~~ **In the case of a suspected *Norovirus* outbreak as defined in A. above, a shellfish harvest area will be considered to pose a risk to human health that is sufficient to warrant public health action when either of the two following criteria is met:**

1. **The outbreak occurs at one location; PCR-confirmed *Norovirus* is found in at least one person with symptoms compatible with *Norovirus* illness; food histories of ill persons include consumption of the shellfish during the proper time frame; shellfish eaten were harvested from the same harvest area; and either;**
   a. **an epidemiologic association is found between illness and consumption of shellfish and there is no indication of contamination by food handlers; or**
   b. ***Norovirus* of the same genotype is found in both the shellfish and at least one person;**
2. **The outbreak occurs at more than one location; PCR-confirmed *Norovirus* is found from at least one person from each location who**

exhibits medical symptoms that are consistent with *Norovirus* illness; and the ill persons share a common food history of eating shellfish that were harvested from the same harvest area.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force II on Proposal 09-205.

Proposal Subject: Extent of Product to be Included Under a Recall and Requirement for Recall Status Reports

Specific NSSP Guide Reference: NSSP Guide Section II. Model Ordinance
Chapter II. Risk Assessment and Risk Management
@. 01. Outbreaks of Shellfish Related Illnesses Sections C., D., and I.

Text of Proposal/ Requested Action

A. When…

B. When…

C. When the investigation outlined in §.02B. does not indicate a post-harvest contamination problem, or illegal harvesting from a closed area, the Authority shall:

(1) Immediately place the implicated portion(s) of the harvest area(s) in the closed status;

(2) Notify receiving states and the FDA **Regional Shellfish Specialist** that a potential health risk is associated with shellfish harvested from the implicated growing area;

(3) As soon as determined by the Authority, transmit to the FDA and receiving states information identifying the dealers shipping the implicated shellfish; and

(4) Promptly initiate recall procedures consistent with the Recall Enforcement Policy, Title 21 Code of Federal Regulations Part 7. **The recall shall include all products that have not undergone a 6D thermal process for *Listeria monocytogenes*.**

D. When the investigation outlined in §.02B demonstrates that the illnesses are related to post-harvesting contamination or mishandling, growing area closure is not required. However, the Authority shall:

(1) Notify receiving states **and the FDA Regional Shellfish Specialist** of the problem; and

(2) Promptly initiate recall procedures consistent with the Recall Enforcement Policy Title 21 Code of Federal Regulations Part 7. **The recall shall include all implicated products. that have not undergone a 6D thermal process for *Listeria monocytogenes*.**

E. When …

F. Upon ...

G. Upon …

H. When ...

I. Whenever an Authority or dealer initiates a recall of shellfish products because of public health concerns, the Authority will monitor the progress and success of the recall. The Authority will immediately notify the FDA and the Authorities in other states involved in the recall. **The Authority shall submit weekly recall status reports to the FDA Regional Shellfish Specialist consistent with the Recall Enforcement Policy Title 21 Code of Federal Regulations Part 7, Subpart C, §7.53 (b) (1-6) until such time that the Authority deems the recall to be completed.** Each Authority involved in a recall will implement actions to ensure

removal of recalled product from the market**.** ~~and~~ issue public warnings if necessary to protect public health **and provide weekly reports to the Authority in the state of product origin regarding recall efforts within their state until such time that the Authority in the state of product origin deems the recall to be completed.**. FDA will decide whether to audit or issue public warnings after consultation with the Authority/Authorities, and after taking into account the scope of the product distribution and other related factors. If the FDA determines that the Authority in any state involved in the recall fails to implement effective actions to protect public health, the FDA may classify, publish and audit the recall, including issuance of public warnings when appropriate.

J. The…

Public Health Significance:

The Model Ordinance provides no guidance concerning the extent of products that are to be included in a shellfish recall. Adding language specifying that recalls are to include all products that have not been thermally processed to achieve a 6D treatment for *Listeria monocytogenes* defines the extent of shellfish products to be included in a recall. Although value added products, such as frozen breaded shellfish, are intended for cooking prior to consumption, their associated hazards are required to be controlled prior to their distribution for retail sale. In accordance with the FDA Seafood HACCP Regulation, processors must control food safety hazards before the product is marketed. Processors are not permitted to pass the control of food safety hazards onto the consumer.

While the Model Ordinance states that recalls are to be initiated in accordance with the Federal Recall Enforcement Policy as outlined in 21 Code of Federal Regulations, Part 7, which includes requirements for recall status reports, it is not explicit regarding this requirement. The lack of recall status reports in the past has proven problematic in determining the extent and effectiveness of recalls and has hindered efforts by public health authorities to manage recalls effectively. Including specific Model Ordinance language clearly establishes importance and need for recall status reports.

Cost Information (if available):

Action by 2009 Task Force II:

Recommended adoption of Proposal 09-206 as amended.

C. When the investigation outlined in §.02B. does not indicate a post-harvest contamination problem, or illegal harvesting from a closed area, the Authority shall:

(1) Immediately place the implicated portion(s) of the harvest area(s) in the closed status;

(2) Notify receiving states and the FDA Regional Shellfish Specialist that a potential health risk is associated with shellfish harvested from the implicated growing area;

(3) As soon as determined by the Authority, transmit to the FDA and receiving states information identifying the dealers shipping the implicated shellfish; and

(4) Promptly initiate recall procedures consistent with the Recall Enforcement Policy, Title 21 Code of Federal Regulations Part 7. The recall shall include all **implicated** products**.** ~~that have not undergone a 6D thermal process for *Listeria monocytogenes*.~~

D. When the investigation outlined in §.02B demonstrates that the illnesses are related to post-harvesting contamination or mishandling, growing area closure is not required. However, the Authority shall:

(1) Notify receiving states and the FDA Regional Shellfish Specialist of the problem; and

(2) Promptly initiate recall procedures consistent with the Recall Enforcement Policy Title 21 Code of Federal Regulations Part 7. The recall shall include all **implicated** products~~, that have not undergone a 6D thermal process for *Listeria monocytogenes*~~.

E. When …

F. Upon ...

G. Upon …

H. When ...

I. Whenever an Authority or dealer initiates a recall of shellfish products because of public health concerns, the Authority will monitor the progress and success of the recall. The Authority will immediately notify the FDA and the Authorities in other states involved in the recall. The Authority shall submit ~~weekly~~ **periodic** recall status reports to the FDA Regional Shellfish Specialist consistent with the Recall Enforcement Policy Title 21 Code of Federal Regulations Part 7, Subpart C, §7.53 (b) (1-6) until such time that the Authority deems the recall to be completed. Each Authority involved in a recall will implement actions to ensure removal of recalled product from the market**,** issue public warnings if necessary to protect public health and provide ~~weekly~~ **periodic** reports to the Authority in the state of product origin regarding recall efforts within their state until such time that the Authority in the state of product origin deems the recall to be completed.. FDA will decide whether to audit or issue public warnings after consultation with the Authority/Authorities, and after taking into account the scope of the product distribution and other related factors. If the FDA determines that the Authority in any state involved in the recall fails to implement effective actions to protect public health, the FDA may classify, publish and audit the recall, including issuance of public warnings when appropriate.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force II on Proposal 09-206.

Proposal Subject: *Vibrio vulnificus* Risk Management for Oysters

Specific NSSP Guide Reference: NSSP Guide Section II. Model Ordinance
Chapter II. Risk Assessment and Risk Management

Text of Proposal/ Requested Action The *Vibrio* Management Committee recommends that the existing language for Section @.04 *Vibrio vulnificus* Risk Management for Oysters remain in the 2009 update of the Guide.

The Committee also recommends that a new section be added to the 2009 update of the NSSP Guide as follows.

Effective January 1, 2012:

@.04 *Vibrio vulnificus* Risk Management for Oysters

~~(2)~~A. For states having 2 or more etiologically confirmed shellfish-borne *Vibrio vulnificus* illnesses since 1995 traced to the consumption of commercially harvested raw or undercooked oysters that originated from the waters of that state (Source State), the Authority shall develop and implement *a Vibrio vulnificus* **Risk** Management Plan.

~~(3)~~B. The Source State's *Vibrio vulnificus* **Risk** Management Plan shall define the administrative procedures and resources necessary to accomplish (i.e. establish and maintain) involvement by the state in a collective illness **risk** reduction program. The goal of the *Vibrio vulnificus* **Risk** Management Plan will be to reduce the **risk per serving to a 60% illness rate reduction for**~~rate of~~ etiologically confirmed shellfish-borne *Vibrio vulnificus* septicemia illnesses reported collectively by California, Florida, Louisiana, and Texas, from the consumption of commercially harvested raw or undercooked oysters **to a level equivalent to a 60% illness rate reduction from 1995 – 1999 baseline average illness rate of 0.278 per million.** ~~by 40 percent for years 2005 and 2006 (average) and by 60 percent for years 2007 and 2008 (average) from the average illness rate for the years 1995 -1999 of 0. 303/million. The list of states (California, Florida, Louisiana, Texas) used to calculate rate reduction may be adjusted if after a thorough review, epidemiological and statistical data demonstrates that it would be appropriate. The illness rate shall be calculated as the number of illnesses per unit of population. The goal may be reevaluated prior to the year 2006 and adjusted in the event that new science, data, or information becomes available. State's compliance with the Plan will require States to maintain a minimum of 60% reduction in years subsequent to 2008. Determination and compliance after 2008 will be based on two-year averages beginning in 2009.~~

~~(4)~~C. The Source State's *Vibrio vulnificus* **Risk** Management Plan shall include, at a minimum:

(1) The ISSC Consumer Education Program targeted toward individuals who consume raw oysters and whose health condition(s) increase their risk for *Vibrio vulnificus* illnesses;
(2) A process to collect~~ed~~ standardized information for each *Vibrio vulnificus* illness: including underlying medical conditions; knowledge of disease status; prior counseling on avoidance of high risk foods, including raw oysters; existence of consumer advisories at point of purchase or consumption; and, if possible, whether consumer was aware and understood the advisories;
(3) A standardized process for tracking products implicated in *Vibrio vulnificus*

illnesses; **and**

(4) Identification and **implementation of the controls, or equivalent controls, which produced an illness per serving equivalent to a 60% illness rate reduction in the core states.** ~~preparation for achieving a goal of post harvest processing capacity of 25 percent of all oysters intended for the raw, half-shell market during the months of May through September harvested from a Source State by the end of the third year (December 31, 2004). The percentage of post harvest processing will include the capacity of all operational plants and the capacity of plants under construction;~~

(5) ~~Identification and preparation for implementation of required post harvest processing capacity of 50% of all oysters intended for the raw, half-shell market during the months of May through September, harvested from a Source State, which shall be implemented should the 40 percent illness reduction goal not be achieved by December 31, 2006. The percentage of post harvest processing will include the capacity of all operational plants and the capacity of plants under construction. In the alternative, the state may utilize the control measures, or equivalent control measures, listed in @.04, (C), (6) (a), (b), (c), and (d) below for such periods of time which, in combination with post harvest processing, will provide equivalent outcomes. This portion of the plan shall be completed no later than December 31, 2005; and~~

(~~4~~6) ~~Identification and preparation for implementation of one or more of~~ the ~~following controls, or equivalent controls, which shall be implemented should the 60 percent rate of illness reduction goal not be achieved collectively by 2008. The control measures identified in the plan shall be appropriate to the state and reflect that state's contribution to the number of Vv illnesses and the controls that have been implemented by each state. This portion of the Plan shall be completed no later than December 2007. The temperature and month of the year parameters identified in the following controls may be adjusted by the ISSC Executive Board as recommended by the Vibrio Management Committee (VMC) on a state by state basis, as needed to achieve the established illness reduction goal. The adjustment to the State's plan can take into account the illness rate reduction that has occurred since the last review of the plan.~~

~~(a) Labeling all oysters, "For shucking by a certified dealer", when the Average Monthly Maximum Water Temperature exceeds 75°F;~~

~~(b) Subjecting all oysters intended for the raw, half-shell market to an Authority- approved post harvest processing that reduces the *Vibrio vulnificus* levels to <30 MPN/gram when the Average Monthly Maximum Water Temperature exceeds 75°F;~~

~~(c) Closing shellfish growing areas for the purpose of harvest of oysters intended for the raw, half-shell market when the Average Monthly Maximum Water Temperature exceeds 75°F;~~

~~(d) Labeling all oysters, "For shucking by a certified dealer", during the months of May through September, inclusive;~~

~~(e) Subjecting all oysters intended for the raw, half-shell market to a post harvest processing that is both approved by the Authority and reduces the *Vibrio vulnificus* levels to <30 MPN/gram during the months of May through September, inclusive; and~~

~~(f) Closing shellfish growing areas for the purpose of harvesting oysters intended for the raw, half-shell market during the months of May through September, inclusive.~~

Public Health Significance:	None submitted.
Cost Information (if available):	None submitted.
Action by 2009 Task Force II:	Recommended adoption of Proposal 09-207 as submitted.
Action by 2009 General Assembly	Adopted recommendation of 2009 Task Force II on Proposal 09-207.

Proposal Subject: Revising Post Harvest Processing Control Measure for a *Vibrio parahaemolyticus* Control Plan

Specific NSSP Guide Reference: NSSP Guide Section II. Model Ordinance
Chapter II. Risk Assessment and Risk Management
@.05 *Vibrio parahaemolyticus* Control Plan

Text of Proposal/ Requested Action

B. Control Plan

(4) For States required to implement *Vibrio parahaemolyticus* Control Plans, the Plan shall include the administrative procedures and resources necessary to accomplish the following:

(a) Establish one or more triggers for when control measures are needed. These triggers shall be the temperatures in § B. (2) where they apply, or other triggers as determined by the risk evaluation.

(b) Implement one or more control measures to reduce the risk of *Vibrio parahaemolyticus* illness at times when it is reasonably likely to occur. The control measures may include:

(i) Post harvest processing using a process that has been validated to **achieve a 2 log reduction in the** ~~ensure that~~ levels of total *Vibrio parahaemolyticus* **for Gulf and Atlantic Coast oysters and a 3 log reduction for Pacific Coast oysters** ~~after processing do not exceed the average levels found in the area at times of the year when the State has determined that *Vibrio parahaemolyticus* illness is not reasonably likely to occur~~;

(ii) Closing the area to oyster harvest;

(iii) Restricting oyster harvest to product that is labeled "For Cooking Only;"

(iv) Limiting time from harvest to refrigeration to no more than five hours, or other times based on modeling or sampling, as determined by the Authority in consultation with FDA;

(v) Limiting time from harvest to refrigeration such that the levels of total *Vibrio parahaemolyticus* after the completion of initial cooling to 60 °F (internal temperature of the oysters) do not exceed the average levels from the harvest water at time of harvest by more than 0.75 logarithms, based on sampling or modeling, as approved by the Authority;

Other control measures that based on appropriate scientific studies are designed to ensure that the risk of *Vp* illness is no longer reasonably likely to occur, as approved by the Authority.

Public Health Significance:	Levels of *Vibrio parahaemolyticus* in time temperature abused oysters are not likely to commonly exceed 100,000/gram. Subjecting temperature abused oysters to post harvest processing that achieves a 2 log reduction for the Gulf and Atlantic and a 3 log reduction for the Pacific would ensure that levels in processed oysters are somewhat more protective of public health than levels under control measures listed in Chapter II. @.05 B. (4) (b) (iv) and (v) but likely less protective than levels under (ii) and (iii). As such, it is consistent with the ISSC goal of substantial risk reduction, rather than that of near elimination of risk. The existing language is substantially more protective still, and is more in keeping with the level of control needed for "for added safety" labeling of post harvest processed product.
Cost Information (if available):	None.
Action by 2009 Task Force II:	Recommended adoption of Proposal 09-208 as submitted.
Action by 2009 General Assembly	Adopted recommendation of 2009 Task Force II on Proposal 09-208.

Proposal Subject: Time Requirement for Achieving Internal Oyster Temperature of 50°F (10°C)

Specific NSSP Guide Reference: NSSP Guide Section II. Model Ordinance
Chapter II. Risk Assessment and Risk Management
@.05 *Vibrio parahaemolyticus* Control Plan

Text of Proposal/ Requested Action Insert new item and re-letter subsequent items.

B. Control Plan

(4) For States required to implement *Vibrio parahaemolyticus* Control Plans, the Plan shall include the administrative procedures and resources necessary to accomplish the following:

(a) Establish one or more…

(b) Implement one or more…

(c) Require the original dealer to cool oysters to an internal temperature of 50°F (10°C) or below within 10 hours or less as determined by the Authority after placement into refrigeration during periods when the risk of *Vibrio parahaemolyticus* illness is reasonably likely to occur. The dealer's HACCP Plan shall include controls necessary to ensure, document and verify that the internal temperature of oysters has reached 50°F (10°C) or below within 10 or less as determined by the Authority hours of being placed into refrigeration. Oysters without proper HACCP records demonstrating compliance with this cooling requirement shall be diverted to PHP or labeled "*for shucking only*", or other means to allow the hazard to be addressed by further processing.

~~(c)~~ **(d)** Evaluate the effectiveness…

~~(d)~~ **(e)** Modify the Control Plan when…

~~(e)~~ **(f)** Optional cost benefits analysis…

Public Health Significance: *Vibrio parahaemolyticus* is the leading cause of bacterial illnesses associated with consumption of raw molluscan shellfish in the U.S. The ISSC adopted a *Vibrio parahaemolyticus* Control Plan for oysters in August 2007. The Plan was fully implemented by states as of June 20, 2008. The major control measure under the plan is a reduction in the time between exposure of oysters to air and initial placement into refrigeration. Once placed under refrigeration, the only Model Ordinance requirement is that the refrigeration unit be maintained at 45°F (7.2°C) or less. There is no requirement for reducing product temperature to a specified level within a specified period of time. The scientific literature indicates that *Vibrio parahaemolyticus* can grow in oysters at temperatures above 50°F (10°C). The FDA *Vibrio parahaemolyticus* Risk Assessment assumes that oysters are cooled to 50°F (10°C) within 10 hours after placement in refrigeration and that controlling growth after initial refrigeration is a key factor affecting the risk of illness. However, cooling systems for shellstock are diverse and little is known about their individual cooling performance under the variety of circumstances in which they are used. According to scientists involved in refrigeration technology, the time required to drop product temperature to 50°F (10°C) on refrigerated vehicles can take as long as 100 hours depending on initial product temperature. According to manufacturers of refrigerated truck compressors, cooling systems generally used on refrigerated trucks are only intended to maintain product temperature, not reduce it. Therefore, product, such as shellfish, needs to be prechilled to the desired temperature prior to truck loading and

transport. Additionally, the FDA/ISSC 2007 Retail Oyster Study indicated levels of both *Vibrio parahaemolyticus* and *Vibrio vulnificus* frequently exceeded 100,000 MPN per gram, further strengthening the need for mandatory time to temperature requirements following placement under refrigeration.

A major premise of the *Vibrio parahaemolyticus* Control Plan is that 10 hours represents the maximum time to cool oysters to 50°F (10°C). Therefore, it is critical that the Model Ordinance support a system to ensure that the 10 hour cool down time is met. Without measures to ensure that oyster shellstock is cooled to 50°F (10°C) within 10 hours, the level of protection intended by the ISSC, the *Vibrio parahaemolyticus* Control Plan will not be achieved.

Cost Information (if available):

Potential costs associated with this action:

1. Cost to upgrade and operate effective refrigeration systems at processing plants.
2. Loss of product value due to withdrawal from raw consumption market

Potential savings that may result from this proposal include:

1. Reduction in cost to individuals or society from fewer illnesses
2. Avoidance of product recall and loss of consumer confidence associated with recalls and recall press
3. Longer shelf life for properly chilled product
4.

Action by 2009 Task Force II

Recommended adoption of Proposal 09-209 as submitted.

Action by 2009 General Assembly

Adopted recommendation of 2009 Task Force II on Proposal 09-209.

Proposal Subject: *Vibrio parahaemolyticus* Control Plan

Specific NSSP Guide Reference: NSSP Guide Section II. Model Ordinance
Chapter II. Risk Assessment and Risk Management
@.05 *Vibrio parahaemolyticus* Control Plan

Text of Proposal/ Requested Action In accordance with the ISSC Constitution, Bylaws, and Procedures and in keeping with the spirit and intent of the Conference, the ISSC Executive Board approved interim guidance on September 11, 2008, as follows:

> Insert the following after "for cooking only": **<u>or for shucking by a certified dealer, or other mechanism such as a variance, to allow the hazard to be addressed by further processing.</u>**

This proposal, as amended by the *Vibrio* Management Committee at its meeting on May 6, 2009, is submitted to the Conference for adoption as required by the ISSC Constitution, Bylaws, and Procedures.

@.05 *Vibrio parahaemolyticus* Control Plan

The goal of the Control Plan is to reduce the probability of occurrence of *Vibrio parahaemolyticus* illness during periods that have been historically associated with annual illnesses. The Plan is to be implemented as part of a comprehensive program which includes all the time and temperature requirements contained in the Model Ordinance.

A. Risk Evaluation.

Every State from which oysters are harvested shall conduct a *Vibrio parahaemolyticus* risk evaluation annually. The evaluation shall consider each of the following factors, including seasonal variations in the factors, in determining whether the risk of *Vibrio parahaemolyticus* infection from the consumption of oysters harvested from an area (hydrological, geographical, or growing) is reasonably likely to occur: (For the purposes of this section, "reasonably likely to occur" shall mean that the risk constitutes an annual occurrence)

(1) The number of *Vibrio parahaemolyticus* cases epidemiologically linked to the consumption of oysters commercially harvested from the State; and

(2) Levels of total and tdh+ *Vibrio parahaemolyticus* in the area, to the extent that such data exists; and

(3) The water temperatures in the area; and

(4) The air temperatures in the area; and

(5) Salinity in the area; and

(6) Harvesting techniques in the area; and

(7) The quantity of harvest from the area and its uses i.e. shucking, half shell, PHP.

B. Control Plan

(1) If a State's *Vibrio parahaemolyticus* risk evaluation determines that the risk of *Vibrio parahaemolyticus* illness from the consumption of oysters harvested from a growing area is reasonably likely to occur, the State shall develop and implement a *Vibrio parahaemolyticus* Control Plan; or

(2) If a State has a shellfish growing area in which harvesting occurs at a time when average monthly daytime water temperatures exceed those listed below, the State shall develop and implement a *Vibrio parahaemolyticus* Control Plan. The average water temperatures representative of harvesting conditions (for a period not to exceed thirty (30) days) that prompt the need for a Control Plan are:

(a) Waters bordering the Pacific Ocean - 60°F.

(b) Waters bordering the Gulf of Mexico and Atlantic Ocean (NJ and south) - 81°F.

(c) However, development of a Plan is not necessary if the State conducts a risk evaluation, as described in §A., that determines that it is not reasonably likely that *Vibrio parahaemolyticus* illness will occur from the consumption of oysters harvested from those areas.

(i) In conducting the evaluation, the State shall evaluate the factors listed in §A. for the area during periods when the temperatures exceed those listed in this section;

(ii) In concluding that the risk is not reasonably likely to occur, the State shall consider how the factors listed in §A differ in the area being assessed from other areas in the state and adjoining states that have been the source of shellfish that have been epidemiologically linked to cases of *Vibrio parahaemolyticus* illness; or

(3) If a State has a shellfish growing area that was the source of oysters that were epidemiologically linked to an outbreak of *Vibrio parahaemolyticus* within the prior five (5) years, the State shall develop and implement a *Vibrio parahaemolyticus* Control Plan for the area.

(4) For States required to implement *Vibrio parahaemolyticus* Control Plans, the Plan shall include the administrative procedures and resources necessary to accomplish the following:

(a) Establish one or more triggers for when control measures are needed. These triggers shall be the temperatures in § B. (2) where they apply, or other triggers as determined by the risk evaluation.

(b) Implement one or more control measures to reduce the risk of *Vibrio parahaemolyticus* illness at times when it is reasonably likely to occur. The control measures may include:

(i) Post harvest processing using a process that has been validated to ensure that levels of total *Vibrio parahaemolyticus* after processing do not exceed the average levels found in the area at times of the year when the State has determined that *Vibrio parahaemolyticus* illness is not reasonably likely to occur;

(ii) Closing the area to oyster harvest;

(iii) Restricting oyster harvest to product that is labeled ~~"For Cooking Only" or~~ for shucking by a certified dealer, or other **means** ~~mechanism such as a variance,~~ to allow the hazard to be addressed

by further processing.

(iv) Limiting time from harvest to refrigeration to no more than five hours, or other times based on modeling or sampling, as determined by the Authority in consultation with FDA;

(v) Limiting time from harvest to refrigeration such that the levels of total *Vibrio parahaemolyticus* after the completion of initial cooling to 60 °F (internal temperature of the oysters) do not exceed the average levels from the harvest water at time of harvest by more than 0.75 logarithms, based on sampling or modeling, as approved by the Authority;.

(vi) Other control measures that based on appropriate scientific studies are designed to ensure that the risk of *Vp* illness is no longer reasonably likely to occur, as approved by the Authority.

(c) Evaluate the effectiveness of the Plan.

(d) Modify the Control Plan when the evaluation shows the Plan is ineffective, or when new information is available or new technology makes this prudent as determined by the Authority.

(e) Optional cost benefits analysis of the *Vibrio parahaemolyticus* Control Plan.

C. The Time When Harvest Begins

For the purpose of time to temperature control, time begins once the first shellstock harvested is no longer submerged.

Public Health Significance:

Cost Information (if available):

Action by 2009 Task Force II: Recommended adoption of Proposal 09-210 as submitted.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force II on Proposal 09-210.

Proposal Subject: Continuing Education Requirement for Licensed Shellfish Harvesters

Specific NSSP Guide Reference: NSSP Guide Section II. Model Ordinance
Chapter VIII. Control of Shellfish Harvesting
@.01 Control of Shellstock Growing Areas

Text of Proposal/ Requested Action

C. Licensing of Harvesting

(1) The Authority shall assure that a license is required to commercially harvest shellstock, including shellstock harvested from aquaculture.

(2) Each license shall:

(a) Not be valid for more than one year;

(b) Require the harvester to complete 3 hours annually of continuing education hours to attain a harvester license from the Authority Continuing Education hours could include attendance at ISSC meetings, attendance at regional shellfish sanitation conferences, attendance at regional shellfish association meetings, or any other conference or meeting approved by the Authority.

(~~b~~**c**) Require the harvester to sell only to dealers listed on the Interstate Certified Shellfish Shippers List; and

(~~c~~**d**) Allow the harvester, at his discretion, to place shellstock in containers for transport of shellstock from a growing area to land or to a dealer.

Public Health Significance: This requirement will better inform licensed shellfish harvesters of new guidelines set forth in the NSSP.

Cost Information (if available): The cost would include registration fee and certification certificate for the licensed harvester to attend a continuing education course.

Action by 2009 Task Force II: Recommended referral of Proposal 09-211 to an appropriate committee as determined by the Conference Chairman.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force II on Proposal 09-211.

Proposal Subject: New Food Safety Training Requirements for Harvesters and Dealers

Specific NSSP Guide Reference: NSSP Guide Section II. Model Ordinance
Chapter VIII. Control of Shellfish Harvesting

Text of Proposal/ Requested Action .02 Shellstock Harvesting and Handling.

A. Harvesters. Any harvester who engages in-shellfish packing as defined in this Ordinance shall:
(1) Be a dealer; or
(2) Pack shellstock for a dealer.

B. Harvester/Dealer Education

Requirement for States that have determined, through a *Vibrio* risk assessment, that *Vibrio* illnesses are reasonably likely to occur.

(1) If a harvester or dealer elects to harvest oysters intended for raw consumption during months that are typically associated with *Vibrio* illnesses, the harvester or dealer shall obtain a minimum of two hours of training in harvest and post-harvest practices, held bi-annually; or an equivalent level of training, as determined by the State Authority.

(2) The training shall cover all phases of harvest and post harvest handling likely to result in temperature abuse or growth of *Vibrio* bacteria. The training shall include harvest and post harvest practices, transportation and handling and processing methods designed to minimize the growth of Vibrio and to reduce the risk of illness from *Vibrios*.

(3) Based upon harvest practices and environmental conditions, the State Authority may determine the exact requirements of the training program, including the length and frequency of the training session.

(4) Harvesters and dealers must receive a certificate for training that has been approved by the Authority prior to issuance of a new license, or before a license shall be renewed.

(5) At least one representative from each company with a harvester or dealer license shall obtain the training.

(6) The Authority may provide the required training course, or approve other training classes or courses provided by other government agencies, educational institutes, academic meetings, private institutions, non profit organizations or trade associations.

~~B~~**C**. Non-Vessel Harvesting
~~C~~**D**. Vessels
~~D~~**E**. Disposal of Human Sewage from Vessels
~~E~~**F**. Shellstock Washing
~~F~~**G**. Shellstock Identification

Public Health Significance:	The risk of *Vibrio* illness can be greatly reduced through appropriate harvesting, post harvesting, transportation, handling, and processing of oysters intended for raw consumption. Because harvesters are not required to obtain HACCP training, it has been recognized that critical information about temperature abuse and the growth of *Vibrio* bacteria is not being conveyed to a large number of growers that only have a harvester's license. Further, it is recognized that dealers will benefit from learning more about the advantages of utilizing certain harvest, post harvest, transportation, handling and processing techniques designed to prevent the growth of *Vibrio* bacteria.
Cost Information (if available):	Undetermined cost implications. Recommend ISSC assistance in providing training materials or support.
Action by 2009 Task Force II:	Recommended referral of Proposal 09-212 to an appropriate committee as determined by the Conference Chairman.
Action by 2009 General Assembly	Adopted recommendation of 2009 Task Force II on Proposal 09-212.

Proposal Subject: In-shell Product Transportation Requirements

Specific NSSP Guide Reference: NSSP Guide Section II. Model Ordinance
Chapter IX. Transportation
Sections:

@.02 C.	.02 C. (4)
@.03 A. (3)	.04 B. (3)
@.04 B. (2)	.04 B. (4)

Text of Proposal/ Requested Action

@.02 Shipment Acceptability

C. Shucked ~~or post harvest processed~~ shellfish **and in-shell product** are cooled to a temperature of 45° Fahrenheit (7.2 ° Centigrade) or less; and

@.03 Shipment Rejection

A. Shellfish shall be rejected when:

(3) Shucked shellfish **temperature or the internal body temperature of in-shell product** exceeds 50° Fahrenheit (10° Centigrade); or

@.04 Bacteriological Examination of Shellfish Shipments.

B. Bacteriological examination shall be made of the shellfish shipment if:

(2) The shucked shellfish temperature **or the internal body temperature of in-shell product** exceeds 45° Fahrenheit (7.2° Centigrade) and is less than or equal to 50° Fahrenheit (10° Centigrade);

.02 Receiving Shellfish

C. The dealer shall:

(4) Ensure that shucked shellfish **and in-shell product** are not permitted to remain without ice, mechanical refrigeration, or other approved means of maintaining shellfish temperature at 45° Fahrenheit (7.2° Centigrade) or less; and

.04 Cargo Protection From Cross Contamination.

B. Shellfish Cargo Only.

(3) **In-shell product shipments shall be shipped on pallets.**

(~~3~~**4**) If the conveyance does not have a channeled floor, pallets shall be used for all shellfish.

Public Health Significance: This proposal is one of several that are part of an effort to incorporate the concept of in-shell product throughout the Model Ordinance.

Cost Information (if available): None.

Action by 2009 Task Force II: Recommended adoption of Proposal 09-213 as submitted.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force II on Proposal 09-213.

Proposal Subject: Research Need for Suitable Time-Temperature Monitoring Devices for Shipping Times Greater than Four Hours

Specific NSSP Guide Reference: NSSP Guide Section II. Model Ordinance
Chapter IX. Transportation .05 Shipping Times

Text of Proposal/ Requested Action The Pacific Rim Shellfish Sanitation Conference requests that the ISSC create an educational committee with the purpose of establishing criteria, plus research and review of suitable time-temperature monitoring devices to adequately monitor the temperature of shellstock during shipping. The educational committee will also post and maintain a clearinghouse showing potential time-temperature monitoring devices on the ISSC organization website so as to support dealers who ship shellfish.

B. Shipping Time is Greater Than Four Hours.

(1) When the shipping …
  (a) Mechanically refrigerated conveyances …
  (b) Containers with an …

(2) Unless the dealer has an approved HACCP plan with an alternate means of monitoring time-temperature, the initial dealer shall assure that a suitable time temperature recording device accompanies each shipment of shellfish.

(3) The initial dealer shall note the date and time on the temperature-indicating device, if appropriate.

(4) Each receiving dealer shall write the date and time on the temperature-indicating device, if appropriate, when the shipment is received and the doors of the conveyance or the containers are opened.

(5) The final receiving dealer shall keep the time-temperature recording chart or other record of time and temperature in his files and shall make it available to the Authority upon request.

(6) An inoperative temperature-indicating device shall be considered as no recording device.

Public Health Significance: Shellfish dealers are required by the NSSP to ensure that shellfish is shipped under proper temperature control to prevent possible pathogen growth. Natural marine pathogens such as *Vibrio vulnificus* and *Vibrio parahaemolyticus* show substantial growth when temperature increases. Pathogen growth has a logarithmic relationship to temperature; therefore, maintaining proper temperature control during shipping can lessen or restrict the growth of these pathogens.

Dealers have requested guidance on what time-temperature devices and technologies are available and suitable for industry use. With ever-changing technologies, a central educational clearinghouse would best serve the conference and its members.

Cost Information (if available): None – research request

Proposed Specific Research Need/Problem to be Addressed:

Research into appropriate time-temperature monitoring devices in order to monitor the temperature of shellstock during shipping. The current problem to be addressed focuses on whether or not shellstock is being kept at proper

and controlled temperatures during shipping in order to suppress or restrict the growth of pathogens such as *Vibrio vulnificus* and *Vibrio parahaemolyticus*. These time-temperature devices could serve to inform the receiver if the product before them is safe for human consumption and the grower on whether or not their product is being shipped as agreed.

How will addressing this research support/improve the mission/role of the ISSC/NSSP/Industry? Support need with literature citations as appropriate.

This research support will improve the mission of the ISSC/NSSP/Industry by increasing the monitoring of shellstock once it leaves the growing area. Time to Temperature controls have been instituted and measured in the growing areas and people are still getting sick. The industry and regulators in the Pacific Rim are asking the questions: how can we measure whether or not the shellstock temperatures are being maintained during shipping? How can we collect this data to help narrow down where the pathogen growth may be occurring? By narrowing in on possible avenues for growth and collecting sound data to support the possibility, public health will be better served.

Relative Priority Rank in Terms of Resolving Research Need:

Immediate	☐	**Important**	☒
Required	☐	**Other**	☐
Valuable	☐		

Estimated Cost:

Proposed Sources of Funding/Support:

Time Frame Anticipated:

Action by 2009 Task Force II Recommended adoption of Proposal 09-214 as submitted.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force II on Proposal 09-214.

Proposal Subject: Shucked Shellfish Labeling

Specific NSSP Guide Reference: NSSP Guide Section II. Model Ordinance
Chapter X. General Requirements for Dealers
.06 Shucked Shellfish Labeling A. Shellfish Labeling (5)

Text of Proposal/ Requested Action: Proposal 03-205 discussed and passed, at the 2005 ISSC Conference, the removal of redundant language dealing with shucked shellfish labeling.

(5) The dealer shall assure that ~~:(a) T~~the shucker-packer's or repacker's certification number is on the label of each package of fresh or frozen shellfish;

~~(b) Packages containing less than 64 fluid ounces have:~~

~~(i) A "SELL BY DATE" which is a reasonable subsequent shelf-life or the words "BEST IF USED BY" followed by a date when the product would be expected to reach the end of its shelf-life; and~~

~~(ii) The date as a month and day of the month.~~

~~(c) Packages containing 64 fluid ounces or more have on the lid and sidewall or bottom the "DATE SHUCKED" indicated as the number of the day of the year or the month and day of the month.~~

Public Health Significance: Removal of redundant language in Chapter X. General Requirements for Dealers per Proposal 03-205 passed in 2005. Items (b) and (c) above are addressed in Chapter X. General Requirements for Dealers .06 A. (6) and (7) respectively.

Cost Information (if available): No additional cost.

Action by 2009 Task Force II: Recommended adoption of Proposal 09-215 as submitted.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force II on Proposal 09-215.

Proposal Subject: Revising Wet Storage and Depuration Labeling Requirements for In-shell Product to be Consistent with Chapter X. .05. B. (2) (e) Shellstock Tagging.

Specific NSSP Guide Reference: NSSP Guide Section II Model Ordinance
Chapter X. General Requirements for Dealers Section
.07 In-Shell Product or Post Harvest Processed In-Shell Labeling

Text of Proposal/ Requested Action

B. In-Shell Product Tags

(1) The dealer tag on in-shell product shall contain the following indelible, legible information in the order specified below:

(a) The dealer's name and address;

(b) The dealer's certification number as assigned by the Authority;

(c) The original shellstock shipper's certification number. If depurated the original shellstock shipper's certification number is not required.

(d) A "SELL BY DATE" which is a reasonable subsequent shelf-life or the words "BEST IF USED BY" followed by a date when the product would be expected to reach the end of its shelf-life. The date shall include, month, day and year;

(e) **When the shellstock processed for in-shell product has been wet stored or** ~~If~~ depurated, the **wet storage or** depuration cycle number or lot number**. The wet storage lot number shall begin with the letter "w"**;

(f) The most precise identification of the harvest location as is practicable including the initials of the state of harvest, and the Authority's designation of the growing area by indexing, administrative or geographic designation. If the Authority has not indexed growing areas, then an appropriate geographical or administrative designation must be used (e.g. Long Bay, Decadent County, lease number, bed, or lot number).

(g) When the **stock processed for** in-shell product has been transported across state lines and placed in wet storage in a dealer's operation, the statement: **"THIS PRODUCT IS A PRODUCT OF (NAME AND STATE) AND WAS WET STORED AT (FACILITY CERTIFICATION NUMBER) FROM (DATE) TO (DATE)";**

(h) The type and quantity of in-shell product; and

(i) The following statement in bold capitalized type on each tag: "THIS TAG IS REQUIRED TO BE ATTACHED UNTIL CONTAINER IS EMPTY OR IS RETAGGED AND THEREAFTER KEPT ON FILE FOR 90 DAYS."

(j) All in-shell product intended for raw consumption shall include a consumer advisory. The following statement, from Section 3-603.11 of the Current Food Code, or an equivalent statement, shall be included on all shellstock: "Consuming raw or undercooked meats, poultry, seafood, shellfish or eggs may increase your risk of foodborne illness, especially if you have certain medical conditions."

(k) The statement "Keep Refrigerated" The statement "Keep Refrigerated" or an equivalent statement must be included on the tag.

(2) If the in-shell product is removed from the original container, the tag on the new container shall meet the requirements in §.07 B.

Public Health Significance: This proposal is to correct the confusion about in-shell product and wet storage by clarifying that the shellstock used in the processing of in-shell product that has been wet stored or depurated should be labeled. In-shell product once processed will not be wet stored or depurated.

Additionally, this correction will make wet storage and depuration labeling for in-sh product consistent with shellstock wet storage and depuration tagging.

Cost Information (if available): None.

Action by 2009 Task Force II: Recommended no action on Proposal 09-216.

Rationale: Insufficient need.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force II on Proposal 09-216.

Proposal Subject: Master Carton Labeling

Specific NSSP Guide Reference: NSSP Guide Section II. Model Ordinance
Chapter X. General Requirements for Dealers
.07 In-shell Product or Post Harvest Processed In-shell Labeling

Text of Proposal/ Requested Action

A. The dealer shall label all in-shell product with tags meeting the requirements of Chapter X .05. B. (1)

B. In-Shell Product Tags.

(1) The dealer shall label all in-shell product with tags meeting the requirements of Chapter X .05. B. (1)
(a) – (k)…

(2) If the in-shell product is removed from the original container, the tag on the new container shall meet the requirements in § .07 B.

(3) Country of origin information (USDA 2004) may be included on the shucker-packer or reshipper tag.

(4) When in-shell product intended for retail sale are packed in containers of 5 pounds or less and shipped in a master container which includes a tag in compliance with Chapter X .05 B.(1), the individual containers of 5 pounds or less shall not require tags as specified in Chapter X .05 B.(1) but may be labeled in some other manner with indelible, legible, information which at a minimum is adequate to trace the in-shell shellfish back to the lot of in-shell product it is part of. Consumer advisory information identified in Chapter X.07.B.(1)(j) shall be included on each retail package.

Public Health Significance: New language provides for consistent language in regards to master carton shipping containers as identified in Chapter X. .05 and .06.

Cost Information (if available): No additional cost.

Action by 2009 Task Force II: Recommended adoption of Proposal 09-217 as submitted.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force II on Proposal 09-217.

Proposal Subject: Incorporating In-shell Product Concept into Model Ordinance Chapter XI.

Specific NSSP Guide Reference: NSSP Guide Section II. Model Ordinance
Chapter XI. Shucking and Packing
Sections:

.01 A.	.02 A. (2)	.03 A. (5) (c)
.01 C.	.02 C. (1) (b)	.03 B. (2) (a)
.01 D.	.02 E. (4) (c)	.03 F. (11)
.01 E.		

Text of Proposal/ Requested Action

.01 Critical Control Points

A. Receiving Critical Control Point - Critical Limits. The dealer shall shuck and pack only ~~shellstock which is~~:

(1) **Shellstock** ~~O~~**o**btained from a licensed harvester who has:
(a) Harvested…
(b) Identified…

(2) **Shellstock** ~~O~~**o**btained from a dealer other than the original harvester who has:
(a) Shipped…
(b) Identified…

(3) In-shell product obtained from a dealer who has:
(a) Shipped the in-shell product adequately iced; or in a conveyance at or below 45°F (7.2°C) ambient air temperature; or 45°F (7.2) internal temperature or less [C];
(b) Identified the in-shell product with a tag on each container. [C]

C. In-shell Product Storage Critical Control Point - Critical Limits. The dealer shall ensure that in-shell product shall be:

(1) Iced; or [C]
(2) Placed and stored in a storage area or conveyance maintained at 45° F (7.2° C) or less. [C]

~~C~~**D**. Processing Critical Control Point - Critical Limits. The dealer shall ensure that:

(1)...
(5) For in-shell product the internal temperature of meats does not exceed 45° F (7.2° C) for more than 2 hours during processing. [C]

~~D~~**E.** Shucked Meat Storage Critical Control Point...

.02 Sanitation

A. Safety of Water for Processing and Ice Production.

(2) Ice Production. Any ice used in the processing, storage, or transport of ~~shellstock or shucked~~ shellfish shall be made on-site from potable water in a commercial ice machine; or **[C]**

C. Prevention of Cross Contamination.

(1) Protection of Cross Contamination.
(b) ~~Shucked s~~**S**hellfish shall be protected from contamination. **[S$^{C/K}$]**

E. Protection from Adulterants

(4) Protection of ice used in shellfish processing.
(c) Any ice used in the processing, storage, or transport of ~~shellstock or shucked~~ shellfish shall come from a facility sanctioned by the Authority or the appropriate regulatory agency. **[C]**

.03 Other Model Ordinance Requirements

A. Plants and Grounds

(5) Plant Interior
(c) All wet area floors used in areas to store **shellfish**~~shellstock~~, process food, and clean equipment and utensils shall be constructed of easily cleanable, impervious, and corrosion resistant materials which:

B. Plumbing and Related Facilities
(2) Adequate floor drainage, including backflow preventers such as air gaps, shall be provided where floors are:
(a) Used in **shellfish** ~~shellstock~~ storage;

F. Shellfish Storage and Handling

(11) Not commingle in-shell product during shucking. [K]

Public Health Significance:

This proposal is one of several that are part of an effort to incorporate the concept of in-shell product throughout the Model Ordinance Aspects of the proposal pertaining to establishing critical limits related to in-shell product temperature control are based on language in Model Ordinance Chapter XVI. C. and Chapter XII. .01 B.

Chapter XVI. C. (Post Harvest Processing) reads:

"For the purposes of refrigeration, if the product is dead, the product shall be treated as shucked product. If the product is live, the product shall be treated as shellstock."

Chapter XII. .01 B. (Repacking of shucked shellfish) reads:

"Processing Critical Control Point - Critical Limits. The dealer shall ensure that repacked shucked shellfish do not exceed an internal temperature of 45° F (7.2° C) for more than 2 hours. **[C]** "

Since in-shell product is dead, it is proposed that aspects of the proposal pertaining to establishing critical limits related to in-shell product temperature control be consistent with the Chapter XVI.C concept of treating dead product as shucked product for the purposes of refrigeration. That includes proposing a processing time/temperature critical limit consistent with that of repacking of shucked product.

Cost Information (if available): None.

Action by 2009 Task Force II: Recommended adoption of Proposal 09-218 as amended.

.01 Critical Control Points

A. Receiving Critical Control Point - Critical Limits. The dealer shall shuck and pack only:
(1) Shellstock obtained from a licensed harvester who has:
(a) Harvested…
(b) Identified…
(2) Shellstock obtained from a dealer other than the original harvester who has:
(a) Shipped…
(b) Identified…
(3) In-shell product obtained from a dealer who has:
(a) Shipped the in-shell product adequately iced; or in a conveyance at or below 45°F (7.2°C) ambient air temperature; or 45°F (7.2) internal temperature or less [C];
(b) Identified the in-shell product with a tag on each container. [C]

C. In-shell Product Storage Critical Control Point - Critical Limits. The dealer shall ensure that in-shell product shall be:
(1) Iced; or [C]
(2) Placed and stored in a storage area or conveyance maintained at 45° F (7.2° C) or less. [C]

D. Processing Critical Control Point - Critical Limits. The dealer shall ensure that:
(1)...
(5) For in-shell product the internal temperature of meats does not exceed 45° F (7.2° C) for more than 2 hours during processing. [C]

E. Shucked Meat Storage Critical Control Point...

.02 Sanitation

A. Safety of Water for Processing and Ice Production.
(2) Ice Production. Any ice used in the processing, storage, or transport of shellfish shall be made on-site from potable water in a commercial ice machine; or [C]

C. Prevention of Cross Contamination.
(1) Protection of Cross Contamination.
(b) Shellfish shall be protected from contamination. [$S^{C/K}$]

E. Protection from Adulterants

(4) Protection of ice used in shellfish processing.
(c) Any ice used in the processing, storage, or transport of shellfish shall come from a facility sanctioned by the Authority or the appropriate regulatory agency. [C]

.03 Other Model Ordinance Requirements

A. Plants and Grounds

(5) Plant Interior

(c) All wet area floors used in areas to store shellfish, process food, and clean equipment and utensils shall be constructed of easily cleanable, impervious, and corrosion resistant materials which:

B. Plumbing and Related Facilities

(2) Adequate floor drainage, including backflow preventers such as air gaps, shall be provided where floors are:

(a) Used in shellfish storage;

F. Shellfish Storage and Handling

~~(11) Not commingle in-shell product during shucking. [K]~~

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force II on Proposal 09-218.

Proposal Subject: Food Contact Equipment Storage

Specific NSSP Guide Reference: NSSP Guide Section II Model Ordinance
Chapter XI. Shucking and Packing .02 B. (2) (d)

Text of Proposal/ Requested Action

.02 Sanitation

B. Condition and Cleanliness of Food Contact Surfaces

(2) Cleaning and sanitizing of food contact surfaces.

(a) Food contact surfaces of equipment, utensils and containers shall be cleaned and sanitized to prevent contamination of shellfish and other food contact surfaces. The dealer shall:

(i) Provide adequate cleaning supplies and equipment, including three compartment sinks, brushes, detergents, and sanitizers, hot water and pressure hoses shall be available within the plant; **[K]**

(ii) Sanitize equipment and utensils prior to the start-up of each day's activities and following any interruption during which food contact surfaces may have been contaminated; **[K]**

(iii) Wash and rinse equipment and utensils at the end of each day. **[K]**

(b) Shellfish shall be protected from contamination by washing and rinsing shucking containers and sanitizing before each filling. **[K]**

(c) Containers which may have become contaminated during storage shall be washed, rinsed, and sanitized prior to use or shall be discarded. **[K]**

(d) ~~Shucked shellfish shall be packed in clean covered containers and stored in a manner which assures their protection from contamination.~~

Shucked shellfish shall be packed in clean covered containers:

(i) Fabricated from food grade materials; and [K]

(ii) Stored in a manner which assures their protection from contamination. [K]

(e) If used, the finger cots or gloves shall be:

(i) Made of impermeable materials except where the use of such material is inappropriate or incompatible with the work being done; **[O]**

(ii) Sanitized at least twice daily; **[K]**

(iii) Cleaned more often, if necessary; **[K]**

(iv) Properly stored until used; and **[K]**

(v) Maintained in a clean, intact, and sanitary condition. **[K]**

Public Health Significance: New language clarifies confusion in regards to where to cite improperly stored food contact equipment. This added language will also be consistent with language currently in Chapter XII. Repacking of Shucked Shellfish .02 Sanitation B. (2) (c).

Cost Information (if available): No additional cost.

Action by 2009 Task Force II: Recommended adoption of Proposal 09-219 as submitted.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force II on Proposal 09-219.

Proposal Subject: Plumbing and Related Facilities

Specific NSSP Guide Reference: NSSP Guide Section II. Model Ordinance
Chapter XI. Shucking and Packing .03 B. (1);
Chapter XII. Repacking of Shucked Shellfish .03 B.(1);
Chapter XIII. Shellstock Shipping .03 B. (1);
Chapter XIV. Reshipping .03 B. (1); and
Chapter XV. Depuration .03 B. (1)

Text of Proposal/ Requested Action Chapters XI. XII. XIII. XIV. and XV

.03 Other Model Ordinance Requirements

B. Plumbing and Related Facilities

(1) All plumbing and plumbing fixtures shall be **properly** designed, installed, modified, repaired, and maintained**. The** ~~to provide a~~ water system **shall provide** ~~that is~~ **an** adequate ~~in~~ quantity **of water** ~~and~~ under pressure, and include~~s~~ cold and warm water at all sinks.

Public Health Significance: New language clarifies confusion in regards to where to cite improperly designed, installed, modified, repaired, and maintained plumbing fixtures.

Cost Information (if available): No additional cost.

Action by 2009 Task Force II: Recommended approval of Proposal 09-220 as submitted.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force II on Proposal 09-220.

Proposal Subject: Frozen Shellfish Storage and Handling

Specific NSSP Guide Reference: NSSP Guide Section II Model Ordinance
Chapter XI. .03 Other MO Requirements F. Shellfish Storage & Handling (9);
Chapter XII. .03 Other MO Requirements F. Shellfish Storage & Handling (g);
Chapter XIII. .03 Other MO Requirements F. Shellfish Storage & Handling (5); and
Chapter XIV. .03 Other MO Requirements F. Shellfish Storage & Handling (4)

Text of Proposal/ Requested Action

Chapter XI. .03 Other MO Requirements
F. Shellfish Storage & Handling
(9) ~~Store packaged shellfish, if they are to be frozen, at an ambient temperature of 0°F (17.8°C) or less; and frozen solid within twelve hours following the initiation of freezing.~~ **During storage frozen shellfish shall be maintained frozen. [SK/0]**

Chapter XII. .03 Other MO Requirements
F. Shellfish Storage & Handling
(1) The dealer shall:
(g) ~~Store packaged shellfish, if they are to be frozen, at an ambient temperature of 32° F (0° C) or less and frozen solid within twelve hours following the initiation of freezing.~~ **During storage frozen shellfish shall be maintained frozen. [SK/0]**

Chapter XIII. .03 Other MO Requirements
F. Shellfish Storage & Handling
(5) During storage frozen shellfish shall be maintained frozen.

Chapter XIV. .03 Other MO Requirements
F. Shellfish Storage & Handling
(4) During storage frozen shellfish shall be maintained frozen.

Public Health Significance: New language provides consistency in regards to frozen shellfish storage across all plant chapters in the Model Ordinance.

Cost Information (if available): No additional cost.

Action by 2009 Task Force II: Recommended adoption of Proposal 09-221 as submitted.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force II on Proposal 09-221.

Proposal Subject: Remove References to Shellstock from Chapter XII. Repacking of Shucked Shellfish

Specific NSSP Guide Reference:
NSSP Guide Section II. Model Ordinance
Chapter XII. Repacking of Shucked Shellfish
.02 Sanitation Sections A. (2) and E. (4) (c)
.03 Other Model Ordinance Requirements Sections A. (5) (c); B. (2) (a); and E. (4)

Text of Proposal/ Requested Action

.02 Sanitation

A. Safety of Water for Processing and Ice Production.

(2) Ice Production. Any ice used in the processing, storage, or transport of ~~shellstock or shucked~~ shellfish shall be made on-site from potable water in a commercial ice machine; or **[C]**

E. Protection from Adulterants.

(4) Protection of ice used in shellfish processing:
(c) Any ice used in the processing, storage, or transport of ~~shellstock or shucked~~ shellfish shall come from a facility sanctioned by the Authority or the appropriate regulatory agency. **[C]**

.03 Other Model Ordinance Requirements

A. Plants and Grounds.

(5) Plant Interior.
(c) All wet area floors used in areas to store **shellfish**~~shellstock~~, process food, and clean equipment and utensils shall be constructed of easily cleanable, impervious, and corrosion resistant materials which:

B. Plumbing and Related Facilities.

(2) Adequate floor drainage, including backflow prevention such as air gaps, shall be provided where floors are:
(a) Used in **shellfish**~~shellstock~~ storage; **[K]**

E. Equipment Condition, Cleaning, Maintenance, and Construction of Non-food Contact Surfaces.

~~(4) All conveyances and equipment which come into contact with stored shellstock shall be cleaned and maintained in a manner and frequency as necessary to prevent shellstock contamination.~~ **~~[O]~~**

Public Health Significance: The Model Ordinance is organized according to activity. The Repacking of Shucked Shellfish activity does not involve shellstock. A dealer certified as a Repacker may engage in shellstock Shipping and/or Reshipping of shellstock. However, if a Repacker does engage Shellstock Shipping and/or Reshipping of shellstock, that Repacker must meet the requirements of Chapter XIII. (Shellstock Shipping) and/or Chapter XIV. (Reshipping) while doing so.

Cost Information (if available):	None.
Action by 2009 Task Force II:	Recommended adoption of Proposal 09-222 as submitted.
Action by 2009 General Assembly	Adopted recommendation of 2009 Task Force II on Proposal 09-222.

Proposal Subject: Incorporating In-shell Product Concept into Model Ordinance Chapter XIII.

Specific NSSP Guide Reference: NSSP Guide Section II. Model Ordinance
Chapter XIII. Shellstock Shipping Sections:

.01 A. (1) (2) (3)	.02 A. (2)	.03 A. (5) (c)
.01 C.	.02 C. (1) (b)	.03 B. (2) (a)
	.02 E. (5) (c)	

Text of Proposal/ Requested Action

.01 Critical Control Points

A. Receiving Critical Control Point - Critical Limits. The dealer shall ship or repack only ~~shellstock that is~~:

(1) **Shellstock** ~~O~~**o**btained from a licensed harvester who has:

(a) Harvested the shellstock from an Approved or Conditionally Approved area in the open status as indicated by the tag; and **[C]**

(b) Identified the shellstock with a tag on each container or transaction record on each bulk shipment; or **[C]**

(2) **Shellstock** ~~O~~**o**btained from a dealer other than the original harvester who has:

(a) Shipped the shellstock adequately iced; or in a conveyance at or below 45°F (7.2°C) ambient air temperature; or 50°F (10°C) internal temperature or less; or in a conveyance capable of lowering the temperature of the shellstock and will maintain it at 50°F (10°C) or less; **[C]**; and

(b) Identified the shellstock with a tag on each container or transaction record with each bulk shipment. **[C]**

(3) In-shell product obtained from a dealer who has:

(a) Shipped the in-shell product adequately iced; or in a conveyance at or below 45°F (7.2°C) ambient air temperature; or 45°F (7.2°C) internal temperature or less; [C] and

(b) Identified the in-shell product with a tag on each container. [C]

C. In-shell Product Storage Critical Control Point – Critical Limits. The dealer shall ensure that in-shell product shall be:

(1) Iced; [C] or

(2) Placed and stored in a storage area or conveyance maintained at 45°F (7.2°C) or less. [C]

.02 Sanitation

A. Safety of Water for Processing and Ice Production

(2) Ice Production. Any ice used in the processing, storage, or transport of ~~shellstock or shucked~~ shellfish shall be made on-site from potable water in a commercial ice machine; or **[C]**

C. Prevention of Cross Contamination.

(1) Protection of Shellfish

(a) Shellstock…

(b) ~~Shucked s~~**S**hellfish shall be protected from contamination. **[S$^{C/K}$]**

E. Protection from Adulterants

(5) Protection of ice used in-shellfish processing.

(c) Any ice used in the processing, storage, or transport of ~~shellstock or shucked~~ shellfish shall come from a facility sanctioned by the Authority or the appropriate regulatory agency. **[C]**

.03 Other Model Ordinance Requirements

A. Plants and Grounds.

(5) Plant Interior.

(c) All wet area floors used in areas to store **shellfish**~~shellstock~~, process food, and clean equipment and utensils shall be constructed of easily cleanable, impervious, and corrosion resistant materials which:

B. Plumbing and Related Facilities.

(2) Adequate floor drainage, including backflow preventers such as air gaps, shall be provided where floors are:

(a) Used in **shellfish**~~shellstock~~ storage;

Public Health Significance: This proposal is one of several that are part of an effort to incorporate the concept of in-shell product throughout the Model Ordinance.

Cost Information (if available): None.

Action by 2009 Task Force II: Recommended adoption of Proposal 09-223 as submitted.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force II on Proposal 09-223.

Proposal Subject: Remove Reference to Shucking from Chapter XIII. Shellstock Shipping

Specific NSSP Guide Reference: NSSP Guide Section II Model Ordinance
Chapter XIII. Shellstock Shipping
.03 Other Model Ordinance Requirements

Text of Proposal/ Requested Action

F. Shellfish Storage Handling

(1) The dealer shall:

(a) Assure that shellstock is:

(i) Alive; **[K]**

(ii) Reasonably free of sediment **[O]**; and

(iii) Culled; **[K]**

~~(b) The dealer shall not commingle shellstock during shucking unless the dealer is included in the Authority's commingling plan.~~ **[K]**

Public Health Significance: Chapter XIII. contains the requirements for shellstock shipping. Shucking is not part of the shellstock shipping process.

Cost Information (if available): None.

Action by 2009 Task Force II: Recommended adoption of Proposal 09-224 as submitted.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force II on Proposal 09-224.

Proposal Subject: Incorporating In-shell Product Concept into Model Ordinance Chapter XIV. Reshipping

Specific NSSP Guide Reference: NSSP Guide Section II. Model Ordinance
Chapter XIV. Reshipping

.01 A. (1) (b) (c)	.01 C.	.01 D.
.02 A. (2)	.02 C. (1) (b)	.02 E. (4) (c)
.03 A. (5) (c)	.03 B. (2) (a)	.03 F. (2) (a)

Text of Proposal/ Requested Action

.01 Critical Control Points

A. Receiving Critical Control Point – Critical Limits
(1) Originated from a dealer other than the original harvester who has:
(a) Shipped the shellstock ...
(b) Shipped the **shucked** shellfish **and/or in-shell product** iced or in a conveyance at or below 45°F (7.2°C) ambient air temperature; **[C]** and
(c) Identified the shellstock with a tag as outlined in Chapter X. .05**, identified the in-shell product with a tag as outlined in Chapter X. .07, and/**~~or a~~ **identified the shucked** shellfish **with a** label as outlined in Chapter X. .06. **[C]**

B. Shellstock Storage Critical Control Point – Critical Limits...
(1) Iced; or…
(2) Placed in a storage…
(3) Not permitted to…

C. In-shell Product Storage Critical Control Point – Critical Limits. The dealer shall ensure that in-shell product shall be:
(1) Iced; or [C]
(2) Placed and stored in a storage area or conveyance maintained at 45°F (7.2°) or less. [C]

~~C~~**D**. Shucked Meat Storage Critical Control Point - Critical Limit. The dealer shall store shucked shellfish at an ambient temperature of 45 ° F (7.2 ° C) or less. **[C]**

.02 Sanitation

A. Safety of Water for Processing and Ice Production.
(1) Water Supply
(a) The dealer shall…
(b) If the water…
(i) Prior to use…
(ii) Every six months…
(iii) After the water…
(2) Ice Production. Any ice used in the processing, storage, or transport of ~~shellstock or shucked~~ shellfish shall:

C. Prevention of Cross Contamination
(1) Shellstock shall be stored in a manner to protect shellstock from contamination in dry storage and at points of transfer. **[$S^{C/K}$]**
(a) Shellstock shall be…

(b) ~~Shucked s~~**S**hellfish shall be protected from contamination. **[S$^{C/K}$]**
(c) Equipment and utensils…

E. Protection from Adulterants
(1) Shellfish shall be…
(2) Any lighting fixtures…
(3) Food contact surfaces…
(4) Protection of ice used in-shellfish reshipping.
(a) Any ice which…
(b) Ice shall be…
(c) Any ice used in the processing, storage, or transport of ~~shellstock or shucked~~ shellfish shall come from a facility sanctioned by the Authority or the appropriate regulatory agency. **[C]**
(5) Adequate ventilation shall…

.03 Other Model Ordinance Requirements

A. Plants and Grounds

(5) Plant Interior.
(a) Sanitary conditions shall be maintained throughout the facility. **[O]**
(b) All dry area floors shall be hard, smooth, easily cleanable; and **[O]**
(c) All wet area floors used in areas to store **shellfish**~~shellstock~~, process food, and clean equipment and utensils shall be constructed of easily cleanable, impervious, and corrosion resistant materials which:
(i) Are graded to provide adequate drainage; **[O]**
(ii) Have even surfaces, and are free from cracks that create sanitary problems and interfere with drainage; and **[O]**
(iii) Have sealed junctions between floors and walls to render them impervious to water.; and **[O]**
(d) Walls and Ceilings. Interior surfaces of rooms where shellfish are stored, handled, processed, or packaged shall be constructed of easily cleanable, corrosion resistant, impervious materials **[O]**.

B. Plumbing and Related Facilities.

(2) Adequate floor drainage, including backflow preventer such as air gaps, shall be provided where floors are:
(a) Used in **shellfish** ~~shellstock~~ storage; **[K]**
(b) Used for food holding units (e.g. refrigeration units); **[K]**
(c) Cleaned by hosing, flooding, or similar methods; **[K]** and
(d) Subject to the discharge of water or other liquid waste including three compartment sinks on the floor during normal activities.

F. Shellfish Storage and Handling

(2) The dealer shall not:
a) Commingle, sort, or repack ~~shellstock or shucked~~ shellfish; or **[K]**
(b) Remove or alter any existing tag or label. **[K]**

Public Health Significance:	This proposal is one of several that are part of an effort to incorporate the concept of in-shell product throughout the Model Ordinance.
Cost Information (if available):	None.
Action by 2009 Task Force II:	Recommended adoption of Proposal 09-225 as submitted.
Action by 2009 General Assembly	Adopted recommendation of 2009 Task Force II on Proposal 09-225.

Proposal Subject: Delete Requirement to Add Name and Certification Number to Shellstock When Reshipping Only

Specific NSSP Guide Reference: NSSP Guide Section II. Model Ordinance
Chapter XIV. Reshipping
.03 Other Model Ordinance Requirement

Text of Proposal/ Requested Action

F. Shellfish Storage and Handling.

(1) The dealer shall~~:~~

~~(a)~~ B**b**uy shellfish only from sources certified by the Authority or listed in the ICSSL**.**~~; and~~ **[K]**

~~(b) Add his name and certification number to the package of shellstock.~~ **[K]**

Public Health Significance: This proposal allows the original tag or label to remain with the package of shellstock until the container is repackaged or empty. The shellstock will still be traced through the required invoices outlined in Chapter IX. This proposal will no longer require reshippers to breakdown pallets or other large shipments of shellstock to add the current dealer's name and certification number to individual containers which could cause cross contamination or temperature abuse during the process.

Cost Information (if available): No additional cost. Possible cost savings in time and effort by the reshipping dealer.

Action by 2009 Task Force II: Recommended adoption of Proposal 09-226 as submitted.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force II on Proposal 09-226.

Proposal Subject: Exemption for Distribution Centers Operating in States Not Participating in the NSSP that Only Reship Processed and Packaged Shellfish from Certified Shippers Listed in the ICSSL

Specific NSSP Guide Reference: NSSP Guide Section II. Model Ordinance
Chapter XIV. Reshipping
.03 Other Model Ordinance Requirements

Text of Proposal/ Requested Action

F. Shellfish Storage and Handling.

(1) The dealer shall not:
- (a) Commingle, sort, or repack shellstock or shucked shellfish; or **[K]**
- (b) Remove or alter any existing tag or label. **[K]**

(2) A dealer whose activity consists of trucks only shall:
- (a) Have his own facility for the storage of shellfish; or **[K]**
- (b) Have arrangements with a facility approved by the Authority for the storage of shellfish; and **[K]**
- (c) Have a permanent business address at which records are maintained and inspections can be performed. **[K]**

The Conference for Food Protection (CFP) approved CFP Issue 2008-I-014, Interstate Shipment of Shellfish at their 2008 conference. The issue recommended that the Conference send a letter to the FDA requesting that:

1. The FDA work with the NSSP to remove reshippers from the ICSSL listing requirement. Shellfish will still need to come from an approved source for harvesting/packing, but once the product is packaged and shipped, the requirement for reshippers (i.e., distribution facilities performing no handling or processing) to be listed on the ICSSL is dropped. (Note: annual verification of a seafood HACCP program still remains); or
2. The FDA provides direct inspection coverage and listing for reshippers in the [then]17 states not currently participating; and/or
3. The FDA accepts an inspection from an accredited 3rd-party auditing firm for distributors/reshippers within the 17 non-participating states, and provides listing on the ICSSL.

The CFP Executive Board, in discussions with the FDA, realized that the issue involves procedures approved under the NSSP and the administration of the ICSSL and therefore this issue is being submitted to the 2009 ISSC as a proposal.

Public Health Significance: The following information was presented in support of the issue that was submitted to the 2008 Conference for Food Protection (CFP) and was considered by CFP Council 1 during deliberation with the conclusion being the recommendation that is being submitted to the ISSC per the CFP Executive Board recommendations:

Certain seafood products are controlled under the National Shellfish Sanitation Program (NSSP), which includes the Interstate Certified Shellfish Shippers List (ICSSL). Any business or individual involved in the sale or resale of shellfish across a state or international border must be included in the ICSSL. The FDA has formalized this in the FDA Model Food Code for a number of years in "Section 3-201.15 (B): that states Molluscan Shellfish received in interstate commerce shall be from sources that are listed

in the Interstate Certified Shellfish Shippers Guide".

While the rules and requirements of the National Shellfish Sanitation Program are federal in scope, they are administered by the individual states. Participation by each state is voluntary, i.e., there is no requirement that a state administer the program, and in fact only 67% of states plus the District of Columbia currently do so with those states involved with growing, harvesting and processing shellfish comprising the predominant percentage of members. Any food distributor or wholesaler located in the states that choose not to participate in the ICSSL for one reason or another, cannot be listed on the ICSSL, and therefore cannot legally ship products covered by this act across state lines.

The laws requiring that purveyors of shellfish must have in place adequate and verifiable food safety measures for inclusion on the ICSSL have worked well to improve the safety of seafood in the U.S. The list itself provides an excellent clearinghouse for registered shellfish shippers by acting as a repository for shellfish dealers and shippers with certified HACCP food safety programs in place. The current system does not recognize the major food distribution centers operating under regulated food safety and sanitation program apart from the NSSP that act as nothing more than a throughput of shellfish that have already met sanitation and food safety requirements under the NSSP. These products are passed through to customers without any additional handling or processing and under regulated time-temperature controls in other non-NSSP programs since they are never opened. Customers receiving these products can verify the certified dealer that initially shipped the product, assure all required shellfish tags, product labeling to meet compliance with the NSSP remain intact and the product delivered under required temperatures set by Law. The Distribution Centers maintain excellent food safety and sanitation programs to include operating under the Seafood HACCP program with oversight from Federal, State or local regulatory officials as applicable. There only discrepancy is that, through no fault of their own, they are operating in a state that has not voluntarily participated in the NSSP from distributing across state lines regardless of the safety of the product.

This issue does not propose elimination of the ICSSL, nor does it recommend that HACCP/food safety standards and controls be eliminated. Therefore, there is no "public health" impact. Eliminating this discrepancy and allowing all legally permitted Distribution Centers to distribute unopened shellfish products without being listed in the ICSSL will not create more of a risk since the same standards are required and met whether a state participates or not.

Cost Information (if available):

Action by 2009 Task Force III: Recommended referral of Proposal 09-227 and the substitute (see below), which was submitted by the presenter, to an appropriate Committee as determined by the Conference Chairman with instructions to consider the impact this proposal would have on non-producing state participation in the ISSC and the NSSP.

Proposal Subject:
Alternate means of Certification of Shellstock reshippers operating in states not participating in the NSSP that only reship processed and packaged shellfish from certified shippers listed in the ICSSL.

Specific NSSP Guide Reference:
NSSP Guide Section II. Model Ordinance
Chapter XIV. Reshipping

Text of Proposal/ Requested Action
The Conference for Food Protection (CFP) believes that consumers throughout the United States should be able to enjoy the benefits of safe, wholesome certified raw molluscan shellfish. We also believe that any business that complies with the requirements for certification and desires to be listed in the ICSSL should be listed. However, consumers are being denied the opportunity to consume certified shellfish and many businesses are unable to sell certified shellfish because the local regulatory authority does not verify compliance with the NSSP requirements and does not recommend to FDA the listing of any firms in the ICSSL. Unfortunately, some firms choose, of necessity, to handle raw molluscan shellfish without being certified. This puts consumers and retailers at risk because there is no assurance that the shellfish they consume is safe and wholesome. It harms competitors who choose not to handle raw molluscan shellfish in facilities that are not certified even though they would do so if certification were available.

The CFP approved Issue 2008-I-014, Interstate Shipment of Shellfish at their 2008 conference. The issue recommended that the Conference send a letter to the FDA requesting that:

1. The FDA work with the NSSP to remove reshippers from the ICSSL listing requirement. Shellfish will still need to come from an approved source for harvesting/packing, but once the product is packaged and shipped, the requirement for reshippers (i.e., distribution facilities performing no handling or processing) to be listed on the ICSSL is dropped. (Note: annual verification of a seafood HACCP program still remains); or
2. The FDA provides direct inspection coverage and listing for reshippers in the [then]17 states not currently participating; and/or
3. The FDA accepts an inspection from an accredited 3rd-party auditing firm for distributors/reshippers within the 17 non-participating states, and provides listing on the ICSSL.

Therefore, CFP is now coming before the ISSC for the purpose of asking that the NSSP Guidelines be amended to provide for a method of certification and listing of firms (shellstock reshippers in particular) which are located in states that do not have a shellfish sanitation program. The CFP does not presume to suggest specific amendments to the NSSP Guidelines to accomplish this because the NSSP Guidelines are under the exclusive purview of the ISSC, which has far greater knowledge and expertise in this area than CFP.

Public Health Significance:
Certain seafood products are controlled under the National Shellfish Sanitation Program (NSSP), which includes the Interstate Certified Shellfish Shippers List (ICSSL). Any business or individual involved in the sale or resale of shellfish across a state or international border must be included in the ICSSL. The FDA has formalized this in the FDA Model Food Code for a number of years in “Section 3-201.15 (B): that states Molluscan Shellfish received in interstate commerce shall be from sources that are listed in the Interstate Certified Shellfish Shippers Guide".

While the rules and requirements of the National Shellfish Sanitation Program are federal in scope, they are administered by the individual states. Participation by each state is

voluntary, i.e., there is no requirement that a state administer the program, and in fact only 67% of states plus the District of Columbia currently do so with those states involved with growing, harvesting and processing shellfish comprising the predominant percentage of members. Any food distributor or wholesaler located in the states that choose not to participate in the ICSSL for one reason or another, cannot be listed on the ICSSL, and therefore cannot legally ship products covered by this act across state lines.

The laws requiring that purveyors of shellfish must have in place adequate and verifiable food safety measures for inclusion on the ICSSL have worked well to improve the safety of seafood in the U.S. The list itself provides an excellent clearinghouse for registered shellfish shippers by acting as a repository for shellfish dealers and shippers with certified HACCP food safety programs in place. The current system does not recognize the major food distribution centers operating under regulated food safety and sanitation program apart from the NSSP that act as nothing more than a throughput of shellfish that have already met sanitation and food safety requirements under the NSSP. These products are passed through to customers without any additional handling or processing and under regulated time-temperature controls in other non-NSSP programs since they are never opened. Customers receiving these products can verify the certified dealer that initially shipped the product, assure all required shellfish tags, product labeling to meet compliance with the NSSP remain intact and the product delivered under required temperatures set by Law. The Distribution Centers maintain excellent food safety and sanitation programs to include operating under the Seafood HACCP program with oversight from Federal, State or local regulatory officials as applicable. Their only discrepancy is that, through no fault of their own, they are operating in a state that has not voluntarily participated in the NSSP from distributing across state lines regardless of the safety of the product.

This issue does not propose elimination of the ICSSL, nor does it recommend that HACCP/food safety standards and controls be eliminated. Therefore, there is no "public health" impact. Eliminating this discrepancy and allowing all legally permitted Distribution Centers to distribute unopened shellfish products without being listed in the ICSSL will not create more of a risk since the same standards are required and met whether a state participates or not.

Cost Information (if available):
N/A

Action by 2009 General Assembly

Voted no action on Proposal 09-227.

Proposal Subject: Remove References to Shucked Product from Chapter XV. Depuration

Specific NSSP Guide Reference: NSSP Guide Section II. Model Ordinance
Chapter XV. Depuration (Requirements for Dealers)
.02 Sanitation A. (2); C. (3) (b); and E. (6) (c)

Text of Proposal/ Requested Action

A. Safety of Water for Processing and Ice Production

(2) Ice production. Any ice used in the processing or storage of ~~shucked~~ shellfish shall:

C. Prevention of Cross Contamination.

(3) Employee practices.
(a) The dealer shall require…
(b) ~~Any employee handling shucked shellfish shall be required to:~~
~~(i) Wear effective hair restraints;~~ **~~[O]~~**
~~(ii) Remove any hand jewelry that cannot be sanitized or secured;~~ **~~[O]~~**
~~(iii) Wear finger cots or gloves if jewelry cannot be removed; and~~ **~~[O]~~**
~~(iv) Wear clean outer garments, which are rinsed or changed as necessary to be kept clean~~ **~~[O]~~**
~~(v)~~ In any area where shellfish are **stored**~~shucked or packed~~ and in any area which is used for the cleaning or storage of utensils, the dealer shall not allow employees to:
~~a.~~**(i)** Store clothing or other personal belongings; [O]
~~b.~~**(ii)** Eat or drink; [K]
~~c.~~**(iii)** Spit; and [K]
~~d.~~**(iv)** Use tobacco in any form. **[K]**

E. Protection from Adulterants

(6) Protection of ice used in shellstock shipping.
(a) Any ice which…
(b) Ice shall be …
(c) Any ice used in the processing, storage, or transport of shellstock ~~or shucked shellfish~~ shall come from a facility sanctioned by the Authority or the appropriate regulatory agency. **[C]**

Public Health Significance: The Model Ordinance is organized according to activity. The depuration activity does not involve shucked shellfish or in-shell product.

Cost Information (if available):

Action by 2009 Task Force II: Recommended adoption of Proposal 09-228 as submitted.

Action by 2009 General Assembly Adopted recommendation of Task Force II on Proposal 09-228.

Proposal Subject: Post Harvest Processing

Specific NSSP Guide Reference: NSSP Guide Section II Model Ordinance
Chapter XVI. Post Harvest Processing

Text of Proposal/ Requested Action

A. If a dealer elects to use a process to reduce the level(s) of one target pathogen or some target pathogens, or all pathogens of public health concern in shellfish, **and wishes to make labeling claims regarding the reduction of pathogens,** the dealer shall:

(1) Have a HACCP plan approved by the Authority for the process that ensures that the target pathogen(s) are at safe levels for the at risk population in product that has been subjected to the process. **The HACCP Plan shall include:**

(a) **Process controls to ensure that the end point criteria are met for every lot; and** ~~The dealer must demonstrate that the process reduces the level of *Vibrio vulnificus* in the processed product to non-detectable (<30 MPN/gram) and the process achieves a minimum 3.52 log reduction, to be determined by use of the *Vibrio vulnificus* FDA approved EIA procedure of Tamplin, et al, as described in Chapter 9 of the FDA *Bacteriological Analytical Manual*, 7th Edition, 1992, or other method approved for NSSP use.~~

(b) **A sampling program to periodically verify that the end point criteria are met.** ~~The dealer must demonstrate that the process reduces the level of *Vibrio parahaemolyticus* in the processed product to non-detectable (<30 MPN/gram) and the process achieves a minimum~~

(c) ~~For processes that target other pathogens the dealer must demonstrate that the level of those pathogens in processed product has been reduced to levels below the appropriate FDA action level, or, in the absence of such a level, below the appropriate level as determined by the ISSC.~~

~~(d) The ability of the process to reliably achieve the appropriate reduction in the target pathogen(s) shall be validated by a study as outlined in Guidance Documents Chapter IV Naturally Occurring Pathogens, Section .04 approved by the Authority, with the concurrence of FDA.~~

~~(e) The HACCP plan shall include:~~
~~(i) Process controls to ensure that the end point criteria are met for every lot; and,~~
~~(ii) A sampling program to periodically verify that the end point criteria are met.~~

~~(2) Package and label all shellfish in accordance with all requirements of this Ordinance. This includes labeling all shellfish which have been subjected to the process but which are not frozen in accordance with applicable~~

~~shellfish tagging and labeling requirements in Chapter X.05 and X.06.~~

~~(3) Keep records in accordance with Chapter X.07.~~

(2) Validate the Process by demonstrating that the process will reliably achieve the appropriate reduction in the target pathogen(s). The process shall be validated by a study as outlined in Guidance Documents Chapter IV, Naturally Occurring Pathogens, Section .04 and be approved by the Authority, with concurrence of FDA.

(a) The dealer must demonstrate that the process reduces the level of *Vibrio vulnificus* and/or *Vibrio parahaemolyticus* in the processed product to non-detectable (<30MPN/gram) and the process achieves a minimum 3.52 log reduction. Determination of *V. vulnificus* and/or *V. parahaemolyticus* levels must be done using the MPN protocols described in Guidance Documents, Chapter IV, Naturally Occurring Pathogens, Section .04 followed by confirmation using methods approved for use in the NSSP.

(b) For processes that target other pathogens the dealer must demonstrate that the level of those pathogens in processed product has been reduced to levels below the appropriate FDA action level, or, in the absence of such a level, below the appropriate level as determined by the ISSC.

(3) Conduct verification sampling to verify that the validated process is working properly. Verification sampling shall be at least equivalent to the verification protocol found in Guidance Documents, Chapter IV, Naturally Occurring Pathogens, Section .04 as determined by the Authority and shall be reviewed annually by the Authority.

(4) Package and label all shellfish in accordance with all requirements of this Ordinance. This includes labeling all shellfish which have been subjected to the process but which are not frozen in accordance with applicable shellfish tagging and labeling requirements in Chapter X.05 and X.06.

(5) Keep records in accordance with Chapter X.07.

Public Health Significance: Requirements for Post Harvest Processes to reduce the levels of pathogen(s) were re-organized for better flow.

Guidance for validation and verification of a process used to reduce levels of pathogen(s) has been developed and appear in the Guidance Documents, Chapter IV. This proposal provides specific requirements of the dealer and SSCA to ensure that companies wishing to use labeling claims concerning the reduction of pathogen(s) validate and verify the process using a protocol that is at least equivalent in public health protection as that listed in the Guidance Document.

Cost Information (if available):

Action by 2009 Task Force II

Recommended adoption of Proposal 09-229 as submitted with the following additional recommendations.

Recommended adoption of recommendation to verify references for NSSP Guide Section II Model Ordinance Chapter X. @.05 Post Harvest Processing A. Sections 4. and 5. (Editorial – does not require Conference action.)

Recommended adoption of recommendation to have Executive Board verify the exclusion of frozen shellfish in the labeling requirements in NSSP Guide Section II Model Ordinance Chapter X. Post Harvest Processing Section A. Section 4. (Editorial – does not require Conference action.)

Recommended adoption of recommendation that an "Additional Guidance Box" be added at (2) (a) to include a reference to the methods that have been approved by the Conference for use in the NSSP. The specific Guide reference to be included in the box is Section IV. Guidance Documents, Chapter II. Growing Areas .10 Approved National Shellfish Sanitation Program Laboratory Tests: Microbiological and Biotoxin Analytical Methods. (Editorial – does not require Conference action.)

Recommended adoption of recommendation that approved changes as a result of Proposal 09-229 will be reflected in the update for the 2009 NSSP Guide for the Control of Molluscan Shellfish Section III. Public Health Reasons and Explanations. (Editorial – does not require Conference action.)

Action by 2009 General Assembly

Adopted recommendation of Task Force II on Proposal 09-229.

Proposal Subject: Post Harvest Process Validation

Specific NSSP Guide Reference: NSSP Guide Section II. Model Ordinance
Chapter XVI. Post Harvest Processing A. (1) (d)

Text of Proposal/ Requested Action

A. If a dealer elects to use a process to reduce the level(s) of one target pathogen or some target pathogens, or all pathogens of public health concern in shellfish, the dealer shall:

(1) Have a HACCP plan approved by the Authority for the process that ensures that the target pathogen(s) are at safe levels for the at risk population in product that has been subjected to the process.

(a) The dealer must demonstrate that the process reduces the level of *Vibrio vulnificus* in the processed product to non-detectable (<30 MPN/gram) and the process achieves a minimum 3.52 log reduction, to be determined by use of the *Vibrio vulnificus* FDA approved EIA procedure of Tamplin, et al, as described in Chapter 9 of the FDA *Bacteriological Analytical Manual*, 7th Edition, 1992, or other method approved for NSSP use.

(b) The dealer must demonstrate that the process reduces the level of *Vibrio parahaemolyticus* in the processed product to non-detectable (<30 MPN/gram) and the process achieves a minimum 3.52 log reduction.

(c) For processes that target other pathogens the dealer must demonstrate that the level of those pathogens in processed product has been reduced to levels below the appropriate FDA action level, or, in the absence of such a level, below the appropriate level as determined by the ISSC.

(d) The ability of the process to reliably achieve the appropriate reduction in the target pathogen(s) shall be validated by a study as outlined in Guidance Documents Chapter IV. Naturally Occurring Pathogens, Section .04 approved by the Authority, with the concurrence of FDA. **Analytical results used for validation and verification of a PHP shall come from an analytical laboratory that is evaluated by the State and/or FDA and found to be in compliance with applicable NSSP laboratory requirements.**

(e) The HACCP plan shall include:
(i) Process controls to ensure that the end point criteria are met for every lot; and,
(ii) A sampling program to periodically verify that the end point criteria are met.

Public Health Significance: Laboratory results used in the NSSP should come from laboratories that have been evaluated and found to comply with NSSP laboratory requirements. Existing laboratory requirements and checklists do not include *Vibrio* analyses and may need to be revised to include evaluation criteria for laboratories conducting *Vibrio* analyses.

Cost Information (if available):

Action by 2009 Task Force III: Recommended adoption of Proposal 09-230 as submitted and further requests the Executive Board to work with states and the FDA to development requirements and a timetable for all laboratories providing data for NSSP requirements to become compliant with the laboratory requirements of the Model Ordinance.

Action by 2009 General Assembly Adopted recommendation of 2209 Task Force III on Proposal 09-230.

Proposal Subject: Post Harvest Handling

Specific NSSP Guide Reference: NSSP Guide Section II Model Ordinance Definitions and New Chapter XVII.

Text of Proposal/ Requested Action

Action #1:

Add a new definition to B. Definition of Terms for Post Harvest Handling and renumber Definitions Section accordingly.

Post Harvest Handling means a control(s) employed by a dealer to further reduce, beyond controls currently in place under the NSSP, the post harvest growth of naturally occurring pathogens for the purposes of handling product outside of existing NSSP management plans.

Action #2:

Add a new chapter to the NSSP Guide Section II. Model Ordinance as follows:

Chapter XVII. Post Harvest Handling

A. If a dealer elects to use a post harvest handling control(s) to reduce the levels of a naturally occurring pathogen(s) of public health concern in shellfish, the dealer shall:

(1) Have a HACCP plan (approved by the Authority) for the control(s) that reduces post harvest growth of the target pathogen(s).

(a) The dealer must validate that the post harvest handling control(s) reduces the post harvest growth of naturally occurring pathogen(s). The validation study must be approved by the State Shellfish Control Authority with FDA concurrence.

(b) The ability of the post harvest handling control(s) to reliably achieve the appropriate reduction in post harvest growth of the target pathogen(s) shall be routinely verified at a frequency determined by the State Shellfish Control Authority.

(2) Package and label all shellfish in accordance with the requirements of this Ordinance.

(3) Keep records in accordance with Chapter X. 07.

Public Health Significance: The changes recommended by this proposal provide added opportunities for shellfish dealers to meet the required State Control Plans for naturally occurring pathogens.

Cost Information (if available):

Action by 2009 Task Force II: Recommended referral of Proposal 09-231 to an appropriate committee as determined by the Conference Chairman.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force II on Proposal 09-231.

Proposal Subject: Post Harvest Handling

Specific NSSP Guide Reference: Section II. Model Ordinance
Chapter XVII. Post Harvest Handling

Text of Proposal/ Requested Action

Post Harvest Handling

A. If a dealer elects to use a post harvest handling process to reduce post harvest growth of some target pathogens of public health concern in shellfish, the dealer shall:

(1) Have a HACCP plan approved by the Authority for the process that reduces post harvest growth of the target pathogen(s).

(a) The dealer must demonstrate that the post harvest handling process reduces the post harvest growth of Vibrio vulnificus in the product to be determined by the State Shellfish Authority or other method approved for NSSP use.

(b) The dealer must demonstrate that post harvest handling process reduces the post harvest growth of Vibrio parahaemolyticus in the product to be determined by the State Shellfish Authority or other method approved for NSSP use.

(c) For handling procedure that target other pathogens the dealer must demonstrate that the level of those pathogens in the post harvest handled product has reduced post harvest growth to an adequate action level determined by the ISSC or SSCA.

(d) The ability of the post harvest handling to reliably achieve the appropriate reduction of growth in the target pathogen(s) shall require the certified dealer to conduct an annual validation study approved by the SSCA with the concurrence of FDA.

(e) The HACCP plan shall include:

(i) Post harvest handling controls to ensure that the end point criteria are met for every lot; and,

(ii) A sampling program to periodically verify that the end point criteria are met.

2. Package and label all shellfish in accordance with all requirements of this Ordinance.

3. Keep records in accordance with Model Ordinance Chapter X.07.

Public Health Significance: It is well documented that a HACCP based approach to handling oysters during and following harvest will reduce the growth of bacteria that may cause illnesses.

Cost Information (if available): The cost associated with this proposal is far less than those that currently exist to meet guidelines set in the *Vibrio vulnificus* and *parahaemolyticus* Management Plans for oysters.

Action by 2009 Task Force II: Recommended referral of Proposal 09-232 to an appropriate committee as determined by the Conference Chairman.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force II on Proposal 09-232.

Proposal Subject: Validation and Verification for Process Studies for Time and Temperature Requirements Related to *Vibrio* Management Plan Controls

Specific NSSP Guide Reference: NSSP Guide Section IV. Guidance Documents
Chapter IV. Naturally Occurring Pathogens
.04 Post Harvest Processing (PHP) Validation/Verification Guidance for *Vibrio vulnificus* and *Vibrio parahaemolyticus*

Text of Proposal/ Requested Action

A. Process Validation

B. Equipment Validation

C. Initial Load Testing

D. Verification

9. Time to temperature controls shall be evaluated using standard protocols for measuring temperatures in shellfish. A protocol shall include the number of samples, when and where samples will be collected and temperatures measured. The protocols will include procedures that can be included in harvester and dealer record keeping to document compliance with time to temperature requirements.

Public Health Significance: Implementation of the *Vibrio vulnificus* Management Plans will require establishing times to refrigeration and times to cool down to 55 degrees. It is important that standardized measuring methods be agreed upon in order to be sure that time to temperature requirements are being accomplished by harvesters and dealers. Additionally, agreed upon validation and verification activities will lead to greater uniformity in implementing *Vibrio* controls.

Cost Information (if available): None available

Action by 2009 Task Force II: Recommended approval of substituted language for Proposal 09-233.

.05 Guidance for Demonstrating the Effectiveness of Time to Temperature Reduction Criteria for *Vibrio vulnificus* and ***Vibrio parahaemolyticus***

Time-to-Temperature Protocol

(1) Identify the target time/temperature requirements for the specific cooling system/unit.
(2) Demonstrate that each cooling method and unit is capable of meeting the target time/temperature by conducting a process study under worst case conditions for that unit. The following parameters should be considered and utilized in conducting the process study:
   a. maximum load for the cooling unit
   b. initial product temperature (studies have demonstrated that measurement of the external temperature and the internal meat temperatures are comparable and either can be used)
   c. location of hot spot(s)
   d. thermostat setting(s)

e. cooling method(s) used
f. method of loading the cooling unit
(3) Include a description of the process; a record of the process study conducted; and monitoring records in a HACCP Plan.
(4) The protocol should be applied at the first point of refrigeration

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force II on Proposal 09-223.

Proposal Subject: *Vibrio parahaemolyticus* Control Plan Guidance

Specific NSSP Guide Reference: Section IV. Guidance Documents Chapter IV. Naturally Occurring Pathogens
.03 *Vibrio parahaemolyticus* Control Plan Guidance

Text of Proposal/ Requested Action In accordance with the ISSC Constitution, Bylaws, and Procedures and in keeping with the spirit and intent of the Conference, the ISSC Executive Board approved Interim Guidance on September 11, 2008, as follows:

> Insert the following after "for cooking only": **or for shucking by a certified dealer, or other mechanism such as a variance, to allow the hazard to be addressed by further processing.**

This proposal, as amended by the *Vibrio* Management Committee at its meeting on May 6, 2009, is submitted to the Conference for adoption as required by the ISSC Constitution, Bylaws, and Procedures.

.03 *Vibrio parahaemolyticus* Control Plan Guidance

I. Risk Evaluation

The determination of Reasonably Likely to Occur should be conducted as follows:

1. A risk evaluation as described in Proposal 07-202 (with the understanding that ISSC has not adopted nor endorsed the FDA *Vp* Risk Assessment); or

2. The risk factor decision tree under development by the VMC using the risk factors included in Proposal 07-202; or

3. Other approaches approved by the State Authority that provide at least an equivalent level of protection and reduce the risk so that it no longer constitutes an annual occurrence.

II. *Vibrio parahaemolyticus* Control Plan

A. Triggers

A plan for an area(s) or a state must include control measures for the month(s) in which:

1. The total number of *Vp* illnesses is two or more in a three (3) year period; or

2. The area was epidemiologically linked to an outbreak within the prior five (5) years and the plan must also apply to the period 30 days prior to the first day of harvest of the outbreak and 30 days after the last day of harvest associated with the outbreak; or

3. The average water temperatures representative of harvesting conditions exceed 60 °F for states bordering the Pacific Ocean and 81 °F for states bordering the Gulf of Mexico and Atlantic Ocean (New Jersey and south). See exemption in the NSSP Model Ordinance Chapter II.@.05.B.2.; or

The regulatory authority to administer this plan is [To be filled in by the Authority].

B. Control Measures

1. Post Harvest Processing (PHP).

2. Closing the area to oyster harvest.

3. Restrict oyster harvest to product labeled ~~"For Cooking Only".~~ **for shucking by a certified dealer, or other means to allow the hazard to be addressed by further processing.**

4. Limit time from harvest to refrigeration to no more than five (5) hours or other times based on modeling and sampling in consultation with FDA.

5. Limit time from harvest to refrigeration such that levels of total *Vp* after completion of cooling to 60 °F do not increase more than 0.75 log from levels at harvest. Calculations for 0.75 log increase can be based on the table as shown below or based on validation studies. The authority may use the FDA Risk Assessment to determine the initial "at harvest" levels.

6. The term refrigeration is storage in a container that is capable of dropping and maintaining ambient air temperature of 45 °F (7.5 °C).

7. Other control measures based on appropriate scientific studies.

C. Plan Effectiveness as Demonstrated by:

1. Post Harvest Processing.

Conduct end product testing consistent with PHP verification protocol as provided in the NSSP Guide for the Control of Molluscan Shellfish. Test results shall demonstrate the level of total *Vp* in the final product does not exceed the average levels found in the area at times of the year the state had determined *Vp* illness is not reasonably likely to occur.

Data may be shared between states or other entities as may be appropriate considering the characteristics of the harvest area(s), such as temperature, hydrological patterns, etc. In the absence of such state data, use 100/gm for the Pacific and 1000/gm for the Atlantic/Gulf as provided in the FDA Risk Assessment.

Note: These levels are significantly higher than those allowed in validation/verification to non-detectable. Labeling "for added safety" would not be permitted unless the lower levels were reached.

2. Closing the area to oyster harvest.
Issue a legally binding closure order(s). Conduct Patrol and maintain Patrol records for the area(s) in accordance with the NSSP MO requirements.

3. Restrict oyster harvest to product labeled ~~"For Cooking Only"~~ **for shucking by a certified dealer, or other means to allow the hazard to be addressed by further processing** or "For PHP Only".

   The authority must notify harvesters and dealers of those areas restricted to harvest ~~"For Cooking Only"~~ **for shucking by a certified dealer, or other means to allow the hazard to be addressed by further processing** or "For PHP Only." Harvesters must include on the tag of all product harvested in these areas the statement ~~"For Cooking Only"~~ **for shucking by a certified dealer, or other means to allow the hazard to be addressed by further processing** or "For PHP Only." Dealers must establish a ~~"For Cooking Only"~~ **for shucking by a certified dealer, or other means to allow the hazard to be addressed by further processing** or "For PHP Only" labeling Critical Limit as part of their HACCP plan for receiving. A shipping Critical Control Point must include ~~"For Cooking Only"~~ **for shucking by a certified dealer, or other means to allow the hazard to be addressed by further processing** or "For PHP Only" labeling requirement.

4. Limit time from harvest to refrigeration to no more than five (5) hours or other times based on modeling and sampling in consultation with FDA. Compliance may be documented by State restriction orders, harvester records, dealer records, field records, storage records, harvester education/inspections, records of capable and operating refrigeration.

5. Limit time from harvest to refrigeration such that levels of total Vp after completion of cooling to 60 °F do not increase more than 0.75 log from levels at harvest. Calculations for 0.75 log increase can be based on the table as shown below or based on validation studies. The authority may use the FDA Risk Assessment to determine the initial "at harvest" levels.

6. The term refrigeration is storage in a container that is capable of dropping and maintaining ambient air temperature of 45°F (7.5°C).

7. Other control measures based on appropriate scientific studies

D. Plan Modification

Cost Benefit Analysis (Optional)

Public Health Significance:

Cost Information (if available):

Action by 2009 Task Force II: Recommended adoption of Proposal 09-234 as submitted.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force II on Proposal 09-234.

Temperature Specific *Vp* Growth Rates and Doubling Times for Calculating Cumulative Growth Based on Hourly Temperature Observations					
Oyster Temperature (degree F)	Growth Rate (logs/hr)	Doubling Time (hrs)	Oyster Temperature (degree F)	Growth Rate (logs/hr)	Doubling Time (hrs)
50	0.008	35.8			
51	0.011	28.4	76	0.147	2.05
52	0.013	23.1	77	0.156	1.93
53	0.016	19.2	78	0.165	1.83
54	0.019	16.1	79	0.174	1.73
55	0.022	13.8	80	0.183	1.64
56	0.025	11.9	81	0.193	1.56
57	0.029	10.4	82	0.203	1.48
58	0.033	9.14	83	0.213	1.41
59	0.037	8.11	84	0.224	1.34
60	0.042	7.24	85	0.235	1.28
61	0.046	6.50	86	0.246	1.23
62	0.051	5.87	87	0.257	1.17
63	0.056	5.33	88	0.268	1.12
64	0.062	4.86	89	0.280	1.07
65	0.068	4.45	90	0.292	1.03
66	0.074	4.09	91	0.304	0.99
67	0.080	3.77	92	0.317	0.95
68	0.086	3.49	93	0.330	0.91
69	0.093	3.24	94	0.343	0.88
70	0.100	3.01	95	0.356	0.85
71	0.107	2.81	96	0.370	0.81
72	0.115	2.63	97	0.383	0.79
73	0.122	2.46	98	0.397	0.76
74	0.130	2.31	99	0.412	0.73
75	0.139	2.17	100	0.426	0.71

Note: Growth rate (in logs/hr) = (0.01122*Temp – 0.4689) ^2

Proposal Subject: Approval of the Use of End-Product Testing as an Alternative to Validation of Post Harvest Processes

Specific NSSP Guide Reference: NSSP Guide Section IV. Guidance Documents
Chapter IV. Naturally Occurring Pathogens

Text of Proposal/ Requested Action

.04 Post Harvest Processing (PHP) Validation/Verification Guidance for *Vibrio vulnificus* and *Vibrio parahaemolyticus*

C. **End Product Testing**

Used as an alternative to validation of new shellfish processes to ensure that the end-product contains less than 30 MPN/g of Vv and/or Vp.

Prior to adding labeling claims to the product, the processor must analyze each lot of the finished product in accordance with the NSSP guidance document.

Only lots having less than 30 MPN/g will be allowed to be labeled as PHP. Processor must incorporate the sampling and testing into their HACCP plan and maintain records of HACCP controls as well as laboratory analytical results for all lots tested.

~~C~~D. Initial Load Testing

Initial level of *Vibrios* in shellfish for each lot of shellfish used in validation shall be 10,000 MPN per gram or greater based on the adjusted geometric mean (AGM) of the MPNs/g of four samples where the AGM is given by:

AGM = the geometric mean of the 4 MPNs/g multiplied by an adjustment factor of 1.3

Note: If 4 samples from a lot of shellfish with a true density of 100,000 cells per gram are examined by the MPN procedure, the probability of the geometric mean of the MPNs showing 100,000 or greater is about 50%. In an attempt to improve the probability of samples being accepted when the true density is 100,000/g an adjustment factor of 1.3 was selected based upon statistical analysis.

~~D.~~ E. Verification

Public Health Significance: None

Cost Information (if available): None

Action by 2009 Task Force II: Recommended referral of Proposal 09-235 to an appropriate committee as determined by the Conference Chairman.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force II on Proposal 09-235.

Proposal Subject: Illness Outbreak Investigation and Recall

Specific NSSP Guide Reference: NSSP Guide Section IV. Guidance Documents

Text of Proposal/ Requested Action Add a New Chapter V. titled "Illness Outbreak Investigation and Recall". In this proposed Chapter V. add all new language:

.01 Guidance for Investigating an Illness Outbreak and Conducting Recall; and

.02 Guidance for a Time-Temperature Evaluation of a Shellfish Implicated Outbreak (moved from Chapter III. .05 Guidance For a Time-Temperature Evaluation of a Shellfish Implicated Outbreak to New Chapter V. Illness Outbreak Investigation and Recall.

Add an Additional Guidance box with specific reference to: NSSP Guide Section IV. Guidance Documents, Chapter V. Illness Outbreak Investigation and Recall. .02 Guidance For a Time-Temperature Evaluation of a Shellfish Implicated Outbreak in the NSSP Guide Section II. Model Ordinance Chapter X. 03. B. (1).

(Documents Attached)

Public Health Significance: This proposal is to provide authorities and dealers with guidance on what is required during an illness outbreak and product recall. Guidance provided follows Title 21 Code of Federal Regulations (CFR) Part 7 as required in the NSSP MO Chapter II.@.01.D.(2).

Expeditious follow-up on reported illnesses and effective recall of implicated product are requisite to protecting public health. This guidance document is to assist in that effort.

Cost Information (if available): None – guidance for existing requirement in NSSP Model Ordinance.

Action by 2009 Task Force II: Recommended approval of Proposal 09-236 as amended.

- Under new chapter 5.01A Requirements for the Authority; strike first sentence of paragraph 7. [~~**An immediate closure of a growing area or lease area may not be appropriate when an illness outbreak investigation reveals that the illnesses occurred weeks or months in advance with no subsequent illnesses.** ~~]
- Under new Chapter 5.01 A Requirements for the Authority; strike first bulleted statement in paragraph 10. [~~**Notification of a confirmed illness outbreak that occurred weeks or months prior to notification with no other illnesses revealed in the preliminary investigation**~~]
- Revise paragraph 10 to make one sentence: A product recall may not be appropriate when an illness outbreak investigation reveals, but is not limited to, the implicated product is no longer available in the market.
- Insert new paragraph 15 under Chapter 5.01 A. Requirements for the Authority; **Pursuant to the Model Ordinance Chapter II @01. (C)(4) and (D)(2) an Authority initiated recall shall include procedures consistent with The Recall Strategy as provided in 21 CFR Part 7.41, 7.42 and 7.50 as listed below: [for purposes of this guidance "the Authority" will be substituted for "the agency for a Food and Drug Administration"]**

- Amend old paragraph 15 further to strike duplicate language and create a header for 21 CFR Part 7.41: [**~~"The type of recall needed for any particular situation cannot be specified and is determined by the nature of the recall, as set forth in~~ 21 CFR Part 7.41:**
- CFR citations are italicized and in parens.
- Strike paragraph 17 [**~~The Recall Strategy as provided in 21 CFR Part 7.42]~~**
- Delete paragraph 4 under section B. Requirements for Dealers: [**~~[Press Release Committee – Mike Hickey, Bill Kramer, Kirk Wiles, Lori Howell, Bill Dewey, etc. procedures and states use to respond to public in the case of a recall/illness investigation (get in touch with Mike and Ken].~~**
- Add at the end of section B. Requirements for Dealers a note to direct the committee as determined by the Task Force to reconcile guidance with the Reportable Food Registry requirements.
- Amend text under section C. 2. Requirements for FDA [**Inform other FDA offices as appropriate ~~the Office of Food Safety and Division of Cooperative Programs~~ as new or pertinent recall information from the Authority becomes available; and**
- Revise appendixes A-G to make forms and other examples generic and blank.

Action by 2009 General Assembly

Adopted recommendation of 2009 Task Force II on Proposal 09-236.

NOTE: Editorial changes will be made to Appendices A through G to reflect the intent of Task Force II that Appendices A through G be generic and not include any reference to any particular States or any particular policy or actions with those states.

Section II. Model Ordinance
Chapter III - Harvesting, Handling, Processing, and Distribution

.01 Shellfish Industry Equipment Construction Guide

.02 Shellfish Plant Inspection Standardization Procedures/NSSP Standardized Shellfish Processing Plant Inspection Form

.03 Dealer Certification and the Interstate Certified Shellfish Shippers List

.04 Shellstock Tagging

Section II Model Ordinance
Chapter X. General Requirements for Dealers

.03 Other Model Ordinance Requirements.

A. Each dealer shall comply with the requirements specified in Chapter XI .03, Chapter XII .03, Chapter XIII .03, Chapter XIV .03 and Chapter XV .03 that are appropriate to the plant and the food being processed. However, monitoring and record keeping for these conditions and practices is not required, unless specifically stated.

B. Recalls.

Additional Guidance Section IV. Guidance Documents, Chapter V. Illness Outbreak Investigation and Recall, .02 Guidance For a Time-Temperature Evaluation of a Shellfish Implicated Outbreak

(1) Dealers shall adopt written procedures for conducting recalls of adulterated misbranded shellfish products. These written procedures for conducting recalls shall be based on, and complementary to, the FDA Enforcement Policy on Recalls, CFR Title 21, Chapter 1, Subchapter A., Part 7-Enforcement Policy, (2002 NSSP Guide for the Control of Molluscan Shellfish, Federal Regulations).

(2) Dealers shall follow their written recall procedures to include timely notification of the SSCA of a situation requiring recall, timely notification of consignee who received the affected product, and effective removal or correction of the affected product.

Chapter V. Illness Outbreaks and Recall Guidance

.01 Guidance for Investigating an Illness Outbreak and Conducting Recall

A. Requirements for the Authority.

Shellfish are filter feeders and therefore have the ability to concentrate microorganisms, including human pathogens and toxigenic micro-algae, from the water column if these organisms are present in the growing area. Concentrations in the shellfish may be as much as 100 times that found in the water column. If the microorganisms concentrated are harmful to humans, and if, in the case of human pathogens, the shellfish are consumed raw or partially cooked, human disease can result. Shellfish can also be contaminated during transport and post harvest treatment; i.e. wet storage, etc. Shellfish can be mishandled during processing which can contribute to the growth of existing microorganisms to the point where consumption can cause illness.

Documentation of the information supporting growing area classification, proper tagging and record keeping, expeditious follow-up on reported illnesses, effective recall of implicated product and public warning announcements are all requisite to protecting public health. Shellfish growing areas implicated through epidemiological association between illness and shellfish consumption must be closed immediately to prevent additional implicated product from reaching the consumer. In addition, shellfish product from the implicated growing areas may be detained and an effective recall of product initiated if the investigation determines that it is necessary to protect public health.

When an illness outbreak investigation indicates that there is an epidemiological association between shellfish consumption and the illnesses, the investigating state Authority shall immediately inform the producing state Authority of the illnesses, the stage of the investigation, and epidemiological link to consumption of molluscan shellfish. Prompt reporting, even in the initial stages of an investigation, will allow the producing state Authority to conduct its investigation (in accordance with Chapter II @ .01 B.) and determine whether harvest area closure, notification, and recall are required.

When an illness outbreak has occurred, immediate closure of the implicated growing area(s) will significantly reduce the chance of additional illnesses during the investigatory process. Immediate closure for the purposes of this guidance document means within 24 hours of notification of the illness (Chapter IV. @03. (A)(1)). If a preliminary investigation reveals that the growing area is not implicated, an immediate closure is not necessary. Additional information concerning investigation of an outbreak of shellfish related illness believed to be associated with a naturally occurring pathogen can be found in the NSSP Model Ordinance Guidance Documents: *Guidance for a Time-Temperature Evaluation of a Shellfish Implicated Outbreak* (ISSC/FDA, 2002). Additional information concerning the disease causing potential of shellfish can be found in the NSSP Model Ordinance Guidance Documents: *Sanitary Survey and the Classification of Growing Waters, Guidance for Developing Marine Biotoxin Contingency Plans,* and *Shellstock Relay* (ISSC/FDA, 2002).

The Authority should assign an Illness Investigation/Recall Coordination Lead (the Lead) for the agency to be listed on the ISSC website as the agency contact person. The Lead will be the agency contact for the duration of the event.

During and after the immediate closure, the Authority must be in the process of investigating, evaluating and conducting increased surveillance. Immediate closures will not always result in an immediate recall of product. It is imperative that the Authority communicate with state epidemiologists, local health officials, pertinent state agencies, industry and others as necessary to complete a thorough investigation.

~~An immediate closure of a growing area or lease area may not be appropriate when an illness outbreak investigation reveals that the illnesses occurred weeks or months in advance with no subsequent illnesses.~~ Additionally, immediate closures may not be necessary if the investigation reveals that the illness outbreak was caused by a specific activity by a single entity which can be controlled through a product recall and an immediate corrective action in the processing or transport of product.

An illness outbreak investigation must include an evaluation of the health hazard presented and consideration of the following factors, including but not limited to:

1. Immediately send staff members out to perform growing area reconnaissance,
2. Review documentation of the information supporting growing area classification, review environmental sample trends, secure additional shellstock and/or water samples if necessary
3. Review toxin sample trends, sampling protocol and supporting information for biotoxin closures, secure additional shellstock and/or water samples if necessary
4. Interview local sources regarding any anecdotal or factual information on the origin of contaminants (large passenger vessels, point and non-point sources),
5. Immediately send staff members out to interview certified dealer(s), restaurant staff members or retail establishment staff members to secure additional details regarding tagging, record keeping, refrigeration temperatures, handling practices, shipping and receiving information and where and from whom the shellfish products were purchased, name and telephone number of contact person,
6. When possible, interview harvesters in the area of concern to determine handling practices and specific harvest area(s)
7. Determine the identity of the product involved, the extent of distribution of implicated product, total amount of the suspected product, total amount in distribution chain, distribution information and proposed recall strategy.

A product recall is appropriate when an illness outbreak investigation reveals the following, including but not limited to:

- When the etiological and epidemiological evidence confirms that shellfish from a specific growing area or lease area are the cause of the illnesses
- When it has been determined that a specific process conducted by a dealer is the cause of the illnesses

A product recall may not be appropriate when an illness outbreak investigation reveals ~~the following, including~~ but <u>is</u> not limited to <u>the implicated product is no longer available in the market</u>:

- ~~Notification of a confirmed illness outbreak that occurred weeks or months prior to notification with no other illnesses revealed in the preliminary investigation~~
- ~~If the outbreak investigation reveals that the implicated product is no longer available in the market~~

When the source of the illness is found to be the distribution and processing system, shellfish product should be also detained and an effective recall of product initiated, and the problem immediately corrected. Under these circumstances no closure of the growing waters is warranted in accordance with Model Ordinance, Chapter II, @.01D.

An area which was closed due to an illness outbreak can be reopened using the criteria outlined in the Model Ordinance, Chapter IV @ 03 (A) (5) (c):

(c) Reopened Status. A growing area temporarily placed in the closed status (as provided in (b) above), shall be returned to the open status only when:

(i) The emergency situation or condition has returned to normal and sufficient time has elapsed to allow the shellstock to reduce pathogens or poisonous or deleterious substances that may be present in the shellstock to acceptable levels. Studies establishing sufficient elapsed time shall document the interval necessary for reduction of contaminant levels in the shellstock to pre-closure levels. In addressing pathogen concerns, the study may establish criteria for reopening based on coliform levels in the water; or

(ii) The requirements for biotoxins or conditional area management plans as established in §.04 and §.03, respectively, are met; and

(iii) Supporting information is documented by a written record in the central file.

Whenever an Authority initiates a recall of shellfish products because of public health concerns, the Authority will monitor the progress and success of the recall. The Authority will immediately notify the FDA, Authorities in other states/countries, ISSC and industry involved in the recall. Each Authority involved in a recall will implement actions to ensure removal of recalled product from the market and issue public warnings if necessary to protect public health.

Pursuant to the Model Ordinance Chapter II. @ 01 © (4) and (D) (2) an Authority initiated recall shall include procedures consistent with The Recall Strategy as provided in 21 CFR Part 7.41, 7.42 and 7.50 as listed below: [for purposes of this guidance "the Authority" will be substituted for "the agency for a Food and Drug Administration"]

FDA will decide whether to audit or issue public warnings after consultation with the Authority(ies), and after taking into account the scope of the product distribution and other related factors. After consultation with the Authority(ies) and after taking into account the scope of the product distribution and other related factors, FDA may audit and/or issue public warnings. If the FDA determines that any Authority involved in the recall fails to implement effective actions to protect public health, the FDA may audit, classify the severity of and publish the recall, including the issuance of public warnings when appropriate.

~~The type of recall needed for any particular situation cannot be specified and is determined by the nature of the recall, as set forth in~~ 21 CFR Part 7.41:

Health hazard evaluation and recall classification.

(a) An evaluation of the health hazard presented by a product being recalled or considered for recall will be conducted by an ad hoc committee of Food and Drug Administration scientists and will take into account, but need not be limited to, the following factors:

(1) Whether any disease or injuries have already occurred from the use of the product.

(2) Whether any existing conditions could contribute to a clinical situation that could expose humans or animals to a health hazard. Any conclusion shall be supported as

completely as possible by scientific documentation and/or statements that the conclusion is the opinion of the individual(s) making the health hazard determination.

(3) Assessment of hazard to various segments of the population, e.g., children, surgical patients, pets, livestock, etc., who are expected to be exposed to the product being considered, with particular attention paid to the hazard to those individuals who may be at greatest risk.

(4) Assessment of the degree of seriousness of the health hazard to which the populations at risk would be exposed.

(5) Assessment of the likelihood of occurrence of the hazard.

(6) Assessment of the consequences (immediate or long-range) of occurrence of the hazard.

(b) On the basis of this determination, the Food and Drug Administration will assign the recall a classification, i.e., Class I, Class II, or Class III, to indicate the relative degree of health hazard of the product being recalled or considered for recall.

~~*The Recall Strategy as provided in 21 CFR Part 7.42*~~

§ 7.42 Recall strategy.

(a) General.

(1) A recall strategy that takes into account the following factors will be developed by the agency for a Food and Drug Administration-requested recall and by the recalling firm for a firm-initiated recall to suit the individual circumstances of the particular recall:

(i) Results of health hazard evaluation.

(ii) Ease in identifying the product.

(iii) Degree to which the product's deficiency is obvious to the consumer or user.

(iv) Degree to which the product remains unused in the market place.

(v) Continued availability of essential products.

(b) Elements of a recall strategy. A recall strategy will address the following elements regarding the conduct of the recall:

(1) Depth of recall. Depending on the product's degree of hazard and extent of distribution, the recall strategy will specify the level in the distribution chain to which the recall is to extend, as follows:

(i) Consumer or user level, which may vary with product, including any intermediate wholesale or retail level; or

~~(i)~~(ii) Retail level, including any intermediate wholesale level; or

~~(ii)~~(iii) Wholesale level.

Means of notification, methods of collecting related information, and summary of findings. Recall notification procedures should be standardized to assure compliance with Title 21 CFR, §7.42:

(2) Public warning. The purpose of a public warning is to alert the public that a product being recalled presents a serious hazard to health. It is reserved for urgent situations where other means for preventing use of the recalled product appear inadequate. The Food and Drug Administration in consultation with the recalling firm will ordinarily issue such publicity. The recalling firm that decides to issue its own public warning is requested to submit its proposed public warning and plan for distribution of the warning for review and comment by the Food and Drug

Administration. The recall strategy will specify whether a public warning is needed and whether it will issue as:

(i) General public warning through the general news media, either national or local as appropriate, or

~~(i)~~(ii) Public warning through specialized news media, e.g., professional or trade press, or to specific segments of the population such as physicians, hospitals, etc.

§ 7.50 Public Notification of Recall.

The Food and Drug Administration will promptly make available to the public in the weekly FDA Enforcement Report a descriptive listing of each new recall according to its classification, whether it was Food and Drug Administration-requested or firm-initiated, and the specific action being taken by the recalling firm.

Organization of the recall procedures must take into consideration the need for work week, weekend, and holiday notifications. Various recall notification strategies may be used depending on the nature of the illness outbreak and recall. (See attached Appendix and supporting forms for example of a Recall Standard Operating Procedure)

Complete removal of shellfish from interstate and intrastate commerce is vital for effective recall reaction. Timely notification and reaction by public health officials utilizing the Title 21 CFR, Part 7 requirements and associated State procedures must provide a safeguard against contaminated shellfish reaching the market. In some cases, duplication of the federal requirements by states may be the method selected to assure standardization of necessary steps to ensure effective recalls.

Educational programs should be developed for both industry and the public describing the public health necessity for effective recall notifications and eliminating potentially unsafe shellfish products from the market place. Programs developed specifically for participation of key industry people may be especially helpful in eliciting cooperative efforts of the entire industry. Such programs should focus on incentives to standardize the procedures for effective and timely recall activities.

The adequacy of state procedures as a basis for assuring rapid and thorough reaction to illness outbreaks and product recall efforts is an important component of this activity. Shellfish recall will be ineffective and/or compromised if State procedures are so written or interpreted that effective reaction can not successfully initiated. It is important that consistent recall expectations and notification procedures be standardized by participating public health Authorities in order to effectively safeguard the general public from potentially hazardous food.

When a recall of shellfish products is initiated, the Authority shall:

1. Immediately notify the appropriate FDA Regional Shellfish Specialist of the recall and provide a recall status report every five (5) working days after the initiation of the recall. Subsequent recall monitoring reports should be provided as information is acquired. The recall monitoring report, which may be verbal or written notification, will include the following information:
   a. The name and address of the recalling dealer(s), plus certification numbers;
   b. The identity of the affected product;
   c. The reason for the recall;
   d. Any other actions deemed appropriate to address the recall such as closing the growing area, conducting surveys, conducting monitoring and contacting other agencies, tribes and stakeholders, in regard to possible growing area closures and investigation of the

situation requiring the recall including but not limited to sanitary or shoreline survey activities, water quality factors, and other environmental factors under consideration;

e. All relevant product identification (harvest date, harvest location, date shucked, lot code, quantity etc.); and

f. Distribution and redistribution of all shipments of the suspected lots.

2. Establish procedures that ensure support staff members who are conducting investigation efforts will report provide results of the investigation activities to the Lead to be added to the progress updates and final recall summary report. Activities include:

a. Review illness investigation reports
b. Review facility inspection reports
c. Review harvest site applications/information
d. Review Survey of pollution sources
e. Review marine water quality test results
f. Review Biotoxin test results
g. Draft a summary of growing area findings for pollution, biotoxins, etc. as needed.

3. Prepare a complete recall summary that determines the effectiveness of the recall. The Authority will forward the recall summary documents to the appropriate FDA Regional Shellfish Specialist within five (5) working days of the completion of the recall. The recall summary will include:

a. The quantity, type, and status of recalled products returned to or recovered by the recalling dealer(s);
b. The quantity, type and status (if known) of recalled products not returned to or not recovered by the recalling dealer;
c. The reason for initiating the recall;
d. The date the recall was initiated;
e. The date the recall was completed;
f. Dealer inspection results or other evidence where appropriate; and
g. A listing, in chronological order, of any complaints or injuries associated with the product.
h. Final disposition of all recalled product.
i. All other actions taken to address the recall such as closing the growing area, conducting surveys, conducting monitoring, contacting other agencies, tribes and stakeholders, etc. relating to possible growing area closures and investigation of the situation requiring the recall, such as sanitary or shoreline survey activities, water quality factors, and other environmental factors for consideration.

4. Provide a summary of the details involving the recall to the appropriate state authorities upon conclusion of the recall. Each respective element of the recall activities will be described in sufficient detail to provide adequate trace back information and/or account for providing public health protection as a result of the recall. Upon approval of the report, copies will be provided via email and or hard copy to the FDA Regional Shellfish Specialist and other agencies needing the information.

B. Requirements for Dealers.

When an illness has occurred or has been reported to a certified dealer or harvester, they shall immediately notify the Authority. Immediate notification to the appropriate agency will significantly reduce the chance of additional illnesses and will limit the duration and extent of any precautionary growing area closures and product recalls.

The Authority will provide the contact information for the Illness Investigation/Recall Coordination Lead (the Lead) for the agency. The Lead will be the contact for the duration of the event.

The affected industry must cooperate with the Authority during the investigation and evaluation. It is imperative that the industry and the Lead communicate as necessary to complete a thorough investigation.

~~[Press Release Committee – Mike Hickey, Bill Kramer, Kirk Wiles, Lori Howell, Bill Dewey, etc. procedures and states use to respond to public in the case of a recall/illness investigation (get in touch with Mike and Ken].~~

If the investigation reveals that the source of the illness is found to be the distribution and processing system, shellfish product should be detained and an effective recall of product initiated. The investigation may reveal a problem with the processing of product, if that is the case, the Authority should work with the processor to immediately correct the problem.

Whenever a certified dealer conducts a recall of shellfish products, the dealer shall:

1. Follow the written recall procedures adopted in accordance with Model Ordinance, Chapter X, .03 B.(1) and (2);
2. Immediately notify the Authority which is responsible for the enforcement of shellfish sanitation, unless directed initially by the Authority; that a product recall has been initiated; and
3. Immediately notify the receiving shipper(s) or other receiver/user that a product recall has been initiated;
4. Provide the Authority and the receiver of the product with:
    a. The type and quantity of shellfish being recalled,
    b. the name and license or permit number of each harvester or shipper certification number, as necessary,
    c. The harvest area, and
    d. The date(s) of harvest and shipment as they appear on the shipping tag or invoice;
5. Direct each receiver of the recalled product to examine their receiving records and invoices and report:
    a. The quantity of product received,
    b. The quantity remaining,
    c. The quantity shipped and to whom, including name, address, phone number and date of reshipment, and
    d. All product being held and considered embargoed;
6. Advise the receiver that:
    a. The product is not to be sold or shipped;
    b. Unless advised otherwise by the Authority, the product is to remain on the premises until the Authority representative or other designee arrives;
    c. When appropriate, they should notify their customers who received the product about the recall; and
    d. All receiving and shipping records and invoices for implicated products are to be available for inspection by the Authority's officials.
7. Provide a recall status report to the Lead every five (5)) working days after the initiation of the recall. Subsequent recall monitoring report, which may be verbal or written notification. Unless other wise specified or inappropriate in a given recall case, the recall progress update should contain the following information:
    a. Number of consignees notified of the recall and the date and method of notification;

b. Number of consignees responding to the recall communication and quantity of products on hand at the time it was received;
c. Number of consignees that did not respond (if needed, the identity of non-responding consignees may be requested by the Authority and the Food and Drug Administration);
d. Number of products returned or corrected by each consignee contacted and the quantity of products accounted for;
e. Number and results of effectiveness checks that were made; and
f. Estimated time frames for completion of the recall.

The dealer must fulfill any additional reporting requirements in accordance with the FD&C Act (need specific citation when it becomes available).The recalling dealer has the initial responsibility for determining if the recall is progressing satisfactorily. It is also the obligation of all recalling dealers to determine the effectiveness of their recall. Effectiveness checks aid in verifying that all known, affected consignees received notification about the recall and have taken appropriate action.
Reconcile with Reportable Food Registry information.

C. Requirements for FDA.

Whenever a certified dealer conducts a recall of shellfish products, the FDA Regional Shellfish Specialist shall:

1. Monitor the Authority and FDA actions ensure that the product recall is consistent with the requirements of the NSSP Model Ordinance;
2. Inform other FDA offices as appropriate ~~the Office of Food Safety and Division of Cooperative Programs~~ as new or pertinent recall information from the Authority becomes available; and
3. Coordinate all FDA and other federal assistance provided, as necessary, to affected states.

D. Dispute Resolution.

The ISSC recognizes that states should be allowed to appropriately respond to public health emergencies that could restrict interstate shipment of shellfish. In instances where prudent action is not taken by a state during recall or illness outbreak situations, an Authority or FDA must notify the Executive Board regarding the state's decision and rationale for taking an action or failure to take an action. The Authority should provide the rationale for the proposed action by describing, at a minimum:

- The potential effect on the public health within that state;
- The potential effect on the public health in other states;
- The potential economic impact on states;
- The necessity for the action within the proposed timeframe

The ISSC will consider the rationale of the Authority and the Executive Board may decide to contact the appropriate agency head or Governor in order to secure prudent public health protection. In the event that action is not taken after deliberation between the Conference and the State, the ISSC may recommend the State as an unresolved issue under the ISSC Constitution, By-Laws and Procedures, Procedure IX. Section 3.

.02 Guidance for a Time-Temperature Evaluation of a Shellfish Implicated Outbreak

Because shellfish are filter feeders, they can concentrate microorganisms, marine biotoxins and poisonous or deleterious substances from the water column when these substances are present in the growing area. In addition, shellfish, like any other food product, can become unfit for human consumption through the introduction of contaminants during handling, storage, transport, distribution and processing.

Furthermore, improper handling and storage can contribute to the increase of naturally occurring pathogens to hazardous levels in-shellfish meats. The intrinsic risk from illness induced by microorganisms associated with consumption of raw or partially cooked shellfish products compels the shellfish control authority to act quickly and effectively when shellfish are implicated in a food-borne outbreak. When illness has occurred, the Authority needs to immediately begin an investigation before critical evidence is inadvertently lost or destroyed.

Currently, the NSSP Model Ordinance does not call for any action if illness is limited to only one person. This is appropriate for molluscan shellfish borne illness caused by microorganisms associated with pollution events. However, when naturally occurring marine bacteria such as *Vibrio vulnificus* or *Vibrio parahaemolyticus* are suspected to cause the illness an evaluation of the possibility of time-temperature abuse of the product is critical to understanding how the illness may have been prevented. A time-temperature audit provides information regarding the time-temperature experience of the product implicated as well as the health conditions of any ill persons which may have contributed to their susceptibility to the disease. Although the gathering of this data has been a public health focus for several years, there has been no effort to standardize how or what data are gathered during an illness investigation. When naturally occurring marine bacteria are believed to be the source of the shellfish implicated illness or outbreak, the time-temperature history of the product and the health of the persons may be more relevant than the traditional investigatory focus on tracing the origin of the product back to the shellfish growing area.

For additional information concerning the *Vibrio* organisms, see Watkins and McCarthy (1994) and the NSSP Guidance Documents contained within Chapter IV- Naturally Occurring Pathogens..

Time-Temperature Evaluation of a Shellfish Implicated Outbreak

The Authority should promptly conduct an audit of the time-temperature history of the implicated product in a shellfish disease outbreak to the extent practicable. The Authority should use all records from any measuring devices in conveyances or coolers used to transport the product, or any records of conditions associated with the implicated product as it moved from harvest to consumption. Where necessary, the Authority in the state of shellfish product origin should be contacted to provide assistance in gathering information. The audit must include the retail market or restaurant where the victim bought the shellfish product, the facility of the person who sold the product that the retail market or restaurant, the facilities of all dealers and common carriers who handled the product following its harvest, and the practices and facilities of the person who harvested the shellfish. The audit should include, but should not be limited to, the following points.

In the retail market or restaurant implicated in the shellfish illness outbreak, the Authority should, at a minimum:

Record the ambient temperature in the establishment; observe the time-temperature control in the establishment, i.e. how the product was handled:

Examine the establishment's records for the temperature of the storage device or facility used for the implicated product while at the establishment, or observe and record the temperature of the storage device or facility during the investigation; observe and record the temperature and age of the remaining product at the establishment. The age of the product must be cross checked with transaction records;

Observe the controls to prevent cross contamination of the implicated product; and provide for the immediate sampling and testing for the suspect organism(s) of any remaining product from the retail or food service location implicated in the outbreak.

The Authority should determine if the dealer or person who sold the product to the retail market or the restaurant is on the ICSSL. If the person is not on the ICSSL, the Authority should gather any pertinent information regarding the status of time-temperature controls practiced by this person such as:

- Inspection reports for the person's facility;
- Observed temperature of the person's conveyance used to transport shellfish product; and
- Presence or absence of adequate refrigeration capability in the person's conveyance.

If the dealer is on the ICSSL, the Authority should conduct an inspection of the dealer's facility and records for purposes of gathering data from time-temperature control procedures and practices at that facility including:

- The presence or absence of adequate refrigeration capability of the dealer's conveyance;
- The presence or absence of temperature records for the delivery conveyance;
- The observed temperature and time-temperature control practices on the dealer's loading dock;

The transaction records demonstrating the product's age from the date of harvest of the implicated product; and

- The dealer's observed product rotation practice (i.e., the existence of product of widely differing ages).

For additional information concerning the ICSSL, see the NSSP Guidance Document, Chapter III .03: *Dealer Certification and the Interstate Certified Shellfish Shippers List*. The Authority should gather data similar to that above from all dealers or common carriers (certified or uncertified) between the point of first receipt from the harvester and the retail market or restaurant.

The Authority should inspect the original dealer's facility (i.e. the point of first receipt from the harvester). If the original dealer's facility is in another state, the Authority should request the appropriate Authority in that state to perform an audit and to share the results of the audit. This audit should, at a minimum:

- Determine if there are adequate provisions for product refrigeration;
- Observe temperature and/or records of temperature for the dealer's refrigeration facility;
- Observe general time-temperature control procedures and practices; and
- Observe the temperature and age of shellfish product on-site under receipt from harvesters or under storage.

To the extent practicable, the Authority should gather information concerning the time-temperature control capability of the harvester of record for the implicated product. If the product was harvested in another state, the Authority should request the appropriate Authority in that state to perform an audit and to share the results of the audit. This audit should, at a minimum, determine:

- If adequate shading was provided for harvested shellfish product;
- The existence of mechanical refrigeration for storage of harvested product; and
- If records of prior enforcement actions against the harvester exist.

In cases where *Vibrio* species are the suspected organisms causing the illness or outbreak, the Authority should investigate the health status of the victim(s) to determine:

- If there were underlying health problems which may have contributed to the occurrence of the illness(es);
- If the victim(s) was aware of his underlying condition;
- If the victim(s) was aware of his high-risk status;
- If the victim(s) had been advised not to consume raw shellfish; and
- If the establishment had posted point-of-sale information for high-risk consumers.

References

- Watkins, W. and S. McCarthy. 1994. *Proceedings of the 1994 Vibrio vulnificus Workshop.* U.S. Department of Health and Human Services, Public Health Service, Office of Seafood (HFS-400), Shellfish Sanitation Branch, 200 C Street, SW, Washington, D.C. 175 pages.

APPENDIX A.

CHECKLIST FOR RECALLS, CLOSURES AND SPECIAL EVENTS		
Specific Event: Date of Event:	Date Office Notified:	Date Office Action Initiated:

Task	Staff Initials	Date
Initial shellfish related illness outbreak/hazardous event reported by: Name: Title: Phone: Organization:		
Office Director informed of outbreak/event: ☐ No ☐ Yes		
Food Safety Manager informed of outbreak/ hazardous event: ☐ No ☐ Yes		
Growing Area Manager informed of outbreak hazardous/event: ☐ No ☐ Yes		
Licensing and Certification Manager informed of outbreak/ hazardous event: ☐ No ☐ Yes		
Recall Required: ☐ No ☐ Yes Initiated on date:		
Assistant Secretary informed of outbreak/event: ☐ No ☐ Yes		
Notification to FDA Regional Shellfish Specialist (within 24 hours of Notice): ☐ No ☐ Yes		
Alert to Media ☐ No ☐ Yes (If yes, attach press release)		

Notification to Epidemiology / Public Health Laboratory: (obtain tracking #) Phone: (enter number)

Person Contacted	Tracking Number(s)	Staff Initials	Date

Notification to Local Health Jurisdiction(s) or Tribes (if more space is needed, attach page – Attachment 1)

LHJ /Tribe	Phone #	Person Contacted	Staff Initials	Date
a.				
b.				
c.				
d.				
e.				
f.				

Notification to Receiving State(s) / Country(s) (if more space is needed, attach page – Attachment 2)

State/Country	Phone #	Person Contacted	Staff Initials	Date
a.				
b.				
c.				
d.				

e.				
f.				
Notification of Involved Companies (if more space is needed, attach page – Attachment 3)				
Growers/Dealers				
Company	Phone #	Person Contacted	Staff Initials	Date
a.				
b.				
c.				
d.				
e.				
f.				
Food Safety Investigation				
Item		Person Responsible	Staff Initials	Date
a.	Illness report summary			
b.	Biotoxin Results survey			
c.	Alert Notifications to Retail (Industry List-Serve)			
License & Certification Investigation				
Item		Person Responsible	Staff Initials	Date
a.	Facility Inspection Survey			
b.	Harvest Site Survey			
c.	Recall actions/Report Summary			
d.	Laboratory Sample Submission /Results with EPI/PHL tracking Number			
Growing Area Investigation:				
Item		Person Responsible	Staff Initials	Date
a.	Pollution Source Survey			
b.	Marine Water Quality Results			
c.	Fresh Water Quality Results			
Item		Person Responsible	Staff Initials	Date
Closure Order Actions coordinated with ACO / AAG offices / Assistant Secretary				
Closure Order Issued on date:				
Closure Order Lifted on date:				
Final Report Summary completed				

Distribution of Final Report			
Signature Verifying that all activities for this recall have been completed			Date
ACTION	COMMENTS	Staff Initials	Date

APPENDIX B

(DATE)

(Example Effective Area) Recall Investigation Summary Report

SUMMARY:

Starting at approximately 6 PM on Tuesday (DATE), the operator of the (Example) wastewater treatment plant (WWTP) noted elevated color in the influent and elevated flows from storm inflow and infiltration (I/I). These were occurring due to a rain storm. The plant collected a fecal coliform effluent water sample at 3 p.m. on (DATE) and the result was 'too numerous to count' (TNTC). On (DATE) at 11:20 AM the plant called the department and reported the high result. The operator started injecting chlorine to supplement the normal UV disinfection upon getting the results. The operator stated that all treatment hardware was in good working order and speculated that the TNTC result was due to the elevated color in the influent interfering with UV disinfection. He also reported that influent flows for (DATE) were about 50% above permitted maximum month design flows for the facility. The insufficient disinfection impacted an estimated 1.2 million gallons of sewage in a 48-hr period.

Based on the fecal coliform sample result collected on (DATE) the (EXAMPLE AREA) growing area Conditionally Approved Area Management Plan was implemented and the department closed the Conditionally Approved area for five days from (DATE) until (DATE). Growers were notified of the closure by 12:30 PM on (DATE).

All shellfish products harvested after 12:01AM on (DATE) were recalled. Licensed companies involved were; (EXAMPLE COMPANIES INVOLVED with certification numbers). The two (EXAMPLE COMPANIES) licensed companies did not harvest on that date. (EXAMPLE COMPANY) shipped products to 19 customers in XX State. (EXAMPLE COMPANY) also shipped to 16 customers in 12 other receiving states. A total of 13 states were involved in this recall. All states were notified by email on (DATE) at 9:00 AM via email by the Department of Health.

The amount of (state) product recalled was 3,910 lbs of mussels, 190 lbs of clams and 370 dz oysters. The amount of out of state product recalled was 750 lbs of mussels, 925 lbs of clams and 1,110 dz oysters. Total amount of product recalled was 4,660 lbs of mussels, 1,115 lbs of clams, and 1,480 dz oysters. Out-of-state shellfish products shipped to 11 receiving states have been destroyed by the receiving states. Shellfish products shipped to and located in (state) and (state) have been picked up, returned to the dealer or destroyed on site. Of the shellfish returned back to the dealer, the mussels were destroyed by the dealer at the local landfill and the returned oysters and clams were placed back into wet storage in (EXAMPLE COMPANY).

ILLNESS REPORT SUMMARY:

Not Applicable – no reported illnesses involved.

HARVEST SITE REVIEW:

The source of the product harvested was verified as licensed and certified by the (Authority), as (EXAMPLE COMPANY).

RECALL OF PRODUCT:

Recall of product was initiated Thursday, (DATE) at 12:20 PM following notification by the department. States involved in the recall are listed below:

US States Shellfish Shipments

STATE	Dealer & Retail company receiving product	Species	Quantity	Product Disposition
Total				

XX State Shellfish Shipments

STATE	Dealer & Retail company receiving product	Species	Quantity	Product Disposition
Total				

GROWING AREA INVESTIGATION:

There was no post-closure shoreline survey.

Water Quality Testing: The (EXAMPLE COMPANY) growing area CAAMP was implemented and the Conditionally Approved area was closed for five days from January 7 (when the TNTC sample was taken). Growers were notified of the closure by 12:30 p.m. on (DATE).

Growing Area Classification Review: Not applicable.

Growing Area Closure: On (DATE) (EXAMPLE COMPANY) growing area was formally closed (e-mail listserve notice); starting (DATE)

Water Quality Results: Not applicable.

SHELLFISH OPERATIONS/FACILITIES INVESTIGATION: Not applicable.

BIOTOXIN MONITORING RESULTS: Not Applicable.

CONCLUSION AND SUMMARY OF ACTIONS:

Recall of Product Confirmation: B & out of state shellfish products recalled.

Reopening of (EXAMPLE) growing area: The (EXAMPLE) growing area was re-opened on (DATE). The 5-Day closure was based on the CAAMP implemented for (EXAMPLE) Growing Area by the department.

Questions should be directed to (EXAMPLE) Point of Contacts.

Name and Title of reporting person and/or State Lead

APPENDIX C

Authority
(Name and Address)
Standard Operating Procedure

1. SUBJECT: Shellfish Recall Program

2. REFERENCES:

   a. Title 21 CFR, Part 7, Enforcement Policy
   b. NSSP 2007 Model Ordinance, Chapter II Risk Assessment and Risk Management
   c. (enter appropriate Authority Rule)

3. PURPOSE: The purpose of this Standard Operating Procedure (SOP) is to provide specific instructions for assigned staff performing a recall of bi-valve molluscan shellfish product.

   3.1 Recalls will be determined based on whether a product's wholesomeness is questioned by:
      a. Pollution events
      b. Biotoxin events/Vibrio parahaemolyticus events
      c. Illness events

d. Post-harvest contamination

4. PROCEDURES:

4.1 Establishment of Recall Control:

Shellfish product recalls are of paramount importance. The Authority will assign a Recall Coordination Lead for each situation involving a shellfish recall. Coordination of support staff needed will be made by the Recall Coordinator. Assigned support staff will be responsive to recall activities and will participate as directed by the Authority. Support staff is expected to accomplish work related to a recall in an expeditious manner and with a great sense of urgency. Recall activities will take priority over normally assigned work. The Authority and Recall Coordination Lead will assure that the following are promptly notified:

4.2 Notifications:

4.2. a) The Office Director:

This can be in person, by email or telephone with sufficient detail indicating either harvest or post-harvest origin to confirm the need for a recall. With confirmation by the Director, the appropriate Manager and the Recall Coordination Lead will specify the type of recall classification per 21 CFR, Part 7.

4.2 .b) The Assistant Secretary;

This can be done in person, by email or by telephone with sufficient detail to provide for awareness of the situation.

4.2. c) The Growing Area Section Manager:

This can be done in person, by email or by telephone with sufficient detail to assist in determining appropriate actions such as closing the growing area, conducting surveys, conducting monitoring, contacting other agencies, tribes and stakeholders, etc. relating to possible growing area closures and investigation of the situation requiring the recall, such as sanitary or shoreline survey activities, water quality factors, and other environmental factors for consideration.

4.2.d) The appropriate shellfish dealers and/or growers:

The industry will be contacted in the most expedient manner concerning recall instructions. The Recall Coordination Lead will organize staff to immediately notify each shellfish grower involved with the recall in person, by email or telephone. The Harvest Site Program Lead will provide involved staff a printed copy of each Dealer/Grower involved in the recall and a clear and detailed script of the recall message to provide to each grower.

4.2.e) The Food and Drug Administration (FDA):

This can be in person, by email or telephone within 24 hours with sufficient detail to provide for awareness of the situation. The FDA will be notified of all interstate commerce distribution by providing the list of receiving states and/or foreign countries receiving the product. The FDA will notify foreign countries and non-Interstate Shellfish Sanitation Conference member states upon request by the Office of Shellfish and Water Protection. The Recall Coordinator will provide appropriate Recall Status Reports in accordance with CFR Part 7 to FDA as required.

4.2.f) The Interstate Shellfish Sanitation Conference (ISSC):

This can be by email or telephone within 24 hours with sufficient detail to provide for awareness of the situation. The purpose of ISSC notification is for their assistance in notifying all identified receiving states. The FDA will be an addressee on this email for notification of receiving states and/or countries that a potential health risk is associated with recalled shellfish involved with the recall.

4.2.g The Public Health Laboratory (PHL) and Communicable Disease Epidemiology:

This can be in person, by email or telephone with sufficient detail to provide for assistance in the tracking or special sampling of illness sources for laboratory support. PHL will assign a tracking number for clinical samples for tracking purposes. Sample collection and submission is coordinated by the Recall Coordination Lead. Samples are tested at the Authority Public Health Laboratory.

4.2.h) The appropriate Local Health Jurisdictions:

This can be in person, by email or telephone with sufficient detail to provide for awareness and/or assistance in the recall.

4.2.i) The Recreational Shellfish Program Lead:

This can be in person, by email or telephone with sufficient detail to provide for assistance in posting an advisory message on the Program website, coordinating signage with local health jurisdictions, and providing educational materials to local health jurisdictions and other stakeholders.

4.2.j) The Food Safety Program:

This can be in person, by email or telephone with sufficient detail to provide for awareness of the situation. The Recall Coordination Lead will notify the appropriate Food Safety Program, of the recall. The Recall Coordination Lead will provide sufficient details to allow the Food Safety Program to determine how best to assist the retail food industry for awareness of the recall and any supportive assistance from local health jurisdictions at the retail level.

4.2.k) The Communications Office:

This can be in person, by email or telephone with sufficient detail to provide for awareness of the situation. The Office Director may decide to issue a News Release announcing a recall. Coordination with the Communications Office will be made prior to any news release. Joint effort will be made with the Communications Office to provide a clear and concise news release providing the details of the situation. The Office Director, Section Manager and Recall Coordination Lead will work closely with the communications staff to develop the news release in a timely manner.

4.3 Recall Activities:

4.3.a) The Recall Coordination Lead will promptly provide information relevant to a recall to the shellfish industry by using the shellfish list serve contact email system and/or by official mail. The recall Coordination Lead will provide sufficient details to ensure clear directions and expectations for Dealer/Growers to provide swift disposition of product within 48 hours to the office.

4.3.b) The Recall Coordination Lead will monitor the progress of the recall and ensure prompt contact with other state agencies, appropriate agencies in other states (with assistance from the ISSC), and the ministries of health or appropriate ministries according to protocol in foreign countries (with assistance from the USFDA), and with shellfish companies involved.

4.3.c) The Recall Coordination Lead will maintain detailed records of the recall, to include records of product destroyed and/or recalled. The Recall Coordination Lead will coordinate with staff in the completion of related recall notification contact forms and other summary reports related to the recall. The Recall Coordinator will maintain all related records when completed on file both in hard copy and electronically on the shared drive.

4.3.d) The Harvest Site Lead will assist in providing the current list of Dealers/Growers involved in the recall. A printed list will be provided to the Recall Coordination Lead and support staff involved in the notification process.

4.3.e) The Recall Coordination Lead will ensure that support staff who are conducting investigation efforts will provide summaries of the review to be added to the final recall summary report. Activities include:

a) Review illness investigation reports
b) Review facility inspection reports
c) Review harvest site applications/information
d) Review Survey of pollution sources
e) Review marine water quality test results
f) Review Biotoxin test results
g) Drafting a summary of growing area findings for pollution, biotoxins, etc. as needed.

4.4 Enforcement:

4.4.a) The Section Manager and Recall Coordination Lead will work with the Enforcement Coordinator, Growing Area staff and Administration support staff in coordination of recall and/or growing area closure orders (if needed) with the ACO/AAG Offices.

4.4.b) The Section Manager and Recall Coordination Lead will coordinate the publishing of an abatement order for any licensed shellfish operations that are involved as to the cause of a recall with the Section Administrative Assistant (AA) to contact the Adjudicative Service Unit (ASU) for a docket number to identify the order.

4.4.c) The Section Manager and Recall Coordination Lead will coordinate with the Enforcement Coordinator to draft the needed abatement order and will provide the draft to the AA for final preparation and submission to the Office Director for review and approval signature. Upon approval and signature the order will be mailed by certified mail to each grower involved.

4.4.d) The Section Manager and Recall Coordination Lead will coordinate any needed amendment of any abatement order based on situational changes such as re-opening, extensions and/or modifications. The AA will contact the Adjudicative Service Unit (ASU) for a new docket number to identify the changed order. The AA will draft the amended order for final preparation and submission to the Director for review and signature. Upon approval and signature the order will be mailed by certified mail to each grower involved.

4.5 Final Recall Summary Report:

The Recall Coordination Lead will complete the recall summary report. A summary of the details involving the recall will be made and provided to the Office Director upon conclusion of the recall. Each respective element of the recall activities will be described in sufficient detail to provide adequate trace back information and/or account for providing public health protection as a result of the recall. Upon approval of the report, copies will be provided via email and or hard copy to the FDA Regional Shellfish Specialist and other agencies needing the information. Hard copies will be filed according to the office retention schedule and kept electronically on the shared drive under the Recall Program.

5. RELATED FORMS:

a. Authority Checklist for Recall Notification/Events
b. Harvest Site Dealer/Grower list(s)
c. Support Staff Recall Script
d. Investigation Summary Reports (Facility/Growing Area/Laboratory)
e. Recalled Product Disposition Summary Sheets
f. Final Recall Summary Report

6. RELATED DATABASES:

Shared Drive EH/SF/Recall Program

7. AUTHORITY:

Name of SSCA Authority

APPENDIX D

[Insert Name of State] State Licensed Shellfish Company

RECALL PROCEDURES

Company Name: _____ Certification Number: _____

This recall procedure is to be kept on file by your company in an easily-accessible location.

Should the (Authority) or a Dealer/Grower (Firm) initiate a recall of shellfish product because of public health concerns, the Authority will monitor the progress and success of the recall. The Authority will immediately notify the Food and Drug Administration (FDA) and the Authorities in other states if products involved in the recall have been distributed outside of Washington State. Each Authority involved in a recall will implement actions to ensure removal of recalled product from the market and issue public warnings if necessary to protect public health. The FDA will decide whether to audit or issue public warnings after consultation with the Authority(s) and after taking into account the scope of the product distribution and other related factors. If the FDA determines that the Authority in any state involved in the recall fails to implement effective actions to protect public health, the FDA may classify, publish and audit the recall, including issuance of public warnings when appropriate.

The Authority will monitor the progress and success of all recalls within (enter State).

Should there be a need to initiate a recall either by direction of the Authority or by a licensed shellfish company, you are required to adhere to the following:

1. Promptly follow the directions of the Authority in reacting to a recall and/or promptly notify the Authority by telephone when any situations come to your attention which could warrant initiating a recall. These situations could be any reports of illness, biotoxin closures, sewage spills, petroleum products spills, etc.

2. Once informed that a Authority directed recall or a Firm-initiated recall is implemented promptly contact each of your customers by telephone or in person and notify them about the recall. Direct your customers to stop all sales and secure any products involved in the recall that may still be on hand.

3. Properly identify each bag/container of shellstock involved in the recall with an On-Hold for Recall placard or marker with date and separate them from other products not involved in the recall. These recall products must be properly secured.

4. Properly identify each container of shucked meats involved in the recall with an On-Hold for Recall placard or marker with date and separate them from other products not involved in the recall. These recall products must be properly secured.

5. Request that your customers report back to you as soon as possible, but no later than 24 hours, where the recalled products were distributed and whether your customers still have any product on hand. Maintain an accurate Recall Account Summary Report of products sold to each of your customers and the current disposition of the products:

    - Amount sold to each customer during the recall period
    - Amount still on hand at your facility
    - Amount still on hand at each of your customers facilities
    - Amount already sold and consumed and not returnable by each of your customers

6. If there is recalled product, you will instruct your customers to return the product to you for proper securing of it in your facility or to hold it in a separate location at their facility and clearly mark it as not for sale and wait for final disposition instructions.

7. You will promptly notify the Authority as to where the entire recalled product is located. You will coordinate with the Authority or the local health jurisdiction in your area to witness destruction of the product. If required, all product returned to you will be destroyed in the presence of a witness from the Authority or a local or state health jurisdiction, or if approved, you may place shellstock product back in the original growing area on an approved harvest site. You will provide a Recall Account Summary Report of the recalled product to the Authority within 48 hours.

8. A list of your current direct customers and their telephone numbers will be maintained in your records for recall notification.

The Authority contact telephone numbers for recall notification purposes are (enter telephone number) during business hours and (enter telephone number and or pager number) during non-business hours.

The following customer notification list is for your use in contacting your customers.

RECALL CUSTOMER NOTIFICATION LIST

Shellfish Customer	Manager or Contact Person	Phone Number

RECALL SUMMARY ACCOUNT REPORT EXAMPLE

The following Recall Summary Account Report is an example of the information required by the Authority when completing recall notifications. Each company directly involved in distribution of shellfish included in a recall is required to provide this type of summary account report. Reports will be faxed to (enter fax number) and an original copy mailed to the Authority.

EXAMPLE:

Date:

From: Name of Company
Address of Company
Certification Number: i.e. WA-0000-SS

To: State
Attention: Recall Coordinator
Address

Subject: Recall Summary Account Report for (List Area and Date)

Attached is the final Recall Summary Accounting Report for (insert name of company) providing the final disposition of all shellfish products involved and distributed in the recall of (enter date).

Recall Summary Account Report for (enter location and date)					
Product	Customer Shipped To	Quantity Shipped to Customer's Location	Quantity Still on hand at Customer's Location	Quantity Returned Or Destroyed	% Returned Or Destroyed
Mediterranean Mussels *(pounds)*					
Manila Clams *(pounds)*					
Geoduck Clams *(pounds)*					
Razor Clams *(pounds)*					
Kumamoto Oysters *(dozen)*					
Pacific Oysters *(dozen)*					
Shucked Oyster Meat *(pounds/ounces)*					
Other Species (if applicable)					

Any questions should be directed to (insert name and telephone number of person and email address and fax number).

Signature Block of Company Owner/Manager

APPENDIX E

NAME OF CLOSED GROWING AREA:	
DATE OF CLOSURE:	
REASON FOR CLOSURE:	

CHECKLIST FOR RECALL EVENTS

Growers/Dealers Identified	Person Contacted	Phone Number	Harvest Status	Staff Initials	Date

APPENDIX F

NAME OF CLOSED GROWING AREA:	
DATE OF CLOSURE:	
REASON FOR CLOSURE:	

 CHECKLIST FOR RECALL EVENTS

LHJ / Tribe	Person Contacted	Phone Number	Staff Initials	Comments	Date	Time

APPENDIX G

NAME OF CLOSED GROWING AREA:	
DATE OF CLOSURE:	
REASON FOR CLOSURE:	

CHECKLIST FOR RECALL EVENTS

State/Country	Person Contacted	Phone Number	Staff Initials	Comments	Date	Time

Proposal Subject: Post Harvest Processing

Specific NSSP Guide Reference: NSSP Guide Section III. Public Health Reasons and Explanations

Text of Proposal/ Requested Action Add a new section for Post Harvest Processing.

Chapter XVI.

Background & Performance of Post Harvest Processing (PHP) Validation/ Verification Protocols

BACKGROUND:

A post harvest process (PHP) to reduce the levels of pathogenic *vibrios* in shellfish, must be capable of reducing potentially high summer levels to a level that presents a negligible health risk. Cook et al 2002 indicated that a concentration of *Vibrio parahaemolyticus* or *Vibrio vulnificus* of 100,000 per gram was not uncommon in market oysters harvested from the Gulf Coast during summer months. A WHO/FAO (2005) risk assessment indicated that a *Vibrio vulnificus* concentration of below 30 per gram is a negligible health risk. Therefore, in an attempt to validate a post harvest process to be used throughout the year, the ISSC adopted as interim guidance, a protocol to assure that the process is capable of reducing levels of *vibrios* from an initial MPN level of 100,000/gram to <30/gram.

Obtaining an initial level of 100,000/gram was difficult to achieve consistently in some locations (even with temperature abuse) except during the hottest part of the summer. This limited the time that a validation could be conducted to 3 months of the year or less. In an attempt to allow validation during other times of the year, the ISSC proposed a validation procedure based upon a 3.52 log reduction (this is equivalent to reducing from 100,000 to 30) regardless of the initial level. A new validation protocol was developed which specified an initial level between 10,000 and 100,000 and reduction by 3.52 logs resulting in a final concentration of <30.

VALIDATION:

Validation is the initial check of a PHP to assure that the process can reduce the concentration of *V. vulnificus* and *V. parahaemolyticus* in shellfish by 3.52 logs and to levels <30 as shown in table 1. Determining the log reduction for validation uses knowledge of both the initial and final concentrations. The interval containing the initial concentration determines a test on a single sample for the final concentration. A multiple dilution test is preferred for finding a concentration and the single dilution for indicating whether the concentration is above a threshold. For the initial concentration, a serial dilution with three tubes at each of three or four dilutions was chosen. Four samples are taken to determine the initial concentration and the adjusted geometric mean is used to combine the MPN results. If four samples from a lot of shellfish with a true concentration of 100,000 per gram are examined by the MPN procedure, the probability of the geometric mean of the MPNs showing 100,000 or greater is about 50%. In an attempt to improve the probability of samples being accepted when the true concentration is 100,000 per gram, an adjustment factor of 1.3 was selected based upon examining tables of the probability of getting various results from simulated outcomes.

For a process to be validated, no more than three samples out of 30 may fail. Depending upon the initial load, failure of a single sample is determined according to the table below.

TABLE 1

AGM Interval	Grams Per Tube	Positive Tubes Allowed
59,995 or Greater	.01	2
37,174 – 59,994	.01	1
23,449 – 37,173	.1	4
12,785 – 23,448	.1	3
10,000 – 12,784	.1	2

The choice of intervals for each test in table 1 tried to keep the probabilities near the original test. The original test used .01 grams/tube and allowed 2 of the 5 tubes to have growth. It tried to test for 30 cfu/gram. At 30cfu/gram the probability of a tube with .01 grams of homogenate not having growth equals exp (-30*.01) from a Poisson. From putting this value into a binomial at a final concentration of 30 cfu/gram a single sample has a probability of passing the original test of .88656. The table gives initial concentrations which can be converted to target final concentrations by multiplying by 30/100,000 ≈ -3.52 $\log_{10}$.

A change from one test to another was done at a concentration where the probability of passing both tests was the same distance from the probability of passing the original test. For example, an initial concentration of 59,995 becomes a target final concentration of

(30/100,000) * 59,995 = 17.9985

At this target final concentration and .01 grams/tube the probability of 2 or fewer growth tubes equals .96562. The probability of 1 or fewer growth tubes equals .80751. Since .96562 - .88656 and .88656 - .80751 are equal up to rounding, the initial concentration of 59,995 was chosen as the value to change between these two tests.

Since validation tries to assure that a PHP gives the desired log reduction and gets the final concentration below 30 per gram, an operational characteristic curve is used to determine how well the process works. For an initial concentration, an operational characteristic curve indicates the probability of passing validation for various final concentrations.

The probability of passing validation for each pair depends on the initial and final concentrations. The initial concentration indicates which of the five tests in the validation procedure is used. The final concentration and the test used give the probability of the sample passing.

The probability of passing any of the five tests in the validation procedure is calculated from the final concentrations. In addition, simulations generated outcomes from the initial concentration. The adjusted geometric means for the

MPNs of these outcomes indicate the probability of each of the five tests given the initial concentration. The product of the probability of each test times the probability of passing with the test were added over all five tests. This gives the probability a sample would pass. Calculating with a binomial gave the probability that at most 3 of the 30 samples would fail for a validation. The following table (table 2) gives the probability of passing validation with various combinations of initial and final concentrations.

TABLE 2

INITIAL CONC.	FINAL CONCENTRATIONS																
	3	6	9	12	15	18	21	24	27	30	33	36	39	42	45	48	51
10,000	.96	.14	.00														
20,000	1	.93	.39	.04	.00												
30,000	1	.99	.89	.56	.21	.05	.01	.00									
40,000		1	.97	.85	.62	.36	.18	.07	.03	.01	.00						
50,000		1	.99	.96	.86	.69	.49	.31	.17	.09	.04	.02	.01	.00			
60,000			1	.99	.95	.87	.73	.56	.38	.24	.13	.06	.03	.01	.00		
70,000				1	.98	.93	.84	.70	.53	.36	.22	.12	.06	.03	.01	.00	
80,000				1	.99	.96	.90	.78	.63	.45	.29	.17	.09	.05	.02	.01	.00
90,000				1	.99	.97	.92	.82	.68	.51	.34	.20	.11	.06	.03	.01	.00
100,000					1	.98	.93	.84	.70	.53	.36	.23	.13	.06	.03	.01	.00

Highlighted areas represent a 3.52 log reduction between initial concentration and final concentration.

The original reason for using 30 samples for validation was to be able to select one each week for 30 weeks during the warm weather. This would have given an idea how the post harvest process performed under various conditions throughout the summer. In order for this to be more feasible for industry, this arrangement was changed to 10 measurements on a single lot on each of 3 days.

VERIFICATION:

After initial validation of a PHP, verification of the process must be done monthly. In the verification process, the output of the PHP is tested to determine if it is below 30 per gram. If a PHP fails verification, then it has to be revalidated in order to use labeling claims as approved by the ISSC. Any verification that is not excessively burdensome may miss some problems with the process. Consequently, if other evidence indicates a problem then action may be needed regardless of verification results.

Samples can be taken throughout a month on different lots of product. Although testing different lots could help find intermittent problems, a small processor during a slow month may not be able to test many different lots. Consequently, the decision of how many lots are tested for verification may be left up to the processor with the approval of the state SSCA.

In order to determine the probability of verification failures that would result in revalidation, 1000 simulations were run with each simulation mimicking nine months and counting the number of passes. Nine months represents the number of months in a year that oysters might be expected to have high vibrio counts. The count for 9 months that passed indicates how likely the post harvest process would

be of not needing revalidation.

Based upon a verification procedure that requires 30 tubes per month be tested with no more than 11 of the 30 tubes being positive for the process to be verified for that month and assuming that all months are independent and identically distributed, the table (table 3) below indicates the probability of failing verification in at least one of nine months and at least twice in nine months for various final concentrations.

TABLE 3

Final Concentration	Probability of 1 failure in 9 months	Probability of 2 failures in 9 months
20	4	0
25	17	1
30	45	11
35	76	39
40	93	73

Example: If a final concentration of 30 has been achieved by the Post Harvest Process, there is an 11% chance that revalidation will be required based upon two verification failures within a 9 month period. Likewise, at a final concentration of 30, there is a 45% chance that one failure would occur within 9 months.

Cook, D.W., P. O'Leary, J.C. Hunsucker, E.M. Sloan, J.C. Bowers, R.J. Blodgett, and A. DePaola. 2002. *Vibrio vulnificus* and *Vibrio parahaemolyticus* in U.S. retail shell oysters: A national survey June 1998 to July 1999. J. Food Prot. 65:79-87.

FAO and WHO. Risk assessment of Vibrio vulnificus in raw oysters: Interpretative summary and technical report. 2005. Rome, Italy, FAO. Microbiological Risk Assessment Series No. 8.

Public Health Significance: This information provides an explanation of the development of the validation/ verification guidance given for post harvest processing.

Cost Information (if available): No additional cost.

Action by 2009 Task Force II: Recommended adoption of Proposal 09-237 as submitted.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force II on Proposal 09-237.

Proposal Subject: Laboratory Methods

Specific NSSP Guide Reference: NSSP Guide Model Ordinance Chapter XVI. Post-Harvest Processing A. (1) (a)

Text of Proposal/ Requested Action

2003 NSSP Model Ordinance Chapter XVI Post Harvest Processing A (1) (a)

For processes that target *Vibrio vulnificus*, the level of *Vibrio vulnificus* in the product that has been subjected to the process shall be non-detectable (<30 MPN/gram), to be determined by the use of ~~the *Vibrio vulnificus* FDA approved EIA procedure of Tamplin, et al., as described in Chapter 9 of the FDA *Bacteriological Analytical Manual*, 7th Edition, 1992, or other~~ method**s** approved **by the Laboratory Methods Review Committee** for NSSP use.

It has been reported by laboratories that the reagents for the Tamplin EIA test are not readily available. Other testing procedures are needed to do perform the analysis of *Vibrio vulnificus*. However, since not all methods listed in the *Bacteriological Analytical Manual* (BAM) are collaboratively tested and approved, methods that appear in the BAM cannot be accepted into the program based solely on the method's inclusion in the BAM. The Laboratory Methods Review Committee must review laboratory methods that are to be accepted into the ISSC program.

Public Health Significance: Laboratory methods detecting the direct or indirect presence of human pathogens must be proven to consistently work at various laboratories throughout the country and in participating MOU countries. Detailed review of scientific data (preferably from collaborative studies) by the Laboratory Methods Review Committee must be done.

Cost Information (if available): None

Action by 2005 Task Force III Recommended referral of Proposal 05-305 to the Executive Board to investigate ISSC approaches to adopting laboratory methods for use in the NSSP.

Action by 2005 General Assembly Adopted recommendation of 2005 Task Force III.

Action by ISSC Executive Board August 19, 2005 Recommended appointment of a workgroup to determine what the role of the ISSC should be in adoption of laboratory methods. The workgroup is also directed to look at similar conferences' procedures regarding laboratory methods approval. The workgroup will report their findings to the Executive Board at the March 2006 meeting.

Action by USFDA Concurred with Conference action.

Action by 2007 Laboratory Methods Review Committee Recommended referral of Proposal 05-305 to an appropriate committee as determined by the Conference Chairman.

Action by 2007 Task Force III Recommended adoption of Laboratory Methods Review Committee recommendation on Proposal 05-305.

Action by 2007 General Assembly	Adopted recommendation of 2007 Task Force III.
Action by USFDA	December 20, 2007 Concurred with Conference action.
Action by 2009 Laboratory Methods Review Committee	Recommended no action on Proposal 05-305. Rationale: The proposed new language is inconsistent with the ISSC Constitution, Bylaws and Procedures and the remainder of the proposal is more appropriately addressed in Proposals 07-103 and 09-229.
Action by 2009 Task Force III	NOTE: The action taken by Task Force III was to only address the proposed new language in Proposal 05-305. Recommended adoption of the Laboratory Methods Review Committee recommendation regarding proposed new language in Proposal 05-305. Task Force III did not take action on the remainder of Proposal 05-305. The remainder of Proposal 05-305 was addressed by Task Force I.
Action by 2009 Task Force I	Recommended no action on the remainder of Proposal 05-305. Rationale: The remainder of Proposal 05-305 is more appropriately addressed by Task Force I and II action on Proposals 07-103 and 09-229.
Action by 2009 General Assembly	Adopted recommendations of Task Force III and Task Force I on Proposal 05-305.

Proposal Subject: Laboratory Methods

Specific NSSP Guide Reference: Section III Public Health Reasons and Explanation, Chapter III Laboratory @ .02 Methods

Text of Proposal/ Requested Action American Public Health Association (APHA) Recommended Procedures for the Examination of Seawater and Shellfish shall be followed for the collection, transportation, and examination of samples of shellfish and shellfish waters. The official references of the NSSP for the examination of shellfish for Vibrio cholerae, V. vulnificus, and V. parahaemolyticus ~~is the FDA Bacteriological Analytical Manual (BAM)~~**are the methods approved by the Laboratory Methods Review Committee and listed in Guidance Documents Chapter II. Growing Areas .10 Approved National Shellfish Sanitation Program Laboratory tests: Microbiological and Biotoxin Analytical Methods.**

Since not all methods listed in the Bacteriological Analytical Manual (BAM) are collaboratively tested and approved, methods that appear in the BAM cannot be accepted into the program based solely on the method's inclusion in the BAM. The Laboratory Methods Review Committee must review laboratory methods that are to be accepted into the ISSC program.

Public Health Significance: Laboratory methods detecting the direct or indirect presence of human pathogens must be proven to consistently work at various laboratories throughout the country and in participating MOU countries. Detailed review of scientific data (preferably from collaborative studies) by the Laboratory Methods Review Committee must be done.

Cost Information (if available): None

Action by 2005 Task Force III Recommended referral of Proposal 05-306 to the Executive Board to investigate ISSC approaches to adopting laboratory methods for use in the NSSP.

Action by 2005 General Assembly Adopted recommendation of 2005 Task Force III.

Action by ISSC Executive Board August 19, 2005 Recommended appointment of a workgroup to determine what the role of the ISSC should be in adoption of laboratory methods. The workgroup is also directed to look at similar conferences' procedures regarding laboratory methods approval. The workgroup will report their findings to the Executive Board at the March 2006 meeting.

Action by USFDA Concurred with Conference action.

Action by 2007 Laboratory Methods Review Committee Recommended referral of Proposal 05-306 to an appropriate committee as determined by the Conference Chairman.

Action by 2007 Task Force III Recommended adoption of Laboratory Methods Review Committee recommendation on Proposal 05-306.

Action by 2007 General Assembly Adopted recommendation of 2007 Task Force III.

Action by USFDA	December 20, 2007 Concurred with Conference action.
Action by 2009 Laboratory Methods Review Committee	Recommended adoption as amended. American Public Health Association (APHA) Recommended Procedures for the Examination of Seawater and Shellfish shall be followed for the collection, transportation, and examination of samples of shellfish and shellfish waters. The official references of the NSSP for the examination of shellfish for Vibrio cholerae, V. vulnificus, and V. parahaemolyticus are the methods ~~approved by the Laboratory Methods Review Committee~~ <u>approved for use in the NSSP</u> and listed in Guidance Documents Chapter II. Growing Areas .10 Approved National Shellfish Sanitation Program Laboratory tests: Microbiological and Biotoxin Analytical Methods.
Action by 2009 Task Force III	NOTE: The action taken by Task Force III was only to delete "approved for use in the NSSP and". The remaining proposed language of Proposal 05-306 was addressed by Task Force I. Recommended adoption of Laboratory Methods Review Committee recommendation on Proposal 05-306.
Action by 2009 Task Force I	Recommended no action on the remaining proposed language of Proposal 05-306. Rationale: Proposal 05-306 is more appropriately addressed by Task Force I and II action on Proposals 07-103 and 09-229.
Action by 2009 General Assembly	Adopted recommendations of Task Force III and Task Force I on Proposal 05-306.

Proposal Subject:	ISSC Policy Statement on the "Consumption of Raw Oysters"
Specific NSSP Guide Reference:	Section VI. NSSP Policy Setting Documents, ISSC Policy Statement, Paragraph 3
Text of Proposal/ Requested Action	"Certain medically compromised individuals are at increased risk from common marine bacteria that are unrelated to pollution. Therefore, it may not be possible to address this risk through environmental controls. Although the reported number of illnesses and fatalities from these bacteria in the United States each year is small in comparison with other food borne illnesses, **shellfish that have been processed to reduce the levels of all pathogens of public health concern to safe levels can be eaten by the at-risk population or the at-risk population should eat molluscan shellfish fully cooked or,** total abstinence from raw molluscan shellfish is the best advice for medically compromised."
Public Health Significance:	This new ISSC policy setting language for the consumption of raw oysters will confirm the use of the labeling allowed for PHP shellfish listed in Chapter XVI. This new policy statement language will show the ISSC supports PHPs and that medically compromised individuals can choose safer post harvest processed shellfish rather than consume other raw shellfish that has not undergone a PHP and/or eat shellfish fully cooked.
Cost Information (if available):	None
Action by 2005 Task Force III	Recommended referral of Proposal 05-308 to an appropriate committee as determined by the Conference Chairperson to investigate the possibility of a change to the ISSC Policy Statement on the Consumption of Raw Molluscan Shellfish.
Action by 2005 General Assembly	Adopted recommendation of 2005 Task Force III.
Action by USFDA	Concurred with Conference action.
Action by 2007 Executive Board	Executive Board directed the Executive Director to discuss the ISSC Consumption Policy with the FDA. These discussions were not productive in identifying meaningful language for incorporating changes to the Policy Statement.
Action by 2007 Task Force III	Recommended no action on the proposed changes to the ISSC Policy Statement on the Consumption of Raw Molluscan Shellfish but, recommends the Executive Board continue to pursue ways to acknowledge Post Harvest Processing in the National Shellfish Sanitation Program.
Action by 2007 General Assembly	Adopted recommendation of 2007 Task Force III.
Action by USFDA	December 20, 2007 Concurred with Conference action.
Action by 2009 ISSC Executive Board	The Executive Board has concluded that it is inappropriate to consider changes to the ISSC Policy Statement on the Consumption of Raw Molluscan Shellfish until the future of PHP is more clearly defined.

Action by 2009 Task Force III	Recommended no action on Proposal 05-308. Rationale: The Conference is in a state of transition regarding PHP approval and it is inappropriate at this time to consider changes to the policy.
Action by 2009 General Assembly	Adopted recommendation of 2009 Task Force III on Proposal 05-308.

Proposal Subject: Guidance on Equivalence Criteria for Food

Specific NSSP Guide Reference: N/A

Text of Proposal/ Requested Action

Under Article 4 of the World Trade Organization (WTO) Agreement on the Application of Sanitary and Phytosanitary Measures (SPS) (the SPS Agreement), each member nation of the WTO, including the United States, is obligated to accept as equivalent a food regulatory system of another country if it provides the same level of health protection as is provided to consumers by its own system.

Equivalent regulatory systems need not be identical. Under the concept of equivalence, the "sanitary or phytosanitary measures" used by an exporting country may differ from the measures applied domestically by an importing country as long as these measures "achieve the importing Member's appropriate level of sanitary or phytosanitary protection".

Under the SPS Agreement, the burden of demonstrating that equivalence exist rest with the exporting country. The exporting country has the right to decide for itself whether the regulatory system of the exporting country is equivalent to its own or is inadequate to achieve "the importing Member's appropriate level of sanitary or phytosanitary protection," or that inadequate evidence has been provided to demonstrate equivalence.

One of the roles of the USFDA in the National Sanitation Shellfish Program (NSSP) is the evaluation of foreign programs and the establishment of MOUs with countries that meet the requirements of the NSSP. This responsibility of FDA is outlined in IV. A. 4. of the ISSC/FDA Memorandum of Understanding, March 14, 1984. Article 4 of the WTO Agreement obligates the FDA to accept equivalency in foreign programs. The Agreement requires that the USFDA consider acceptance of foreign shellfish safety programs that, while having a system of sanitary measures that differ from those applied domestically, are recognized as providing an equivalent level of public health protection.

The FDA is seeking input from the ISSC for purposes of incorporating the concept of equivalency into the NSSP. Recognizing that FDA has a clear obligation under the WTO Agreement to take responsibility for equivalency determination, it is important to the Agency that this responsibility be recognized within the NSSP.

Public Health Significance: N/A

Cost Information (if available): N/A

Action by 2007 Task Force III Recommended referral of Proposal 07-303 to Executive Board for developing short term and long term approaches to incorporating equivalency into the NSSP and the ISSC.

Action by 2007 General Assembly Adopted recommendation of 2007 Task Force III.

Action by USFDA December 20, 2007
Concurred with Conference action.

Action by 2009 Executive Board	Recommended the Executive Board continue discussions with FDA to address equivalency of food programs.
Action by 2009 Task Force III	Recommended adoption of the Executive Board recommendation on Proposal 07-303.
Action by 2009 General Assembly	Adopted recommendation of 2009 Task Force III on Proposal 07-303.

Proposal Subject: Press Releases

Specific NSSP Guide Reference: NSSP Section II Model Ordinance Chapter II. Risk Assessment and Risk Management

Text of Proposal/ Requested Action The US FDA issued press releases associated with outbreaks in the Pacific Northwest in the summer of 2006 and in Texas in March of 2007. These press releases created concern regarding the appropriateness and effectiveness of press releases as a public health measure to address an illness outbreak.
Use of press is to inform consumers.

The ISSC Executive Board discussed the issuance of these press releases and directed the formation of a working group to further investigate and review the use of press by state and federal agencies. The workgroup is to look for ways to coordinate use of press and provide recommendations for discussion at the 2007 Biennial Meeting.

Public Health Significance:

Cost Information (if available):

Action by 2007 Use of Press Committee Recommended that this Committee continue its deliberations and that a meeting be held in January 2008 in conjunction with appropriate FDA officials and report back to the Executive Board in March 2008. In the interim FDA will consult with the involved state regulatory agency on the content and timing of the release of press.

Action by 2007 Task Force III Recommended adoption of the Press Release Committee recommendation on Proposal 07-305.

Action by 2007 General Assembly Adopted recommendation of 2007 Task Force III.

Action by USFDA December 20, 2007
Concurred with Conference action.

Action by 2009 Use of Press Committee The Committee held a conference call on March 13, 2008, and planned a meeting in Washington, DC for April 30, 2008. The plans for this meeting were reported to the Executive Board on April 3, 2008.

On April 30, 2008, several members of the Committee and the ISSC Executive Director met with FDA officials at FDA headquarters and discussed agency procedures regarding use of press. The discussions of this meeting were presented to the Executive Board at the September 11, 2008, Executive Board meeting. The Committee reported that it is working to develop a press protocol for use in addressing press releases associated with outbreaks and product recall

The Committee held a meeting at the 2009 Biennial Conference and is continuing to develop a press protocol. The Committee will continue to fine tune a list of issues to be considered when use of press is contemplated. This list should be incorporated into NSSP Guidance Documents that address outbreaks and product recall.

Action by 2009 Task Force III	Recommended adoption of the Use of Press Committee recommendations on Proposal 07-305. Additionally, the Task Force recommended the Committee address the use of press in situations where significant time lapses have occurred between the last reported illness and the proposed use of press. The protocol should address the rationale for using press in situations where product is not likely to still be available for consumption. Task Force III further recommended the Use of Press Committee complete the protocol and present the protocol to the Executive Board at the 2010 Spring Meeting. In the interim, as noted in the March 13, 2008, Use of Press Committee report, FDA should be requested to continue to consult with the involved State regulatory agencies on the content and timing of press releases.
Action by 2009 General Assembly	Adopted recommendation of 2009 Task Force III on Proposal 07-305.

Proposal Subject: Approval of Shellfish Shippers/Reshippers in Non-Participatory States

Specific NSSP Guide Reference:
1. ISSC Constitution, Bylaws, and Procedures Definitions
2. NSSP Guide Section II. Model Ordinance Definitions B. Definition of Terms (7)
3. NSSP Guide Section II. Model Ordinance Chapter I. Shellfish Sanitation Program @.01 Administration B. State Laws & Regulations
4. NSSP Guide Section II. Model Ordinance Chapter I. Shellfish Sanitation Program @.02 Dealer Certification A. General (1)

Text of Proposal/ Requested Action

1. ISSC Constitution, Bylaws, and Procedures Definitions

   Add a new definition:
   NON-PARTICIPATING STATE – any state that chooses not to participate in the NSSP. [Subsequent definitions will need to be renumbered.]

2. NSSP Guide Section II. Model Ordinance Definition of Terms B. Definition of Terms

   (7) Authority means the State or local shellfish control authority or authorities or its designated agents, which are responsible for the enforcement of this code. **In NON-PARTICIPATING STATES, Authority shall mean the FDA Office of Seafood or its designated agents.**

3. NSSP Guide Section II. Model Ordinance Chapter I. Shellfish Sanitation Program @.01 Administration

   B. State Laws and Regulations.
   The Authority shall have laws and regulations which provide an adequate legal basis for the safety and sanitary control of all program elements including but not limited to the elements outlined in @.01 A. **If the Authority is the FDA Office of Seafood, the laws and regulations shall be the NSSP Model Ordinance.**

4. NSSP Guide Section II. Model Ordinance Chapter I. Shellfish Sanitation Program @.02 Dealer Certification A. General (1)

   (1) A person requesting certification shall be subject to a comprehensive, onsite inspection and meet the criteria in §B. or §C., as appropriate. The plant inspection shall be conducted by the state shellfish standardization inspector, using the appropriate inspection form, within the 120 day period. **In NON-PARTICIPATING STATES, the inspection shall be conducted by an agent of the FDA Office of Seafood.**

Certain seafood products are controlled under the National Shellfish Sanitation Act (NSSA), which includes the Interstate Certified Shellfish Shippers List (ICSSL). Any business or individual involved in the sale or resale of shellfish across a state or international border must be included in the ICSSL. The FDA has formalized this in the FDA Model Food Code for a number of years in Section 3-201.15 (B) that states "Molluscan Shellfish received in interstate commerce shall be from sources that are listed in the Interstate Certified Shellfish Shippers Guide".

While the rules and requirements of the Shellfish Sanitation Act are federal in scope, they are administered by the individual states. Participation by each state is voluntary- there is

no requirement that a state administer the program, and in fact only 35 states plus the District of Columbia currently do so. Any food distributor or wholesaler located in the remaining 15 states that do not participate cannot be listed on the ICSSL, and therefore cannot legally ship products covered by this act across state lines.

While there are several possible solutions, the most efficient appears to be allowing FDA Shellfish Specialists to approve facilities in states that do not participate. While the FDA prefers that all states participate, non-producing states have little incentive to incur the expense and time of training (or simply do not have the personnel). The changes below are designed to change the regulations to allow such activities by the FDA.

Public Health Significance:

Currently, a shipper or reshipper in a non-participating state has no recourse. The only option is to not ship across any state lines, thereby causing a loss of business both directly and indirectly.

Or, a shipper can ignore the law and hope they do not get caught. This latter recourse means shellfish are being shipped interstate from facilities that have not been approved for such actions This reduces the effectiveness of the law, designed to ensure safe shellfish handling from harvest through consumption.

Further, the flaw in this rule, which sets a requirement but does not ensure the ability to meet such requirement, weakens the overall opportunity for industry and regulators to work as partners in protecting public health. Both the International Food Distributors Association (IFDA) and the National Conference for Food Protection (NCFP) are supporting this issue.

Therefore we are requesting that ISSC work with the FDA so that any reshipper desiring to move product across state lines is afforded the opportunity for inspection and inclusion on the ICSSL.

Cost Information (if available):

The only cost is to the FDA which will be required to provide inspections to firms in non-participating states.

Action by 2009 Task Force III

Recommended referral of Proposal 09-300 to an appropriate committee as determined by the Conference Chairman.

Action by 2009 General Assembly

Voted no action on Proposal 09-300.

Proposal Subject: Shellfish Sanitation Program Records

Specific NSSP Guide Reference: Section II. Model Ordinance Chapter I. Shellfish Sanitation Program Requirements for the Authority @.01 Administration C. Records

Text of Proposal/ Requested Action

A. Scope. The Authority shall establish a statewide shellfish safety and sanitation program to regulate:
   (1) The classification of shellfish growing areas;
   (2) The harvesting of shellfish;
   (3) Shellfish processing procedures and facilities;
   (4) Product labeling;
   (5) Storage, handling and packing;
   (6) Shellfish shipment in interstate commerce;
   (7) Shellfish dealers; and
   (8) Bivalve aquaculture.

B. State Laws and Regulations. The Authority shall have laws and regulations which provide an adequate legal basis for the safety and sanitary control of all program elements including but not limited to the elements outlined in @.01 A.

C. Records. The Authority shall maintain records to demonstrate the effective administration of a statewide shellfish safety and sanitation program. These records shall be maintained in a central file and made available **physically and/or electronically** to any interested person upon request, consistent with appropriate state and federal law.

(1) Electronically means in a common electronic file format for text, spreadsheet, portable document format (PDF), and geographic (map) information.

(2) Records to be made available electronically include:

(a) Outbreak Growing Area Summary Report (Chapter II. @.01 H. (3))
(b) Growing Area Investigation Report (Chapter II. @.02 B.)
(c) Growing Area Risk Assessment (Chapter II. @.02 D. (1) and (2) (a) (i)
(d) Growing Area Management Plan – Human Pathogens (Chapter II. @.02 D. (2) (d) (i) (iii) and (3) (b) (iii))
(e) Growing Area Management Plan – Toxic Substances (Chapter II @.02 D.)
(f) Sanitary Survey and Supporting Documentation (Chapter IV. @.01 A. (3))
(g) Pollution Sources (Chapter IV. @.01 A. (4))
(h) Growing Area List and Maps (Chapter IV. @.01 A. (5))
(i) Shoreline Survey (Chapter IV. @.01 D.)
(j) Growing Area Classification (Chapter IV. @.03)
(k) Growing Area Management Plan - Conditional Area (Chapter IV. @.03 C. (2))
(l) Marine Biotoxin Contingency Plans (Chapter IV. @.04 A. (1) and E.)
(m) Shellfish Aquaculture Records (Chapter VI. @.01)

D. Shared Responsibilities. If more than one agency is involved in the administration of the statewide shellfish safety and sanitation program, memoranda of agreement shall be developed between the agencies to define each agency's responsibilities.

E. Administrative Procedures. The Authority shall have administrative procedures sufficient to:
   (1) Regulate shellfish harvesting, sale, or shipment; and
   (2) Ensure that all shellfish shipped in interstate commerce originate from a dealer located within the state from which the shellstock are harvested or landed, unless

the Authority has a memorandum of understanding with the Authority in another State to allow dealers from its state to purchase the shellstock.

(3) Detain, condemn, seize, and embargo shellfish.

(4) Assure compliance with Shellfish Plant Inspection Standardization.

F. Epidemiologically Implicated Outbreaks of Shellfish-Related Illness. The Authority shall have procedures for investigating incidents of shellfish borne disease.

G. Commingling.

(1) Except for any shellstock included in the Authority's commingling plan, the Authority shall not permit the commingling of shellstock.

(2) If the Authority permits shellstock commingling, the Authority shall develop a commingling management plan. The plan shall:

(a) Minimize the commingling dates of harvest and growing areas;

(b) Define a primary dealer;

(c) Limit the practice of commingling to primary dealers;

(d) Limit commingling to shellstock harvested from specific growing areas within the State as identified by the Authority and purchased directly from harvesters; and

(e) Define how the commingled shellstock will be identified.

Public Health Significance: N/A

Cost Information (if available): N/A

Action by 2009 Task Force III Recommended adoption of Proposal 09-301 as amended.

A. Scope. The Authority shall establish a statewide shellfish safety and sanitation program to regulate:

(1) The classification of shellfish growing areas;

(2) The harvesting of shellfish;

(3) Shellfish processing procedures and facilities;

(4) Product labeling;

(5) Storage, handling and packing;

(6) Shellfish shipment in interstate commerce;

(7) Shellfish dealers; and

(8) Bivalve aquaculture.

B. State Laws and Regulations. The Authority shall have laws and regulations which provide an adequate legal basis for the safety and sanitary control of all program elements including but not limited to the elements outlined in @.01 A.

C. Records. The Authority shall maintain records to demonstrate the effective administration of a statewide shellfish safety and sanitation program. These records shall be maintained in a central file and made available physically and/or electronically to any interested person upon request, consistent with appropriate state and federal law.

(1) Electronically means in a common electronic file format for text, spreadsheet, portable document format (PDF), and geographic (map) information.

~~(2) Records to be made available electronically include:~~

~~(a) Outbreak Growing Area Summary Report (Chapter II. @.01 H. (3))~~

~~(b) Growing Area Investigation Report (Chapter II. @.02 B.)~~

~~(c) Growing Area Risk Assessment (Chapter II. @.02 D. (1) and (2) (a) (i))~~

~~(d) Growing Area Management Plan Human Pathogens (Chapter II. @.02 D.~~

~~(2) (d) (i) (iii) and (3) (b) (iii))~~
~~(e) Growing Area Management PlanToxic Substances (Chapter II @.02 D.)~~
~~(f) Sanitary Survey and Supporting Documentation (Chapter IV. @.01 A. (3))~~
~~(g) Pollution Sources (Chapter IV. @.01 A. (4))~~
~~(h) Growing Area List and Maps (Chapter IV. @.01 A. (5))~~
~~(i) Shoreline Survey (Chapter IV. @.01 D.)~~
~~(j) Growing Area Classification (Chapter IV. @.03)~~
~~(k) Growing Area Management Plan - Conditional Area (Chapter IV. @.03 C. (2))~~
~~(l) Marine Biotoxin Contingency Plans (Chapter IV. @.04 A. (1) and E.)~~
~~(m) Shellfish Aquaculture Records (Chapter VI. @.01)~~

D. Shared Responsibilities. If more than one agency is involved in the administration of the statewide shellfish safety and sanitation program, memoranda of agreement shall be developed between the agencies to define each agency's responsibilities.

E. Administrative Procedures. The Authority shall have administrative procedures sufficient to:
   (1) Regulate shellfish harvesting, sale, or shipment; and
   (2) Ensure that all shellfish shipped in interstate commerce originate from a dealer located within the state from which the shellstock are harvested or landed, unless the Authority has a memorandum of understanding with the Authority in another State to allow dealers from its state to purchase the shellstock.
   (3) Detain, condemn, seize, and embargo shellfish.
   (4) Assure compliance with Shellfish Plant Inspection Standardization.

F. Epidemiologically Implicated Outbreaks of Shellfish-Related Illness. The Authority shall have procedures for investigating incidents of shellfish borne disease.

G. Commingling.
   (1) Except for any shellstock included in the Authority's commingling plan, the Authority shall not permit the commingling of shellstock.
   (2) If the Authority permits shellstock commingling, the Authority shall develop a commingling management plan. The plan shall:
      (a) Minimize the commingling dates of harvest and growing areas;
      (b) Define a primary dealer;
      (c) Limit the practice of commingling to primary dealers;
      (d) Limit commingling to shellstock harvested from specific growing areas within the State as identified by the Authority and purchased directly from harvesters; and
      (e) Define how the commingled shellstock will be identified.

Action by 2009 General Assembly Adopted recommendation of Task Force III on Proposal 09-301.

Proposal Subject: Qualifications for Standardization

Specific NSSP Guide Reference: NSSP Guide Section IV. Guidance Documents
Chapter III. Harvest, Handling, Processing, Distribution
.02 Shellfish Plant Inspection Standardization Procedures

Text of Proposal/ Requested Action

Chapter 3 – Qualifications for Standardization

- Classroom Training – Prior to field standardization, the *Candidate* must successfully complete the following courses:
  - 3 or 2 day Seafood Alliance HACCP (Basic Seafood HACCP)
  - ~~2 day Seafood Regulators Training~~
  - FD 1040 Basic Shellfish Plant Sanitation; and
  - FD 2041 Shellfish State Standardization Officer Training (not recommended for State Standardized inspectors unless specifically offered)

Public Health Significance: The 2-Day Seafood Regulator Training course has been replaced with FD249 Conducting Seafood Inspections. This is a completely revamped course that now focuses on training FDA and state contracted inspectors on the proper way to conduct a Seafood HACCP inspection. This new course does not mention Molluscan shellfish, nor does it help train a standardization officer candidate on how to conduct a shellfish inspection. Currently all FDA Shellfish Specialists, as well as, State Standardization Officers are required to successfully complete the FD241 Shellfish State Standardization Officer Training course. This course teaches attendees how to conduct a shellfish inspection, as well as, how to properly mark the NSSP Shellfish Inspection Form. Therefore, the Conducting Seafood Inspections course will be costly and ineffective based on the cooperative design and implementation aspects of the Shellfish Program. FDA does however believe the new FD249 Conducting Seafood Inspections course to be a very well laid out and good course and would encourage all FDA Shellfish Specialists and State Standardization Officers to take the training when possible.

Cost Information (if available): No additional cost. This will save states and FDA money by not having to send inspectors to this training.

Action by 2009 Task Force III Recommended adoption of Proposal 09-302 as submitted.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force III on Proposal 09-302.

Proposal Subject:	ISSC Region Change
Specific NSSP Guide Reference:	Specific ISSC Constitution, By-Laws and Procedures Reference: Amendment to Constitution: Definitions
Text of Proposal/ Requested Action	ISSC REGION - geographical grouping of shellfish producing states with similar characteristics and interests, established to provide for fairly distributed representation. The ISSC Regions shall be: **Region 1 - Maine, New Hampshire, Massachusetts, Rhode Island, Connecticut, New York, New Jersey, Maryland, Delaware, Virginia, North Carolina, South Carolina, Georgia** **Region 2 - Florida, Alabama, Mississippi, Louisiana, Texas** **Region 3 - Alaska, Washington, Oregon, California, Hawaii**
Public Health Significance:	The region changes submitted in this proposal reflect the true regions of the shellfisheries the U.S. This distribution of regional representation will be more accurate and fair as this conference currently exists.
Cost Information (if available):	No cost to implement change in Constitution.
Action by 2009 Task Force III	Recommended referral of Proposal 09-303 to an appropriate committee as determined by the Conference Chairman.
Action by 2009 General Assembly	Voted no action on Proposal 09-303.

Proposal Subject: Task Force I Membership Change

Specific NSSP Guide Reference: Specific ISSC Constitution, By-Laws and Procedures Reference: Amendment to By-Laws: ARTICLE I. Section 2. & subdivision b.

Text of Proposal/ Requested Action

Section 2. Each Task Force shall have a total voting membership of eight (8) members, **except for Task Force I which shall have a voting membership of six (6)** to be appointed by the Board Chairperson with the approval of the Board.

Subdivision b. Three (3) of the state shellfish control authority members shall be from producing states and one (1) shall be from a non-producing state, except for Task Force I where ~~at least four (4)~~ **three (3)** shellfish control authority members **and three (3) industry members** shall be from producing states. Prior to the March Board meeting, the industry and regulatory Board member from each region may submit a list of Task Force nominees of up to three (3) candidates each per Task Force to the Board Chairperson. The Board Chairperson shall appoint a member from each ISSC Region to each Task Force from the list of candidates submitted. The Board shall approve the candidates selected. In the absence of any nominees submitted from a region, the Board Chairperson, with Board approval, shall appoint the Task Force member.

Public Health Significance: This proposal will correct membership for Task Force I that deals with growing waters that affect the producing States.

Cost Information (if available): No cost to implement change in Constitution.

Action by 2009 Task Force III Recommended referral of Proposal 09-304 to an appropriate committee as determined by the Conference Chairman.

Action by 2009 General Assembly Voted no action on Proposal 09-304.

Proposal Subject:	Board Membership Change
Specific NSSP Guide Reference:	Specific ISSC Constitution, By-Laws and Procedures Reference: Amendment to Constitution: ARTICLE IV. Section 2.
Text of Proposal/ Requested Action	The Board shall be comprised of ~~eighteen (18)~~ **twelve (12)** voting members selected as follows:(a) ~~six (6)~~ **three (3)** state shellfish control authority members elected from the producing states, one (1) from each of the ISSC Regions; (b) three (3) state shellfish control authority members elected at large from the non-producing states; (c) ~~six (6)~~ **three (3)** members elected from industry, one (1) from each of the ISSC Regions; (d) one (1)member designated by the United States Food and Drug Administration; (e) one (1)member designated by the National Marine Fisheries Service; and (f) one (1) member designated by the United States Environmental Protection Agency.
Public Health Significance:	This proposal will reduce the size of the Board while it will reflect the true regions of the shellfisheries in the U.S. This distribution of regional representation will be more accurate and fair as this conference currently exists.
Cost Information (if available):	No cost to implement change in Constitution.
Action by 2009 Task Force III	Recommended referral of Proposal 09-305 to an appropriate committee as determined by the Conference Chairman.
Action by 2009 General Assembly	Voted no action on Proposal 09-305.

Proposal Subject:	ISSC Board Term Limits
Specific NSSP Guide Reference:	Specific ISSC Constitution, By-Laws and Procedures Reference: Amendment to Constitution: Article IV. Executive Board, Officers, Committees, Section 7.
Text of Proposal/ Requested Action	Section 7. Elected Board members shall serve four-year terms. Terms of the elected Board members shall expire at the end of the voting general assembly of the regular Biennial Conference meeting. **Board members may succeed themselves, unless re-election would extend the total terms of consecutive service to more than twelve (12) years. This requirement shall become retroactive following the close of the 2009 Bi-Annual ISSC meeting.**
Public Health Significance:	Many members of the ISSC Board have been on the Board for over 3 terms. A requirement to have term limits for Board members would encourage members other than those currently on the Board to take a more active role in the ISSC. Term limits would also encourage fresh thinking and new ideas as well as reduce the power of a few members that have served on the Board for over 10 years. This action would copy the requirement set forth under the National Conference on Interstate Milk Shipments Constitution.
Cost Information (if available):	No cost to implement change in Constitution.
Action by 2009 Task Force III	Recommended no action on Proposal 09-306. Rationale: The ISSC Constitution, Bylaws and Procedures do not preclude regions from limiting terms. Adoption of this proposal could create challenges in identifying industry representation on the Executive Board.
Action by 2009 General Assembly	Adopted recommendation of 2009 Task Force III on Proposal 09-306.

Proposal Subject: ISSC Constitutional Cost-Benefit Requirement for New Proposals that have a Significant Financial Impact on the States and Shellfish Industry

Specific NSSP Guide Reference: Specific ISSC Constitution, By-Laws and Procedures Reference: Amendment to Constitution Article XIII New Section 4.

Text of Proposal/ Requested Action Section 3. Proposals submitted by any Conference participants requiring Conference action are to be referred to the Executive Director for assignment to the appropriate Task Force.

Section 4. Proposals submitted by any Conference participant that may have a significant cost to implement by either the SSCA or the Shellfish Industry must include an independent Cost-Benefit Analysis and an Economic Impact Study.

Section ~~4~~ 5. The Executive Director shall review and assign all problems or proposals received for Task Force and Conference deliberation. Problem or proposal assignment shall be made according to subject matter and in accordance with Article XIII. **Section 4.,** Section 5., Section 6., and Section 7. of the Constitution of the Conference.

Public Health Significance: Cost-Benefit Analyses and Economic Impact Studies are required by Federal and State Agencies prior to imposing new regulations. For too many years the ISSC through amendments made to the NSSP without any regards to the costs imposed on the SSCA and Shellfish Industry to implement the new guidelines.

Cost Information (if available): The cost to conduct Cost-Benefit Analyses and Economic Impact Studies will be much less on the SSCA'S and Shellfish Industry than the cost to implement by the SSCAs or the Shellfish Industry.

Action by 2009 Task Force III Recommended no action on Proposal 09-307.

Rationale: The Conference has previously discussed this concept and has chosen to request cost information only if available.

Action by 2009 General Assembly Adopted recommendation of 2009 Task Force III on Proposal 09-307.

Proposal Subject:	Executive Board, Officers, Committees
Specific NSSP Guide Reference:	Constitution, Bylaws, and Procedures, Article IV. Section 8.
Text of Proposal/ Requested Action	Section 8. The Board shall elect a Chairperson and Vice-Chairperson for a two (2) year term at the Executive Board meeting following the voting general assembly of the regular Biennial Conference meeting. New officers shall take office at the beginning of the ~~March~~ **Spring** Executive Board meeting.
Public Health Significance:	N/A
Cost Information (if available):	No cost to implement change in Constitution.
Action by 2009 Task Force III	Recommended adoption of Proposal 09-308 as submitted.
Action by 2009 General Assembly	Adopted recommendation of 2009 Task Force III on Proposal 09-308.

Resolution Subject: Educational Outreach Common Carrier Associations

Text of Resolution

Whereas, the National Shellfish Sanitation Program, (NSSP), was developed in 1925 when the U. S. Public Health Service responded to a request for assistance from local and state public health officials in controlling disease associated with the consumption of raw shellfish,and

Whereas, each shellfish shipping state has adopted adequate laws and regulations for sanitary control of the shellfish industry, completed sanitary surveys of harvest areas, delineated and patrolled restricted areas, inspected shellfish plants, and conducted such additional inspections, laboratory investigations, and control measures as were necessary to insure that the shellfish reaching the consumer had been grown, harvested and processed in a sanitary manner, and

Whereas, the shellfish industry has cooperated by obtaining shellfish from safe sources, by providing plants which met the agreed upon sanitary standards, by maintaining sanitary operating conditions, by placing the proper certificate number on each package of shellfish,and by keeping and making available to the control authorities records which showed the origin and disposition of all shellfish, and

Whereas, in 1982, a delegation of state officials met in Annapolis, Maryland and formed the Interstate Shellfish Sanitation Conference (ISSC), that is composed of state shellfish regulatory officials, industry officials, FDA, and other federal agencies, and

Whereas, the ISSC has provided a forum for state shellfish regulatory officials, industry officials, FDA, and other federal agencies, to establish uniform national guidelines and to exchange information regarding sources of safe shellfish, and

Whereas, under the guidance of the ISSC, the NSSP has lead the United States in the prevention of food-borne illnesses by requiring the States, to monitor bacteriological water quality of shellfish growing areas, to label shellfish with exact growing area information for trace-back purposes, to inspect and certify shellfish processing facilities, to require refrigeration of shellfish to reduce the growth of food-borne pathogens, and

Whereas, shellfish dealers are required by the NSSP to ensure that shellfish is shipped under proper temperature control to prevent possible pathogen growth, especially natural marine pathogens such as *Vibrio vulnificus* and *Vibrio parahaemolyticus* that have substantial growth based on temperature, and

Whereas, common carriers are exempt from the time-temperature control requirements of the Model Ordinance, causing most dealers to be concerned if the shellfish products shipped via these carriers are maintained at proper temperatures and

Be it Resolved that the ISSC acknowledge that managing pathogen growth comes from proper temperature control and maintaining this proper temperature control is of concern for dealers who ship shellfish including shipping via common carriers,

Be it Further Resolved that the ISSC will extend an educational outreach to Common Carrier Associations to express these concerns in a letter to Common Carrier Associations detailing the need for proper temperature control for shipping shellfish,

Be it Further Resolved that the ISSC will extend an educational outreach to Common Carrier Associations to express these concerns by promoting the attendance of an Executive Board member to attend Common Carrier Association Conferences as they may occur and as executive board budget allows.

Action by 2009 Resolutions Committee Recommended adoption of Resolution 09-001 as submitted.

Action by 2009 Task Force II Recommended adoption of Resolutions Committee recommendation on Resolution 09-001.

Action by 2009 Executive Board Recommended adoption of 2009 Task Force II recommendation on Resolution 09-001.

Action by 2009 General Assembly Adopted recommendation of ISSC Executive Board on Resolution 09-001.

Resolution Subject Guy C. Jackson Memorial Resolution

Text of Resolution

Whereas, Guy Cade "Jack" Jackson, III, was born on August 16, 1931, in San Antonio, Texas and passed away at the age of 77 in Anahuac, Texas on February 23, 2009; and

Whereas, Jack was 1948 graduate of Anahuac High School and went on to earn his BBA at Texas A&M in May 1952 and graduated from the University of Texas School of Law in January 1958. Jack, like his father before him was a man who got things done or sometimes tried his best to get things done. He liked the public arena and he savored causes; and

Whereas, Jack was admitted to practice before the Supreme Court of Texas; U.S. District Courts for Southern and Eastern Districts of Texas; U.S. 5th Circuit Court of Appeals and the United States Supreme Court; and

Whereas, Jack was a retired Lieutenant Colonel and served in the United States Army for 23 years of active and reserve service, including two active-duty tours with principal assignments. He received a Meritorious Service Medal, an Army Commendation Medal, a United Nations Service Medal, and Korean Service Medal; and

Whereas, Jack was an exemplary gentleman of many talents and interests and a versatile community leader, he was highly respected for his accomplishments and the significant role he played in so many organizations; and

Whereas, Jack was known for his engaging personality and for his integrity and generosity; he gave unselfishly of his time and energy to others and was a distinguished, hardworking citizen; and

Whereas, Jack was the Executive Secretary of the Coastal Oyster Leaseholders Association and a member of the Interstate Shellfish Sanitation Conference; and

Whereas, Jack was beloved by his family and friends, and he leaves behind memories that will be deeply treasured by all who were privileged to share in this life; now

Be It Therefore Resolved, that the Interstate Shellfish Sanitation Conference extends its gratitude for Jack's leadership and lasting contributions to the organization; and

Be It Therefore Further Resolved that the Interstate Shellfish Sanitation Conference acknowledges his contribution by a copy of this Resolution to his family.

Action by 2009 Resolutions Committee Recommended adoption of Resolution 09-002 as submitted.

Action by 2009 Executive Board Recommended adoption of Resolutions Committee recommendation on Resolution 09-002.

Action by 2009 General Assembly Adopted recommendation of Executive Board on Resolution 09-002.

Resolution Subject P. Roger Holbrook Memorial Resolution

Text of Resoluion

Whereas, P. Roger Holbrook was born in Salt Lake City, Utah on April 8, 1943 and died 64 years later on October 6, 2007 in Elizabeth, Colorado; and

Whereas, Roger obtained his undergraduate degree in Zoology with a teaching certificate from Weber State College in Utah and after teaching for a year went on to Colorado State University to pursue a degree in Environmental Science; and

Whereas, Roger began work with the Colorado Department of Health in the Consumer Protection Division in March of 1974 as an inspector in the Wholesale Food Program; and

Whereas, Roger was one of Colorado's original shellfish inspectors beginning in the mid 1980's and was instrumental in the development and passage of Colorado's Shellfish Dealer Certification Act that modeled Colorado's shellfish program on the National Shellfish Sanitation Program; and

Whereas, Roger was an active and involved member of the ISSC and served on the Executive Board and in numerous other capacities for the Interstate Shellfish Sanitation Conference. He was instrumental in convincing the ISSC to grant full voting privileges to non-producing states; and

Whereas, Roger, always the gracious host, welcomed the participants of the ISSC to his ranch when the annual meeting was held in nearby Denver, Colorado; and

Whereas, Roger became the Assistant Director of the Consumer Protection Division in September 1993 and retired as Acting Director in January 2001; and

Whereas, Roger continued his passion for life, friends, animals and the outdoors in retirement as was recalled during his funeral service.

Be It Therefore Resolved, that the Interstate Shellfish Sanitation Conference formally recognizes Roger Holbrook's dedication to advancing public health standards for consumers of shellfish and his contribution to the National Shellfish Sanitation Program, and;

Be It Therefore Further Resolved that the executive board, executive committee, office staff and membership of the Interstate Shellfish Sanitation Conference wish to extend their deepest sympathies and condolences to his wife, Diane Holbrook.

Action by 2009 Resolutions Committee Recommended adoption of Resolution 09-003 as submitted.

Action by 2009 Executive Board Recommended adoption of Resolutions Committee recommendation on Resolution 09-003.

Action by 2009 General Assembly Adopted recommendation of Executive Board on Resolution 09-003.

Resolution Subject John D. "Jack" Pingree Memorial Resolution

Text of Resolution

Whereas John D. "Jack" Pingree was born in Wilmington, Delaware on October 17, 1958, and grew up in the Oakland's development in Newark, Delaware.

Whereas Jack attended Newark public schools from the 1st to the 12th grade and graduated with honors in 1976.

Whereas Jack received his undergraduate degree from the University of Delaware in 1980 and was a member of Pi Kappa Alpha, better known as the "Pikes" at the University of Delaware.

Whereas John D. known as Jack was the Manager of the Shellfish and Recreational Water Branch for the State of Delaware-Department of Natural Resources and Environmental Control.

Whereas Jack was a well known environmentalist and a negotiator of many of the state's ocean and bay beaches and shellfish agreements.

Whereas Jack was a pioneer in implementing the State of Delaware's Beach Water Program and an advocate of the shellfish industry.

Whereas Jack was a man of diplomacy, a leader and most of all made a difference in the many lives that he touched and a man that would help anyone with a project, caring to a fault.

Whereas Jack was a renaissance man, loved nature and enjoyed working to protect the environment, as well as when hunting with his buddies at MJP & M Hunting.

Whereas Jack enjoyed writing, painting, music, cooking history, New Orleans and most of all Key West.

Whereas Jack was an advocate for the shellfish industry and belonged to many organizations, including serving on the Interstate Shellfish Sanitation Conference Executive Board representing the State of Delaware and the Shellfish Industry.

Whereas Jack enjoyed politics, supporting animal and environmental endeavors and was secretary of the Sussex County League of Women Voters and held a Director's position with the Dewey Beach Lions Club.

Whereas Jack looked forward to working the Piping Competition as a Piping Steward and bartender each year with the Scottish Games.

Whereas Jack was a man of dreams, looking for the best in everyone and the nice thing is he always found it. Being an optimist, Jack would always look for the silver lining. With energy, passion and humor, Jack could save the day, and always got the job done. Most of all he loved his animals, both wild and domestic.

Whereas Jack will always be sadly missed by everyone who crossed his path in life.

Be It Therefore Resolved that the Interstate Shellfish Sanitation Conference extends its gratitude for Jack's leadership and lasting contributions to the organization; and

Be It Further Resolved, that the Interstate Shellfish Sanitation Conference acknowledges his contributions by a copy of this Resolution to his family.

Action by 2009 Resolutions Committee Recommended adoption of Resolution 09-004 as submitted.

Action by 2009 Executive Board Recommended adoption of Resolutions Committee recommendation on Resolution 09-004.

Action by 2009 General Assembly Adopted recommendation of Executive Board on Resolution 09-004.

Resolution Subject Daniel R. Leonard Memorial Resolution

Text of Resolution

Whereas, Daniel R. Leonard was born in Providence, Rhode Island on March 31, 1947, and died 61 years later on September 22, 2008; and

Whereas, Dan loved the shellfish business and worked with distinction in both Rhode Island and Florida and was affectionately known as the "Clammah"; and

Whereas, Dan helped commercial fishermen in southwest Florida recover from the devastating effects of the 1994 Net Ban by having the vision and drive to help launch the hard clam industry by obtaining the first aquaculture lease in southwest Florida in 1993; and

Whereas, Dan and his wife Carol owned and operated Bull Bay Clam Farm; and

Whereas, Dan's tireless work helped the hard clam industry in Florida grow from infancy to one that generated tens of millions of dollars in economic impact and employed hundreds of people; and

Whereas, Dan volunteered his time to both the clam industry and the oyster industry in Florida, through his work with the Harbor Branch Oceanographic Institute's shellfish aquaculture training program, the Statewide Clam Industry Taskforce, and many other groups; and

Whereas, Dan served as Region 4 Industry Representative to the Interstate Shellfish Sanitation Conference Executive Board; and

Whereas, fishermen, scientists and public health professionals on the Interstate Shellfish Sanitation Conference all grew to respect Dan's passion for shellfish, his smile and his love for life,

Be It Therefore Resolved that the Interstate Shellfish Sanitation Conference formally recognizes Dan Leonard's dedication to advancing public health standards for consumers of shellfish and his contribution to the National Shellfish Sanitation Program.

Be It Therefore Further Resolved that the Executive Board, Executive Committee, Office Staff and Membership of the Interstate Shellfish Sanitation Conference wish to extend their deepest sympathies and condolences to Dan's family.

Action by 2009 Resolutions Committee Recommended adoption of Resolution 09-005 as submitted.

Action by 2009 Executive Board Recommended adoption of Resolutions Committee recommendation on Resolution 09-005.

Action by 2009 General Assembly Adopted recommendation of Executive Board on Resolution 09-005.

Resolution Subject Miles Motes Memorial Resolution

Text of Resolution

Whereas, Miles Lafayette Motes, Jr. was born in Sylacauga, Alabama on November 20th, 1950 and passed away on June 3rd, 2009; and

Whereas, Miles graduated from Auburn University August 24th, 1973, with a Bachelor of Science in Marine Biology and went on to receive his Master of Science in Fisheries Biology; and

Whereas, Miles was an avid Auburn fan until the day he passed away, just like his father Miles L. Motes, Sr. and mother Eva P. Motes; and

Whereas, Miles had four siblings who are also Auburn fans, Margaret Mugg, Julian Motes, David Motes, Carol Headley, and also his wife of three years Lisa B. Motes; and

Whereas, Miles spent twenty years as a research biologist at the FDA Gulf Coast Seafood Laboratory in Dauphin Island, Alabama and twelve years as an FDA Southeast Region Shellfish Specialist. He retired from the FDA in January 2008; and

Whereas, Miles had numerous peer reviewed publications relating to *Vibrios* and molluscan shellfish which significantly contributed to our current understanding and mitigation of *Vibrio* illnesses in the United States; and

Whereas, Miles read his daily devotional every day and was a spiritual beacon to his community and colleagues; and

Whereas, Miles loved the outdoors, running, the mountains, fishing, turkey hunting and had mastered an expert turkey call; and

Whereas, Miles was a very good man with a big heart and a great sense of humor who imparted a lasting warm impression on everyone he met and worked with.

Be It Therefore Resolved that the Interstate Shellfish Sanitation Conference formally recognizes Miles Motes' dedication to advancing public health standards for consumers of shellfish and his contributions to the National Shellfish Sanitation Program.

Be It Therefore Further Resolved that the Executive Board, Executive Committee, Office Staff and Membership of the Interstate Shellfish Sanitation Conference wish to extend their deepest sympathies and condolences to Mile's wife and family.

Action by 2009 Resolutions Committee Recommended adoption of Resolution 09-006 as submitted.

Action by 2009 Executive Board Recommended adoption of Resolutions Committee recommendation on Resolution 09-006.

Action by 2009 General Assembly Adopted recommendation of Executive Board on Resolution 09-006.

Resolution Subject John H. Volk Memorial Resolution

Text of Resolution

Whereas, John H. Volk was born in Milford, Connecticut on December 1, 1946, and died 60 years later on November 12, 2007, in Milford, Connecticut; and

Whereas, John started his career as the principal biologist for Long Island Oyster Farms from 1978-1982; and

Whereas, John became the Director for the State of Connecticut Department of Agriculture's Bureau of Aquaculture from 1982 to 2003; and

Whereas, John was described by many as being "the right person for the job" as a leader in the promotion of the Connecticut shellfish industry and protection of the Long Island Sound; and

Whereas, John took an active role in many organizations including: NESSA, the Atlantic States Marine Fisheries Commission, Connecticut Coves and Embayment Advisory Board, the Connecticut Sea Grant Review Panel, the Long Island Sound Assembly and was chosen as a United States aquaculture expert in the "People to People Citizen Ambassador Program" in the People's Republic of China in 1987; and

Whereas, John was a major advisor supporting Connecticut's effort to build two regional vocational aquaculture schools; and

Whereas, John received several awards of achievement and commendation including; several state awards for Excellent Managerial Performance, an Honorary Future Farmers of America Degree, the Rhode Island House of Representatives Citation for Expertise in Aquaculture, and the Governor's Award for service to the state, publishing numerous articles along the way; and

Whereas, John effectively represented the Connecticut shellfish program for many years on the ISSC Aquaculture Committee and chaired the Legislative Aquaculture Commission Study laying the framework for shellfish aquaculture development throughout the country; and

Whereas, John was well liked, respected and considered by many to be a person of knowledge, integrity and skilled foresight working together with officials at Federal, State and local levels; and

Whereas, John's accomplishments will not be forgotten as the Connecticut Legislature has passed a bill to rename the current Bureau of Aquaculture vessel to the "John H. Volk" in his memory; and

Whereas, his presence is greatly missed due to his passing; now

Be It Therefore Resolved that the Interstate Shellfish Sanitation Conference extends its gratitude for John's leadership and lasting contributions to the organization; and

Be It Further Resolved, that the Interstate Shellfish Sanitation Conference acknowledges his contributions by a copy of this Resolution to his family.

Action by 2009 Resolutions Committee	Recommended adoption of Resolution 09-007 as submitted.
Action by 2009 Executive Board	Recommended adoption of Resolutions Committee recommendation on Resolution 09-007.
Action by 2009 General Assembly	Adopted recommendation of Executive Board on Resolution 09-007.

Resolution Subject Resolution of Appreciation

Text of Resolution

Whereas, the twenty-third meeting of the Interstate Shellfish Sanitation Conference convened October 17, 2009, at the Radisson Hotel, Manchester, New Hampshire; and

Whereas, several individuals were instrumental in contributing to the outstanding success of this meeting.

Be It Therefore Resolved that the Interstate Shellfish Sanitation Conference goes on record expressing appreciation to:

The Staff of the Radisson Hotel Manchester, particularly,

Kim Roy, General Manager
Shawn Monahan
Carol Meyer
Chef Brian Sullivan
Annie Harrison
Natalia Androsovych
Peter Palandri
Scott Calson
Korkut Tuter
Ryan Meyer
Omid Sheyhikian
Elizabeth Embree
Cristy Bergeron

The Volunteer ISSC Staff

Office Manager, Bill Eisele, Retired

And Be It Finally Resolved, that the Interstate Shellfish Sanitation Conference directs the Executive Director to write a letter of appreciation to each of the above mentioned individuals and organizations.

Action by 2009 Resolutions Committee Recommended adoption of Resolution 09-008 as submitted.

Action by 2009 Executive Board Recommended adoption of Resolutions Committee recommendation on Resolution 09-008.

Action by 2009 General Assembly Adopted recommendation of Executive Board on Resolution 09-008.

Resolution Subject Resolution of Appreciation

Text of Resolution

Whereas, the twenty-third meeting of the Interstate Shellfish Sanitation Conference convened October 17, 2009, at the Radisson Hotel, Manchester, New Hampshire; and

Whereas, the following industry sponsors, companies, and individuals were instrumental in contributing to the outstanding success of the Interstate Shellfish Sanitation Conference Chairman's Reception.

Be It Therefore Resolved that the Interstate Shellfish Sanitation Conference goes on record expressing appreciation to:

A. C., Inc. Albert Carver	Beals, Maine
A. E. Phillips & Son, Inc.	Fishing Creek, Maryland
Bay Hundred Seafood	McDaniel, Maryland
Bivalve Packing	Port Norris, NJ
Blaine & Virginia Olsen	Stonington, Maine
Blount Fine Foods	Warren, Rhode Island
Charles Parks & Son	Fishing Creek, Maryland
Cherrystone Aquafarms	Cheriton, Virginia
Even Young	Hancock, Maine
Fishers Island Oyster Farm	Fishers Island, NY
Glidden Point Sea Farm	Edgecomb, Maine
G. W. Hall & Son	Fishing Creek, Maryland
J M Clayton Company	Cambridge, Maryland
Kool Ice & Seafood Company	Cambridge, Maryland
Metompkin Bay Oyster Co.	Crisfield, Maryland
Norm Bloom & Son	East Norwalk, CT
Northcoast Seafoods	Boston, Massachusetts
Russell Hall Seafood	Fishing Creek, Maryland
Seawatch International, Ltd.	Easton, Maryland
Spinney Creek Shellfish	Eliot, Maine
W T Ruark & Co.	Fishing Creek, Maryland

And Be It Finally Resolved, that the Interstate Shellfish Sanitation Conference directs the Executive Director to write a letter of appreciation to each of the above mentioned individuals and organizations.

Action by 2009 Resolutions Committee Recommended adoption of Resolution 09-009 as submitted.

Action by 2009 Executive Board Recommended adoption of Resolutions Committee recommendation on Resolution 09-009.

Action by 2009 General Assembly Adopted recommendation of Executive Board on Resolution 09-009.

Resolution Subject Resolution of Appreciation

Text of Resolution ***Whereas***, Ken B. Moore attended his first Interstate Shellfish Sanitation Conference meeting in Cherry Hill, New Jersey in August 1985; and

Whereas, Ken was elected Chairman of the Interstate Shellfish Sanitation Conference in Stamford, Connecticut in July 1989; and

Whereas, Ken was selected by the Interstate Shellfish Sanitation Conference Executive Board to serve as the first Executive Director in McLean, Virginia in August 1993; and

Whereas, Ken has dedicated over sixteen years as the Executive Director of the Interstate Shellfish Sanitation Conference serving to promote shellfish safety; and

Whereas, Ken's unyielding dedication to improving public health has strengthened the National Shellfish Sanitation Program; and

Whereas, Ken's ability to bring together the Shellfish Industry, State Shellfish Control Authorities, and Federal Regulatory Agencies has guided the Interstate Shellfish Sanitation Conference through many difficult issues; and

Whereas, Ken's stewardship has enabled the Conference to mature and grow into an internationally recognized authority on molluscan shellfish safety; and

Whereas, Ken's unique ability to unite the Conference membership has guided the organization through some difficult and controversial challenges, including

Creation of the Model Ordinance;
Incorporation of HACCP into the National Shellfish Sanitation Program;
Development of the *Vibrio vulnificus* and *Vibrio parahaemolyticus* Plans;
Education of the at-risk consumer;
Online continuing education courses for the medical profession;
Negotiation of unresolved issues;
Development of Conference procedures;
Conference action to develop NSSP Shellfish Standardization Officer training and certification programs; and
Conference efforts to establish a standardized Control of Harvest evaluation process; and

Whereas, Ken ensured that each member of this organization was provided the opportunity to participate and express their opinion; and

Whereas, Ken's boundless energy, candor, and sense of humor has provided inspiration and relief when needed most; and

Whereas, Ken has mastered the trait of the classic American storyteller which he has used to express his view or to make a point; now

Be It Therefore Resolved that the Interstate Shellfish Sanitation Conference extends its appreciation, gratitude, and indebtedness for his commitment to advance the goals of this organization and to strengthen the National Shellfish Sanitation Program; and

Be It Further Resolved, that the Interstate Shellfish Sanitation Conference will always value Ken's lasting contributions and wishes him success in all of his future endeavors; and

Be It Finally Resolved, that the Interstate Shellfish Sanitation Conference acknowledges Ken B. Moore's legacy of excellence by a copy of this Resolution.

Action by 2009 Resolutions Committee Recommended adoption of Resolution 09-010 as submitted.

Action by 2009 Executive Board Recommended adoption of Resolutions Committee recommendation on Resolution 09-010.

Action by 2009 General Assembly Adopted recommendation of Executive Board on Resolution 09-010.