Interstate Shellfish Sanitation Conference

Task Force I

(Growing Area)

2011 Biennial Meeting

Proposals for Consideration

October 1 – 7, 2011

11-113	Mississippi Department of Marine Resources • Addition to the Requirements for the Authority during a suspected shellfish related outbreak	TF-I-233
11-114	Mississippi Department of Marine Resources • Addition to the Requirements for the Authority during a suspected oyster related outbreak of *Norovirus*	TF-I-235
11-115	Mississippi Department of Marine Resources • Addition to the Requirements for the Authority during a suspected shellfish related outbreak	TF-I-237
11-116	Debbie Rouse, John M. Hickey, Eric M. Hickey, John Mullen, Joseph Migliore, Darcie Couture, USFDA • Control of Marine Biotoxins	TF-I-238
11-117	ISSC Executive Office, USFDA • Recall Notification	TF-I-246

Proposal for Consideration at the Interstate Shellfish Sanitation Conference 2011 Biennial Meeting	☒ **Growing Area** ☐ **Harvesting/Handling/Distribution** ☐ **Administrative**
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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 the rule is far less burdensome to the egg industry than the *Vibrio vulnificus* Risk Management Plan for Oysters is on the Gulf oyster industry, an exemption should be given to oyster 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	Recommended no action. Rationale: No data presented.

Research Guidance Committee	
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.
Action by USFDA 02/16/2010	Concurred with Conference action on Proposal 05-100 with the following comments and recommendations for ISSC consideration. While FDA agrees to participate in Vibrio Management Committee discussions to identify approaches for gathering information that may further deliberation on Proposal 05-100, it is the Agency's current thinking that exemption of any harvester or dealer, regardless of operational size, from NSSP *Vv* controls is not an appropriate public health approach. FDA considers it essential that all harvesters and all dealers employ NSSP *Vv* control measures. Any allowance for exemption would be contrary to the food safety and public health protection initiatives of the NSSP. In consideration of the ongoing and developing efforts to address *Vv* illnesses and deaths, FDA believes it would be more prudent for the Executive Board to take no action on Proposal 05-100, or at least table further consideration pending consideration of Proposal 00-201.

Proposal for Consideration at the Interstate Shellfish Sanitation Conference 2011 Biennial Meeting	☒ **Growing Area** ☐ **Harvesting/Handling/Distribution** ☐ **Administrative**
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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.
Action by USFDA 02/16/2010	Concurred with Conference action on Proposal 05-109.

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 recognise 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 homogenised 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 digitised 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 recognised 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.

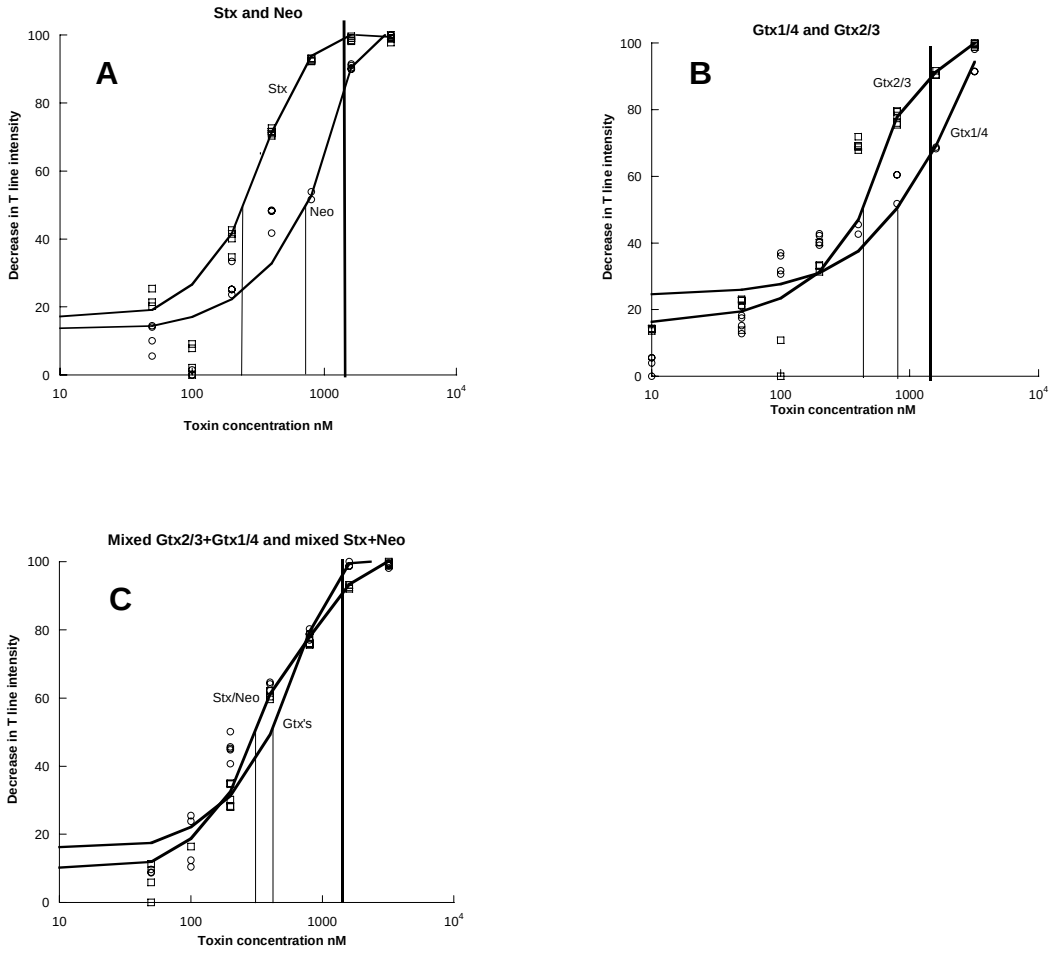

Figure 1.

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 digitising (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.

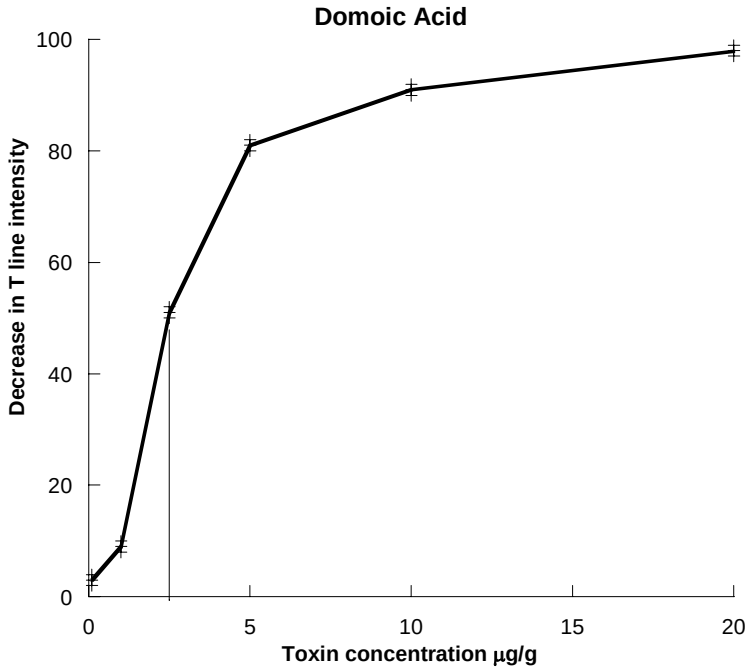

Figure 2.
Non-toxic mussel homogenate was extracted into four volumes of 50% methanol a sample spiked with domoic acid to 20 µg/g homogenate. Serial dilutions were made with non-toxic extract and mixed with ASP running buffer solution. A sample (100 µl) of each solution was applied to each test strip. 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.

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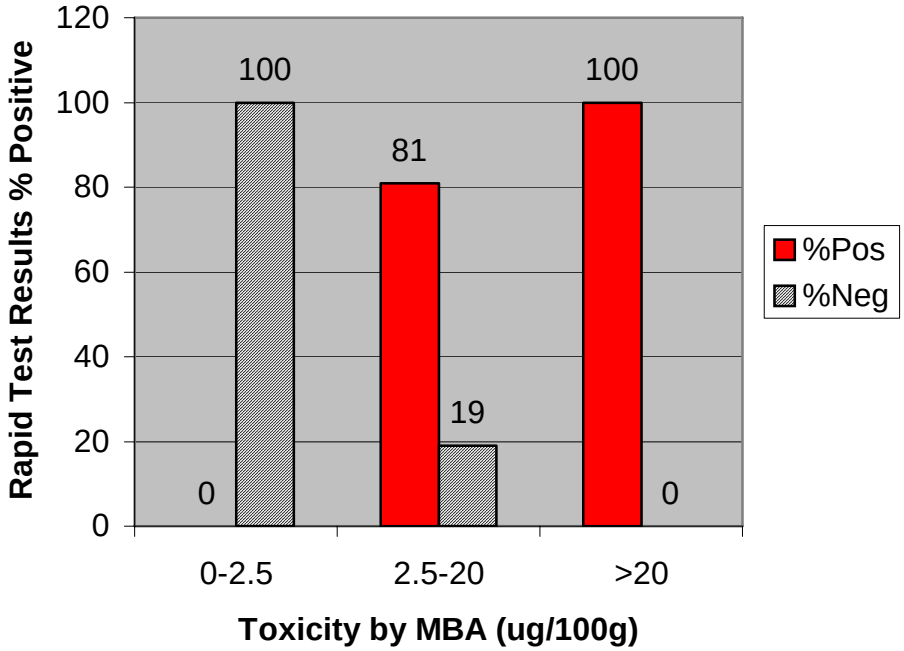
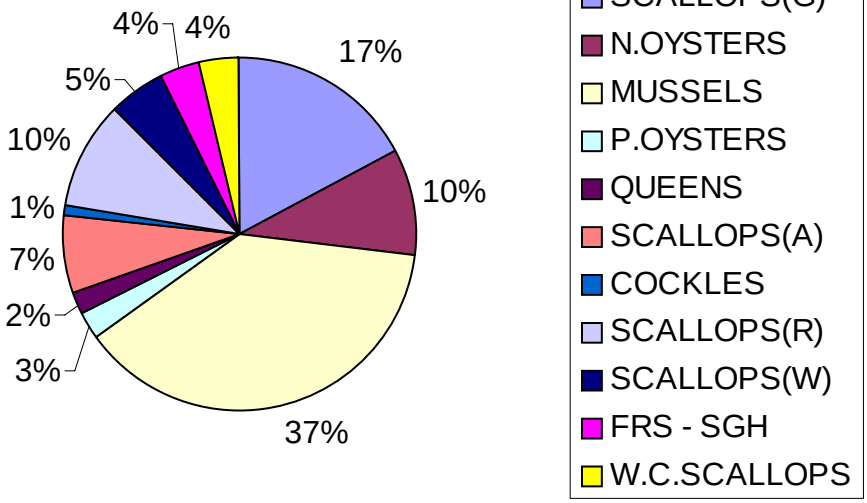
Detection of ASP in Shellfish Tissue
from UK



Tissue Types from UK
(n= 111)



Proposal for Consideration at the Interstate Shellfish Sanitation Conference 2011 Biennial Meeting	☒ **Growing Area** ☐ **Harvesting/Handling/Distribution** ☐ **Administrative**
Name of Submitter:	Joanne Jellett
Affiliation:	Jellett Rapid Testing Ltd.
Address:	4654 Route 3, Chester Basin Nova Scotia, Canada B0J 1K0
Phone: **Fax:** **Email:**	902-275-5104 902-275-2242 jjellett@ns.sympatico.ca
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.
Action by USFDA 02/16/2010	Concurred with Conference action on Proposal 05-111.

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	1/4/05	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 for Consideration at the Interstate Shellfish Sanitation Conference 2011 Biennial Meeting	☒ **Growing Area** ☐ **Harvesting/Handling/Distribution** ☐ **Administrative**
Name of Submitter:	Kenneth F. Micciche, Director of Marketing
Affiliation:	Advanced Instruments, Inc.
Address:	Two Technology Way Norwood, MA 02062
Phone: **Fax:** **Email:**	781-320-9000 781-320-8181 kenm@aicompanies.com
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	Recommended adoption of Laboratory Methods Review Committee recommendation on

Task Force I	Proposal 05-115.
Action by 2009 General Assembly	Adopted recommendation of 2009 Task Force I on Proposal 05-115.
Action by USFDA 02/16/2010	Concurred with Conference action 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

LEAST COST FORMULATIONS, LTD.
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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)

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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.
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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-?.
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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 for Consideration at the Interstate Shellfish Sanitation Conference 2011 Biennial Meeting	☒ **Growing Area** ☐ **Harvesting/Handling/Distribution** ☐ **Administrative**
Name of Submitter:	US Food and Drug Administration
Affiliation:	US Food and Drug Administration
Address:	5100 Paint Branch Parkway College Park, MD 20740
Phone: **Fax:** **Email:**	(301) 436-1410 (301) 436-2601 Paul.Distefano@fda.hhs.gov
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 biotoxin 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 (c) 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 Poison**ing** (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. comm.). *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 organisation 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.

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Twiner, M.J., Rehmann, N., Hess, P., Doucette, G.J. 2008. Azaspiracid shellfish poisoning: a review on the chemistry, ecology, and toxicology with an emphasis on human health impacts. 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.
Action by USFDA 02/16/2010	Concurred with Conference action on Proposal 09-101.

Proposal for Consideration at the Interstate Shellfish Sanitation Conference 2011 Biennial Meeting	☒ **Growing Area** ☐ **Harvesting/Handling/Distribution** ☐ **Administrative**
Name of Submitter:	Anita Wright
Affiliation:	University of Florida – Aquatic Food Products Lab
Address:	105 AFPL – P.O. Box 110375 Gainesville, FL 32611
Phone: **Fax:** **Email:**	352-392-1991 Ext. 311 352-392-8594 vmga@ufl.edu
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.
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.
Action by USFDA 02/16/2010	Concurred with Conference action on Proposal 09-102.

Research Need for Consideration at the Interstate Shellfish Sanitation Conference 2011 Biennial Meeting	
Name of Submitter:	Anita Wright
Affiliation:	University of Florida
Address:	Bldg 475 Newell Dr. Gainesville, FL 32611
Phone: **Fax:** **Email:**	352-392-1991 Ext. 311 352-392-9467 acw@ufl.edu

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

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 vibrio 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.

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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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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 accruate 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 expresssed difficulty with DNA probe product reliability and plating problems related to "spreading" colonies that interfer 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 havested from the Gulf of Mexico receive some type of validated post havrest 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 perfomred 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.

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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 innoculated 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.

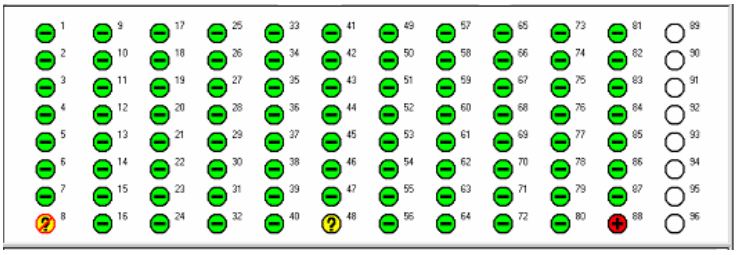
12.4 *Warm up cycler/detector* - Select RUN FULL PROCESS from the menu bar of the application window to heat the instrument to the set temperature (90ºC for the block, 100ºC for the lid).

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	18 cfu/g	5	5	5	100		0	0	-

(25g)									
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
DD10156		Unknown	*Staphylococcus aureus*	Neg	Neg	Neg
DD7426		Unknown	*Staphylococcus aureus*	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*
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.

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Proposal for Consideration at the Interstate Shellfish Sanitation Conference 2011 Biennial Meeting	☒ **Growing Area** ☐ **Harvesting/Handling/Distribution** ☐ **Administrative**
Name of Submitter:	Thomas Stewart
Affiliation:	Mercury Science Inc
Address:	4802 Glendarion Dr Durham, NC 27713
Phone: **Fax:** **Email:**	(866) 861-5836 (407) 557-3720 tom@mercuryscience.com
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

Research Need for Consideration at the Interstate Shellfish Sanitation Conference 2011 Biennial Meeting

Name of Submitter:	Thomas Stewart
Affiliation:	Mercury Science Inc
Address:	4802 Glendarion Dr Durham, NC 27713
Phone: **Fax:** **Email:**	(866) 861-5836 (407) 557-3720 tom@mercuryscience.com

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.
Action by USFDA 02/16/2010	Concurred with Conference action 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.

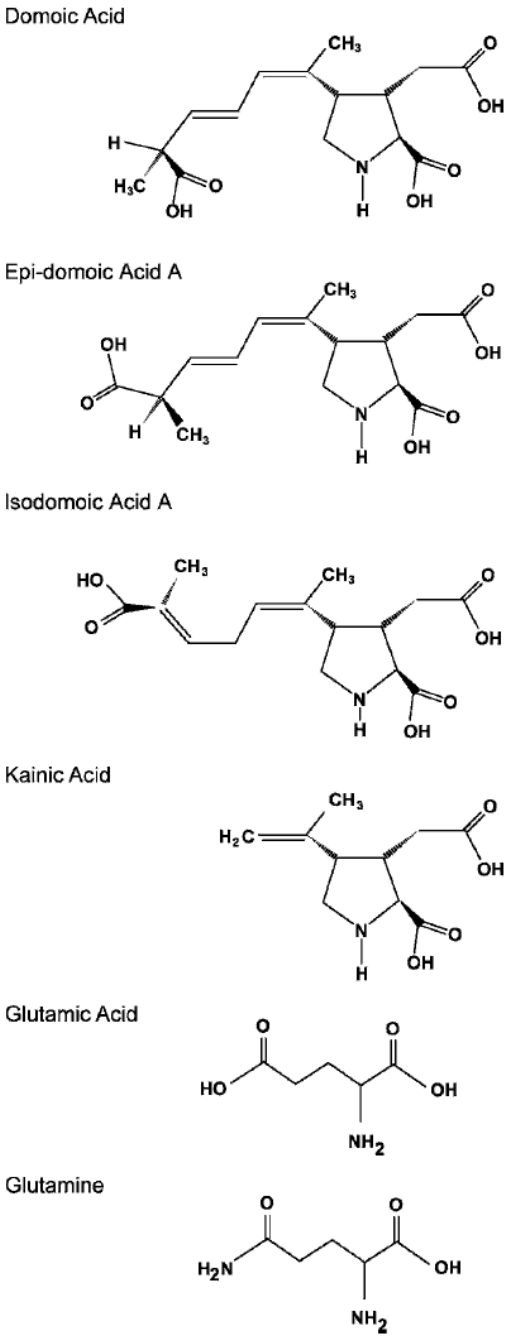

Figure 1. Structure of domoic acid, the isomers epi-domoic acid, isodomoic acid, and two analogues kainic acid and glutamic acid.

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

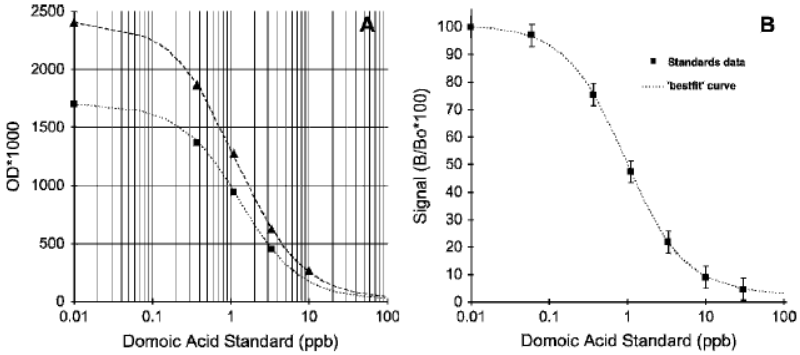

Figure 2. (A) Representative dose response curves for domoic acid analyzed on different days. It should be noted that B_o (the average of the maximal 450 nm absorbance values from the first two wells of a strip to which no DA is added) can vary noticeably between assays depending on differences in ambient 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.

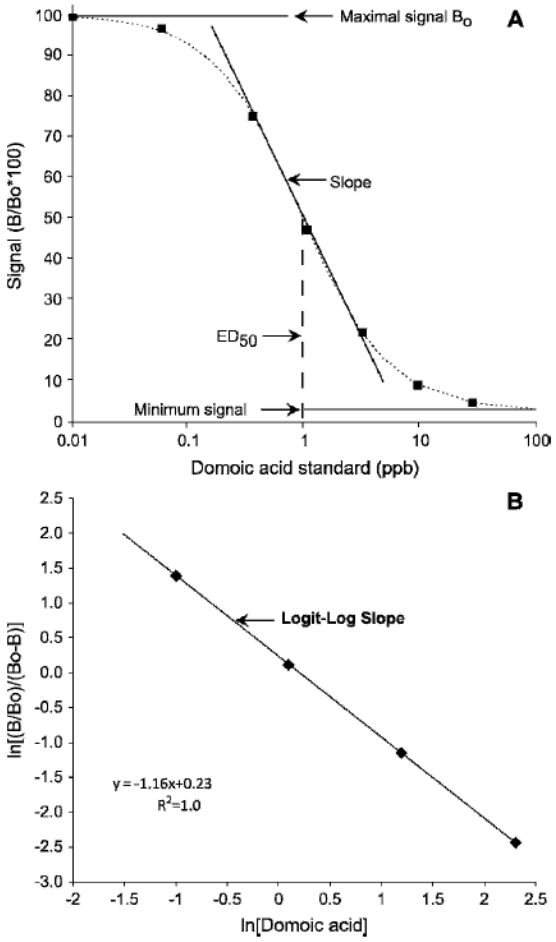

Figure 3. (A) DA concentrations versus the corresponding ELISA absorbance values, which were normalized by dividing by maximal (B_o) absorbance value. (B) Log-logit transform of the data shown in Fig. 3A. From this analysis it was possible to calculate the parameters needed to accurately calculate domoic 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,

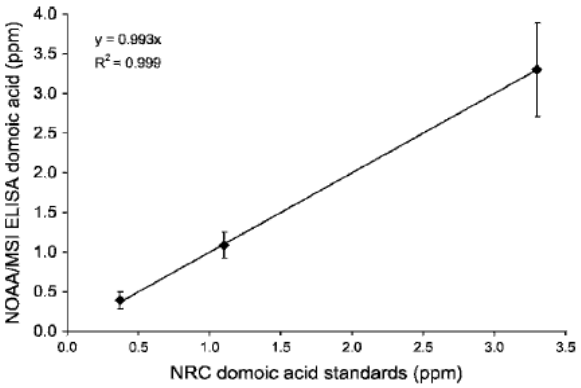

Figure 4. Relationship between various concentrations of National Research Council of Canada (NRC) domoic acid standards and the resultant NOAA/Mercury Science (NOAA/MSI) ELISA values determined using 10 different plates.

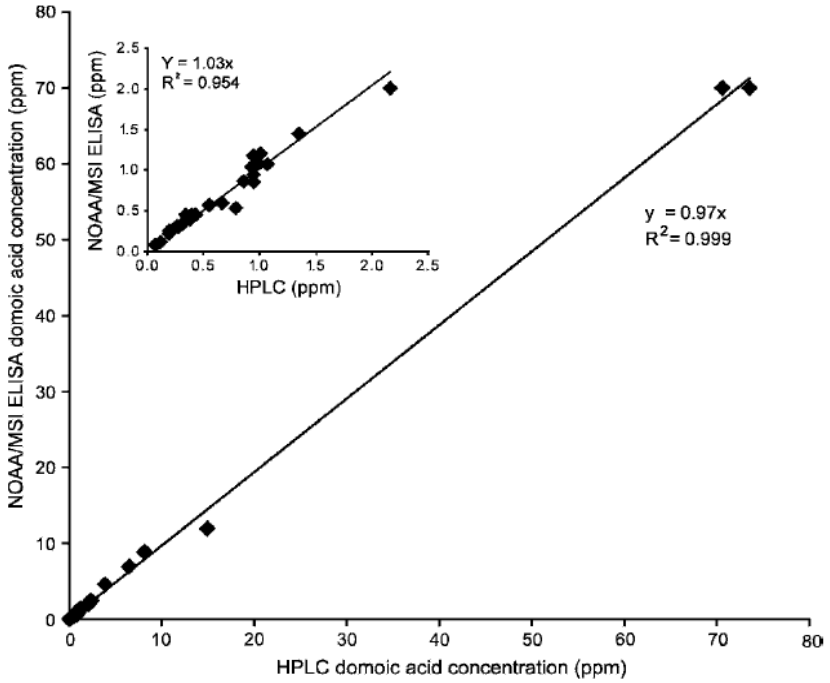

Figure 5. Domoic acid concentrations in razor clam tissues determined from replicate tissue extracts analyzed using HPLC and NOAA/Mercury Science (NOAA/MSI) ELISA. The inset shows an expanded version of the regression analysis for sample containing less than 2.5 ppm domoic acid.

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.

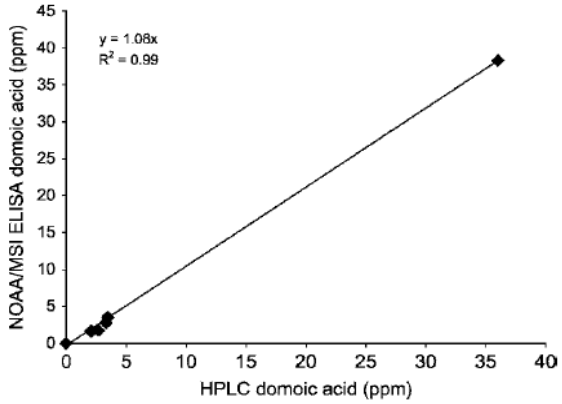

Figure 6. Domoic acid concentrations in mussel tissues determined using HPLC and the NOAA/Mercury Science (NOAA/MSI) ELISA. Aliquots from each sample were run simultaneously.

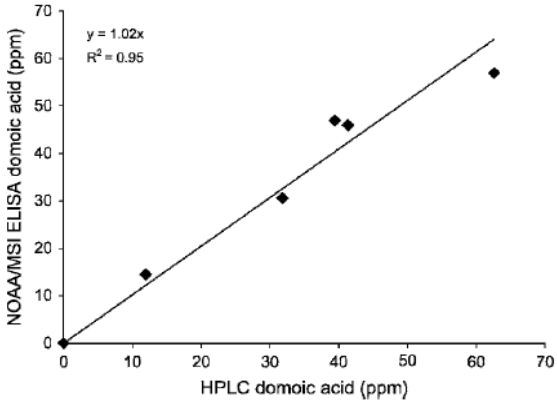

Figure 7. Concentration of domoic acid in scallop tissues extracted from the scallop (*Pecten maximus*) using the standard NOAA/Mercury Science (NOAA/MSI) protocol.

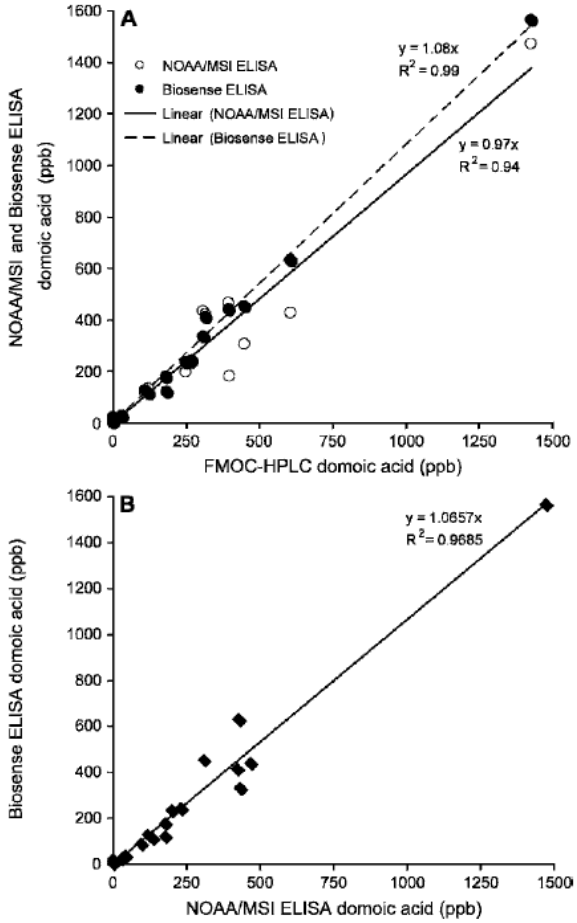

Figure 8. (A) Domoic acid concentrations measured from split phytoplankton sample extracts, which were measured within 24 h by FMOC-HPLC and either the NOAA/Mercury Science (NOAA/MSI) or Biosense ELISAs. (B) Comparison of domoic acid concentrations measured in split samples by either HPLC or ELISA.

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.

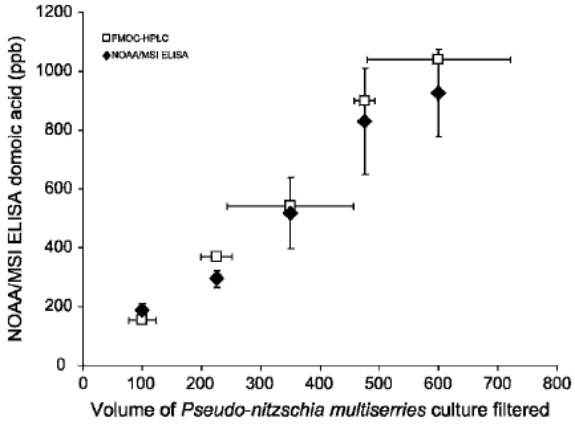

Figure 9. Comparison of the variability between phytoplankton extracts measured using FMOC-HPLC (□) and the NOAA/Mercury Science ELISA (♦). Error bars indicate ±1 SD. Because the error bars largely overlap, for clarity, the standard deviation for the FMOC-HPLC is plotted in the horizontal direction and 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

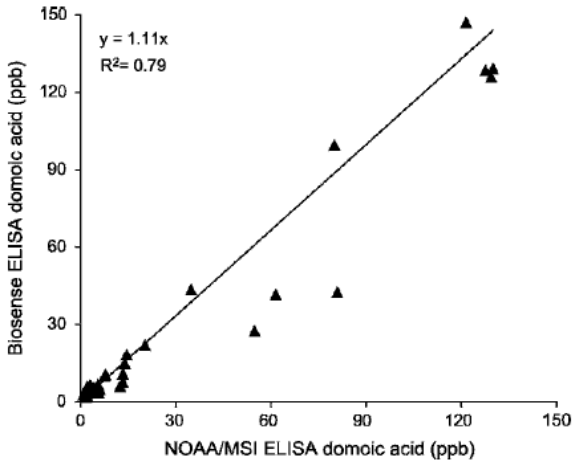

Figure 10. NOAA/MSI ELISA versus Biosense ELISA for phytoplankton samples when the two assays were run several weeks apart showing the increased variability, caused by differential degradation or absorption rates, when samples are not measured within the same 24 h period.

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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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

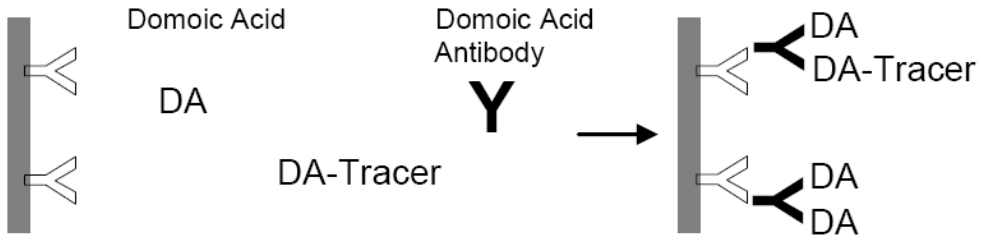
Domoic Acid Screening Test Kit

For Scientific Research Use Only.
This product is not to be used for In Vitro or In Vivo Diagnosis.

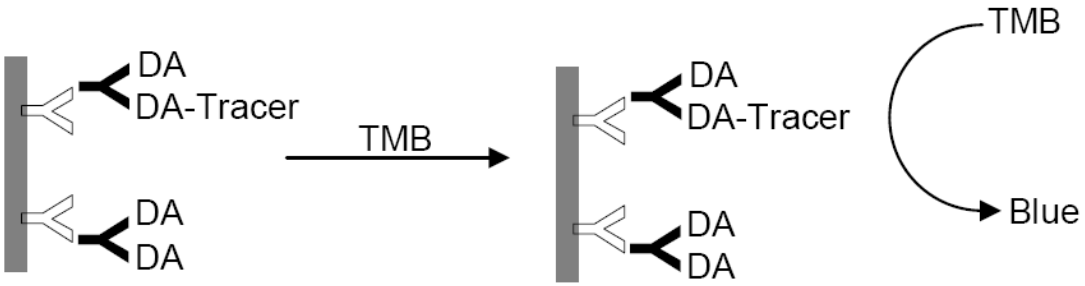
PRINCIPLES OF THE ASSAY
This product contains an antibody (Ab) that binds Domoic Acid and has been developed for the semi-quantitative detection of Domoic Acid in sample extracts. The signal of samples and a control are compared to determine the amount of Domoic Acid present.

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:

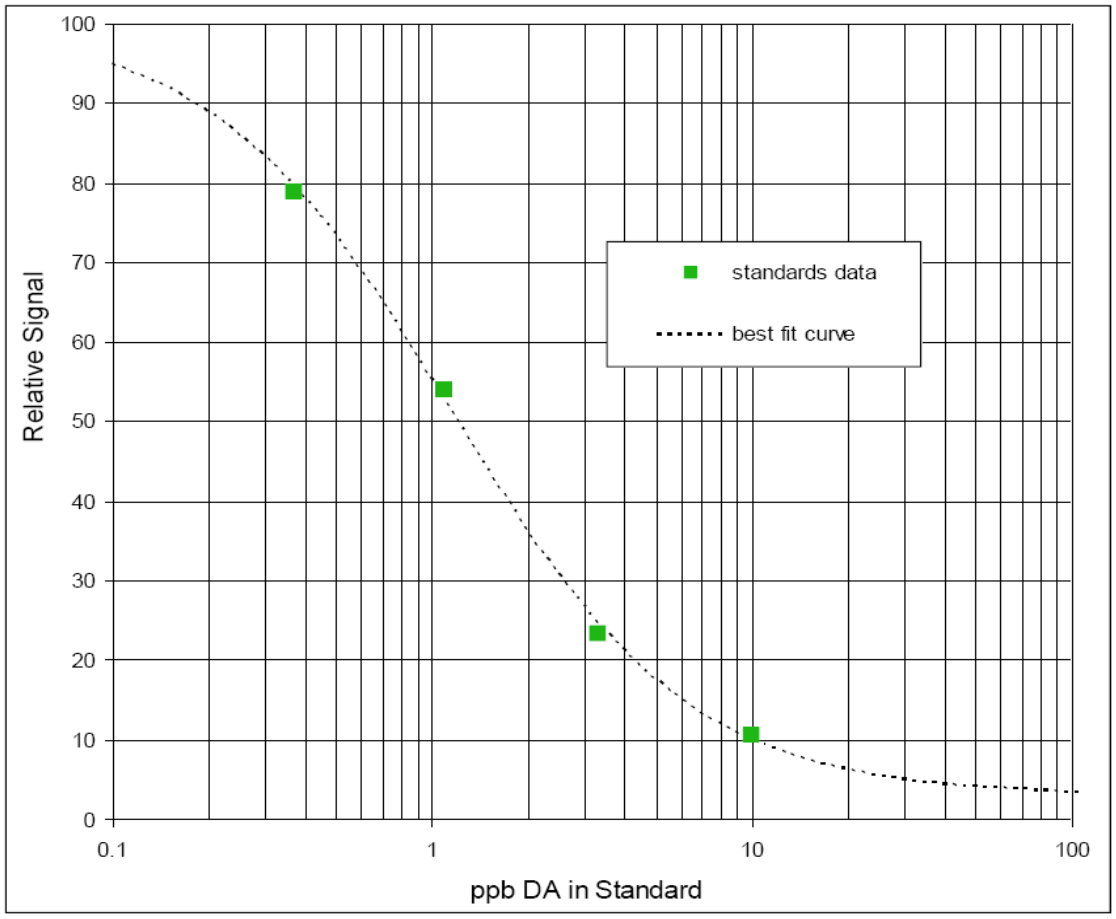
http://mercuryscience.com/Domoic Acid Quantitation 8Well Strip.xls

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.

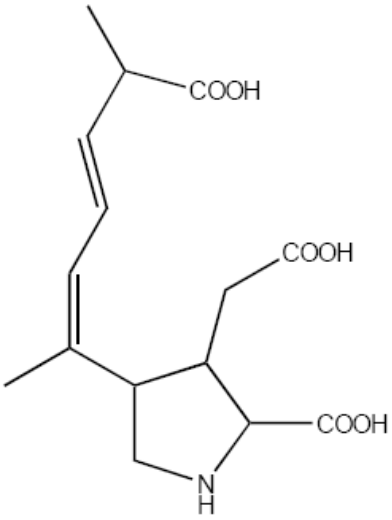
1. Microtiterplate Reader able to measure Absorbance at 450 nm
2. Platewasher
3. Plate Shaker
4. 8 Channel pipet
5. Pipetmen (P10, P200 and P1000)

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

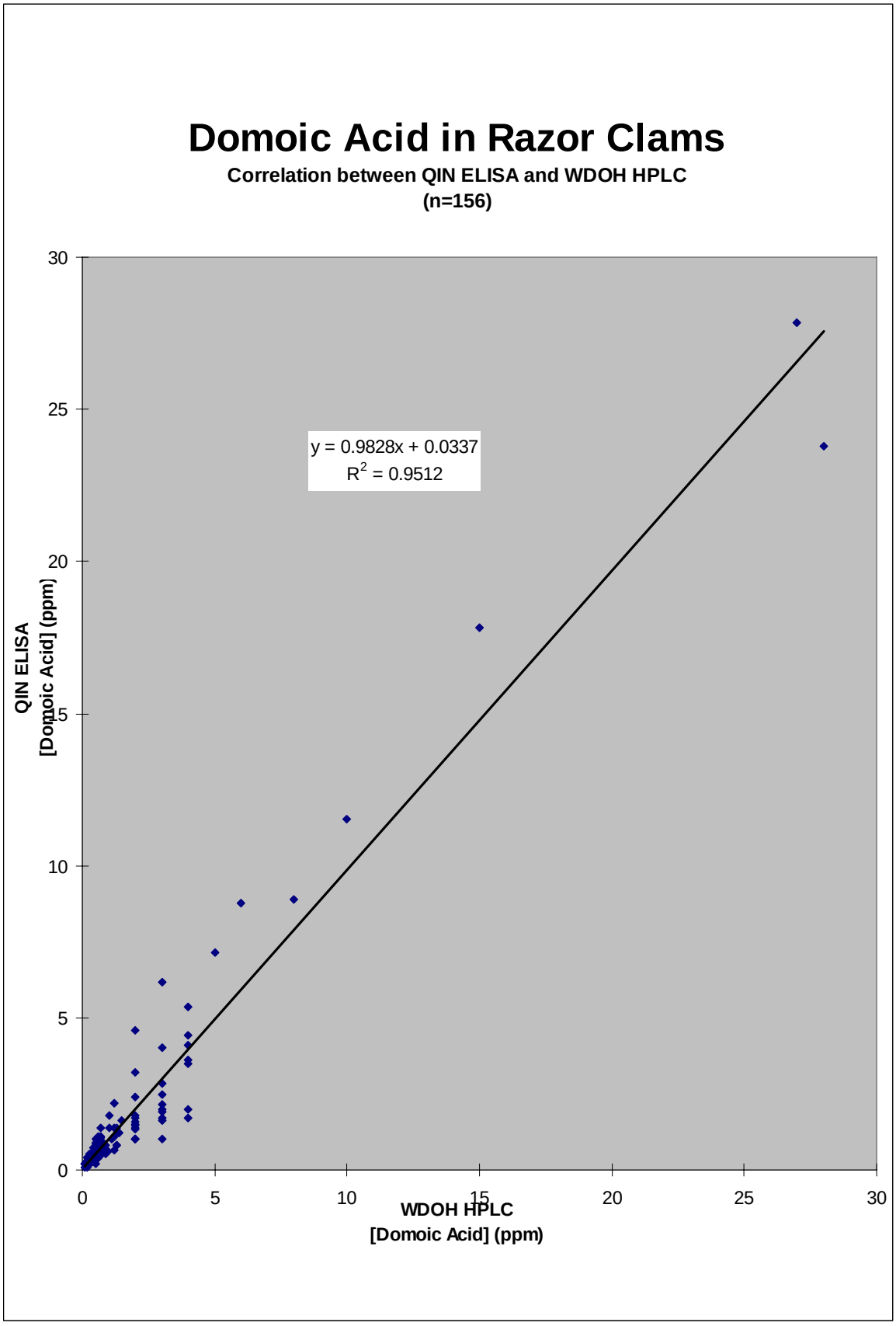


Domoic Acid Test Kit

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%

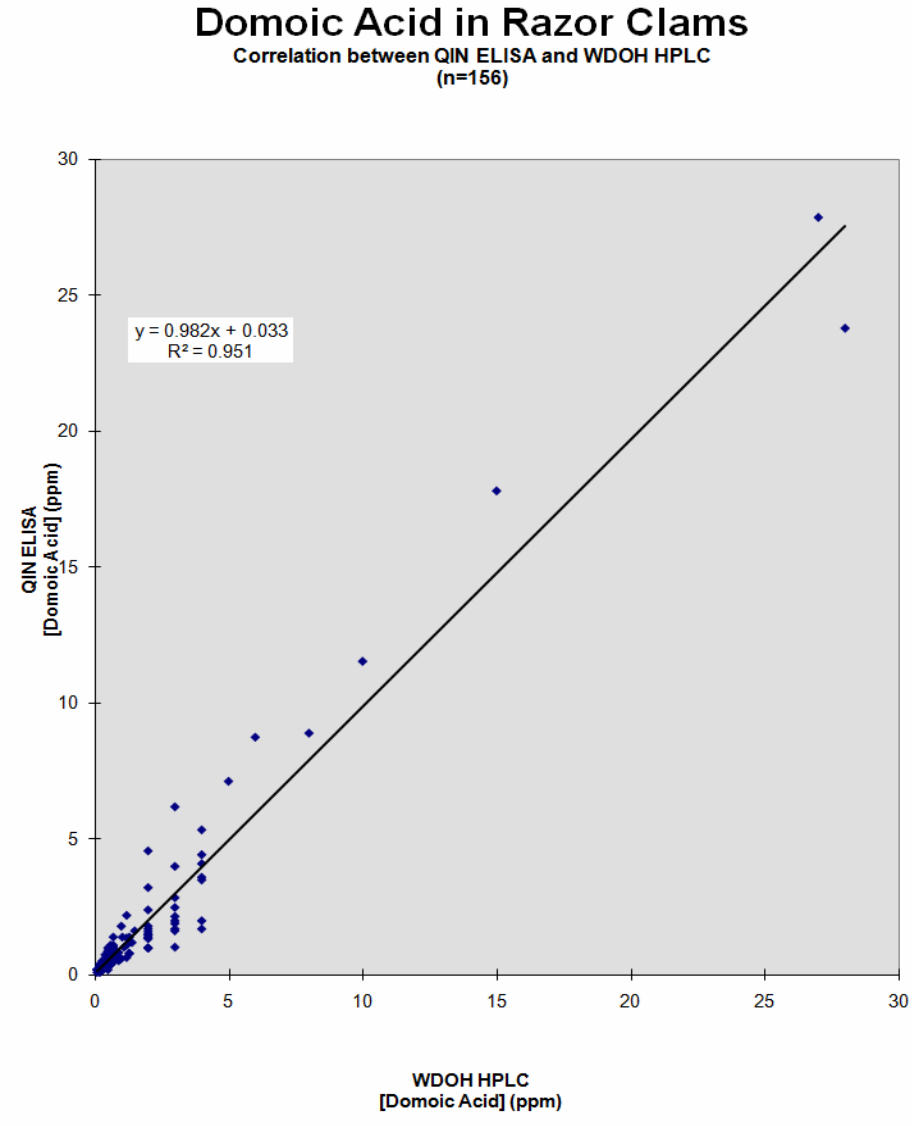
Domoic Acid standard solutions can be run as needed to QC the accuracy of the assay. QC protocols can be developed on a case-by-case basis with assistance provided by Mercury Science Inc.

• 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

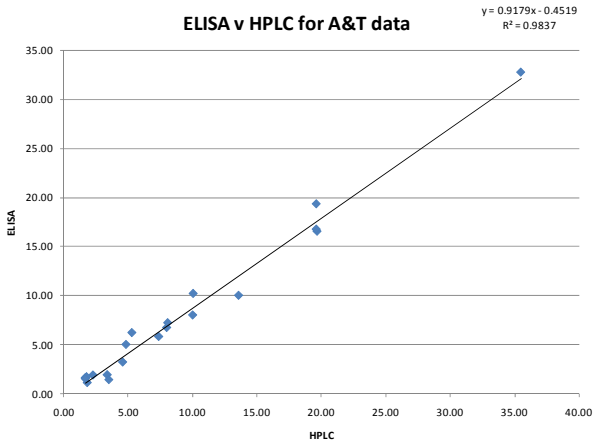
1. ~1.4mL from each tube were transferred into a 2mL microfuge tube
2. Samples in microfuge tubes centrifuged at 14,000 rpm for 5 min
3. Aliquots of the resulting supernatant were diluted with ELISA kit sample dilution buffer with a 2 step dilution series so each extract contained ~2ppb
4. Diluted extracts 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.

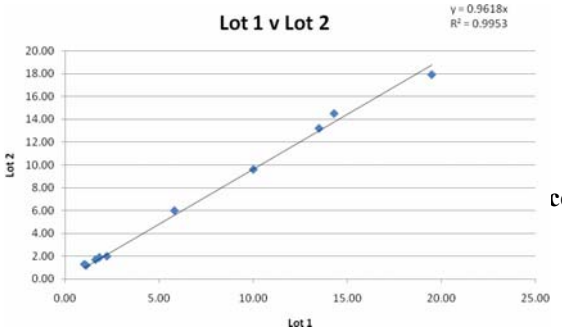
Procedure: This procedure is applicable for use with either growing waters or shellfish tissue. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish type of interest use a minimum of 10 – 12 animals. For each sample take two (2) aliquots of either 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 for Consideration at the Interstate Shellfish Sanitation Conference 2011 Biennial Meeting	☒ **Growing Area** ☐ **Harvesting/Handling/Distribution** ☐ **Administrative**
Name of Submitter:	Darcie Couture and Bruce Chamberlain
Affiliation:	Maine Department of Marine Resources
Address:	P O Box 8 West Boothbay Harbor, Maine 04575
Phone: **Fax:** **Email:**	207 633 9570 207 633 9570 darice.couture@maine.com
Proposal Subject:	Post Harvest Processing
Specific NSSP Guide Reference:	2009 NSSP Section II Model Ordinance Chapter IV Shellstock Growing Areas @.03 Growing Area Classification D (1)(a)(ii)
Text of Proposal/ Requested Action	D. Restricted Classification. (1) General (a) A growing area may be classified as restricted when: (i) A sanitary survey indicates a limited degree of pollution; and (ii) Levels of fecal pollution, human pathogens, or poisonous or deleterious substances are at such levels that shellstock can be made safe for human consumption by either relaying, depuration or low acid-canned food processing <u>or by other verifiable processes.</u>
Public Health Significance:	Including new technology for safe processing of shellstock from restricted areas will result in expanded industry access to resource, while maintaining public health standards.
Cost Information (if available):	N/A

Proposal for Consideration at the Interstate Shellfish Sanitation Conference 2011 Biennial Meeting	☒ **Growing Area** ☐ **Harvesting/Handling/Distribution** ☐ **Administrative**
Name of Submitter:	Thomas L. Howell
Affiliation:	Spinney Creek Shellfish, Inc
Address:	PO Box 310 Eliot, ME 03903
Phone: **Fax:** **Email:**	207-439-2719 207-439-7643 tlhowell@spinneycreek.com
Proposal Subject:	Re-opening Conditional Areas using Male-specific Coliphage after WTP Malfunction
Specific NSSP Guide Reference:	NSSP 2009 Section II Model Ordinance Chapter IV Shellstock Growing Areas @ .03 Growing Area Classification A. (5) (c) (ii)
Text of Proposal/ Requested Action	(ii) For emergency closures ~~(not applicable for conditional closures)~~ of harvest areas caused by the occurrence of raw untreated sewage or <u>partially treated sewage</u> 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 coliphage 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
Public Health Significance:	Male-specific Coliphage (MSC) is an RNA virus of E. coli present in high numbers in raw sewage (on the order of 10^5 PFU/100gm). MSC is similarly resistant to chlorine disinfection as are norovirus and hepatitis A viruses, which are the viral pathogens of primary concern in sewage. MSC is a good surrogate or marker for these enteric viruses. Raw or partially treated sewage accidentally discharged into a growing area by sewage by-pass from pump station failures, broken sewage lines, or malfunctions at the wastewater treatment facilities represent a serious public health risk and require emergency closure of adjacent conditional growing areas. These closures are typically 21 days after the wastewater treatment system returns to normal operation. Recent work has shown that persistence of viruses in the growing waters is much lower in the summer months than in the winter months. Likewise, bio-accumulation rates and retention of enteric viruses in molluscan shellfish is much lower in the summer months than the winter months. MSC can be a useful tool for state shellfish programs to mitigate the negative effect of prolonged conditional closures due to wastewater treatment system failures. This approach is most appropriate in the late-spring and summer months to shorten these closures from 21 to 7 days.
Cost Information (if available):	The Male-specific Coliphage (MSC) Method is an inexpensive double-agar pour plate method that can be run in any state-certified microbiological laboratory. A refrigerated centrifuge capable of 9,000G is required which costs $10K to $12K (USD). Re-opening after 7 days using MSC method is optional for state shellfish control agencies.

Proposal for Consideration at the Interstate Shellfish Sanitation Conference 2011 Biennial Meeting	☒ **Growing Area** ☐ **Harvesting/Handling/Distribution** ☐ **Administrative**
Name of Submitter:	Thomas L. Howell
Affiliation:	Spinney Creek Shellfish, Inc
Address:	PO Box 310 Eliot, ME 03903
Phone: **Fax:** **Email:**	207-439-2719 207-439-7643 tlhowell@spinneycreek.com
Proposal Subject:	Using Male-specific Coliphage as a Tool to Refine Determinations of the Size of the Areas to be Classified as Prohibited Adjacent to Each Outfall
Specific NSSP Guide Reference:	NSSP 2009 Section II Model Ordinance Chapter IV Shellstock Growing Areas @.03 Growing Area Classification E. (5)
Text of Proposal/ Requested Action	(c) An assessment of the combined impact of waste water treatment plant outfall and/or ex-filtration (leakage) from sewerage collection systems may be performed using male-specific coliphage assays on shellstock from adjacent growing areas. A male-specific coliphage standard of ≤ 50 PFU/100gm in shellfish meats may be used as the basis for the determination of the size of the adjacent area to be classified as conditionally restricted or approved.
Public Health Significance:	Male-specific Coliphage (MSC) is a RNA virus of E. coli present in high numbers in raw sewage (on the order of 10^5 PFU/100gm). MSC is similarly resistant to chlorine disinfection as are norovirus and hepatitis A viruses, which are the viral pathogens of concern in sewage. MSC is a good surrogate or marker for these enteric viruses and is a powerful tool to assess the impact on a growing area of raw, partially treated and treated sewage on adjacent growing areas. US and EU studies show that during the summer months MSC and associated pathogenic enteric viruses are at seasonal lows. Conversely, the risk of viral disease transmission is significantly higher in the winter months as evidenced by epidemiological studies as well as studies conducted using MSC and molecular detection of target pathogens. A better assessment of the risk of viral contamination at a particular location in an adjacent growing area at a particular time of year can be ascertained directly using MSC assays of the shellstock. Performing and evaluating dye studies on waste water treatment plant outfall evaluation is expensive and complicated. Difficulties assessing ex-filtration and leakage from the sewage collection system are well known. Few tools and less guidance are available to adequately assess the performance of a particular waste water treatment plant design and its operation with respect to virus removal. The advantages of using this specialty viral indicator to assess the overall impact of a municipal wastewater treatment system on a particular growing area are many. In growing areas impacted by waste water treatment systems, positive norovirus detected by molecular methods at significant levels in the shellfish are accompanied by corresponding high levels of MSC. MSC assays are a direct and straightforward method to determine the viral risk or validate traditional assessment techniques.
Cost Information (if available):	The Male-specific Coliphage (MSC) method is an inexpensive double-agar pour plate method, which can be run in any state-certified microbiological laboratory. A refrigerated centrifuge capable of 9,000G is required which costs $10K to $12K (USD). Cost savings and a higher level of public health protection may be realized using MSC assays of shellfish verses the level of effort needed to ascertain the viral risk indirectly through dye studies, 1000:1 dilution line determinations and performance evaluations.

Proposal for Consideration at the Interstate Shellfish Sanitation Conference 2011 Biennial Meeting	☒ **Growing Area** ☐ **Harvesting/Handling/Distribution** ☐ **Administrative**
Name of Submitter:	Thomas L. Howell
Affiliation:	Spinney Creek Shellfish, Inc
Address:	PO Box 310 Eliot, ME 03903
Phone: **Fax:** **Email:**	207-439-2719 207-439-7643 tlhowell@spinneycreek.com
Proposal Subject:	Alternative Male-specific Coliphage Meat Standard for Restricted Classification of Growing Areas Impacted by wastewater treatment plant outfall.
Specific NSSP Guide Reference:	NSSP 2009 Section II Model Ordinance Chapter IV Shellstock Growing Area @ .02 Bacteriological Standards G. – add new section (4)
Text of Proposal/ Requested Action	(4) Exception. If the Male-specific Coliphage indicator is used for supplemental process verification using an end-point meat standard of < 50PFU/100gm and existing fecal coliform testing requirements in Chapter XV .03 J. are used, then FC water quality monitoring is not required for the restricted classification of growing areas affected by point sources such as wastewater treatment plant outfall.
Public Health Significance:	Under shellfish relay, water quality requirements are not needed for the restricted classification when a contaminant reduction study is conducted and a minimum time period of two weeks is used. For depuration, the restricted classification requires water quality monitoring and standards. The reason for these upper FC limits is that FC meat indicator does not adequately reflect the viral risk and/or viral depuration kinetics. Male-specific coliphage is a viral indicator organism to be used in growing areas impacted by point source sewage contamination. MSC demonstrates significant advantages over FC alone for both the assessment of viral contamination and assessment of viral depuration kinetics. Upper FC limits were put into the NSSP to prevent shellfish with higher levels of viruses from being depurated. Several studies clearly show that conventional depuration using FC for process validation is not adequate to protect public health with respect to virus contamination in growing areas with significant wastewater treatment plant and sewage impact. Studies have also shown that viral levels in shellfish impacted by sewage and partially treated sewage detected using MSC and molecular techniques are much lower in the summer months than the winter months. Additionally, the viral depuration rate is higher in the summer with process waters >18°C. Recent studies have also shown that MSC is an appropriate viral indicator to assess viral depuration. Therefore, seasonal viral depuration using male-specific coliphage as well as FC for process verification is a superior approach to taking water samples using FC in a growing area adjacent to wastewater treatment plant outfall. Combining the bacterial indicator of FC and the viral indicator MSC for mitigation strategies that use meat scores is far more direct and effective than water quality sampling in this context.
Cost Information (if available):	The Male-specific Coliphage (MSC) method is an inexpensive double-agar pour plate method that can be run in any state-certified microbiological laboratory. A refrigerated centrifuge capable of 9,000G is required which costs $10K to $12K (USD). Significant cost savings and a higher level of public health protection may be realized using strategies such as seasonal coliphage depuration process validated using MSC and seasonal coliphage relay using MSC in contaminant reduction studies than requiring water quality limits using FC.

Proposal for Consideration at the Interstate Shellfish Sanitation Conference 2011 Biennial Meeting	☒ **Growing Area** ☐ **Harvesting/Handling/Distribution** ☐ **Administrative**
Name of Submitter:	ISSC Executive Office Patti Fowler
Affiliation:	Interstate Shellfish Sanitation Office (ISSC) Department of Environment & Natural Resources
Address:	209-2 Dawson Road Columbia, SC 29223 P.O. Box 769 Morehead City, NC 28557
Phone: **Fax:** **Email:**	803-788-7559 803-788-7576 issc@issc.org 252-808-8147 252-726-8475 patti.fowler@ncdenr.gov
Proposal Subject:	Use of analytical methods other than NSSP methods
Specific NSSP Guide Reference:	2009 NSSP Section II Model Ordinance Chapter III Laboratory @ .02 Methods, Paragraphs A, C, D (1) and (2)
Text of Proposal/ Requested Action	Revise Chapter III @.02 Methods, Paragraphs A, C and D as follows. Chapter III @ .02 Methods A. Microbiological. Methods~~, practices, and procedures~~ for the analyses of shellfish and shellfish growing or harvest waters shall be: (1) the Approved NSSP M~~m~~ethods validated for use in the National Shellfish Sanitation Program under Procedure XVI of the Constitution, Bylaws and Procedures of the ISSC and / or cited in the Guidance Documents, Chapter II. Growing Areas .10 Approved National Shellfish Sanitation Program Laboratory Tests; (2) When there is an immediate or ongoing critical need for a method and no Approved NSSP Method exists, the following may be used: (a) A validated AOAC, BAM, or EPA method; (b) An Emergency Use Method pursuant to .02 D. (1) and (2) below. B. Chemical and Physical. (1) Methods for the analysis of shellfish and shellfish growing or harvest waters shall: (a) Be the current AOAC or APHA method for all physical and chemical measurements; and (b) Express results of all chemical and physical measurements in standard units, and not instrument readings. (2) When there is an immediate or ongoing critical need for a Method and no Approved NSSP Method exist, the following may be used: (a) A Validated AOAC, BAM, or EPA method; (b) An Emergency Use Method pursuant to .02 D. (1) and (2) below. ~~(2) When an AOAC or APHA method is not available, EPA methods may be used.~~ ~~(3) If a method is not approved or validated by AOAC, APHA, or EPA then the method shall be validated in accordance with Procedure XVI of the Constitution, Bylaws and Procedures of the ISSC.~~

	C. Biotoxin. Methods for the analyses of shellfish and shellfish harvest waters shall be: (1) The current AOAC and APHA methods used in the bioassay for paralytic shellfish poisoning toxins : and (2) The current APHA method used in the bioassay for *Karenia brevis* toxins; or (3) Approved NSSP Methods validated for use ~~in the National Shellfish Sanitation Program~~ under Procedure XVI of the Constitution, Bylaws and Procedures of the ISSC and / or cited in the Guidance Documents, Chapter II. Growing Areas .10 Approved National Shellfish Sanitation Program Laboratory Tests. (4) When there is an immediate or ongoing critical need for a method and no Approved NSSP Method exists, the following may be used: (a) A validated AOAC, BAM, or EPA method; (b) An Emergency Use Method pursuant to .02 D. (1) and (2) below. D. Emergency Use ~~Emerging~~ Methods. (1) When there is an immediate or critical need and no Approved NSSP ~~approved m~~ Method~~s~~ exists, ~~and~~ the ISSC Executive Board may grant interim approval to ~~considers allowing~~ an unapproved or non-validated method to be used for a specific purpose.~~,~~ ~~t~~ The following minimum requirements ~~as the Lab Method Review Committee Advisory for Emerging Methods~~ will be provided to the Executive Board prior to granted interim approval ~~and shall contain the following criteria~~: (a) Name of Method (b) Date of Submission (c) Specific purpose or intent of the method for use in the NSSP (d) Step by step procedure including equipment, reagents and safety requirements necessary to run the method (e) Data generated in the development and/or trials of the method and/or comparing to approved methods if applicable (f) Any peer reviewed articles detailing the method (g) Name of developer(s)~~/~~ or SSCA submitter~~s~~ (h) Developer/submitter contact information (2) Within two years of Executive Board interim approval ~~the initial allowed use of~~ the Emergency Use M~~m~~ethod, the entire Single Lab Validation Protocol should be submitted. The Lab Methods Review Committee will report to the Executive Board on the status of the Single Lab Validation Protocol data submission.
Public Health Significance:	
Cost Information (if available):	None

Proposal for Consideration at the Interstate Shellfish Sanitation Conference 2011 Biennial Meeting	☒ **Growing Area** ☐ **Harvesting/Handling/Distribution** ☐ **Administrative**
Name of Submitter:	Laboratory Methods Review Committee/Patti Fowler Chair
Affiliation:	ISSC
Address:	P.O. Box 769 Morehead City, NC 28557
Phone: **Fax:** **Email:**	252-808-8147 252-726-8475 patti.fowler@ncdenr.gov
Proposal Subject:	Use of analytical methods other than NSSP methods
Specific NSSP Guide Reference:	2009 NSSP Section II Model Ordinance Chapter III Laboratory @ .02 Methods, Paragraphs A, C, D (1) and (2)
Text of Proposal/ Requested Action	Revise Chapter III @.02 Methods, Paragraphs A, C and D as follows. Chapter III @ .02 Methods A. Microbiological. Methods~~, practices, and procedures for the analyses of shellfish and shellfish growing or harvest waters shall be the methods validated for use in the National Shellfish Sanitation Program under Procedure XVI of the Constitution, Bylaws and Procedures of the ISSC and/or cited in the Guidance Documents, Chapter II. Growing Areas .10 Approved National Shellfish Sanitation Program Laboratory Tests.~~ for the analyses of shellfish and shellfish harvest waters shall be: (1) The methods validated for use in the National Shellfish Sanitation Program under Procedure XVI of the Constitution, Bylaws and Procedures of the ISSC and listed in the Guidance Documents, Chpater II. Growing Areas .10 Approved National Shellfish Sanitation Program Laboratory Tests. (2) When there is an immediate need for a method of analysis and no NSSP approved analytical method exists, a validated AOAC, BAM or EPA method may be used. (3) When there is an ongoing critical need for a method of analysis and no NSSP approved analytical method exista an emergent method may be used pursuant to .02 D (1) and (2) below. B. Chemical and Physical (1) Methods for the analysis …… (a) Be the current …….. (b) Express results of all …… (2) When an AOAC…….. (3) If a method is not …….. C. Biotoxin. Methods for the analyses of shellfish ~~and shellfish harvest waters~~ shall be: (1) The current AOAC and APHA …… (2) The current APHA method …… (3) Methods validated for use in the National Shellfish Sanitation Program under

	Procedure XVI of the Constitution, Bylaws and Procedures of the ISSC and~~/or cited~~ listed in the Guidance Documents, Chapter II. Growing Areas .10 Approved National Shellfish Sanitation Program Laboratory Tests. (4) When there is an immediate need for a method of analysis and no NSSP approved method exists, a validated AOAC method may be used. (5) When there is an ongoing critical need for a method of analysis and no NSSP approved method exists, an emergent method may be used pursuant to .02 D (1) and (2) below. D. Emerging Methods. (1) When there is an ~~immediate or~~ ongoing critical need for a method of analysis and no NSSP approved method exists, ~~and~~ the ISSC Executive Board may consider~~s~~ allowing an unapproved or non-validated method to be used for a specific purpose~~;~~. The minimum requirements as defined in the Laboratory Methods Review Committee Advisory for Emerging Methods will be provided to the Executive Board and shall contain the following: ~~criteria:~~ Name of Method; Date of Submission; Specific purpose or intent of the method for use in the NSSP; Step by step procedure including equipment, reagents and safety requirements necessary to run the method; Data generated in ~~the development and/or comparing to approved methods if applicable~~ support of the efficacy of the method if available; Any peer reviewed articles detailing the method and its efficacy; Name of the developer(s)~~/submitters~~ or SSCA submitter; Developer/submitter contact information. (2) Within two years of the initial allowed use of the emerging method, the entire Single Lab Validation Protocol should be completed and submitted to the ISSC for consideration as an approved method. The Laboratory Methods Review Committee will review the submission and report to the Executive Board on its Status. ~~of the single Lab Validation data submission.~~.
Public Health Significance:	
Cost Information (if available):	None

Proposal for Consideration at the Interstate Shellfish Sanitation Conference 2011 Biennial Meeting	☒ **Growing Area** ☐ **Harvesting/Handling/Distribution** ☐ **Administrative**
Name of Submitter:	Laboratory Methods Review Committee/Patti Fowler Chair
Affiliation:	ISSC
Address:	P.O. Box 769 Morehead City, NC 28557
Phone: **Fax:** **Email:**	252-808-8147 252-726-8475 patti.fowler@ncdenr.gov
Proposal Subject:	Definitions for Types I, II, III and IV Methods
Specific NSSP Guide Reference:	2009 NSSP Section II - Model Ordinance - Purpose and Definitions
Text of Proposal/ Requested Action	Add the following definitions: (115) **Type I Methods** mean the core methods of analysis used to support established Program requirements within the NSSP. Type I methods have been evaluated and the performance characteristics for specific applications in the NSSP have been determined and found fit for purpose. (116) **Type II Methods** mean permanent methods of analysis used widely within the NSSP as alternative methods to improve turnaround time, cost effectiveness or to develop analytical capacity beyond what is achieved by the core methods. Type II methods are NSSP validated and the performance characteristics for specific applications within the NSSP have been determined and found fit for purpose. (117) **Type III Methods** mean interim methods of analysis used to fill an ongoing NSSP Program need. Type III methods are NSSP validated and the performance characteristics for specific applications within the NSSP have been determined and found fit for purpose. Type III methods are designated for periodic review and assessment by the Laboratory Methods Review Committee for continued use, redesignation or deletion. (118) **Type IV Methods** mean provisional methods of analysis developed to fill an ongoing NSSP Program need. Type IV methods are newly accepted for use in the NSSP and/or not yet used for Program support outside the laboratory in which the method was developed and/or validated. Type IV methods are NSSP validated and the performance characteristics for specific applications within the NSSP have been determined and found fit for purpose. Type IV methods are designated for periodic review and assessment by the Laboratory Methods Review Committee for continued use, redesignation or deletion. ~~(115)~~ (119) **Wet storage** means …….
Public Health Significance:	These definitions help clarify the various categories of analytical methods accepted for use in the NSSP.
Cost Information (if available):	None

Proposal for Consideration at the Interstate Shellfish Sanitation Conference 2011 Biennial Meeting	☒ **Growing Area** ☐ **Harvesting/Handling/Distribution** ☐ **Administrative**
Name of Submitter:	Mark A. Mozola
Affiliation:	Neogen Corporation
Address:	620 Lesher Place Lansing, MI 48912
Phone: **Fax:** **Email:**	517-372-9200 517-367-0514 mmozola@neogen.com
Proposal Subject:	Reveal ASP (Domoic Acid) test kit
Specific NSSP Guide Reference:	2009 NSSP Section IV Guidance Documents Chapter II. Growing Areas .10 Approved NSSP Laboratory Tests, Table 4 - Type III and Type IV Marine Biotoxin Test Methods
Text of Proposal/ Requested Action	We request review of the validation study submission for the Reveal ASP (domoic acid) test kit and consideration of the method for approval as a Type IV marine biotoxin screening method for qualitative determination of domoic acid in shellfish. Add Reveal ASP (domoic acid) test to list of approved Type III and Type IV marine biotoxin methods.
Public Health Significance:	Amnesic shellfish poisoning is caused by the toxin domoic acid, produced by phytoplankton of the genus Pseudonitzschia. It is associated with eating contaminated oysters, clams, mussels, and other shellfish. There have been numerous outbreaks of ASP, and there is evidence that the occurrence of the phytoplankton responsible for ASP is widespread. Current methods for detection of domoic acid consist primarily of instrumental chemistry methods, which are laborious and time-consuming. Methods for rapid screening for domoic acid, in field and laboratory settings, are needed and will assist the industry and public health authorities in responding to this health concern. The Reveal ASP test is a lateral flow immunoassay designed for qualitative determination of domoic acid in shellfish at levels of 10 ppm (mg/kg) and above. The test uses minimal equipment and simple reagents, does not require specialized training, and can provide results in 20 minutes from sample receipt, including sample preparation.
Cost Information (if available):	Approximately $17.00 per test.

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	Reveal ASP (Domoic Acid)
Name of the Method Developer	Neogen Corporation
Developer Contact Information	Mark Mozola, 517-372-9200, mmozola@neogen.com

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.		There is a need for a simple, rapid screening method for domoic acid in shellfish, one that can be used in the field as well as in a laboratory setting.
2. What is the intended purpose of the method?		The method is designed for rapid qualitative screening of shellfish for domoic acid.
3. Is there an acknowledged need for this method in the NSSP?		Simply assays that provide rapid results are needed.
4. What type of method? i.e. chemical, molecular, culture, etc.		Lateral flow immunoassay in dipstick format.
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title		Reveal ASP (Domoic Acid)
Method Scope		Qualitative detection of domoic acid in oysters, clams, and mussels.
References		Study report and kit insert included in this submission.
Principle		Competitive lateral flow immunoassay in dipstick format. Water extraction of analyte from homogenized shellfish tissue.
Any Proprietary Aspects		Yes, commercial test kit.
Equipment Required		Extraction containers with lids (40 mL capacity), timer, bag roller, sample cup rack, pipettes (0.1 mL), result interpretation card.
Reagents Required		Reveal ASP test devices, extraction bags with mesh filter, sample cups, distilled water.
Sample Collection, Preservation and Storage Requirements		Shellfish should be collected according to standard industry practices and stored at 2-8^{o}C before testing.
Safety Requirements		Used test devices, extraction bags, sample cups, and pipettes should be treated as if contaminated with domoic acid and handled accordingly. Gloves and lab coats should be worn while performing the test.
Clear and Easy to Follow Step-by-Step Procedure		Step-by-step procedure in kit insert and study report.
Quality Control Steps Specific for this Method		Test device contains an internal positive control that confirms that it is functioning properly. A domoic acid solution in buffer at a concentration $\geq$ 10 mg/kg can be used as an external positive control if desired.

C. Validation Criteria		
1. Accuracy / Trueness		95.9% overall for oysters, clams, and mussels
2. Measurement Uncertainty		Not applicable.
3. Precision Characteristics (repeatability and reproducibility)		Not applicable.
4. Recovery		Not applicable.
5. Specificity		100%. No impact on test results by potentially interfering compounds – okadaic acid, glutamic acid, glutamine, saxitoxin. No false-positive results on unpiked samples.
6. Working and Linear Ranges		Not applicable.
7. Limit of Detection		≥ 10 ppm
8. Limit of Quantitation / Sensitivity		Not applicable.
9. Ruggedness		No statistically significant differences in results using 2 kit lots and +/- 2 min. variation in test incubation time.
10. Matrix Effects		None observed.

11. Comparability (if intended as a substitute for an established method accepted by the NSSP)		Agreement with LC-UV reference method in testing of mussel tissue samples with incurred domoic acid.
D. Other Information		
1. Cost of the Method		Approx. $17.00 per test.
2. Special Technical Skills Required to Perform the Method		None
3. Special Equipment Required and Associated Cost		None
4. Abbreviations and Acronyms Defined		ppm = parts per million, equivalent to mg/kg
5. Details of Turn Around Times (time involved to complete the method)		The test can be performed in approximately 20 minutes including sample preparation.
6. Provide Brief Overview of the Quality Systems Used in the Lab		
Submitters Signature 	Date: June 3, 2011	
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:

13. 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.
14. IUPAC Technical Report, 2002. Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, Pure Appl. Chem., Vol. 74, (5): 835-855.
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16. MAF Food Assurance Authority, 2002. A Guide for the Validation and Approval of New Marine Biotoxin Test Methods. Wellington, New Zealand.
17. National Environmental Laboratory Accreditation. , 2003. Standards. June 5.
18. 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.

Validation Study of the Reveal® ASP Test for the Qualitative Detection of Domoic Acid in Shellfish

Jill Feldpausch, R. Lucas Gray, Jake Knickerbocker, Mark Mozola*, and Jennifer Rice
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Submitted June 3, 2011

Introduction

Domoic acid, produced by certain species of the diatom *Pseudonitzschia*, is the primary toxin responsible for amnesic shellfish poisoning (ASP) associated with consumption of contaminated shellfish including oysters, clams, and mussels. Current methodologies for detection of domoic acid in shellfish are laborious and time-consuming, consisting primarily of LC-UV, LC-MS, and immunoassay procedures. LC-UV methods [1, 2] have been accepted as quantitative reference methods in many parts of the world. Assays facilitating more rapid determination of domoic acid with simplified procedures are needed by the shellfish industry and regulatory authorities.

In this report, we describe results of a validation study of the Reveal® ASP test for qualitative detection of domoic acid in shellfish. Reveal ASP is a lateral flow immunoassay designed for rapid determination of domoic acid at a level of approximately 10 ppm or greater (one-half the regulatory limit in many countries). The test is easy to use and results can be obtained in less than 20 minutes, including sample preparation.

Principle of the Method

Reveal ASP is a single-step, lateral flow immunochromatographic assay based on the principle of competitive immunoassay. Following a simple distilled water extraction of domoic acid from homogenized shellfish tissue, the dipstick-format Reveal device is placed into the extract. The extract is wicked through a reagent zone containing antibodies specific for domoic acid conjugated to colloidal gold particles. If domoic acid is present, it will be captured by the labeled antibody. Migration of the sample continues through a membrane, which contains a zone of domoic acid conjugated to a protein carrier. This zone captures any unbound antibody- gold conjugate, resulting in a visible line. With increasing amounts of domoic acid in the test sample, less unbound conjugate is available for binding to the test line. Thus, intensity of the test line is inversely proportional to the amount of domoic acid in the sample. The test device also incorporates a control conjugate and which binds to a second line. The control line will form regardless of the amount of domoic acid present in the sample, ensuring that the test device is functioning properly. Test results are interpreted as positive or negative by scoring the intensity of the test line using an interpretation card supplied with the test kit.

Intended Use
For the qualitative detection (at greater than or equal to 10 ppm [mg/kg]) of domoic acid in shellfish, including oysters, clams, and mussels.

Reveal ASP Method
The kit insert is included as Appendix I.

Materials Provided
Starter Kit (Neogen #9563), contains:
- Sample cup rack
- Roller

Reveal ASP kit (Neogen #9560), contains:
- 25 lateral flow test strips
- 25 sample cups
- 25 filter extraction bags
- 50 100 µL disposable pipettes
- Interpretation card

Materials Required but not Supplied (available from Neogen Corp. and other sources)
- Blender and blender jar
- Scale, capable of weighing 0.5-400 g $\pm$ 0.1 g
- Timer
- 50-mL graduated cylinder or bottle-top liquid dispenser
- Distilled water
- Leakproof container with lid, 40 mL capacity

Storage Requirements
Store Reveal ASP kit components at controlled room temperature (18-30^{o}C, 64-86^{o}F). Do not freeze.
Test strips should remain in their original sample tubes until use to maintain shelf life and ensure optimal performance.

Precautions
Do not use test kits beyond their expiration date.
Treat all liquids, including sample extract, and used components as if contaminated with toxin.
Gloves and other protective apparel should be worn at all times.
To avoid cross-contamination, use clean pipettes, extraction bags, and fresh extraction solution for each sample.
A Material Safety Data Sheet (MSDS) is available from Neogen Corp.

Sample Preparation and Extraction
Samples should be collected according to accepted sampling techniques.
1. Obtain a representative sample and shell the sample.
2. Thoroughly rinse with cold water.
3. Homogenize in a high-speed blender.

4. Weigh 1.0 ± 0.1 g of homogenized sample, preferably in a leak-proof container capable of holding 40 mL of liquid.

5. Add 20 mL distilled water to the container with sample.

6. Shake the container vigorously by hand for 30 seconds until all shellfish tissue is in solution (a cloudy appearance and/or bubbles are normal).

7. Number one side of the extraction bag "1" and the other side "2".

8. Pour the sample extract into side 1 of the extraction bag. The extraction bag contains a mesh filter which allows for partial filtration of the sample extract.

9. Seal the extraction bag by positioning the green straw approximately 2-3 inches down from the top of the bag, fold the upper edge of the bag so that it covers the green straw, and firmly clip on the white bag clip. This prevents leakage of the sample extract.

10. Press the roller firmly on the extraction bag, pushing the roller back and forth for 30 seconds to aid in obtaining a homogenous sample extract.

11. Slide out the green straw and remove the white bag clip.

12. Pinch the top of the bag and carefully pour all the bag contents from side 2 back into the original sample container (there may be small pieces of shellfish remaining on side 1 of the bag). Discard the used extraction bag.

13. Shake container vigorously by hand for 30 seconds.

14. Remove 100 µL of the sample extract using the disposable pipette* provided (alternatively by use of a standard pipette), and add to a fresh container containing 20 mL distilled water.

    * To use the disposable pipettes provided, firmly press the top bulb of the pipette, insert the tip into the sample extract, and slowly release the top bulb to draw up the sample extract. Excess volume (above 100 µL) will overflow into the lower bulb, ensuring that 100 µL is available to dispense. Press the top bulb firmly and slowly release the top bulb to dispense the liquid into the container with distilled water. Discard the used pipette.

Assay Procedure

All steps should be performed at controlled room temperature (18-30°C, 64-86°C).

1. Remove the appropriate number of sample cups and place in the sample cup rack.

2. Shake the extracted sample prepared above vigorously by hand for 30 seconds.

3. Remove 100 uL using a fresh pipette and add 100 uL to the sample cup.

4. Remove the required number of Reveal ASP test strips from the container and immediately close the container.

5. Place the Reveal test strip with the sample end down (Neogen logo on top) into the sample cup.

6. Allow the test strip to develop in the sample cup for 10 minutes.

7. Remove the test strip and interpret the results as described below.

Interpretation of Results
Test strips should be interpreted immediately following completion of the 10 minute incubation.
Using the interpretation card provided, score the test line intensity to determine if the sample contains less than 10 ppm or greater than or equal to 10 ppm domoic acid.
Note: The control line should always be present and will be darker than the test line. If no control line is visible, this indicates an invalid result and the sample should be retested using another Reveal device.

Single-Laboratory Validation Study
A single-laboratory validation study was conducted to measure accuracy/trueness, specificity, and ruggedness of the Reveal ASP method, as well as effects of potential interfering compounds. In addition, Reveal ASP results were compared to those of an accepted LC-UV reference method [1]. Matrices tested were oysters, clams, and mussels.

I. Accuracy/trueness and specificity

Methods
Fresh oysters, clams, and mussels were obtained from a local retail market that receives fresh shellfish by air shipment daily. Shellfish were held at 2-8^{o}C before use. Shellfish were shucked and approximately 12-15 animals were combined and homogenized in a blender to produce a bulk sample. The bulk samples were separated into 10 portions of 1 g each. Five served as unspiked controls. One each of the remaining 5 samples was spiked separately at 5, 10, 15, 20 and 40 ppm domoic acid. Certified reference material (CRM-DA-f), obtained from the National Research Council, Canada- Institute for Marine Biosciences (NRC- IMB), was used as the spiking material. The CRM consisted of 101.8 µg/mL domoic acid extracted from contaminated cultured blue mussels and dissolved in a solution of 5% acetonitrile/95% water.
Each sample was then prepared according to the procedures in **Sample Preparation and Extraction** above, and tested with the Reveal ASP assay. Ten replicates of each extracted spiked sample and three replicates of each extracted unspiked sample were tested with the Reveal ASP assay.

Accuracy rates were calculated for each shellfish matrix separately and in combination. A dose-response curve was constructed using the combined data.

Results
Results of the accuracy study are shown in Table 1. Accuracy is defined as the level of agreement between the assay and the expected test results based on the domoic acid spike level.
For oysters, accuracy of the Reveal ASP method was 95.4%. Seven of ten tests at 10 ppm domoic acid were positive. All tests at higher levels of domoic acid were positive. All tests at 5 ppm were negative. There were no false-positive results on unspiked control samples.
For clams, accuracy of the assay was 92.3%. All tests at 10 ppm domoic acid and higher were positive. Five of ten tests at 5 ppm domoic acid were also positive. There were no false positive results on unspiked control samples.

For mussels, accuracy of the assay was 100%. All tests at 10 ppm domoic acid and higher were positive. All tests at 5 ppm were negative. There were no false-positive results on unspiked control samples.
Overall accuracy of the Reveal ASP test was 95.9%. A dose-response curve was constructed using combined data from all three shellfish matrices and is shown in Fig. 1. Based on the dose-response curve, performance of the Reveal ASP test can be characterized as follows:

Zone 1	Positive < 5% of the time	< 2 ppm domoic acid
Zone 2	Positive 5-50% of the time	2-7 ppm domoic acid
Zone 3	Positive 51-95% of the time	8-11 ppm domoic acid
Zone 4	Positive > 95% of the time	> 11 ppm domoic acid

II. Interfering compounds

Methods
Fresh oysters, clams, and mussels were obtained as described above. Approximately 12-15 animals were combined and homogenized in a blender a produce a bulk sample. The bulk samples were separated into 12 portions of 1 g each. The 12 portions were separated into 4 groups each containing three 1-g samples. Samples in each group were spiked individually with one of the following potentially interfering compounds: okadaic acid, 10 ppm; glutamic acid, 100 ppm; glutamine, 100 ppm; or saxitoxin, 5 ppm. One sample in each group was spiked with 10 ppm domoic acid, one sample was spiked with 40 ppm domoic acid, and one sample was left unspiked. All interfering compounds were obtained from Sigma, except saxitoxin which was obtained from NRC-IMB. Domoic acid CRM, described above, was used as the spiking material.
Sample preparation and testing were performed as described above. Five replicates of each extracted sample were tested with the Reveal ASP assay.

Results
Results of testing for effects of potentially interfering compounds on performance of the Reveal ASP assay are shown in Table 2. There was no evidence of interference by okadaic acid, glutamic acid, glutamine, or saxitoxin on assay performance in any of the three shellfish types. All tests produced expected results at levels of 0, 10, and 40 ppm domoic acid.

III. Ruggedness

Methods
Fresh oysters, clams, and mussels were obtained as described above. Approximately 12-15 animals were combined and homogenized in a blender a produce a bulk sample. The bulk samples were separated into 3 portions of 1 g each. One portion was spiked at 10 ppm, one at 40 ppm, and the remaining sample left unspiked. Domoic acid CRM, described above, was used as the spiking material.

Sample preparation and testing were performed as described above. Ten replicates of each extracted sample were tested with the Reveal ASP assay. Each replicate was tested using devices from two different test kit lots (LFD-001 and LFD-002). The devices were interpreted after 8, 10 and 12 minutes to measure potential differences in results at different test incubation times. For each shellfish type, this trial was performed twice, on separate days, by two operators each day.

For each shellfish matrix, results from the two days of testing were pooled. Chi-square analysis (McNemar's test, [3]) was performed to determine if results were significantly different for the two kit lots or three test incubation times evaluated.

Results
Results of assay ruggedness trials with respect to Reveal ASP kit lot and assay incubation period are shown in Tables 3 and 4, respectively. In the trials measuring the effect of kit lot, there were no significant differences in the number of positives obtained with kit lots 1 and 2 at any spike level in any shellfish matrix, as determined by chi-square analysis at $p < 0.05$ (Table 3). Similarly, in the trials measuring the effect of variation in test incubation time, there were no significant differences in the number of positives obtained at incubation times of 8, 10 and 12 minutes at any spike level in any shellfish matrix (Table 4).

IV. Comparison with Reference Method

Methods
Fresh mussels were obtained as described above. Approximately 12-15 animals were combined and homogenized in a blender a produce a bulk sample. Incurred CRM consisting of a thermally stabilized homogenate of mussel tissue containing domoic acid at a concentration of 41 µg/g (ppm) was purchased from NRC-IMB (CRM-ASP-Mus-c). The incurred material was blended 1:1 with clean mussel tissue to obtain a domoic acid level of approximately 20 ppm. From the blended material, 20 samples of 1 g each were prepared. Ten samples were retained and tested in triplicate using the Reveal ASP test. The remaining 10 samples were sent to NRC-IMB for testing by the LC-UV method.

Results
Results of testing of samples of mussel tissue with incurred domoic acid by both the Reveal ASP assay and a reference LC-UV quantitative method are shown in Table 5. All 10 samples tested with the Reveal ASP method produced positive results. Results obtained with the LC-UV method were also positive for all 10 samples, ranging from 11.9 to 16.4 ppm.

Quality Control Testing
Quality control testing of manufactured lots of the Reveal ASP assay is performed at both in-process and finished product stages. In-process testing consists of balancing the antibody-colloidal gold conjugate for optimal test and control line intensity, and testing the device membrane for proper test and control line placement by running negative samples.
For finished product testing, samples are produced by diluting domoic acid (certified reference material CRM-DA-f, NRC-IMB) to concentrations of 2, 10, and 40 ppm in buffer. An unspiked sample is also prepared. Ten Reveal devices, randomly selected from the lot, are run at each concentration. For acceptance of the lot, all tests at 0 and 2 ppm must be negative and all tests at 10 and 40 ppm must be positive.

Discussion
Results of the validation study showed that the Reveal ASP test is an effective procedure for qualitative determination of domoic acid in oysters, clams, and mussels. In the accuracy study, all tests at the accepted action level of 20 ppm were positive. There were no false-positive results on unspiked control samples. The dose-response curve indicates that the test produces a positive result

greater than 95% of the time at a concentration above 11 ppm, 51-95% of the time at a concentration of 8-11 ppm, and less frequently at levels below 8 ppm.
Four compounds, okadaic acid, glutamic acid, glutamine, and saxitoxin, were tested for potential interference with the Reveal ASP assay. None was noted, as all samples produced the expected results at 0, 10, and 40 ppm domoic acid.

Results of ruggedness trials indicated that there was no statistically significant difference in performance between two Reveal ASP kit lots, nor was there any significant difference in performance in assays conducted with variation of +/- 2 minutes around the specified incubation time of 10 minutes.

Results of testing of mussel tissue samples containing incurred domoic acid showed agreement between the Reveal ASP and reference LC-UV methods, with all 10 samples testing positive by Reveal and LC-UV producing results in the range of 11.9-16.4 ppm.
Reveal ASP can be used as an accurate screening test for the rapid determination of domoic acid in shellfish. The test requires little equipment, uses water for sample extraction, and can be performed by personnel with minimal training. The test can be used in a field or laboratory setting, with results available within 20 minutes of sample receipt.
It is recommended that the Reveal ASP test be approved by the Interstate Shellfish Sanitation Conference as a screening method for qualitative determination of domoic acid in oysters, clams, and mussels.

References

1. Quilliam, M.A., Xie, M., & Hardstaff, W.R. (1995) Rapid extraction and cleanup for liquid chromatographic determination of domoic acid in unsalted seafood. *J. AOAC Intl.* **78**,543-554.

2. Lawrence, J.F., Charbonneau, C.F., & Menard, C. (1991) Liquid chromatographic determination of domoic acid in mussels, using AOAC paralytic shellfish poison extraction procedure: collaborative study. *J. Assoc. Off. Anal. Chem*. **74**,68-72.

3. Siegel, S. (1956) *Nonparametric Statistics for the Behavioral Sciences*, McGraw-Hill Book Co., New York, NY.

Acknowledgements

We thank Dr. Michael Quilliam and Kelley Reeves of the National Research Council, Institute for Marine Biosciences, Halifax, Nova Scotia, Canada for performing the LC-UV analyses.

Table 1. Results of accuracy study of the Reveal ASP test.

Sample Type	Level Domoic Acid (ppm)	Number Tests	Number Positive
Oysters	0	15	0
	5	10	0
	10	10	7
	15	10	10
	20	10	10
	40	10	10
Clams	0	15	0
	5	10	5
	10	10	10
	15	10	10
	20	10	10
	40	10	10
Mussels	0	15	0
	5	10	0
	10	10	10
	15	10	10
	20	10	10
	40	10	10
All Data	0	45	0
	5	30	5
	10	30	27
	15	30	30
	20	30	30
	40	30	30

Table 2. Results of interference study for the Reveal ASP test.

Sample Type	Interfering Compound and Level	Level Domoic Acid (ppm)	Number Tests	Number Positive
Oysters	Okadaic acid 10 ppm	0	5	0
		10	5	5
		40	5	5
	Glutamic acid 100 ppm	0	5	0
		10	5	5
		40	5	5
	Glutamine 100 ppm	0	5	0
		10	5	5
		40	5	5
	Saxitoxin 5 ppm	0	5	0
		10	5	5
		40	5	5
Clams	Okadaic acid 10 ppm	0	5	0
		10	5	5
		40	5	5
	Glutamic acid 100 ppm	0	5	0
		10	5	5
		40	5	5
	Glutamine 100 ppm	0	5	0
		10	5	5
		40	5	5
	Saxitoxin 5 ppm	0	5	0
		10	5	5
		40	5	5
Mussels	Okadaic acid 10 ppm	0	5	0
		10	5	5
		40	5	5
	Glutamic acid 100 ppm	0	5	0
		10	5	5
		40	5	5
	Glutamine 100 ppm	0	5	0
		10	5	5
		40	5	5
	Saxitoxin 5 ppm	0	5	0
		10	5	5
		40	5	5

Table 3. Results of assay ruggedness trials for the Reveal ASP test – effect of kit lot.

Sample Type	Level domoic acid (ppm)	Number Tests	Number Positive Lot 1	Number Positive Lot 2	χ^{2a}
Oysters	0	20	0	0	-[b]
	10	20	15	15	-
	40	20	20	20	-
Clams	0	20	0	0	-
	10	20	18	20	0.50
	40	20	20	20	-
Mussels	0	20	0	0	-
	10	20	15	14	0.00
	40	20	20	20	-
All Data	0	60	0	0	-
	10	60	48	49	0.00
	40	60	60	60	-

[a] $\chi^2 > 3.84$ indicates a significant difference at $\rho < 0.05$.
[b] χ^2 not applicable since all results were in agreement.

Table 4. Results of assay ruggedness trials for the Reveal ASP test – effect of incubation time.

Sample Type	Level domoic acid (ppm)	Number Tests	Number Positive 8 min.	Number Positive 10 min.	Number Positive 12 min.	χ^{2a} 8 vs. 10 min.	χ^2 12 vs. 10 min.
Oysters	0	40	0	0	0	-[b]	-
	10	40	36	35	35	0.00	-
	40	40	40	40	40	-	-
Clams	0	40	0	0	0	-	-
	10	40	34	37	38	0.44	0.00
	40	40	40	40	40	-	-
Mussels	0	40	0	0	0	-	-
	10	40	30	29	29	0.00	-
	40	40	40	40	40	-	-
All Data	0	120	0	0	0	-	-
	10	120	100	101	102	0.00	0.00
	40	120	120	120	120	-	-

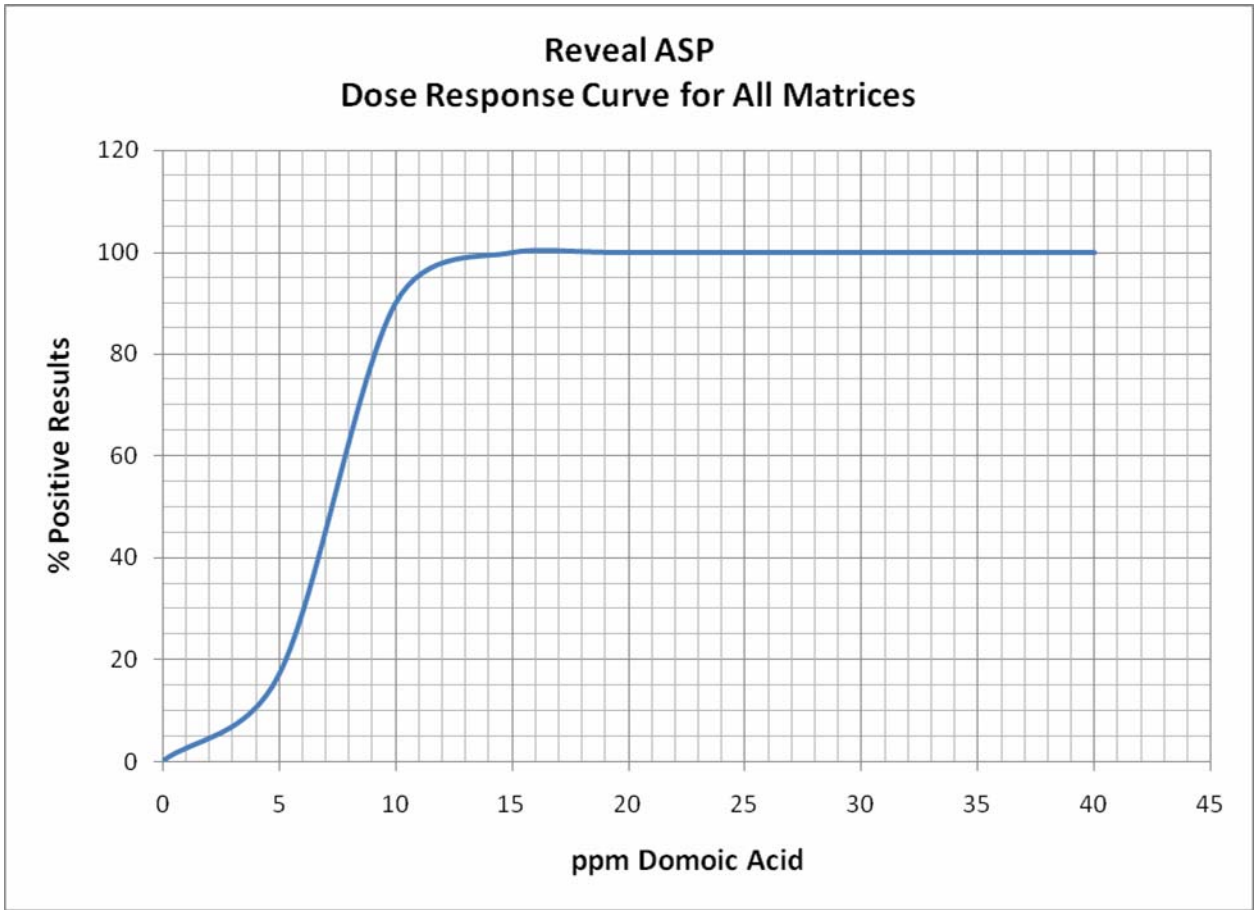
[a] $\chi^2 > 3.84$ indicates a significant difference at $\rho < 0.05$.
[b] χ^2 not applicable since all results were in agreement.

Table 5. Results of testing of domoic acid containing mussel tissue with Reveal ASP and a LC-UV reference method.

Sample No.	LC-UV Method Result ppm Domoic Acid (mean ± SD)[a]	Reveal ASP Result
1	16.4 ± 0.3	Positive
2	15.4 ± 0.1	Positive
3	14.5 ± 0.1	Positive
4	15.7 ± 0.2	Positive
5	15.06 ± 0.04	Positive
6	14.60 ± 0.03	Positive
7	13.65 ± 0.06	Positive
8	15.17 ± 0.08	Positive
9	14.0 ± 0.1	Positive
10	11.92 ± 0.05	Positive

[a] Mean of 3 determinations.

Fig. 1. Dose-response curve for the Reveal ASP test.



Appendix I. Reveal ASP kit insert

Product #9560

Read instructions carefully before starting test

Reveal®
ASP
(Domoic acid)

THE TOXIN

Amnesic shellfish poisoning (ASP) causing toxins are produced mainly by the toxigenic diatom strain *Pseudo-nitzchia multiseries*. The ASP toxins include primarily domoic acid (DA).

In addition to contamination of seafood, these marine biotoxins can result in human and marine wildlife mortality. The clinical toxicological effects attributed to DA can include: permanent loss of short-term memory, nausea, vomiting, headache, disorientation, and loss of balance.

Action limits for DA were established soon after the 1987 domoic acid/mussel crisis in Canada in which over 150 people became ill and four deaths resulted. Many countries have currently established a maximum permitted level of 20 mg DA per kg in whole shellfish (20 ppm).

INTENDED USE/USER

Reveal for ASP is intended for the qualitative screening of shellfish for DA, with the cut-off value for a positive result at approximately 10 ppm (i.e., half of the regulatory limit in many countries). The test kit is designed for use by personnel with an interest in the rapid screening of shellfish samples (further to obtaining a sample homogenate, it takes less than 20 minutes to carry out the sample extraction and obtain the result).

ASSAY PRINCIPLES

Reveal for ASP is a single-step lateral flow immunochromatographic assay based on a competitive immunoassay format. The extract is wicked through a reagent zone, which contains antibodies specific for DA conjugated to coloured particles. These DA-antibody-particle complexes result in the visible signal. If DA is present, it will be captured by the particle-antibody complex. The particle-antibody complex is then wicked onto a membrane, which contains a zone of DA conjugated to a protein carrier. This zone captures any uncomplexed DA antibody, allowing particles to concentrate and form a visible line. As the level of DA in the sample increases, free DA will complex with the particle-antibody complex. This, in turn, allows less particle-antibody complexes to be captured in the test zone. Therefore, as the concentration of DA in the sample increases, the test line decreases. The membrane also contains a control zone where an immune complex present in the reagent zone is captured by the antibody, forming a visible line. The control line will always form regardless of the level of DA, ensuring the strip is functioning properly.

STORAGE REQUIREMENTS

Store kit components at room temperature (18-30°C, 64–86°F) to assure full shelf life. Test strips should remain capped in their original sample tubes until used to assure optimal performance.

PRECAUTIONS

1. The test strips must remain inside the stay-dry tube before use.
2. Store test kit at room temperature (18–30°C, 64–86°F) when not in use, do not freeze.
3. Do not use kit contents beyond expiration date.
4. Treat all liquids, including sample extract, and used components as if contaminated with toxin. Gloves and other protective apparel should be worn at all times.
5. To avoid cross-contamination- use clean pipettors, extraction bags and fresh extraction solutions for each sample.

MATERIALS PROVIDED

9563 - Starter Kit - Reveal ASP (Domoic Acid)	**9560 - Reveal ASP (Domoic Acid)**
1 Reveal sample cup rack	25 ASP lateral flow test strips
1 Roller	25 sample cups
1 Bag-clip (white clip and green straw)	25 filter extraction bags
	50 exact volume pipettors

MATERIALS RECOMMENDED BUT NOT PROVIDED

1. Distilled water
2. 40 mL leakproof container including lids
3. Blender, Oster (Neogen item #9493)
4. Blender Jar, MINI, with blade & cap, 250 mL (Neogen item #9477)
5. Blender Jar, Stainless Steel 1 L (Neogen item #9495)
4. Scale capable of weighing 0.5–400 g ± 0.1 g (Neogen item #9427)
5. Timer (Neogen item #9452)
6. 50 mL Graduated cylinder (Neogen item #9367)
7. Bottle-top dispenser (Neogen item #9448)

EXTRACTION SOLUTION PREPARATION

The required extraction solution for the test is distilled water. A total volume of 40 ml of the solution is required per each sample tested. Fill 2 bottles with 20 mL each of distilled water, and label as SOLUTION 1 and SOLUTION 2, respectively.

Please note: *The solution containers should be capable of holding a volume of 40 mL to effectively carry out the procedure.*

SAMPLE PREPARATION AND EXTRACTION

The sample to be tested should be collected according to accepted sampling techniques.

1. Obtain a representative sample. Shell the samples.
2. Thoroughly rinse with cold water.
3. Homogenise† the shellfish in a high-speed blender.
   IMPORTANT: A good homogenate is essential in order to obtain an accurate result.
4. Weigh 1g (± 0.1g) of homogenized sample, ideally in leak-proof bottle capable of holding ~40 mL of liquid.
5. Pour entire contents of one bottle containing 20 mL of SOLUTION 1 into bottle containing one sample.
6. Shake the sample bottle vigorously by hand for 30 seconds, until all shellfish tissue is in solution (a cloudy appearance or bubbles may form, which do not affect the running of the test).
7. Number both sides of the extraction bag using a marker, so that there is a side labeled "1" and the other side labeled "2". Pour solution/sample mixture into one extraction bag (side 1). The extraction bag contains a mesh filter which allows for partial filtration of the sample.

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8. To seal the bag, position and hold the green straw approximately 2–3 inches down from the top of the bag, fold the upper edge of the bag so that it covers the green straw and firmly clip on the white bag-clip. This prevents leakage of the sample.
9. Press the roller firmly on the sample extraction bag, pushing the roller back and forth for 30 seconds to aid in obtaining a homogenous sample extract.
10. Slide out the green straw and remove the white bag-clip.
11. Pinch the top of the bag and carefully pour all the bag contents from side 2 back into the original sample container (there may be small pieces of shellfish remaining on side "1" of the bag). **Discard the used extraction bag** (***Note:*** *pinching the top of the bag to create a sharp edge allows easy pouring of the sample, preventing any spillage*).
12. Shake bottle with sample extract vigorously by hand for 30 seconds.
13. Remove 100ul of the sample extract using the exact-volume pipettors* provided (alternatively by use of a standard pipettor), and add into a fresh bottle containing SOLUTION 2.

    **To use the exact-volume pipettors, firmly press the top bulb of the pipettor, insert the tip into the sample, slowly release the top bulb to draw up the sample extract. Excess volume (i.e. above 100 µL) will overflow into the lower bulb, ensuring 100µl is ready to dispense. Press the top bulb firmly and slowly release the top bulb to dispense 100 µL into the bottle containing SOLUTION 2. Discard the used pipettor.*

    †Homegenise – to blend or to puree

TEST PROCEDURE

1. Remove the appropriate number of sample cups and place in the sample cup tray.
2. Shake the SOLUTION 2 bottle (which contains 100 µL of the sample extract) vigorously by hand for 30 seconds.
3. Remove 100 µL from the SOLUTION 2 bottle using a fresh pipettor and add 100 µL per sample cup.
4. Remove the required number of strips from the lateral flow device container and immediately close the container.
5. Place the new ASP strip with the sample end down (Neogen logo on top) into the sample cup.
6. Allow the strip to develop in the sample cup for 10 minutes.
7. Remove strip and interpret the results (as described below).

INTERPRETATION OF RESULTS (VISUAL)

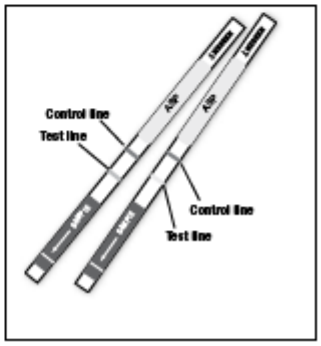
Test strips should be interpreted directly following completion of the 10 minute incubation.

Use the provided interpretation card to determine the level of the toxin present in the sample.

Note: *The control line should always be present and will always be darker than the test line. If there is no control line development, this indicates an invalid result and the sample should be retested using another device.*

MSDS INFORMATION AVAILABLE

Material safety data sheets (MSDS) are available for this test kit, and all of Neogen's Food Safety test kits, at www.neogeneurope.com.



VALIDATED MATRICES

Mussels, scallops, oysters, clams and cockles. Contact your Neogen representative concerning additional commodities.

WARRANTY

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 NE430-0111

Proposal for Consideration at the Interstate Shellfish Sanitation Conference 2011 Biennial Meeting	☒ **Growing Area** ☐ **Harvesting/Handling/Distribution** ☐ **Administrative**
Name of Submitter:	Mercuria Cumbo
Affiliation:	Northeast Laboratory Evaluation Officers and Managers (NELEOM)
Address:	MEDMR Lamoine WQ Laboratory 22 Coaling Station Rd. Lamoine, ME 04605
Phone: **Fax:** **Email:**	207 667-5654 207 664-0592 mercuria.cumbo@maine.gov
Proposal Subject:	Update Microbiology Laboratory Evaluation Checklist
Specific NSSP Guide Reference:	2009 NSSP Section IV. Guidance Documents Chapter II. Growing Areas .11 Evaluation of Laboratories By State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists Laboratory Evaluation Checklist - Microbiology
Text of Proposal/ Requested Action	Update Microbiology Laboratory Evaluation Checklist. Please find the updated Microbiology Laboratory Checklist attached - word document titled "Revised Microbiology Checklist 11-08-2010.doc". A summary of the changes is: • Renumbered checklist items to accommodate proposed additions and deletions and to better identify each checklist item. • Added, deleted or changed language for checklist items to be consistent with the PSP laboratory evaluation checklist. • Deleted the requirement for metals testing on reagent water and the inhibitory residue test for washed labware and increased the requirements for the bromothymol blue test. • Clarified and defined requirements for laboratory equipment, reagents including the bacterial quality control requirements for media productivity and method process control testing. • Update thermometer requirements to accommodate state bans on the use of mercury thermometers. • Updated the sterility check requirements for both in lab sterilized items and purchased pre-sterilized items.
Public Health Significance:	The current microbiology laboratory checklist was last revised in 2009 when the male specific coliphage method was approved and added to the checklist. Deficiencies have been identified while using the microbiology checklist in evaluation of laboratories and the microbiology checklist is inconsistent with some requirements in the PSP checklist. It is important that the checklist items and quality assurance requirements are clear and understandable. It is important that quality assurance requirements among the different laboratory evaluation checklists remain as consistent as possible since many monitoring laboratories perform multiple types of tests and are evaluated using multiple NSSP checklists; inconsistencies among the checklist cause confusion, extra expense and work for the laboratories.
Cost Information (if available):	None

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U.S. FOOD AND DRUG ADMINISTRATION
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~~SHELLFISH SAFETY TEAM~~ SHELLFISH AND AQUACULTURE POLICY BRANCH
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SHELLFISH LABORATORY EVALUATION CHECKLIST

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

LABORATORY REPRESENTED BY:	**TITLE:**

LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:** **REGION:**

OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by:
C- Critical K - Key O - Other NA- Not Applicable Conformity is noted by a "√"

Check the applicable analytical methods:

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

PART 1 - QUALITY ASSURANCE

CODE	REF.	ITEM
K	8, 11	**1.1 Quality Assurance (QA) Plan**

			~~1.~~ 1.1.1 Written Plan (Check those items which apply.)
		☐	a. Organization of the laboratory.
		☐	b. Staff training requirements.
		☐	c. Standard operating procedures.
		☐	d. Internal quality control measures for equipment, their calibration, maintenance, repair, ~~and for~~ performance ~~checks~~ and rejection criteria established
		☐	e. Laboratory safety.
		☐	f. Internal performance assessment.
		☐	g. External performance assessment.
C	**8**	☐	**~~6.~~ 1.1.2 QA Plan Implemented**
K	11	☐	~~7.~~ 1.1.3 The Laboratory participates in a proficiency testing program annually. Specify Program(s)_____
		1.2 Educational/Experience Requirements	
C	State's Human Resources Department	☐	**~~2.~~ 1.2.1 In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory**
K	State's Human Resources Department	☐	~~3.~~ 1.2.2 In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	USDA Microbiology & EELAP	☐	**~~4.~~ 1.2.3 In ~~private~~ commercial laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology, or equivalent discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP	☐	~~5.~~ 1.2.4 In ~~private~~ commercial laboratories, the analyst(s) must have at least a high school diploma and shall have at least three months of experience in laboratory sciences.
		1.3 Work Area	
O	8,11	☐	~~1.~~ 1.3.1 Adequate for workload and storage.
K	11	☐	~~2.~~ 1.3.2 Clean, well lighted.
K	11	☐	~~3.~~ 1.3.3 Adequate temperature control.
O	11	☐	~~4.~~ 1.3.4 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.~~ 1.3.5 Microbiological quality of the air contains fewer than 15 colonies for a 15 minute exposure and determined monthly. The results are recorded and records maintained.
O	11	☐	~~6. Pipette aid used, mouth pipetting not permitted.~~ Moved to equipment 1.4.25
		1.4 Laboratory Equipment	
O	9	☐	~~1.~~ 1.4.1 To determine the pH of prepared media, the pH meter has a standard accuracy of 0.1 units.
O	14	☐	~~2.~~ 1.4.2 pH electrodes consisting of pH half cell and reference half cell or equivalent combination electrode/triode (free from silver/silver chloride (Ag/AgCl) or contains an ion exchange barrier ~~preventing passage of Ag ions into the medium which may effect the accuracy of the pH reading~~) to prevent the passage of silver (Ag) ions into the substance being measured.
K	11	☐	~~3.~~ 1.4.3 The effect of temperature on the pH is compensated for by an ATC probe or by manual adjustment.
K	8	☐	~~4.~~ 1.4.4 pH meter is calibrated daily or with each use ~~and records are maintained.~~ Results are recorded and records maintained.
K	11	☐	~~5.~~ 1.4.5 A minimum of two standard buffer solutions is used to calibrate the pH meter.

			The first must be near the electrode isopotential point (pH 7). The second near the expected sample pH (i.e., pH 4 or pH 10). Standard buffer solutions are used once daily and discarded.
O	8,15	☐	~~6. Electrode effectiveness is determined daily or with each use.~~ ~~Method of determination_____.~~ 1.4.6 Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope. (*Circle the method used.*)
K	9	☐	~~7.~~ 1.4.7 Balance provides a sensitivity of at least 0.1 g at ~~a load of 150 g.~~ weights of use.
K	11,13	☐	~~8. Balance checked monthly using NIST Class S or ASTM Class 1 or 2 weights or equivalent and records are maintained.~~ 1.4.8 Balance calibrations are checked monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use. Results are recorded and records maintained.
K	11	☐	~~9.~~ 1.4.9. Refrigerator temperature (s) monitored at least once daily on workdays ~~and recorded.~~ Results are recorded and records maintained
K	1	☐	~~10.~~ 1.4.10 Refrigerator temperature maintained at 0° to 4°C.
C	**9**	☐	**~~11.~~ 1.4.11 The temperature of the incubator is maintained at 35 ± 0.5°C.**
C	**11**	☐	**~~12.~~ 1.4.12 Thermometers used in the air incubator(s) are graduated at no greater than ~~0.5°~~ 0.1° C increments.**
K	9	☐	~~13.~~ 1.4.13 Working thermometers are located on top and bottom shelves of use in the air incubator(s).
C	**11**	☐	**~~14.~~ 1.4.14 Temperature of the waterbath is maintained at 44.5 ± 0.2°C under ~~any~~ all loading ~~capacity~~ conditions.**
C	**9**	☐	**~~15.~~ 1.4.15 The thermometers used in the waterbath are graduated in 0.1°C increments.**
~~O~~ C	**13**	☐	**~~16.~~ 1.4.16 The waterbath has adequate capacity for workload.**
K	9	☐	~~17.~~ 1.4.17 The level of water in the waterbath covers the level of liquid in the incubating tubes.
K	8, 11	☐	~~18.~~ 1.4.18 Air incubator/waterbath temperatures are taken twice daily ~~and recorded~~ on workdays. The results are recorded and records maintained.
~~K~~	~~13~~	~~☐~~	~~19. Working thermometers are tagged with identification, date of calibration, calibrated temperature and correction factor.~~
~~K~~ C	**4**	☐	**~~20.~~ 1.4.19 All working thermometers are appropriately immersed.**
C	**29**	☐	**1.4.20 Either mercury-in-glass thermometers or non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury are used as working thermometers. In the case of the waterbath, low drift electronic resistance thermometers with an accuracy of +0.05°C may also be used.**
~~K~~ C	**11**	☐	~~21. A standards thermometer has been calibrated by NIST or one of equivalent accuracy at the points 0°, 35° and 44.5° C (45.5° C for ETCP). Calibration records maintained.~~ **1.4.21 A standards thermometer has been calibrated by a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at the points 0°, 35° and 44.5°C (45.5°C for ETCP). These calibration records are maintained.**
K	9	☐	~~22.~~ 1.4.22 Standards thermometers ~~is~~ are checked annually for accuracy by ice point determination. Results recorded and maintained. Date of most recent determination_____.
C	**29**	☐	**1.4.23 Either mercury-in-glass thermometers, non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury or**

			low drift electronic resistance thermometers with an accuracy of < ±0.05°C are used as the laboratory standards thermometer. (*Circle the thermometer type used.*)
K	13	☐	~~23.~~ 1.4.24 Incubator and waterbath working thermometers are checked annually against the standards thermometer at the temperatures at which they are used. Results are recorded and records maintained.
O	11	☐	1.4.25 Appropriate pipet aids are available and used to inoculate samples. Mouth pipetting is not permitted.
		1.5 Labware and Glassware Washing	
O	9	☐	~~1.~~ 1.5.1 Utensils and containers are clean borosilicate glass, stainless steel or other noncorroding materials
K	9	☐	~~2.~~ 1.5.2 Culture tubes are of a suitable size to accommodate the volume for nutritive ingredients and samples
K	9	☐	~~3.~~ 1.5.3 Sample containers are made of glass or some other inert material ~~(ie polypropylene)~~.
O	9	☐	~~4.~~ 1.5.4 Dilution bottles and tubes are made of borosilicate glass or plastic and closed with rubber stoppers, caps or screw caps with nontoxic liners.
K	9	☐	~~5.~~ 1.5.5 Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method is used to ensure appropriate volumes.
~~K~~ C	9	☐	**~~6.~~ 1.5.6 Pipettes used to inoculate the sample deliver accurate aliquots, have unbroken tips and are appropriately graduated. Pipettes larger than 10 mL are not used to deliver 1mL aliquots; nor, are pipets larger than 1mL used to deliver 0.1 mL aliquots.**
K	9	☐	~~7.~~ 1.5.7 Reusable sample containers are capable of being properly washed and sterilized.
K	9	☐	~~8.~~ 1.5.8 In washing reusable pipits, a succession of at least three fresh water rinses plus a final rinse of distilled/deionized water is used to thoroughly rinse off all the detergent.
~~C~~	~~9~~	☐	~~9. In washing reusable sample containers, glassware and plasticware, the effectiveness of the rinsing procedure is established annually and when detergent (brand or lot) is changed by the Inhibitory Residue Test as described in the current edition of Standard Methods for the Examination of Water and Wastewater. Records are kept.~~ ~~Date of most recent testing_____~~ ~~Average difference between Groups A and B_____~~ ~~Average difference between Groups B and D_____~~ ~~Detergent Brand_____ Lot #_____~~
C	2	☐	**1.5.9 An alkaline or acidic detergent is used for washing glassware/labware.**
~~K~~ C	11	☐	~~10. Once during each day of washing several pieces of glassware (pipettes, sample bottles, etc.) from one batch are tested for residual acid or alkali w/aqueous 0.04% bromthymol blue. Records are maintained.~~ **1.5.10 With each load of labware/glassware washed the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali) with aqueous 0.04% bromothymol blue. Results are recorded and records maintained.**
		1.6 Sterilization and Decontamination	
~~O~~ K	9	☐	~~1.~~ 1.6.1 Autoclave(s) are of sufficient size to accommodate the workload.
O	8	☐	~~2.~~ 1.6.2 Routine autoclave maintenance performed ~~(e.g. pressure relief valves, exhaust trap, chamber drain)~~ and the records maintained.
~~O~~	~~8~~	☐	~~3. Autoclave(s) and/or steam generators serviced annually or as needed by qualified technician and records maintained.~~

C	**11, 30**	☐	~~4. Autoclave(s) provides a sterilizing temperature of 121° C (tolerance 121 ± 2° C) as determined weekly using a calibrated working maximum registering thermometer or equivalent (thermocouples, platinum resistance thermometers).~~ **1.6.3 The autoclave provides a sterilizing temperature of 121°C (tolerance 121 + 2°C) as determined for each load using a working maximum registering thermometer concluded to be within temperature tolerance specifications. As an alternative, an appropriate temperature monitoring device is used in place of the maximum registering thermometer when these are unavailable due to the ban on mercury.**
K	11	☐	~~5. An autoclave standards thermometer has been calibrated by the National Institute of Standards and Technology (NIST) or its equivalent at 121° C.~~ 1.6.4 An autoclave standards thermometer has been calibrated by a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at 121°C. Calibration at 100°C, the steam point, is also recommended but not required.
K	16	☐	~~6. The autoclave standards thermometer is checked every five years for accuracy at either 121° C or at the steam point.~~ 1.6.5 The autoclave standards thermometer is checked every five years for accuracy at either 121°C or at 100°C, the steam point, if the thermometer has been previously calibrated at this temperature. Date of most recent determination_____
K	1	☐	~~7.~~ 1.6.6 Working autoclave thermometers are checked against the autoclave standards thermometer at 121°C yearly. Date of last check _____ Method _____
K	11	☐	~~8.~~ 1.6.7 Spore strips/suspensions appropriate for use in an autoclave are used monthly according to manufacturer's instructions to evaluate the effectiveness of the ~~autoclave~~ sterilization process. Results are recorded and the records maintained.
O	11	☐	~~9.~~ 1.6.8 Heat sensitive tape is used with each autoclave batch.
K	11, 13	☐	~~10.~~ 1.6.9 Autoclave sterilization records including length of sterilization, total heat exposure time and chamber temperature are maintained. Type of record: Autoclave log, computer printout or chart recorder tracings. (*Circle appropriate type or types.)*
K	11	☐	~~11.~~ 1.6.10 For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperatures in the range of 160° to 180°C.
K	9	☐	~~12.~~ 1.6.11 A thermometer capable of determining temperatures accurately in the range of 160 to 180°C is used to monitor the operation of the hot-air sterilizing oven ~~when in use.~~
K	13	☐	~~13.~~ 1.6.12 Records of temperatures and exposure times are maintained for the operation of the hot-air sterilizing oven during use.
K	11	☐	~~14.~~ 1.6.13 Spore strips/suspensions are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. Records are maintained.
K	11	☐	~~15.~~ 1.6.14 Reusable sample containers are sterilized for 60 minutes at 170°C in a hot-air oven or autoclaved for 15 minutes at 121°C.
~~O~~ C	1	☐	~~16. The sterility of reusable/disposable sample containers is determined for each batch/lot.~~ **1.6.15 The sterility of reusable sample containers is determined for each load sterilized. The results are recorded and the records maintained.**
C	1	☐	**1.6.16 The sterility of pre-sterilized disposable sample containers is determined for each lot received. Results are recorded and the records maintained.**
K	9	☐	~~17.~~ 1.6.17 Reusable pipettes are stored and sterilized in aluminum or stainless steel canisters ~~or equivalent alternative~~.

K	9	☐	~~18.~~ 1.6.18 Reusable pipettes (in canisters) are sterilized in a hot-air oven at 170°C for 2 hours.
~~O~~ **C**	**2**	☐	~~19. The sterility of reusable/disposable pipettes is determined with each batch/lot. Results are recorded and maintained.~~ **1.6.19 The sterility of reusable pipettes is determined with each load sterilized. Results are recorded and records maintained.**
C	**2**	☐	**1.6.20 The sterility of pre-sterilized disposable pipets is determined with each lot received. Results are recorded and the records maintained.**
K	18	☐	~~20.~~ 1.6.21 Hardwood applicator transfer sticks are properly sterilized. Method of sterilization _____
C	**2**	☐	**1.6.22 The sterility of the hardwood transfer sticks is checked routinely. Results are recorded and the records maintained.**
O	13	☐	~~21. Spent broth cultures and agar plates are decontaminated by autoclaving for at least 30 minutes before conventional disposal.~~ 1.6.23 Spent broth cultures and agar plates are decontaminated before disposal. Method _____.
		1.7 Media Preparation	
K	3, 5	☐	~~1.~~ 1.7.1 Media is commercially dehydrated except in the case of medium A-1 which ~~is~~ must be prepared from the individual components and modified MacConkey agar which may be prepared from its components.
O	11	☐	~~2.~~ 1.7.2 Dehydrated media and media components properly stored in cool, clean, dry place.
O	11	☐	~~3.~~ 1.7.3 Dehydrated media are labeled with the analyst's initials date of receipt and date opened.
C	12	☐	~~4.~~ **1.7.4 Caked or expired media or media components are discarded.**
C	11	☐	~~5. Make-up water is distilled or deionized *(circle one)* and exceeds 0.5 megohm resistance or is less than 2µ Siemens/cm conductivity at 25° C to be tested and recorded monthly for resistance or conductivity *(circle the appropriate)*~~ **1.7.5 Reagent water is distilled or deionized (circle appropriate choice), tested monthly and exceeds 0.5 megohm-cm resistance (2 megohms-cm in-line) or is less than 2.0 µSiemens/cm conductivity at 25°C. (*Circle the appropriate water quality descriptor determined.*) Results are recorded and the records maintained.**
C	11	☐	~~6.~~ **1.7.6** ~~Makeup~~ **Reagent water is analyzed for residual chlorine monthly and is at a non-detectable level (< 0.1 ppm). Results are recorded and the records ~~are~~ maintained.** **Specify method of determination _____.**
~~K~~	~~11~~	☐	~~7. Make-up water is free from trace (<0.05mg/L) dissolved metals, specifically Cd, Cr, Cu, Ni, Pb, and Zn as determined annually with total heavy metal content < or equal to 1.0mg/L and records are maintained.~~
K	11	☐	~~8.~~ 1.7.7 ~~Make up~~ Reagent water contains ~~<1000~~ ≤100 CFU/mL as determined monthly using the heterotrophic plate count method. Results are recorded and the records maintained.
K	11	☐	~~9.~~ 1.7.8 Commercially prepared dehydrated media are sterilized according to the manufacturer's instructions.
K	9	☐	~~10.~~ 1.7.9 The volume and concentration of media in the tube are suitable for the amount of sample inoculated.
C	11	☐	~~11.~~ **1.7.10 Total time of exposure of sugar broths to autoclave temperatures does not exceed 45 minutes.**
C	1	☐	~~12. Media sterility and positive and negative controls are run with each lot of commercially prepared media or are run with each batch of media prepared from its components as a check of media productivity. Results recorded and records~~

			~~maintained.~~ **1.7.11 Media sterility is determined for each load sterilized. Results are recorded and the records maintained.**
C	1	☐	**1.7.12 Media productivity is determined using media appropriate, properly diluted positive and negative control cultures for each lot of dehydrated media received or with each batch of media prepared from the individual components. When an alternative visual temperature monitoring device is used in place of the maximum registering autoclave thermometer, media productivity is determined using media appropriate, properly diluted positive and negative control cultures with each batch of media prepared.**
O	9	☐	~~13.~~ 1.7.13 Sterile phosphate buffered dilution water is used as the sample diluent.
K	11	☐	~~14.~~ 1.7.14 The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer's requirements. Results are recorded and records are maintained.
		1.8 Storage of Prepared Culture Media	
~~O~~ K	9	☐	~~1.~~ 1.8.1 Prepared culture media are stored in a cool, clean, dry space where excessive evaporation and the danger of contamination are minimized.
K	5,11	☐	~~2.~~ 1.8.2 Brilliant green bile 2% broth and A-1 media are stored in the dark.
K	13	☐	~~3.~~ 1.8.3 Stored media are labeled with the storage expiration date or the sterilization date.
O	9	☐	~~4.~~ 1.8.4 Storage of prepared culture media at room temperature does not exceed 7 days.
O	2	☐	~~5.~~ 1.8.5 Storage under refrigeration of prepared broth media with loose fitting closures shall not exceed 1 month.
O	11	☐	~~6.~~ 1.8.6 Storage under refrigeration of prepared culture media with screw-cap closures does not exceed 3 months.
K	17	☐	~~7.~~ 1.8.7 All prepared ~~media~~ MPN broth stored under refrigeration are held at room temperature overnight prior to use. Culture tubes containing any type of precipitate or Durham tubes containing air bubbles are discarded.
PART II - SEAWATER SAMPLES			
		2.1 Collection and Transportation of Samples	
C	**11**	☐	**~~1.~~ 2.1.1 Sample containers are of a suitable size to contain at least ~~100~~ 110 mL of sample and to allow adequate headspace for proper shaking. Seawater samples are collected in clean, sterile, watertight, properly labeled sample containers.**
K	1	☐	~~2.~~ 2.1.2 Samples are identified with collectors name, harvest area, sampling station, time and date of collection.
C	**9**	☐	~~3. After collection, seawater samples shall be kept at a temperature between 0 and 10° C until examined.~~ **2.1.3 Immediately after collection, seawater samples are placed in dry storage (ice chest or equivalent) which is maintained between 0° and 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed in the refrigerator unless processed immediately.**
~~K~~ O	1	☐	~~4.~~ 2.1.4 A temperature blank is used to determine the temperature of samples upon receipt at the laboratory. Results are recorded and maintained.
C	**9**	☐	~~5. Examination of the sample is initiated as soon as possible after collection. However, seawater samples are not tested if they are held beyond 30 hours of refrigeration.~~ **2.1.5 Analysis of the sample is initiated as soon as possible after collection. Seawater samples are not tested if they have been held for more than 30 hours from the time of collection.**
		2.2 Bacteriological Examination of Seawater by the APHA MPN	
C	**9**	☐	**~~1.~~ 2.2.1 Lactose broth or lauryl tryptose broth is used as the presumptive medium.** *(Circle appropriate one.)*

C	**2**	☐	**2. 2.2 The media productivity controls utilized are properly diluted and appropriate for the presumptive medium being used. The results are recorded and the records maintained.** **Positive productivity control Negative productivity control**
C	**9**	☐	**2. 2.2.3 Sample and dilutions of sample are shaken ~~mixed~~ vigorously (25 times in a 12" arc in 7 seconds) before inoculation.**
C	**9**	☐	**3. 2.2.4 In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended).**
C	**6**	☐	**4. 2.2.5 In a single dilution series not less than 12 tubes are used (for depuration at least 5 tubes are used).**
~~K~~ C	**6**	☐	**5. 2.2.6 In a single dilution series, the volumes analyzed ~~examined~~ are adequate to meet the needs of routine monitoring.** **Sample volume inoculated _____** **Range of MPN_____** **Strength of media used_____**
K	9	☐	6. 2.2.72.2.7 Inoculated ~~media~~ tubes are ~~placed in an air incubator~~incubated in air at 35~~+~~± 0.5°C ~~for up to 48 ± 3 hours~~.
~~K~~ C	**2**	☐	7. Positive and negative control cultures accompany samples throughout the procedure. Records are maintained. **2.2.8 Appropriately diluted process control cultures accompany the samples *throughout both the presumptive and confirmed phases of incubation.* Results are recorded and the records maintained.** **Positive process control _____ Negative process control _____**
K	9	☐	8. Inoculated media are read after 24 ± 2 hours and 48 ± 3 hours of incubation and transferred at both intervals if positive for gas. 2.2.9 Inoculated tubes are read after 24+ 2 hours and 48+ 3 hours of incubation and transferred at both time intervals if positive for growth (the presence of turbidity) and gas or effervescence in the culture tube. These tubes are considered presumptive positive requiring further confirmatory testing
		2.3 Confirmed Test for Seawater by APHA MPN	
C	**9**	☐	**1. 2.3.1 Brilliant green bile 2% broth (BGB) is used as the confirmatory medium for total coliforms.**
C	**9**	☐	**2. 2.3.2 EC medium is used as the confirmatory medium for fecal coliforms.**
C	**2**	☐	**2.3.3 The media productivity controls utilized are properly diluted and appropriate for the confirmed medium being used. The results are recorded and the records maintained.** **Positive productivity control Negative productivity control**
K	9, 11	☐	3. 2.3.4 Transfers are made to BGB/EC by either sterile loop or sterile hardwood transfer stick from positive presumptives tubes incubated for 24 and 48 hours as appropriate . *(Circle the method of transfer.)*
~~K~~	~~2~~	☐	4. When the inoculation of both EC and BGB broths is performed using the same loop or transfer stick, the order of inoculation is EC first, followed by BGB.
C	**9**	☐	**5. 2.3.5 BGB tubes are incubated at 35 ± 0.5°C.**
K	9	☐	6. 2.3.6 BGB tubes are read after 48 ± 3 hours of incubation.
C	**9**	☐	**7. 2.3.7 EC tubes are incubated in a circulating waterbath maintained at 44.5 ± 0.2°C ~~for 24 ± 2 hours~~.**
C	**9**	☐	**2.3.8 EC tubes are read after 24 ± 2 hours of incubation.**
C	**9**	☐	**8. 2.3.9 The presence of turbidity and any amount of gas or effervescence in the culture tube constitutes a positive test.**

		2.4 Computation of Results – APHA MPN	
K	9	☐	~~1.~~ 2.4.1 Results of multiple dilution tests are read from tables in *Recommended Procedures for the Examination of Sea Water and Shellfish*,Fourth 4th Edition.
K	7	☐	~~2.~~ 2.4.2 Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
~~K~~C	**7, 9**	☐	**~~3.~~ 2.4.3 Results are reported as MPN/100 mL of sample.**
		2.5 Bacteriological Examination of Seawater by the MA-1 Method	
C	**5**	☐	**2.5.1 A-1 medium complete is used in the analysis.**
C	**2, 31**	☐	**2.5.2 A-1 medium without salicin is used in the analysis. Comparability testing with medium A-1 complete has been undertaken and the results justify exclusion of the salicin from the formulation of medium A-1.**
C	**5**	☐	**~~1.~~ 2.5.3 A-1 medium sterilized for 10 minutes at 121°C.**
C	**2**	☐	**2.5.4 The media productivity controls used are properly diluted and appropriate for use with A-1 medium. The results are recorded and the results maintained.** **Positive productivity control _____ Negative productivity control _____**
C	**9**	☐	**~~2.~~ 2.5.5 Sample and dilutions of sample are shaken ~~mixed~~ vigorously (25 times in a 12" arc in 7 seconds) before inoculation.**
C	**9**	☐	**~~3.~~ 2.5.6 In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended).**
C	**6**	☐	**~~4.~~ 2.5.7 In a single dilution series at least 12 tubes are used.**
~~K~~C	**6**	☐	**~~5.~~ 2.5.8 In a single dilution series, the volumes analyzed ~~examined~~ are adequate to meet the needs of routine monitoring.** **Sample volume inoculated _____** **Range of MPN _____** **Strength of media used _____**
~~K~~C	**2**	☐	~~6. Positive and negative control cultures accompany samples throughout the procedure. Records are maintained.~~ **2.5.9 Appropriately diluted process control cultures accompany the samples *throughout both resuscitation and waterbath incubation* Results are recorded and the records maintained.** **Positive process control _____ Negative process control _____**
C	**2,5**	☐	**~~7.~~ 2.5.10 Inoculated ~~media~~ tubes are placed in an air incubator at 35 ± 0.5°C for 3 ± 0.5 hours of resuscitation.**
C	**5**	☐	**~~8.~~ 2.5.11 After 3 ± 0.5 hours resuscitation at 35°C, inoculated tubes ~~media~~ are incubated at 44.5 ± 0.2° C in a circulating waterbath for the remainder of the 24 ± 2 hours.**
C	**5**	☐	**~~9.~~ 2.5.12 The presence of turbidity and any amount of gas or effervescence in the culture tube constitutes a positive test.**
		2.6 Computation of Results - MPN	
K	9	☐	~~1.~~ 2.6.1 Results of multiple dilution tests are read from tables in *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition.
K	7	☐	~~2.~~ 2.6.2 Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
~~K~~ C	**7, 9**	☐	**~~3.~~ 2.6.3 Results are reported as MPN/100 mL of sample.**
		2.7 Bacteriological ~~Examination~~ Analysis of Seawater by Membrane Filtration	

			(MF) using mTEC Agar -Materials and Equipment
C	**23, 24**	☐	**1. 2.7.1 When used for elevated temperature incubation in conjunction with ethafoam resuscitation, the temperature of the hot air incubator is maintained at 44.5 ± 0.5°C under any loading capacity.**
C	**23**	☐	**2. 2.7.2 When using a waterbath for elevated temperature incubation, the level of the water completely covers the plates.**
C	**23**	☐	**3. 2.7.3 Pre-sterilized plastic or sterile glass culture plates that are clear, flat bottomed, free of bubbles and scratches with tight fitting lids are used.**
C	**2**	☐	**2.7.4 The sterility of pre-sterilized culture plates is determined for each lot received. Results are recorded and the records maintained.**
K	11	☐	4. 2.7.5 Colonies are counted with the aid of magnification.
C	**11, 23**	☐	**5. 2.7.6 Membrane filters are made from cellulose ester material, white, grid marked, 47 mm in diameter with a pore size of 0.45 µm and certified by the manufacturer for fecal coliform analyses.**
O C	**2**	☐	**6. 2.7.7 Lot number, date of receipt and if provided the expiration date of the membrane filters are recorded and records maintained.**
C	**2**	☐	**2.7.8 When initiating monitoring by mTEC or switching brands or types of membrane filters used and no previous lots of filters are available for comparing acceptable performance, an appropriate method for determining the suitability of the lot is developed and the comparison testing implemented. The results are recorded and this record is maintained.**
K	2, 11	☐	7. 2.7.9 New lots of membrane filters are checked by comparing recovery of fecal coliform organisms against membrane filters from previously acceptable lots.
C	**2**	☐	**8. 2.7.10 The sterility of each lot or autoclave batch of membrane filters are checked before use.**
K	2	☐	9. 2.7.11 Membrane filters which are beyond their expiration date are not used.
O	11	☐	10. 2.7.12 Forceps tips are clean.
O	11	☐	11. 2.7.13 Forceps tips are smooth without pitting or corrugations to damage the filters being manipulated.
K	11	☐	12. 2.7.14 Forceps are dipped in alcohol and flame sterilized between sample filters.
K	11	☐	13. 2.7.15 If indelible graduation marks are used on clear glass or plastic funnels to measure sample volumes, their accuracy is checked gravimetrically or with a Class A graduated cylinder before use and periodically rechecked. Funnels having a tolerance greater than 2.5% are not used. Checks are recorded and records maintained
K	11	☐	14. 2.7.16 Membrane filtration units are made of stainless steel, glass or autoclavable plastic free of scratches, corrosion and leaks.
C	**11**	☐	**15. 2.7.17 Membrane filter assemblies are autoclave sterilized for 15 minutes at 121°C prior to the start of a filtration series.**
O	11, 23, 26	☐	16. 2.7.18 A UV sterilization unit is used to disinfect filter assemblies between sample and filtration runs.
K	11	☐	17. 2.7.19 ~~If used,~~ The effectiveness of the UV sterilization unit is determined by biological testing monthly. Results are recorded and records maintained.
K	2	☐	2.7.20 Maintenance of the UV sterilization unit is performed as needed. This maintenance is documented and the records maintained.
			2.8 Media Preparation and Storage– MF using mTEC Agar
K	11	☐	1. 2.8.1 Phosphate buffered saline is used as the sample diluent and filter funnel rinse .
C	**11**	☐	**2. 2.8.2 The phosphate buffered saline is properly sterilized.**
K	23	☐	3. 2.8.3 A sufficient amount of medium (4-5 mL) is used in each plate.
O	11	☐	4. 2.8.4 Refrigerated prepared plates are stored for no more than 2 weeks in sealed plastic bags or containers to minimize evaporation.
			2.9 Sample Analyses -MF using mTEC Agar
C	**24**	☐	**1. 2.9.1 mTEC agar is used.**

C	**2**	☐	**2.9.2 The media productivity controls used are properly diluted and appropriate for use with mTEC medium. The results are recorded and the results maintained.** **Positive productivity control _____ Negative productivity control _____**
C	**23**	☐	**2. 2.9.3 The sample is ~~mixed~~ shaken vigorously (25 times in a 12″ arc in 7 seconds) before filtration.**
C	**23**	☐	**3. 2.9.4 The membrane is placed grid side up within the sterile filter apparatus.**
C	**23, 25**	☐	**4. 2.9.5 Sample volumes tested are consistent with the sampling regime employed (i.e., half log or other appropriate dilutions are used with systematic random sampling).**
C	**23**	☐	**5. 2.9.6 Sample volumes are filtered under vacuum.**
K	26	☐	6. 2.9.7 The pressure of the vacuum pump does not exceed 15 psi.
C	**23, 26**	☐	**7. 2.9.8 The sides of the filter funnel are rinsed at least twice with 20-30 mL of sterile phosphate buffered saline after sample filtration.**
C	**23**	☐	**8. 2.9.9 The membrane filter is removed from the filtering apparatus with sterile forceps and rolled onto mTEC agar so that no bubbles form between the filter and the agar.**
C	**11**	☐	**9. 2.9.10 Blanks are run at the beginning of filtration, after every 10th aliquot and at the end of the filtration run to check the sterility of the testing system (phosphate buffered saline, filter funnel, forceps, membrane filter, media and culture plate).**
~~K~~C	**2, 11**	☐	10. Positive and negative control cultures accompany samples throughout the procedure. Records are maintained. **2.9.11 Appropriately diluted process control cultures accompany the samples throughout both resuscitation and elevated temperature incubation. Results are recorded and the records maintained.** **Positive process control _____ Negative process control _____**
C	**11, 23, 24**	☐	**11. 2.9.12 ~~Innoculated plates are placed inverted wither directly in an air incubator or in a watertight, tightly sealed container at 35 + 0.5°C for 2 hours of resuscitation prior to waterbath incubation or in Ethyfoam for incubation in air at 44.5 + 0.5°C.~~** **Inoculated plates are placed inverted into a watertight, tightly sealed container prior to being placed in the air incubator and incubated at 35 + 0.5°C for 2 hours of resuscitation. Alternatively inoculated plates may be placed in ethafoam prior to air incubation at 44.5 + 0.5°C for 24 + 2 hours .**
C	**11, 23, 24**	☐	**12. 2.9.13 After 2 hours of resuscitation at 35°C, the watertight, tightly sealed containers are transferred to a circulating waterbath at 44.5 + 0.2°C, submerged completely and incubated for 22-24 hours.** ~~Individual plates are transferred inverted to a watertight container, tightly sealed and submerged completely in a circulating waterbath at 44.5 + 0.2°C for 22-24 hours of incubation.~~
		2.10 Computation of Results- MF using mTEC Agar	
C	**23**	☐	**1. 2.10.1 All yellow, yellow-green or yellow-brown colonies are counted.**
C	**23**	☐	**2. 2.10.2 Only plates having 80 or fewer colonies are counted. If it is unavoidable ~~necessary~~ to use plates having more than 80 colonies, counts are given as >80 x 100/the volume of sample filtered.**
C	**2, 11, 23**	☐	**2.10.3 When multiple dilutions are filtered, the laboratory has developed a procedure for assessing the contribution of all positive dilutions to the final count.**
K	23, 11	☐	3. 2.10.4 The number of fecal coliforms is calculated by the following equation: Number of fecal coliforms per 100 mL = [number of colonies counted per plate

			used in the count / volume (s) of sample filtered in ml] x 100.
~~K~~C	23, 11	☐	**~~4.~~ 2.10.5 Results are reported as CFU/100 mL of sample.**
PART III - SHELLFISH SAMPLES			
		3.1 Collection and Transportation of Samples	
C	9	☐	**~~1.~~ 3.1.1 A representative sample of shellstock is collected.**
K	9	☐	~~2.~~ 3.1.2 Shellstock samples are ~~is~~ collected in clean, waterproof, puncture resistant containers loosely sealed .
K	9	☐	~~3.~~ 3.1.3 Shellstock samples are labeled with collector's name, type of shellstock, the source or harvest area, sampling station, time, date and place (if applicable ~~market sample~~) of collection.
C	9	☐	~~4. Shellstock samples are maintained in dry storage between 0 and 10° C until examined.~~ **3.1.4 Immediately after collection, shellfish samples are placed in dry storage (ice chest or equivalent) which is maintained between 0° and 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed under refrigeration unless processed immediately.**
C	1	☐	**~~5.~~ 3.1.5 ~~Examination~~ Analysis of the samples is initiated as soon as possible after collection. ~~However,~~ Shellfish samples are not tested ~~examined~~ if the time interval between collection and analysis ~~examination~~ exceeds 24 hours.**
		3.2 Preparation of Shellfish for Examination	
K	2,11	☐	~~1.~~ 3.2.1 Shucking knives, scrub brushes and blender jars are (autoclave) sterilized for 15 minutes prior to use.
O	2	☐	~~2.~~ 3.2.2 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.~~ 3.2.3 The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2	☐	~~4.~~ 3.2.4 The faucet used ~~to provide the potable water~~ for rinsing the shellstock does not contain an aerator.
K	9	☐	~~5.~~ 3.2.5 Shellstock are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
C	2	☐	**3.2.6 If a water supply is a non-chlorinated private well, the water is tested every six months for total coliforms. Results are recorded and maintained.**
O	9	☐	~~6.~~ 3.2.7 Shellstock are allowed to drain in a clean container or on clean towels prior to opening.
K	9	☐	~~7.~~ 3.2.8 Immediately prior to ~~opening~~ shucking, the hands (or gloved hands) of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol.
~~K~~C	9	☐	**~~8.~~ 3.2.9 Shellstock are not shucked directly through the hinge.**
C	9	☐	**~~9.~~ 3.2.10 Contents of shellstock (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	9	☐	~~10.~~ 3.2.11 At least 200 grams of shellfish meat or a quantity of meat sufficient to cover the blender blades is used for the analysis.
K	9	☐	3.2.12 A representative sample of at least 12 shellfish is used for the analysis.
K	2, 19	☐	~~11.~~ 3.2.13 The sample is weighed to the nearest 0.1 gram and an equal amount by weight of (tempered for ETCP) diluent is added.
O	9	☐	1~~2.~~ 3.2.14 Sterile phosphate buffered dilution water is used as the sample diluent.
~~K~~	~~3~~	☐	~~13. Sterile phosphate buffered saline is used as a sample diluent for the ETCP procedure.~~ Moved to ETCP section
C	9	☐	**~~14.~~ 3.2.15 Samples are blended at high speed for 60 to 120 seconds until homogenous.**
K	9	☐	~~15. For other shellstock, APHA *Recommended Procedures* are followed for the examination of freshly shucked and frozen shellfish meats.~~

			3.2.16 APHA *Recommended Procedures for the Examination of Sea Water And Shellfish*, Fourth Edition is followed for the analysis of previously shucked and frozen shellfish meats.
		3.3 MPN Analysis for Fecal Coliform Organisms, Presumptive Test, APHA	
C	**9**	☐	**~~1.~~ 3.3.1 Appropriate strength lactose or lauryl tryptose broth is used as presumptive media in the analysis. ~~(circle appropriate choice)~~ (Circle the medium used.)**
C	**2**	☐	**3.3.2 The media productivity controls utilized are properly diluted and appropriate for the presumptive medium being used. The results are recorded and the records maintained.** **Positive productivity control_____ Negative productivity control_____**
K	9	☐	~~2.~~ 3.3.3 Immediately (within 2 minutes) after blending, the ground sample is diluted and inoculated into tubes of presumptive media.
C	**9**	☐	**~~3.~~ 3.3.4 No fewer than 5 tubes per dilution are used in a multiple dilution MPN series.**
C	**9**	☐	**~~4.~~ 3.3.5 Allowing for the initial 1:1 dilution of the sample, appropriate portions are inoculated (i.e., 2 ml of original 1:1 dilution for the 1 g portion) and diluted for subsequent inoculation (i.e., 22 ml of 1:1 diluted sample to 88 ml of diluent or the equivalent for 0.1 g portion). All successive dilutions are prepared conventionally.**
K	6	☐	~~5.~~ 3.3.6 In a single dilution series, the volumes examined are adequate to meet the needs of routine monitoring. Sample volume inoculated _____ Range of MPN_____ Strength of media used_____
C	**2**	☐	~~6. Positive and negative control cultures accompany samples throughout the procedure. Records are maintained.~~ **3.3.7 Appropriately diluted process control cultures accompany the samples throughout both the presumptive and confirmed phases of incubation. Results are recorded and the records maintained.** **Positive Process control _____ Negative Process control_____**
K	9	☐	~~7.~~ 3.3.8 Inoculated media are incubated at 35 ± 0.5°C.
K	10	☐	~~8. Presumptive tubes are read at 24 ± 2 hours of incubation and transferred if positive.~~ 3.3.9 Tubes are read after 24+2 hours of incubation and transferred if positive for growth (the presence of turbidity and gas or effervescence in the culture tube). These tubes are considered presumptive requiring further confirmatory testing.
		3.4 Confirmed Test for Fecal Coliforms - APHA	
C	**9**	☐	**~~1.~~ 3.4.1 EC medium is used as the confirmatory medium.**
C	**2**	☐	**3.4.2 The media productivity controls utilized are properly diluted and appropriate for use with EC medium. The results are recorded and the records maintained.** **Positive productivity control_____ Negative productivity control_____**
K	9, 11	☐	~~2.~~ 3.4.3 Transfers are made to EC medium by either sterile loop or hardwood sterile ~~applicator~~ transfer sticks from positive presumptives ~~incubated for 24 hours~~. *(Circle the method of transfer.)*
C	**9**	☐	**~~3.~~ 3.4.4 EC tubes are incubated in a circulating waterbath at 44.5 ± 0.2°C ~~for 24 ± 2 hours.~~**
K	9	☐	~~4.~~ 3.4.5 EC tubes are read for gas production after 24 ± 2 hours of incubation.
C	**9**	☐	**~~5.~~ 3.4.6 The presence of turbidity and any amount of gas and/or effervescence in**

			the Durham tube constitutes a positive test.
		3.5 Computation of Results for MPN Analyses	
K	9	☐	~~1.~~ 3.5.1 Results of multiple dilution tests are read from tables in *Recommended Procedure for the Examination of Sea Water and Shellfish,* 4th Edition and multiplied by the appropriate dilution factor.
K	7	☐	~~2.~~ 3.5.2 Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
~~K~~ C	**9**	☐	**~~3.~~ 3.5.3 Results are reported as MPN/100 grams of sample.**
		3.6 Standard Plate Count Method	
O	20	☐	~~1.~~ 3.6.1 A standard plate count (SPC) analysis ~~is~~ may be performed in conjunction with the analysis for fecal coliform organisms.
K	9	☐	~~2.~~ 3.6.2 In the standard plate count procedure at least four plates are used, duplicates of two dilutions ~~are used to provide 30 to 300 colonies per plate~~. One of the dilutions should produce colonies of 30 to 300 per plate.
K	2	☐	~~3.~~ 3.6.3 Fifteen to 20 mL of tempered sterile plate count agar is used per plate.
~~K~~ C	**9**	☐	**~~4.~~ 3.6.4 Agar tempering bath maintains the agar at 44- 46°C.**
~~O~~ C	**9**	☐	~~5. Temperature control of the plate count agar is used in the tempering bath.~~ **3.6.5 An agar based temperature control having a similar volume and shape as the tempering plate count agar is used in the tempering bath.**
K	9	☐	~~6.~~ 3.6.6 Not more than 1 mL nor less than 0.1 mL of sample or sample dilution is plated.
C	**9**	☐	**~~7.~~ 3.6.7 Samples or sample dilutions to be plated are ~~mixed~~ shaken vigorously (25 times in a 12" arc in 7 seconds) before plating.**
K	11	☐	~~8.~~ 3.6.8 Control plates are used to check air quality and the sterility of the ~~air,~~ agar and the diluent.
K	9,21	☐	~~9.~~ 3.6.9 Solidified plates are incubated at 35 ± 0.5°C for 48 ± 3 hours inverted and stacked no more than four high.
K	9	☐	~~10.~~ 3.6.10 Quebec Colony Counter or its equivalent is used to provide the necessary magnification and visibility for counting plates.
K	1	☐	~~11.~~ 3.6.11 A hand tally or its equivalent is used for accuracy in counting.
		3.7 Computation of Results -SPC	
K	9	☐	~~1.~~ 3.7.1 Colony counts determined in accordance with Part III, A, Sections 4.31 through 4.33 in *Recommended Procedures for the Examination of Sea Water and Shellfish*,, 4th Fourth Edition.
C	**19**	☐	**~~2.~~ 3.7.2 Colony counts are reported as APC/g of sample.**
		3.8 Bacteriological ~~Examination~~ Analysis of Shellfish Using the ETCP	
C	**2,3**	☐	**3.8.1 Prepared modified MacConkey agar is used on the day that it is made.**
~~K~~	~~9~~	~~☐~~	~~1. Sample homogenate is cultured within 2 minutes of blending.~~
K	3	☐	~~2.~~ 3.8.2 Double strength modified MacConkey agar is used.
C	**3**	☐	~~3. Hydrated double strength Modified MacConkey Agar is heated to boiling, removed from the heat, and boiled again. This agar is never autoclaved.~~ **3.8.3 Prepared double strength modified MacConkey agar is heated to boiling, removed from the heat, and boiled again. This agar is never autoclaved.**
K	2, 3	☐	~~4.~~ 3.8.4 Twice boiled, double strength modified MacConkey agar ~~and sterile phosphate buffered saline~~ are maintained in a tempering bath at 45 to 50°C until used. ~~Prepared Modified MacConkey Agar is used on the day it is made.~~
K	2, 3	☐	3.8.5 Phosphate buffered saline is used as the sample diluent in the ETCP.
C	**2, 3**	☐	**3.8.6 The phosphate buffered saline is tempered at 45 - 50°C to prevent premature solidification of the agar.**
C	**9**	☐	**3.8.7 The sample homogenate is cultured within 2 minutes of blending.**

C	**2,3**	☐	~~5. The equivalent of 6 grams of the homogenate is placed into a sterile container and the contents brought up to 60 ml with tempered, sterile phosphate buffered saline.~~ **3.8.8 Six grams of shellfish (12 grams of homogenate if initially diluted 1:1) is placed into a sterile container and the contents brought up to 60 mL with sterile, tempered phosphate buffered saline.**
K	3	☐	~~6.~~ 3.8.9 Sixty (60) mL of tempered, twice boiled double strength Modified MacConkey Agar is added.
K	2,3, 22	☐	~~7.~~ 3.8.10 The container is gently swirled or ~~rotated~~ slowly inverted once to mix the contents, which are ~~then~~ subsequently distributed uniformly over ~~6 to 8~~ ~~petri~~ six plates.
C	**1**	☐	**~~8.~~ 3.8.11 Media and diluent sterility are determined with each use. Results are recorded and the records maintained.**
C	**1**	☐	~~9. To determine media productivity, positive and negative control cultures are pour plated in an appropriate concentration to accompany samples throughout the procedure.~~ **3.8.12 Media productivity is determined using media appropriate properly diluted pour plated positive and negative control cultures for each batch of Modified MacConkey agar prepared.** **Positive control culture _____ Negative control culture _____**
C	**3, 13**	☐	~~10. Plates are incubated inverted within 3 hours of plating in air at 45.5 ± 0.5° C for 18 to 30 hours. Plates are stacked not more than four high.~~ **3.8.13 When solidified the plates are placed inverted into an air incubator at 45.5 ± 0.5°C for 18 to 30 hours of incubation.**
C	**2**	☐	**3.8.14 Plates are stacked no more than three high in the incubator.**
C	**2**	☐	**3.8.15 Appropriately diluted pour plated process control cultures *accompany each set of samples throughout incubation.* The results are recorded and the records maintained.** **Positive process control_____Negative process control_____**
C	**3**		**~~11.~~ 3.8.16 Incubator temperature is maintained at 45.5 ± 0.5°C.**
		3.9 Computation ~~Expression~~ of Results - ETCP	
K	11	☐	~~1.~~ 3.9.1 Quebec Colony counter or its equivalent is used to provide the necessary magnification and visibility for counting.
O	1	☐	~~2.~~ 3.9.2 A hand tally or its equivalent is used to aid in counting.
C	**3, 6**	☐	**~~3.~~ 3.9.3 All brick red colonies greater than 0.5 mm in diameter are totaled over all the plates and multiplied by a factor of 16.7 ~~to report results as CFU/100 grams of sample~~ .**
C	**3**	☐	**3.9.4 Results are reported as CFU/100 grams of sample.**
		Bacteriological Examination of Soft-shelled Clams and American Oysters for Male Specific Coliphage (MSC)	
		3.10 MSC Equipment and Supplies	
K	30	☐	~~1.~~ 3.10.1 Sample containers used for the shucked sample are sterile, made of glass or some other inert material (i.e. polypropylene) and hold 100 – 125 mL.
C	**27, 28**	☐	**~~2.~~ 3.10.2 The refrigerated centrifuge used must have the capacity to accommodate the amount of shellfish sample required for the procedure, perform at 9000 x g and maintain a temperature of 4°C.**
C	**27, 28**	☐	**~~3.~~ 3.10.3 The tempering bath(s) must be able to maintain the temperature within 2°C of the set temperature.**
K	9	☐	~~4.~~ 3.10.4 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.~~ 3.10.5 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.~~ 3.10.6 The sterility of each lot of pre-sterilized syringes and syringe filters is determined. Results are recorded and records maintained.
K	1	☐	~~7.~~ 3.10.7 The sterility of each batch of reusable glass syringes is determined. Results are recorded and records maintained.
C	**27, 28**	☐	**~~8.~~ 3.10.8 The balance used provides a sensitivity of at least 10 mg.**
C	**27, 28**	☐	**~~9.~~ 3.10.9 The temperature of the incubator used is maintained between 35 – 37°C.**
C	**28**	☐	**~~10.~~ 3.10.10 Sterile disposable 50 mL centrifuge tubes are used and their sterility is determined with each lot. Results are recorded and records maintained.**
		3.11 MSC Media Preparation	
K	28	☐	~~1.~~ 3.11.1 Media preparation and sterilization is according to the validated method.
K	27, 28	☐	~~2.~~ 3.11.2 Bottom agar, double strength soft agar and growth broth are prepared from their individual components.
K	27, 28	☐	~~3.~~ 3.11.3 Soft agar is prepared double strength in volumes of 2.5 mL.
C	**27, 28**	☐	**~~4.~~ 3.11.4 The streptomycin and ampicillin solutions are added to tempered bottom agar.**
O	27, 28	☐	~~5.~~ 3.11.5 Storage of the bottom agar under refrigeration does not exceed 1 month.
K	27, 28	☐	~~6.~~ 3.11.6 Unsterilized soft agar is stored at -20°C for up to 3 months.
K	27, 28	☐	~~7.~~ 3.11.7 The soft agar is removed from the freezer and sterilized for 15 minutes at 121°C before use.
K	27, 28	☐	~~8.~~ 3.11.8 Storage of growth broth in the refrigerator in loosely capped tubes/bottles does not exceed 1 month and in screw capped tubes/bottles does not exceed 3 months.
K	27, 28	☐	~~9.~~ 3.11.9 Bottom agar plates are allowed to reach room temperature before use.
		3.12 Preparation of the Soft-Shelled Clams and American Oysters for MSC Analysis	
K	2,11	☐	~~1.~~ 3.12.1 Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2	☐	~~2.~~ 3.12.2 The blades of shucking knives are not corroded.
O	9	☐	~~3.~~ 3.12.3 The hands of the analyst are thoroughly washed with soap and water immediately prior to ~~scrubbing and rinsing~~ cleaning the shells of debris ~~off the shellfish~~.
O	2	☐	~~4.~~ 3.12.4 The faucet used for rinsing the shellfish does not contain an aerator.
K	9	☐	~~5.~~ 3.12.5 The shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
C	**2**	☐	**3.12.6 If a water supply is a non-chlorinated private well, the water is tested every six months for total coliforms. Results are recorded and maintained.**
O	9	☐	~~6.~~ 3.12.7 The shellfish are allowed to drain in a clean container or on clean towels unlayered prior to shucking.
K	9	☐	~~7.~~ 3.12.8 Immediately prior to shucking, the hands (or gloved hands) of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol.
C	**9**	☐	**~~8.~~ 3.12.9 Shellfish are not shucked through the hinge.**
C	**9**	☐	**~~9.~~ 3.12.10 The contents of shellfish (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	9	☐	~~10.~~ 3.12.11 A representative sample of at least 12 shellfish is used for the analysis.
~~C~~ K	2, 19	☐	~~11.~~ 3.12.12 The sample is weighed to the nearest 0.1 gram.
		3.13 MSC Sample Analysis	
C	**28**	☐	**~~1.~~ 3.13.1 *E.coli Famp* ATCC 700891 is the bacterial host strain used in this procedure.**
K	27, 28	☐	~~2.~~ 3.13.2 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.~~ 3.13.3 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.~~ 3.13.4 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.~~ 3.13.5 After inoculation, the host cell growth broth culture is not shaken.**
C	28	☐	**~~6.~~ 3.13.6 A 2:1 mixture of growth broth to shellfish tissue is used for eluting the MSC.**
C	28	☐	**~~7.~~ 3.13.7 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.~~ 3.13.8 The elution mixture is homogenized at high speed for 180 seconds.**
C	28	☐	**~~9.~~ 3.13.9 Immediately after blending, 33 grams of the homogenized elution mixture are weighed into centrifuge tubes.**
C	28	☐	**~~10.~~ 3.13.10 The homogenized elution mixture is centrifuged for 15 minutes at 9000 x g at 4°C.**
C	27, 28	☐	**~~11.~~ 3.13.11 The supernatant is pipetted off, weighed and the weight recorded.**
C	27, 28	☐	**~~12.~~ 3.13.12 The supernatant is allowed to warm to room temperature about 20 to 30 minutes.**
K	27, 28	☐	~~13.~~ 3.13.13 The autoclaved soft agar is tempered and held at 50 – 52°C throughout the period of sample analysis.
K	27, 28	☐	~~14.~~ 3.13.14 Two hundred microliters (0.2 mL) of log phase host strain *E coli* is added to the tempering soft agar immediately prior to adding the sample supernatant.
K	27, 28	☐	~~15.~~ 3.13.15 The sample supernatant is shaken or vortexed before being added to the tempering soft agar.
C	27, 28	☐	**~~16.~~ 3.13.16 2.5 mL of sample supernatant is added to each tube of tempering soft agar.**
C	27, 28	☐	**~~17.~~ 3.13.17 The soft agar/sample supernatant/host cell mixture is gently rolled between the palms of the hands to mix.**
C	27, 28	☐	**~~18.~~ 3.13.18 The soft agar/sample supernatant/host cell mixture is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**
C	28	☐	**~~19.~~ 3.13.19 Ten (10) plates are used, 2.5 mL per plate for a total of 25 mL of supernatant analyzed per sample.**
K	27, 28	☐	~~20.~~ 3.13.20 Negative and positive control plates are prepared and accompany each set of samples analyzed. The results are recorded and records maintained.
K	27, 28	☐	~~21.~~ 3.13.21 Growth broth is used as the negative control or blank.
K	27, 28	☐	~~22.~~ 3.13.22 Type strain MS2 (ATCC 15597) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.
K		☐	~~23.~~ 3.13.23 A negative control plate is plated at the beginning and end of each set of samples analyzed.
K	27, 28	☐	~~24.~~ 3.13.24 The positive control is plated after all the samples are ~~analyzed~~ inoculated and immediately prior to the final negative control.
C	27, 28	☐	**~~25.~~ 3.13.25 All plates are incubated at 35 – 37°C for 16 to 20 hours.**
		3.14 Computation of Results - MSC	
C	27	☐	**~~1.~~ 3.14.1 Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted.**
C	28	☐	**~~2.~~ 3.14.2 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 grams for soft-shelled clams and <7 PFU/ 100 grams for American oysters. If the density exceeds 100 PFU per plate on all plates, the count is given as > 10,000 PFU/100 grams.**
K	28	☐	~~3.~~ 3.14.3 The formula used for determining the density of MSC in PFU/100 grams is: (0.364)(N)(Ws), where N = total number of plaques counted on all 10 plates and Ws = weight of the supernatant used.
O	9	☐	~~4.~~ 3.14.4 The MSC count is rounded off conventionally to give a whole number.

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SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

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

Total # of Critical, Key and Other (O) Nonconformities in Parts I-III	_____

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

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

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

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

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

2. **Provisionally Conforms Status**: The microbiological component of this

laboratory is determined to be provisionally conforming to NSSP requirements if

the number of critical nonconformities is **≥ 1 but ≤ 3**

C. Laboratory Status (*circle appropriate*)

Does Not Conform Provisionally Conforms Conforms

Acknowledgment by Laboratory Director/Supervisor:

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

Laboratory Signature:_____ Date:_____

LEO Signature:_____ Date:_____

NSSP Form LAB-100 Microbiology Rev. 2010-11-08

Proposal for Consideration at the Interstate Shellfish Sanitation Conference 2011 Biennial Meeting	☒ **Growing Area** ☐ **Harvesting/Handling/Distribution** ☐ **Administrative**
Name of Submitter:	Mercuria Cumbo
Affiliation:	Northeast Laboratory Evaluation Officers and Managers (NELEOM)
Address:	MEDMR Lamoine WQ Laboratory 22 Coaling Station Rd. Lamoine, ME 04605
Phone: **Fax:** **Email:**	207 667-5654 207 664-0592 mercuria.cumbo@maine.gov
Proposal Subject:	Update PSP Laboratory Evaluation Checklist
Specific NSSP Guide Reference:	2009 NSSP Section IV. Guidance Documents Chapter II. Growing Areas .11 Evaluation of Laboratories By State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists-Laboratory Evaluation Checklist - PSP
Text of Proposal/ Requested Action	Update PSP Laboratory Evaluation Checklist. Please find the updated PSP Laboratory Checklist attached - word document titled "Revised PSP Cecklist 11-08-2010.doc". A summary of the changes is: • Added the checklist items for Jellett Rapid Test for PSP • Renumbered checklist items to accommodate proposed additions and deletions and to better identify each checklist item. • Added, deleted or changed language for checklist items to be consistent with the microbiology laboratory evaluation checklist including added laboratory education and experience requirements • Deleted the requirement for metals testing on reagent water • Clarified and defined requirements for laboratory equipment, reagents and the mouse bioassay method.
Public Health Significance:	The current PSP laboratory checklist was last revised in 2005. Since that time the Jellett Rapid Test has received approval and is not in the checklist. Deficiencies have been identified while using the PSP checklist in evaluation of laboratories and the PSP checklist is inconsistent with some requirements in the microbiology checklist which has more recently been revised . It is important that the checklist items and quality assurance requirements are clear and understandable. It is important that quality assurance requirements among the different laboratory evaluation checklists remain as consistent as possible since many monitoring laboratories perform multiple types of tests and are evaluated using multiple checklists; inconsistencies among the checklist cause confusion, extra expense and work for the laboratories.
Cost Information (if available):	None

PUBLIC HEALTH SERVICE **U.S. FOOD AND DRUG ADMINISTRATION** **OFFICE OF FOOD SAFETY** **SHELLFISH AND AQUACULTURE POLICY BRANCH** **5100 PAINT BRANCH PARKWAY** **COLLEGE PARK, MD 20740-3835** **TEL. 240-402-2151/2055 FAX 240-402-2601**		
SHELLFISH LABORATORY EVALUATION CHECKLIST		
LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**
LABORATORY REPRESENTED BY:	**TITLE:**	
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:** **REGION:**	
OTHER OFFICIALS PRESENT:	**TITLE:**	
Items which do not conform are noted by: C- Critical K - Key O - Other NA - Not Applicable Conformity is noted by a "√"		
Check the applicable assays performed:		
	Mouse Bioassay (MBA)	
	Jellett Rapid Test (JRT)	
PART I – QUALITY ASSURANCE		
ITEM		
CODE		
		1.1 Quality Assurance (QA) Plan
K	☐	1. 1.1 Written plan adequately covers all the following [check (√) those that apply]
		a. Organization of the laboratory.
		b. Staff training requirements.
		c. Standard operating procedures (SOPs).

		d. Internal quality control measures for equipment, calibration, maintenance repair ~~and~~, performance and rejection criteria established.
		e. Laboratory safety.
		f. ~~Quality assessment.~~ Internal performance assessment.
		g. ~~Proper animal care.~~External performance assessment.
		h. Animal care.
C	☐	**~~2.~~ 1.1.2 QA plan implemented.**
		1.2 Educational/Experience Requirements
C	☐	**1.2.1 In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	☐	1.2.2 In state/county laboratories, the analysts meet the state/county educational and experience requirements for processing samples in a public health laboratory.
C	☐	**1.2.3 In commercial laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or an equivalent discipline with at least two years of laboratory experience.**
K	☐	1.2.4 In commercial laboratories, the analysts must have at least a high school diploma and shall have at least three months of experience in laboratory science.
		1.~~2~~3 Work Area
O	☐	~~1.~~ 1.3.1 Adequate for workload and storage.
O	☐	~~2.~~ 1.3.2 Clean and well lighted.
O	☐	~~3.~~ 1.3.3 Adequate temperature control.
O	☐	~~4.~~ 1.3.4 All work surfaces are nonporous and easily cleaned.
C	☐	**~~5.~~ 1.3.5 A separate, quiet area with adequate temperature control for mice acclimation and injection is maintained.**
		1.~~3~~4 Laboratory Equipment
O	☐	~~1.~~1.4.1 The pH meter has a standard accuracy of 0.1 pH unit.
K	☐	~~1.4.2pH paper in the appropriate range (i.e. 1-4) is used with minimum accuracy of 0.5 pH units.~~ ~~2.~~ 1.4.2 pH paper in the appropriate range (i.e., pH <2 to >4.5) having a minimum accuracy of 0.5 units is used.
K	☐	~~3.~~1.4.3 The pH electrodes being used consist of a pH half cell and reference half cell or equivalent combination electrode/triode free from silver/silver chloride (Ag/AgCl) or contains an ion exchange barrier to prevent the passage of silver (Ag) ions into the ~~medium that may result in inaccurate pH readings~~ substance being measured.
K	☐	~~4.~~1.4.4 pH meter is calibrated daily or with each use. Results are recorded and records maintained.
K	☐	~~5.~~1.4.5 Effect of temperature has been compensated for by an ATC probe, use of a triode or by manual adjustment.
K	☐	~~6.~~1.4.6 A minimum of two standard buffer solutions (pH 2 & pH 7) is used to calibrate the pH meter. Standard buffer solutions are used once and discarded.
K	☐	~~7.~~1.4.7 Electrode ~~efficiency~~acceptability is determined daily or with each use ~~following either slope or~~ by the millivolt procedure or through determination of the slope. (circle the method used.)
K	☐	~~8. The balance provides a sensitivity of at least 0.1g at a load of 150 grams.~~ 1.4.8 The differing sensitivities in weight measurements required by the various steps in the assay are met by the balance/balances being used. a. To prepare the reference solution, the balance used must have a sensitivity of at least 0.1 gram at a load of 1 gram. b. For sample extraction, the balance used must have a sensitivity of

		at least 0.1 gram at a load of 100 grams. c. For gravimetric extract volume adjustment, the balance used must have a sensitivity of at least 0.1 gram at a load of 200 grams. d. To determine the weight of the mice, the balance must have a sensitivity of at least 0.1 gram at a load of 20 grams.
K	☐	~~9. The balance calibration is checked monthly using NIST Class S or ASTM Class 1or 2 weights or equivalent. Records maintained.~~ 1.4.9 Balance calibrations are checked monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use. Results are recorded and records maintained.
K	☐	~~10.~~1.4.10 Refrigerator temperatures ~~is~~are maintained between 0 and 4°C.
O	☐	~~11~~1.4.11 Refrigerator temperatures ~~is~~are monitored at least once daily on workdays. Results are recorded and records maintained.
K	☐	~~12.~~1.4.12 Freezer temperatures ~~is~~ are maintained at ~~20°C~~or below -15°C.
O	☐	~~13.~~1.4.13 Freezer temperatures ~~is~~ are monitored at least once daily on workdays. Results are recorded and records maintained.
O	☐	~~14.~~1.4.14 All glassware is clean.
~~O~~ **C**	☐	~~15. Once during each day of washing, several pieces of glassware from each batch washed are tested for residual detergent with aqueous 0.04% bromthymol blue solution. Records are maintained.~~ **1.4.15 With each load of labware/glassware washed, the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali) with aqueous 0.04% bromthymol blue (BTB) solution. Results are recorded and records maintained.**
C	☐	**1.4.16 An alkaline or acid based detergent is used for washing glassware/labware**
		~~1.4~~1.5 Reagent and Reference Solution Preparation and Storage
C	☐	**1.5.1 Opened PSP ~~reference~~ standard solution (100µg/mL) is not stored.**
K	☐	~~2. PSP working standard solution (1 µg/ml) and all dilutions are prepared with dilute HCl, pH 3 water, using 'Class A' volumetric glassware (flasks and pipettes) or prepared gravimetrically.~~ 1.5.2 PSP reference solution (1µg/mL) is prepared by weight (grayimetrically) with dilute HCl, pH 3 water.
K	☐	~~3. Refrigerated storage of PSP working standard solution (1µg/ml) does not exceed 6 months and is checked gravimetrically for evaporation loss.~~ 1.5.3 Refrigerated storage of PSP reference solution (1µg/mL) in a sealed container is stored indefinitely as long as there is no evaporation loss as checked by weight. If evaporation is detected, the solution is discarded appropriately. Records are maintained.
C	☐	**1.5.4 Dilutions of the 1µg/mL reference solution are prepared by weight or volume using dilute HCl, pH 3 water.**
K	☐	~~4.~~1.5.5 PSP working dilutions(dilutions of the 1µg/mL reference solution) are discarded after use.
K	☐	~~5. Make-up water is distilled or deionized (*circle one*) and exceeds 0.5 megohm resistance or is less than 2 µ Siemens/cm conductivity at 25°C to be tested and recorded monthly for resistance or conductivity *(circle the appropriate).*~~ 1.5.6 Reagent water is distilled or deionized (*circle appropriate choice*), tested monthly and exceeds 0.5 megohm-cm resistance (2 megohms-cm in-line) or is less than 2.0 µSiemens/cm conductivity at 25°C (*circle the appropriate water quality descriptor determined*). Results are recorded and the records maintained.
O	☐	~~6.~~ 1.5.7 ~~Make up~~ Reagent water is analyzed for residual chlorine monthly and is at a nondetectable level (<0.1ppm). Results are recorded and records maintained.
~~K~~	☐	~~7. Make-up water is free from trace (< 0.5 mg/l) dissolved metals specifically Cd,~~

		~~Cr, Cu, Ni, Pb, and Zn as determined annually with total heavy metal content ≤1.0 mg/l. Records maintained.~~
O	☐	~~8.~~1.5.8 ~~Makeup~~ Reagent water contains ~~<1000~~ <100 CFU/mL as determined monthly using the heterotrophic plate count method. Results are recorded and records maintained.
		1.5~~6~~ Collection and Transportation of Samples
O	☐	~~1. Shellstock are collected in clean, waterproof, puncture resistant containers.~~ 1.6.1 Shellfish are collected in clean, waterproof , loosely sealed, puncture resistant containers.
K	☐	~~2.~~1.6.2 Samples are appropriately labeled with the collector's name, harvest area, sampling station and time and date of collection.
K	☐	~~3. Immediately after collection, shellstock samples are placed in dry storage for transport (e.g. cooler) which is maintained between 0 and 10°C. Upon receipt at the lab, samples are placed under refrigeration.~~ 1.6.3 Immediately after collection, shellfish samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10°C with ice or cold packs for transport to the laboratory. Upon receipt at the laboratory, samples are placed under refrigeration.
K	☐	~~4.~~1.6.4 The time from collection to completion of the bioassay should not exceed 24 hours. However, if there are significant transportation delays, then shellstock samples are processed immediately as follows (*circle the appropriate choice*): a. Washed, shucked, drained, frozen until extracted. b. Washed , shucked, drained, homogenized and frozen. c. Washed, shucked, drained, extracted, the supernatant decanted and refrigerated (**best choice**) ; or d. The laboratory has an appropriate contingency plan in place to handle samples which can't be analyzed within 24 hours due to transportation issues.
~~K~~**C**	☐	~~5.~~**1.6.5 Frozen, shucked product or homogenates are allowed to thaw completely and all liquid is included as part of the sample before being processed further.**
Part II – ~~EXAMINATION~~ ANALYSIS OF SHELLFISH FOR PSP TOXINS		
		2.1 Preparation of the Sample
C	☐	**~~1.~~ 2.1.1 At least 12 animals (equivalent to at least 100 g of shellfish meat) are used per sample or the laboratory has a~~n~~ ~~appropriate~~ proven effective contingency plan for dealing with non-typical species of shellfish.**
O	☐	~~2.~~ 2.1.2. The outside of the shell is thoroughly cleaned with fresh water.
O	☐	~~3.~~ 2.1.3 Shellstock are opened by cutting adductor muscles.
O	☐	~~4.~~ 2.1.4 The inside of the shell is rinsed with fresh water to remove sand or other foreign material.
O	☐	~~5.~~ 2.1.5 Shellfish meats are removed from the shell by separating adductor muscles and tissue connecting at the hinge.
K	☐	~~6.~~ 2.1.6 Damage to the body of the mollusk is minimized in the process of opening.
O	☐	~~7.~~2.1.7 Shucked shellfish are drained on a #10 mesh sieve (or equivalent) without layering for 5 minutes.
K	☐	~~8.~~ 2.1.8 Pieces of shell and drainage are discarded.
C	☐	~~**9. Drained meats or thawed homogenates are blended at high speed until homogenous** (60 – 120 seconds).~~ **2.1.9 Drained meats or previously cooled/refrigerated, shucked, drained meats and their drip-loss liquid or thawed, shucked meat with its freeze-thaw liquid or thawed homogenates with their freeze-thaw liquid are blended at high speed until homogenous (60 – 120 seconds).**
		2.2 Extraction
K	☐	~~1.~~ 2.2.1 100 grams of homogenized sample is weighed into a beaker.

K	☐	~~2.~~ 2.2.2 An equal amount of 0.1 N/0.18 N HCl is added to the homogenate and thoroughly mixed. (*circle the appropriate normality*).
C	☐	**~~3.~~ 2.2.3 The pH is checked and, if necessary adjusted to between pH 2.0 and 4.0.**
C	☐	**~~4.~~ 2.2.4 Adjustment of the pH is made by the dropwise addition of either (5 N HCl) or base (0.1 N NaOH) as appropriate while constantly stirring the mixture.**
C	☐	**~~5.~~ 2.2.5 The homogenate/acid mixture is promptly brought to a boil, 100 +1°C then gently boiled for 5 minutes.**
O	☐	~~6.~~ 2.2.6 The homogenate/ acid mixture is boiled under adequate ventilation (i.e., fume hood).
O	☐	~~7.~~ 2.2.7 The extract is cooled to room temperature.
C	☐	**~~8.~~ 2.2.8 The pH of the extract is determined and adjusted if necessary to between pH 2 and 4 preferably to pH 3 with the stirred dropwise addition of 5 N HCl to lower the pH or 0.1 N NaOH to raise the pH.**
K	☐	~~9.~~ 2.2.9 The extract volume~~(or mass)~~ is adjusted to 200 mL (or grams) with dilute HCl, pH 3.0 water.
K	☐	~~10.~~2.2.10 The extract is returned to the beaker, stirred to homogeneity and allowed to settle to remove particulates; or, if necessary, an aliquot of the stirred supernatant is centrifuged at 3,000 RPM for 5 minutes before ~~injection~~ being bioassayed.
K	☐	~~11. If mice cannot be injected immediately then the supernatant should be removed from the centrifuge tubes and refrigerated for up to 24 hours.~~ 2.2.11 If the extract cannot be bioassayed or the Jellett Rapid Test (JRT) for PSP cannot be performed immediately, then the supernatant is removed from the centrifuge tubes and sealed and refrigerated for up to 24 hours.
K	☐	~~12.~~ 2.2.12 Refrigerated extracts are allowed to reach ambient temperature before being bioassayed or tested by the JRT for PSP.
		2.3 Bioassay
O	☐	~~1.~~ 2.3.1 A 26-gauge hypodermic needle is used for injection.
~~K~~**C**	☐	~~2. Healthy mice in the weight range of 17 – 23 grams (19 – 21 grams is preferable) from a stock colony are used for routine assays. Mice are not reused for the bioassay.~~ ~~Stock strain used_____ Source of the mice _____~~ **2.3.2 Healthy mice in the weight range of 17 – 23 grams (19 – 21 grams is preferable) from a stock colony are used for routine assays. Mice are not reused for the bioassay.** **Stock strain used_____ Source of the mice_____**
C	☐	**~~3.~~ 2.3.3 Mice are allowed to acclimate for at least 24 hours prior to injection. In some cases up to 48 hours may be required.**
C	☐	**~~4.~~ 2.3.4 A conversion factor (CF) has been determined as _____. Month and year when current CF determined _____.**
C	☐	**~~5.~~ 2.3.5 CF value is checked weekly if assays are done on several days during the week, or, once each day that assays are performed if they are performed less than once per week.** **Date of most recent CF check_____** **CF verified~~/CF not verified~~; *yes / no*: (*circle the appropriate choice*).**
C	☐	**~~6.~~ 2.3.6 If the CF is not verified, 5 additional mice are injected with the dilution used in the CF check to complete a group of 10 mice. Ten additional mice are also injected with this dilution to produce a second group of 10 mice. The**

		CF is calculated for each group of 10 mice and averaged to give the CF to be used in sample toxicity calculations for the day's or week's work only. All subsequent work must make use of the original laboratory CF value unless this value continues to fail to be verified by routine CF checks.
C	☐	**~~7.~~ 2.3.7 If the CF fails to be verified, the cause is investigated and the situation corrected. If the cause cannot be determined with reasonable certainty and fails >3 times per year, the bioassay is restandardized.**
O	☐	~~8.~~ 2.3.8 Mice are weighed to the nearest ~~0.5 gram~~ 0.1 gram .
C	☐	**~~9.~~ 2.3.9 Mice are injected intrapertioneally with 1 mL of the acid extract.**
K	☐	~~10.~~2.3.10 For the CF check at least 5 mice are used.
C	☐	**~~11.~~ 2.3.11 At least 3 mice are used per sample in routine assays.**
C	☐	**~~12.~~2.3.12 Elapsed time is accurately determined and recorded.**
K	☐	~~13.~~ 2.3.13 If death occurs, the time of death to the nearest second is noted by the last gasping breath.
C	☐	**2.3.14 Mice are continually observed for up to 20 minutes after injection with periodic checks for a total of 60 minutes as appropriate.**
C	☐	**~~14.~~ 2.3.15 If the median death time~~(2 out of 3 mice injected die)~~ is <5 minutes, a dilution is made with dilute HCl, pH 3 water, to obtain a median death time in the range of 5 to 7minutes.**
		2.4 Calculation of Toxicity
C	☐	**~~1.~~ 2.4.1 The death time of each mouse is converted to mouse units (MU) using Sommer's Table (Table 6, *Recommended Procedures for the examination of Sea Water and Shellfish, Fourth*, ~~4~~ 4th ~~-~~Fourth Edition). The death time of mice surviving beyond 60 minutes is considered to be <0.875 MU.**
K	☐	~~2.~~ 2.4.2 A weight correction in MU is made for each mouse injected using Table 7 in *Recommended Procedures for the Examination of Sea Water and Shellfish*, Fourth 4th- Edition.
C	☐	**~~3.~~ 2.4.3 The death time of each mouse in MU is multiplied by a weight correction in MU to give the corrected mouse unit (CMU), the true death time for each mouse.**
C	☐	**~~4.~~ 2.4.4 The median value of the array of corrected mouse units (CMU) is determined to give the median corrected mouse unit (MCMU), median death time.**
C	☐	**~~5.~~ 2.4.5 The concentration of toxin is determined by the formula, MCMU x CF x Dilution Factor (DF) x 200.**
C	☐	**~~6.~~ 2.4.6 Any value greater than 80 µg/100 grams of meat is actionable.**
PART III – JELLETT RAPID TEST (JRT) FOR PSP		
		3.1 Procedure
K	☐	3.1.1 The batch/lot numbers of the test strips and buffers, their expiration dates, date received and date used are recorded.
K	☐	3.1.2 When placed into service, test strips and buffers (PSP & Matrix) are within their respective expiration dates.
C	☐	**3.1.3 When opened, the test strip desiccant pouch is blue in color indicating its suitability for use. Test strips emerging from desiccant pouches which are pink in color are never used.**
K	☐	3.1. 4 Test strips and buffer are stored according to the manufacturer's instructions.
C	☐	**3.1.5 Negative extracts are spiked at a low level concentration (40 – 60 µg/100 grams of sample) or equivalent (a bioassayed extract) and used as a positive control for testing both new batches/lots of kits and buffers. Results are recorded and records maintained.**
C	☐	**3.1.6 Micropippettors capable of accurately delivering volumes of 100 and 400 µL are used to transfer buffer and sample extracts and to inoculate test strips with diluted extract.**
K	☐	3.1.7 Volumes delivered by the micropippettor are checked for accuracy at 100 and 400 µL monthly while in service. Results are recorded and records

		maintained.
C	☐	**3.1.8 400 µL of the buffer supplied with the test kits is accurately transferred to a small tube.**
C	☐	**3.1.9 100 µL of the sample extract is added to the buffer.**
K	☐	3.1.10 The sample/extract is thoroughly mixed with buffer by inserting the tip of the micropippettor into the buffer/sample extract mixture and pipetting up and down at least three (3) times.
C	☐	**3.1.11 100 µL of the thoroughly mixed diluted sample extract is inoculated into the test strip sample well.**
K	☐	3.1.12 Micropippettor tips are not reused.
K	☐	3.1.13 Inoculated test strips are allowed to react with the sample extract for the period of time specified by the manufacturer.
C	☐	**3.1.14 The test is interpreted according to the manufacturer's instruction card which is specific to each batch/lot of test strips.**
K	☐	3.1.15 When invalid tests are repeated, the pH of the sample extract is checked and adjusted as necessary to between pH 2.0 and pH 4.0. An aliquot of Matrix buffer and a fresh test strip is used to reassay the sample.
C	☐	**3.1.16 When a repeated JRT test for PSP gives identical invalid results, the sample contains interfering substances which require the use of the mouse bioassay for testing.**
C	☐	**3.1.17 A positive JRT for PSP is actionable.**

Revised 11 – 08 2010

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6. Title 21, Code of Federal Regulations, Part 58, Good Laboratory Practice for Nonclinical Laboratory Study. U.S. Government Printing Office, Washington, D.C.
7. National Research Council. 1996. *Guide for the Care and Use of Laboratory Animals*. National Academy Press. Washington, D.C.
8. Personal communication with USFDA Seafood Laboratory Branch, Office of Seafood, CFSAN, 1998-1999.
9. JRT Instruction Materials with specified batch/lot number instructions.
10 NELAP – National Environmental Laboratory Accreditation Conference. 2003. Chapter 252. ENVIRONMENTAL LABORATORY ACCREDITATION, 252.302. Qualifications of the Laboratory Supervisor, 252.304. Personnel Requirements.

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

Laboratory Evaluation Checklist - **PSP**

LABORATORY STATUS

LABORATORY:	**DATE:**
LABORATORY REPRESENTATIVE:	

PARALYTIC SHELLFISH TOXIN COMPONENT: PARTS I and II and III

A. Results:

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

B. Criteria for Determining Laboratory Status of the PSP Component

1. **Does not Conform Status**. The PSP component of this Laboratory is not in conformity with NSSP requirements if :
   A. The total # of Critical Nonconformities is **>3 or**
   B. The total # of Key Nonconformities is **>6 or**
   C. The total # of Critical, Key and Other is **>10**

2. **Provisionally Conforms Status.** The PSP component of this Laboratory is determined to be provisionally conforming to NSSP requirements if the number of **Critical** Nonconformities is **< 3 and** the number of **Key** Nonconformities is **<6 and** the number of **Other** Nonconformities is **<4.**

3. **Conforming Status.** The PSP component of this Laboratory is determined to be conforming when it has **no Critical** Nonconformities **and < 6 Key** Nonconformities **and < 4 Other** Nonconformities.

C. Laboratory Status *(circle appropriate choice)*:
Does Not Conform - Provisionally Conforms - Conforms

Revised 11 - 08 – 2010

Proposal for Consideration at the Interstate Shellfish Sanitation Conference 2011 Biennial Meeting	☒ **Growing Area** ☐ **Harvesting/Handling/Distribution** ☐ **Administrative**
Name of Submitter:	Robert Parkinson
Affiliation:	Sole Proprietor of St. Thomas Creek Oysters
Address:	43765 Little Cliffs Road Holywood, MD 20636
Phone: **Fax:** **Email:**	301-751-2114 301-737-2771 bobparkinson@hughes.net
Proposal Subject:	Refinement of Fecal Colliform Sources
Specific NSSP Guide Reference:	2009 NSSP Guide for the Control of Molluscan Shellfish Section II Model Ordinance Chapter IV Shellstock Growing Areas @.02 Bacteriological Standards.
Text of Proposal/ Requested Action	Add the following statement to Note: "Where there is evidence that the fecal coliform strategy for sampling is effected by false positives from decaying vegetation or other bacteria (within 1000 feet of shoreline) that do not indicate a risk to consumer health, the authority is required to perform adequate source testing. The authority shall subtract these false positive results from the fecal coliform result to get an accurate reading of the actual bacteriological quality of the test station."
Public Health Significance:	None. This additional source testng is to refine the source of fecal in a non-point source remote site where there is no other evidence of human pathogens. There is substantial evidence that the bacteria that is involved in the decay of vegitation does test positive for the fecal coliform in the test that is currently the standard. Three documents are attached to provide adequate and sufficient rationale for this change to the NSSP.
Cost Information (if available):	Unkown. It is expected that cost of sampling will be reduced as more accurate sampling will result in less sampling required.

CLINICAL MICROBIOLOGY REVIEWS, Oct. 1994, p. 419–425
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Infectious Diseases Associated with Molluscan Shellfish Consumption

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INTRODUCTION

Raw and partially cooked molluscan shellfish (clams, oysters, and mussels) have a long history as vectors of infectious agents and marine biotoxins. Illnesses associated with these food sources originate principally from bacterial and viral pathogens and from toxin-producing dinoflagellates concentrated by shellfish during the filter-feeding process. Infectious disease outbreaks have been reported in the United States since the late 1800s; since then, more than 400 outbreaks and 14,000 cases have been reported (Table 1). These illnesses are attributed to bacterial and viral agents that are associated either with human wastes (delivered to estuarine and marine environments in sewage effluents that have received variable levels of treatment) or to bacterial pathogens indigenous to coastal marine environments (e.g., *Vibrio* spp.).

Before the 1950s, the most common illness associated with the consumption of raw molluscan shellfish was typhoid fever (Fig. 1). After several large outbreaks of typhoid in the mid-1920s (15), when more than 1,500 cases and 150 deaths were reported in several U.S. cities, the U.S. Public Health Service convened a committee to establish regulations for the sanitary control of shellfish. This committee, a forerunner of the National Shellfish Sanitation Program, made the following recommendations (7). (i) Shellfish should be marketed from growing areas that, on careful examination, are free from any suspicion of dangerous contamination with disease-producing organisms or from any deleterious or offensive substances. (ii) After their removal from the water, shellfish should be handled in a manner that would safeguard them from contamination with pathogenic microorganisms or nonpathogenic agents (e.g., toxins, heavy metals, and organics), deterioration, or alteration that would render them unfit for consumption, either hygienically or aesthetically. (iii) Epidemiological studies should be conducted for all outbreaks (epidemics) that implicate shellfish so that the sources of the shellfish can be promptly and accurately traced and measures can be initiated to prevent further infection.

It was understood at that time that the inappropriate disposal of raw and partially treated sewage was a principal reason for the increasing incidence of shellfish-borne illness, particularly typhoid fever. In addition, the process of "fattening" oysters, whereby the animals absorb water through osmosis when placed in tanks of low salinity, was also of significant public health concern. Under poor sanitary conditions, these tank waters (and shellfish) may have been contaminated with pathogenic microorganisms, including *Salmonella typhi*.

As the National Shellfish Sanitation Program recommendations gradually gained acceptance, the incidence of typhoid began to decline for at least two reasons. First, the technology for treating sewage wastes improved, particularly with regard to the removal of pathogen-associated particulates and disinfection. Second, a water quality standard was developed for classifying shellfish-growing areas on the basis of densities of the total coliform bacterial indicator group. This early classification system was used to determine whether or not shellfish could be harvested from given waters, depending on the levels of the indicator group found therein. The standard, as one aspect of the National Shellfish Sanitation Program, appears to have been effective, since no shellfish-associated typhoid cases have been reported in the United States in almost 40 years (Fig. 1).

In the past two decades, however, the nature of shellfish-vectored illness has changed. This report considers infectious diseases from a historical perspective, leading up to current public health issues associated with consumption of raw shellfish. It deals with problems that result from the contamination of molluscan shellfish resources by infectious agents from human and/or animal fecal wastes, treated and untreated wastewaters, and the marine environment.

Data are presented for outbreak (defined as two or more cases of illness resulting from a common exposure), incident (a report of infectious disease resulting from a given exposure, involving usually one person and an etiological agent of *Vibrio* spp.), and case reports primarily from the United States. Information used for this report was obtained from federal,

TABLE 1. Cases and outbreaks of infectious disease (all agents) resulting from the consumption of molluscan shellfish (1898–1990)

Decade	Outbreaks		Cases	
	No.	% of total	No.	% of total
1900	11	2.6	364	2.5
1910	7	1.7	208	1.4
1920	17	4.0	2,161	14.8
1930	31	7.4	567	3.9
1940	40	9.5	1,840	12.6
1950	6	1.4	134	0.9
1960	48	11.4	1,726	11.9
1970	44	10.5	871	6.0
1980	217	51.5	6,687	45.9

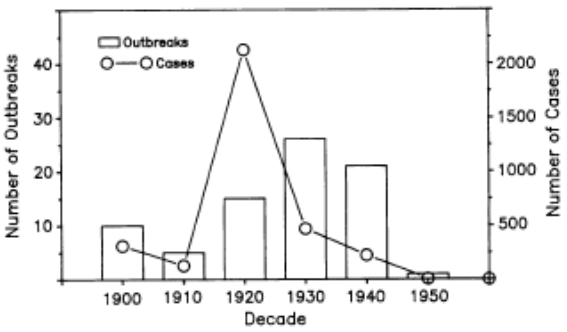

FIG. 1. Number of outbreaks and cases of shellfish-vectored typhoid fever reported since 1894.

state, and local government agencies, research reports, news accounts, and personal communications and does not represent an active, prospective investigation to identify cases of shellfish-associated disease. The data reported here probably represent only a small portion of the actual number of cases that occur annually (10). The true incidence of shellfish-vectored infectious disease may be underestimated as much as 20-fold or more (2). This is true for several reasons. First, because there are no mandatory federal requirements for reporting gastroenteritis of an unspecified nature (i.e., it is not a reportable illness), physicians and state health departments are generally under no obligation to forward case reports to federal authorities. Second, many reported illnesses are cases of relatively mild gastroenteritis; thus, few victims ever seek treatment by a physician. Those reported often describe outbreaks in which relatively large groups of people are affected (e.g., company picnics or gatherings at restaurants). Third, when only a limited number of people are infected, it is very difficult to ascribe the illness to one particular food source. For these reasons, the data may not accurately reflect the true magnitude of the social and economic consequences of illnesses that result in death, that require extended physician and/or hospital care, or, if moderately acute, that prevent individuals from pursuing normal daily activities (2).

ILLNESS ASSOCIATED WITH DISPOSAL OF FECAL WASTES AND SEWAGE INTO THE AQUATIC ENVIRONMENT

Wastewater Disposal Practices and Shellfish-Vectored Illnesses

The association of shellfish consumption and infectious disease has been known or suspected for many years. In 1816, more than 40 years before Pasteur advanced his germ theory of disease, the French physician Pasquier described typhoid fever in a group of people who had consumed oysters harvested from a coastal area contaminated by raw sewage (9, 18). In the United States, infectious bacterial disease associated with molluscan shellfish consumption was first reported in 1894 with two cases of typhoid fever described in Connecticut from shellfish harvested from its coastal waters. No documented cases of infectious disease were reported in the United States before that time, although other types of shellfish-associated illnesses (caused by marine biotoxins) were reported in the late 1700s (16). There are several reasons for this. The construction of storm water or sewerage systems, which began during the mid- to late 1800s in urban centers, resulted in the consolidation of human-derived wastes in collection systems and their eventual release into near coastal environments (8). This practice resulted in the progressive contamination of commercial and recreational shellfish-growing areas and outbreaks of enteric disease associated with shellfish harvested from them. Also, unlike the case for illnesses caused by marine biotoxins, the association between consumption of sewage-contaminated shellfish and infectious disease was not established until the late 1800s. Marine biotoxins, which are produced by dinoflagellates and are a naturally occurring and often highly visible phenomenon, are not associated with sewage contamination of coastal environments. In addition, the occurrence of algal blooms coupled with shellfish-associated human intoxications (generally occurring within several hours of ingestion) establishes an immediately evident relationship. With enteric infections, the relationship is not as clear, since there is no visible measure of water quality and the onset of illness after consumption of contaminated shellfish can be days to weeks.

Until the 1980s, the number of shellfish-associated infectious disease outbreaks was less than 50 outbreaks per decade. Outbreaks and cases by decade as a percentage of the total reported are presented in Table 1. More than 45% of the total historical cases were reported in the 1980s, although there are no obvious reasons for this dramatic increase. However, in the past decade, certain states have adopted aggressive procedures for identifying and describing shellfish-associated disease outbreaks. New York and Florida alone account for more than 50% of the total number of incidents reported nationwide (Table 2). This fact, coupled with increasing consumer awareness of health problems associated with seafood consumption,

TABLE 2. Incidents and cases of shellfish-associated disease (all agents) by principal reporting states

State	Incidents		Cases	
	No.	%[a]	No.	%[b]
Florida	197	26.5	735	5.0
New York	195	26.3	6,611	45.3
Louisiana	48	6.5	195	1.3
Massachusetts	41	5.5	665	4.6
Connecticut	37	5.0	517	3.5
Texas	31	4.2	452	3.1
California	26	3.5	323	2.2
New Jersey	22	3.0	1,989	13.6
Alabama	20	2.7	291	2.0
Georgia	12	1.6	37	0.3

[a] Percentage of the total number of incidents reported nationwide (see text for definitions).
[b] Percentage of the total number of cases reported nationwide.

TABLE 3. Shellfish-borne disease agents occurring in and transmitted by sewage and/or wastewater (1898–1990)

Agent	No. of cases	No. of incidents	No. of outbreaks
Unknown[a]	7,978	277	256
Typhoid	3,270	93	78
Hepatitis A	1,798	51	42
Norwalk virus	311	7	7
Salmonella spp.	130	8	3
Snow Mountain virus	116	4	4
Shigella spp.	111	9	4
Hepatitis[b]	47	5	2
Campylobacter spp.	27	12	1
Plesiomonas spp.	18	3	1
Aeromonas spp.	7	1	1
Staphylococcus aureus	5	1	1
Bacillus cereus	4	1	1
Escherichia coli	2	1	1

[a] No agent isolated or identified.
[b] Type unspecified.

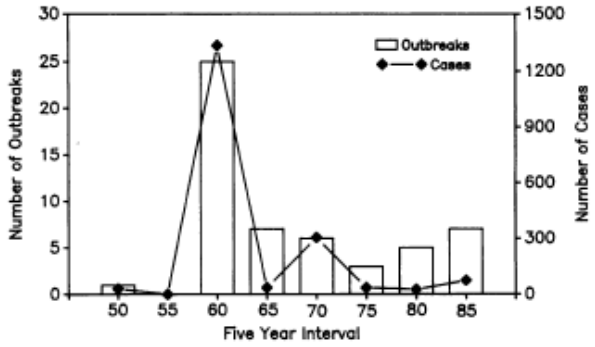

FIG. 2. Shellfish-vectored outbreaks and cases of hepatitis A from 1950 to 1989.

may partially explain the abrupt increase in outbreak and case reports. In addition, shellfish, and particularly oysters, are becoming an increasingly scarce resource as the total acreage of estuarine and marine environments approved or conditionally approved for harvest for direct human consumption decreases with increasing inputs of human-associated contaminants to those areas (14). Also, large areas of potentially productive shellfishing grounds remain closed because they have not been subjected to the sanitary survey work required for proper classification. As a result, there is a strong economic incentive for the illegal harvesting of shellfish from closed but productive growing areas where contaminant loads exceed a generally accepted safe level. This criminal activity is certainly a factor that affects public health, as sewage-contaminated shellfish enter the marketplace. Finally, the rise in case reports may be attributed to deficiencies in current sewage treatment practices (e.g., sewage treatment plants may exceed their design capacity or may have periodic breakdowns which result in inadequate particle removal or disinfection), coupled with the increasing volumes of wastes disposed of in our coastal waters. The use of chlorine to disinfect wastewater effluents is a particular problem in this regard. Certain human enteric viral pathogens (e.g., Norwalk virus) are resistant to the elevated chlorine levels (12) that effectively inactivate vegetative bacterial cells, including the total and fecal coliform indicator groups. Thus, the fecal coliform group, which is the principal indicator of the sanitary quality of most state shellfish-growing waters, may not reliably index the quality of waters that receive chlorine-disinfected effluents. Waters presently considered to be safe for the harvest of molluscan shellfish may, in fact, be contaminated with enteric viral pathogens, and shellfish harvested from those areas may pose an unacceptably high risk of viral illness.

There is no conclusive evidence of an association between contamination derived from animal fecal wastes and the occurrence of shellfish-vectored human illnesses (22). Current assumptions are that illnesses occur primarily from shell stock that accumulate waste from human-associated sources.

Agents of Viral and Bacterial Gastroenteritis

The etiological agents associated with the consumption of raw and lightly cooked molluscan shellfish are listed in Table 3. Most illness reports are ascribed to gastroenteritis, with no causative agent isolated or identified. However, in most cases, the symptoms of disease are very similar. Many reports describe a relatively "mild" gastroenteritis with a typical onset time of 24 to 48 h and a duration of about 2 days. Rarely is a physician's care required. The symptoms, onset, and duration are characteristic of viral gastroenteritis (Norwalk virus has often been implicated). However, since methods for identifying some of these viruses in stools have only recently been developed, and since a limited supply of antigen (obtained from fecal samples of infected individuals) has previously been available only for serological work, these viral pathogens have rarely been identified in shellfish-associated outbreaks. Moreover, there are presently no methods for isolating and culturing viruses from the Norwalk family of agents, including many of the small round viruses.

Bacterial illnesses associated with molluscan shellfish consumption have been infrequently reported since the last case of shellfish-vectored typhoid fever in 1954. Among these bacterial agents (e.g., *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Plesiomonas* spp., *Aeromonas* spp., and *Escherichia coli*), most are found in sewage wastes and are readily inactivated by chlorine disinfection. *Aeromonas* spp. and *Plesiomonas* spp. also occur naturally in freshwater and estuarine environments but appear to present a minimal public health hazard, especially compared with that associated with the environmental *Vibrio* spp. Illnesses caused by *Staphylococcus aureus* or *Bacillus cereus* are most likely a result of postharvest contamination. The recently recognized *E. coli* O157 biotype associated with outbreaks from improperly cooked beef has yet to be associated with a shellfish vector. However, its frequent occurrence in livestock indicates a potential public health problem with shellfish harvest areas affected by farm runoff.

Hepatitis A Infections

Hepatitis A is one of the most serious illnesses associated with shellfish-vectored disease, causing debilitating and chronic infection and even death. The first documented outbreak of shellfish-borne hepatitis occurred in Sweden (19) in 1956, when 629 cases associated with raw oyster consumption were reported. Subsequent to that, hepatitis A cases were reported in the United States (Fig. 2). In 1961, several large outbreaks were reported among consumers of raw oysters in Mississippi and Alabama and consumers of raw clams in New Jersey and Connecticut. In 1964, 20 outbreaks and 743 cases were reported among consumers of both oysters and hard clams and other, unspecified shellfish in several states. In most instances, shellfish harvest areas were not identified for several

reasons. First, the onset time of this illness is 2 to 8 weeks or more, and by then, the implicated shellfish were no longer in the distribution system. Second, the tagging systems used to identify original shellfish harvest sites were, and still are, often unreliable. (The tagging system involves labeling sacks of shellfish with specified information, including harvest area. Tags remain attached to the sacks throughout the distribution network until a specified time after their retail distribution.) There is no standard, nationally accepted tagging system for confidently determining the original harvest area of a given lot of shellfish. Third, the economic incentive for "bootlegging" shellfish (i.e., illegally harvesting animals from unapproved or prohibited areas) is quite compelling. The state patrol procedures needed to deter this illegal activity are often compromised by the lack of financial resources and manpower needed for active and suitable enforcement. In addition, the penalties for these offenses are often not a sufficient deterrent. Thus, shellfish that are not suitable for raw consumption can, and do, enter the marketplace. The magnitude of this problem is not known.

The percentage of hepatitis A virus outbreaks is lower than that caused by certain other infectious agents, and most outbreaks that are reported usually involve a large number of cases. Underreporting of a shellfish-vectored hepatitis A virus outbreak is due to the extended onset period following consumption of the contaminated food and the corresponding difficulty in determining a common food source when only a limited number of individuals are involved. Outbreaks of hepatitis A have been reported consistently since the early 1960s (Fig. 2), and the illness continues to be a public health concern today. Worldwide, the illness is reported frequently. The most disturbing recent incident occurred in China in 1988 (1), when more than 292,000 cases (nine deaths) of hepatitis A (associated with the consumption of uncooked, contaminated cockles) were reported in the urban areas around Shanghai. This outbreak clearly demonstrated the need for effective sanitation programs to prevent the introduction of contaminated shellfish into the marketplace and what can happen when the system breaks down or when there are no effective programs in place.

Seasonality of Illness Reports

Gastroenteritis of an unknown or viral etiology seems to occur more frequently at certain times of the year. When grouped by month, both the outbreak (Fig. 3) and case (Fig. 4) data reveal two periods of increased illness: late spring and late fall. These incidents roughly coincide with times when bioaccumulation rates in shellfish are high. During certain times in spring (4) and fall (5) in temperate waters, hard clams (*Mercenaria mercenaria*) accumulate viruses (and other microbial indicators) at a significantly higher rate than at other times of the year and thus can be periodically contaminated with high levels of sewage-associated microorganisms, including microbial pathogens. This phenomenon is subsequently reflected in the human health effects data. The increased consumption of raw shellfish (particularly hard clams) during these periods may also be coincident with higher illness rates. Although data are lacking on the seasonal incidence of Norwalk and Norwalk-like viruses, they probably show a seasonal occurrence much like that of other viral groups that are epidemic only at certain times of the year. Their input into the marine and estuarine environment would then be intermittent and unpredictable as they pass through the infected individual into the wastewater stream.

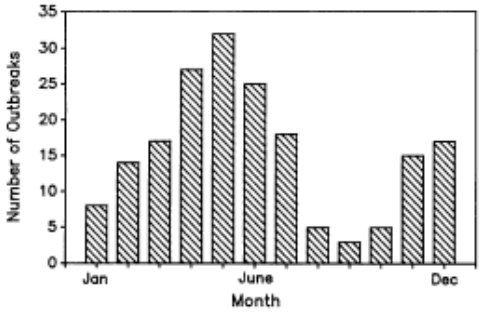

FIG. 3. Number of outbreaks by month of illness ascribed to viral pathogens or to illnesses of undetermined etiology (1894 to 1989).

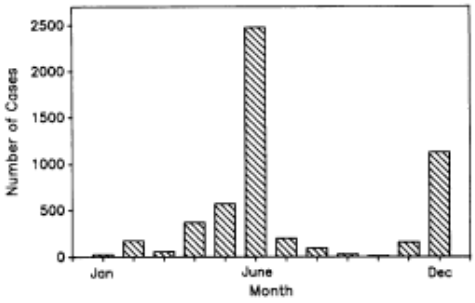

FIG. 4. Shellfish-associated illness cases ascribed to viral pathogens or to illnesses of undetermined etiology (1894 to 1989).

Relative Incidence of Allocthonous Microbial Agents Involved in Disease Outbreaks

The bacterial agents of shellfish-associated disease (Table 4) represent a small proportion of the outbreak (4.0%) and case (3.8%) reports. This may be because the indicator organisms used to assess and classify the sanitary quality of shellfish-growing areas (as open, restricted, or prohibited) effectively protect the health of the shellfish-consuming public against diseases of an allocthonous bacterial origin or because the etiological agents of gastroenteritis associated with shellfish outbreaks are infrequently isolated and identified.

Compared with bacteria, viral agents of shellfish-vectored disease represent a significantly greater proportion (Table 4) of the totals reported. However, those cases in which no agent was isolated represent the bulk of illness reports (more than 75% of the cases and 79% of the outbreaks). If the presumption is correct that most of these "unknowns" can be ascribed

TABLE 4. Shellfish-vectored disease outbreaks and cases by class of agent for sewage- and wastewater-associated pathogens (1905–1990)[a]

Class of agent	Cases		Outbreaks	
	No.	% of total	No.	% of total
Unknown	7,978	75.7	256	79.0
Viral	2,272	21.5	55	17.0
Bacterial	304	3.8	13	4.0

[a] Typhoid fever is not included in this table. The last reported shellfish-vectored case was in 1954.

TABLE 5. Incidents, outbreaks, and cases of shellfish-associated illnesses associated with members of the *Vibrio* genus (1967–1990)

Vibrio species	No. of cases	No. of incidents	No. of outbreaks
V. parahaemolyticus	159	60	14
V. vulnificus	160	133	8
V. cholerae non-O1	143	57	14
V. cholerae[a]	5	3	2
V. cholerae O1	14	14	
V. fluvialis	8	6	1
V. hollisae	15	15	
V. mimicus	14	14	
V. alginolyticus	1	1	
Vibrio spp.	6	4	1

[a] No serotype specified.

to a viral agent (symptomatically), enteric viral pathogens present the principal concern to the public health.

AUTOCHTHONOUS MARINE BACTERIA AS AGENTS OF SHELLFISH-VECTORED ILLNESSES

Several *Vibrio* spp., native to both marine and estuarine environments, have been identified as the causative agents of shellfish-vectored illnesses (Table 5). These halophilic, nonsporeforming bacteria occur in saline aquatic environments in densities that are related, at least in part, to water temperatures and salinity. Other factors that influence their occurrence and distribution in the aquatic environment are not well understood. The severity of human disease caused by the different species (Table 5) varies considerably. *Vibrio vulnificus* infections can result in septicemia with a high mortality rate; cholera has been reported among consumers of raw shellfish for the past two decades. All of the *Vibrio* spp. listed in Table 5, except *V. vulnificus*, are associated with gastroenteritis of varying severity. Among this group, the O1 serogroup of *Vibrio cholerae* is the most serious and debilitating. In general, all of these agents produce a much more severe gastroenteritis than that caused by enteric viral pathogens.

V. vulnificus

Oyster-associated *V. vulnificus* septicemia and death were first reported in 1975 (3). Since then, about ten cases (five deaths) of oyster-borne infections attributable to this species have been reported annually in the United States. The population at risk of developing this illness is well defined because they have certain health problems, such as liver cirrhosis, diabetes, hemochromatosis, and immunosuppressive disorders (17), which predispose them to infectious disease. The case fatality rate averages about 50% among this group. Given the numbers of individuals at risk and the frequency of raw shellfish consumption in certain areas of the United States (13), it is surprising that the number of cases and deaths is not higher. Clearly, the mechanisms of pathogenesis of this organism need further investigation. In addition, temperature abuse (i.e., the holding of shellfish at temperatures in excess of 45°C for prolonged periods of time in transit or in the marketplace) may contribute to illness associated with *Vibrio* spp. (or other bacteria) by providing a condition that would allow these pathogens to multiply in the molluscan shell stock. The significance of the role of temperature abuse in human morbidity or mortality is unknown.

V. vulnificus case reports show a seasonal pattern, with the highest frequencies occurring from midsummer through late

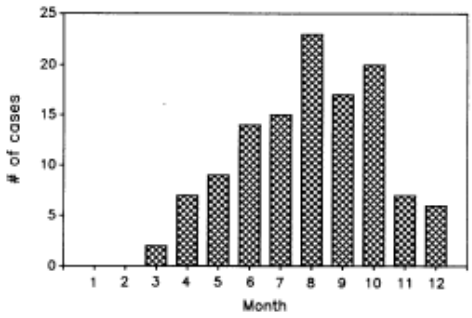

FIG. 5. Cases of shellfish-vectored *V. vulnificus* infections reported by month (1975 to 1989).

fall (Fig. 5). No shellfish-associated cases have been reported in the United States in January or February. Because of its temperature sensitivity, *V. vulnificus* is found in highest densities when water temperatures exceed 15°C (23); below this temperature, environmental densities decline rapidly. *V. vulnificus* is commonly found in all U.S. coastal waters and presumably in all species of near-coastal shellfish in densities that fluctuate with the season. However, case reports associate illness from this organism only with consumption of raw oysters and with shellstock harvested from waters of the U.S. Gulf Coast. The reason for this remains unexplained.

V. cholerae

Cholera was first identified in the United States in 1832, and the illness, involving several large food- and waterborne epidemics (20), was reported periodically until 1911. After that, it was believed to have been eradicated from this country. However, in 1973, a Texas fisherman was diagnosed with the illness (24), although the source of the organism could not be determined. Since that time, *V. cholerae* cases (and deaths), although rare, have been reported sporadically among shellfish consumers (Fig. 6). Both the O1 and other serotypes have been isolated from individuals with relatively severe gastroenteritis. Non-O1 serotypes are reported most frequently, and although the illness caused by them is generally less severe than that caused by O1 serotypes, these organisms have been associated with several oyster-vectored deaths. Non-O1 biotypes are indigenous to marine environments; however, there is no conclusive evidence for an autochthonous marine O1 popula-

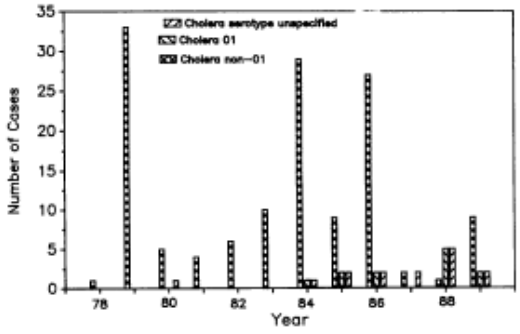

FIG. 6. Shellfish-associated cases of cholera (*V. cholerae* O1, non-O1 cholera, and unspecified serotypes) reported from 1978 to 1989.

TABLE 6. Shellfish-associated outbreaks and cases of infectious illness by type of shellfish (1898–1990)

Species	Agents other than *Vibrio* spp.[a]		*Vibrio* spp.[b]	
	No. of incidents	No. of cases	No. of incidents	No. of cases
Hard clams	232	6,201	4	4
Oysters	155	4,959	279	362
Soft clams	10	43	0	0
Mussels	14	174	1	1
Scallops	1	2	0	0

[a] Includes all illnesses from agents other than those in the *Vibrio* genus and of unknown etiology.

[b] Includes *V. parahaemolyticus*, *V. cholerae* O1 and non-O1, *V. vulnificus*, *V. fluvialis*, *V. mimicus*, and *V. hollisae*.

tion, although this possibility has been advanced (21). No cases of shellfish-vectored, domestically acquired cholera associated with the serotype responsible for the current South American epidemics (6) have been reported in the United States.

Other Vibrios

A number of other *Vibrio* spp. have been associated with shellfish-vectored illness outbreaks (Table 5). *Vibrio parahaemolyticus* cases are reported as frequently as *V. vulnificus* cases. However, illness caused by this bacterium is generally confined to gastroenteritis, although it can be severe and of relatively long duration. A problem in clearly establishing *Vibrio* spp. as etiological agents is that they are all native to marine waters (and presumably shellfish) and may be simply passing through the human gut after the ingestion of uncooked or lightly cooked shellfish. Classical epidemiological methods (e.g., isolation of the organism from the human host followed by reinfection) cannot be used to demonstrate the causality of a suspect organism in a foodborne outbreak. Therefore, the relationship between the isolation of a *Vibrio* sp. from a fecal sample and its role as the causative agent in a case report may be generally assumed but not conclusively established.

SPECIES AND SOURCES OF SHELLFISH FROM ILLNESS REPORTS

Most illness reports in which no causative agent was identified, or the agent was not identified as a *Vibrio* spp., have historically been associated with consumption of hard clams (Table 6). More than 56% of the outbreaks and 54% of the case reports identify hard clams (*M. mercenaria*) as the vector. Oysters (*Crassostrea virginica* and *Ostrea edulis*) are also significant vehicles for infectious illness, associated with 38% of outbreaks and 44% of cases. Soft clams, mussels, and scallops are of minimal public health concern with regard to infectious disease because they are usually cooked before consumption, or only the adductor muscle is usually consumed (scallops). The sources of shellfish (i.e., the original growing areas or last point of water immersion, such as a depuration facility or wet storage area) implicated in these illnesses are predominantly unknown. Of the 412 total outbreaks associated with species other than *Vibrio* spp. (Table 6), 317 (66%) were from shellfish of unknown or questionable origin. New York and Florida, the states most frequently reported as the sources of shellfish implicated in outbreaks, also reported the largest number of outbreaks and cases (Table 2). This association is probably not coincidental.

A completely different history is evident for infections

TABLE 7. Incidents of *Vibrio*-caused illnesses by source of shellfish (1898–1990)

Source of shellfish	No. of incidents[a]
Unknown	198
Louisiana	41
Florida	40
Texas	5
Alabama	5

[a] Incidents are defined as one or more cases from a common source.

associated with certain *Vibrio* spp., particularly *V. vulnificus*, the bacterial species of primary public health concern today. For illnesses caused by these autochthonous bacteria, oysters (principally *C. virginica*) are the predominant vector. More than 98% of the incident reports and 99% of the case reports (Table 6) are associated exclusively with this shellfish species. Most reports involve oysters whose original harvest (or wet storage) sites could not be reliably determined (Table 7); however, positively identified harvest areas were exclusively in Gulf Coast waters. This very interesting fact remains unexplained in light of the limited information available on the marine and estuarine ecology of these aquatic bacteria, particularly since their densities vary widely in the saline environments of all continental U.S. coasts (11, 23). In addition, there is little information on the mechanisms of pathogenesis of these organisms, although the predisposing factors that affect the susceptibility and morbidity of the human host are generally well described.

CONCLUSION

Infectious diseases attributable to the consumption of raw and lightly cooked molluscan shellfish are caused by bacterial agents that are native to the marine environment and by viral and bacterial agents from sewage effluents and other sources that contaminate environmental waters. As filter-feeding organisms, shellfish magnify public health problems associated with environmental contamination because they accumulate microbial pathogens, including viruses, manyfold over the densities found in overlying waters.

The current public health problems of greatest concern to consumers of molluscan shellfish are associated with viral, and suspected viral, pathogens. The numbers of cases and outbreaks caused by these pathogens far exceed those of all other infectious diseases. In terms of the severity of human illness and death, the *Vibrio* genus (specifically *V. vulnificus*) presents a serious problem. Although the number of cases reported yearly is quite low, the high mortality rates involved are of significant public health concern.

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Rate of Occurrence of False-Positive Results from Total Coliform Most-Probable-Number Analysis of Shellfish and Estuaries

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The incidence of confirmed test, false-positive coliform most-probable-number results was compared with environmental parameters and was found to be inversely related to water temperature. It is concluded that the completed coliform test must be done when water temperatures drop below 15°C.

Shellfish harvested from estuarine waters are examined for total numbers of coliforms, along with water and sediment samples from the harvesting areas. The most-probable-number (MPN) analysis (1, 3, 13) is routinely employed and is carried through the presumptive, confirmed, or completed sequence of tests. The completed tests are not always done when the sanitary quality of water is being assessed, notably in the cases of bathing and potable waters (2). To establish a balance between efficiency and accuracy, the incidence of false-positive and false-negative results at each stage of the analysis should be known.

It has been well documented that the presumptive test alone may be of limited reliability (11), historically because of those noncoliforms which may be present and capable of fermenting lactose aerogenically (4, 5, 6, 8, 9, 10, 12). In the study reported here, Chesapeake Bay oysters and oyster beds were examined over a 2-year period. Two sites in Chesapeake Bay, Tolly Point and Eastern Bay, were sampled on a routine basis. These sites were selected because they are commercially important oyster harvest areas and, in addition, the water column of both areas is subject to very little fecal contamination (mean total coliform completed test MPN, 8/100 ml). At approximately 1-month intervals during 1977 and 1978, bottom water samples were collected at one meter above the sediment by means of the Niskin sampler (General Oceanics, Inc.). Sediment samples were collected by using a Petite Ponar grab (Wildlife Supply Co.), and oysters were harvested using a drag-type dredge. All samples were processed within 30 min of collection.

Six oysters, each of which weighed ca. 16 to 20 g, including meat and liquor, after shucking, were scrubbed, rinsed, and aseptically shucked. The oyster tissue was pooled and homogenized in a solution consisting of sterile 0.5% (wt/vol) peptone (Difco Laboratories) in a 1:2 dilution of oyster tissue. Sediment samples were suspended in an estuarine three salts solution (3). Salinity and temperature were measured at the time of collection of the bottom water samples.

A five-tube, total coliform MPN analysis of each of the water, sediment, and oyster samples was performed in duplicate, and the results were normalized for 100 ml (or 100 g) of sample, following procedures recommended by the American Public Health Association (1). Samples (10, 1.0, 0.1, and, for sediment suspension, 0.01 ml) were transferred to appropriate tubes. Lactose broth (Difco), brilliant green bile (2%) broth (Difco), and eosin methylene blue agar (Difco) were employed. Total viable counts (TVC) of aerobic heterotrophic bacteria were enumerated on 30% strength 2216E Marine agar (Difco) (7) plates prepared in triplicate. The TVC plates were incubated at 17 ± 2°C for ca. 15 days before counts were made.

Within each MPN test series, the number of positive results at each successive step (Table 1) was compared, and the proportion of positive presumptive tests which failed to be confirmed as total coliform-positive was defined as the false-positive percent (FP%). The calculation was done using the formula: $FP\% = [(P - C)/P] \times 100$, where P is positive results and C is confirmed (or completed) positive results. This comparison was made for the presumptive-confirmed and also the confirmed-completed test steps. For each sample, the false-positive percentages were, in turn, compared with data for total coliform MPN, TVC, salinity, and temperature. Correlation coefficients were obtained using the Biomedical Computer Programs (BMDP) statistical package on the University of Maryland UNIVAC 1108 computer, and compared with values for critical r (15).

Bottom water salinities ranged from 7.4 to 15.0 ‰. TVC and temperature values are re-

TABLE 1. *Numbers of positive reactions obtained for presumptive, confirmed, and completed coliform MPN tests*

Date	Tolly Point			Eastern Bay			
	Temp (°C)[a]	No. of positive reactions[b]		Temp (°C)[a]	No. of positive reactions		
		Oyster	Bottom water		Oyster	Bottom water	Sediment
24 October 1977	15	21, 20, 20 (—)	12, 8, 8 (1.2E5)	13	10, 1, 1 (1.1E5)	10, 0, 0 (—)	21, 1, 1 (4.4E5)
18 November 1977	11	18, 15, 15 (—)	8, 5, 5 (—)	13	10, 7, 6 (—)	4, 3, 3 (—)	—, —, — (—)
20 December 1977	5	7, 4, 2 (2.0E3)	15, 9, 8 (—)	5	6, 3, 3 (—)	8, 4, 3 (—)	7, 4, 1 (—)
18 January 1978	1	2, 0, 0 (1.7E4)	20, 19, 10 (2.8E4)	1	7, 1, 0 (3.9E4)	9, 4, 1 (9.0E3)	22, 5, 1 (1.3E5)
28 March 1978	6	10, 9, — (5.0E3)	18, 17, — (—)	8	1, 0, — (—)	14, 4, — (—)	23, 0, — (—)
18 April 1978	10	13, 13, 13 (4.0E3)	7, 0, 0 (—)	9	1, 1, 1 (4.0E3)	7, 4, 4 (5.0E3)	23, 10, 3 (4.3E6)
19 May 1978	9	14, 14, 13 (4.7E4)	18, 10, 10 (7.4E4)	9	11, 4, 3 (3.6E5)	12, 3, 0 (6.0E4)	23, 13, 0 (—)
21 July 1978	26	5, 3, 3 (—)	3, 2, 2 (—)	24	5, 1, 1 (—)	1, 0, 0 (—)	4, 0, 0 (—)
6 September 1978	26	9, 7, 7 (9.3E4)	6, 5, 5 (2.0E3)	27	1, 1, 1 (2.7E4)	1, 1, 1 (5.0E3)	20, 1, — (2.3E7)
31 October 1978	14	11, 6, 6 (6.0E3)	9, 5, 5 (2.0E3)	15	17, 14, 14 (3.2E4)	0, 0, 0 (2.0E3)	16, 0, 0 (2.1E5)

[a] Temperature of water 1 to 2 m below surface.

[b] Positive results at each test level: first column is presumptive, second is confirmed, third is completed. Initial observations were recorded for 15 MPN tube series done in duplicate. Number within parentheses is the TVC for the corresponding sample. See text for procedures. —, No data.

ported in Table 1. Oyster total coliform MPN values were consistently low, averaging 81/100 g at Tolly Point and 34/100 g at Eastern Bay. Bottom water total coliform MPN values averaged ca. 12/100 ml and 3.2/100 ml, respectively. Sediment counts at Eastern Bay averaged 13/100 g.

The percent occurrence of false-positive presumptive and confirmed results are presented in Table 2 and Fig. 1, respectively. Overall, some parameters were not found to be correlated (probability, $P < 80\%$). For example, the percent occurrence of false-positive confirmed results did not correlate with: (i) percent occurrence of false-positive presumptive tests ($r = 0.068$); (ii) TVC ($r = 0.193$); or (iii) salinity ($r = -0.125$). Some equivocal correlations ($90\% < P < 95\%$) were noted, and these included total coliform MPN with false-positive percentages, both presumptive ($r = -0.269$) and confirmed ($r = -0.272$), and with salinity ($r = -0.113$).

The most important and definitive relationship detected was that of false-positive and confirmed results and sample temperature (Fig. 1). Although some variations were recorded between the stations as well as for each sample type, it was clear, particularly with regard to sediments (Table 1), that the number of false-positives detected in the confirmation tests increased significantly when the water temperature fell below 10°C. In fact, this relationship was statistically validated for each sample type and station. To cite composite data, water temperatures were found to have a strong negative correlation with percentage of false-positive confirmed tests (−0.593 correlation, significant at

TABLE 2. *Percentage of false-positive presumptive MPN results*

Date	% of false-positive results				
	Tolly Point		Eastern Bay		
	Oyster	Bottom water	Oyster	Bottom water	Sediment
24 October 1977	5	33	90	100	95
18 November 1977	17	38	30	25	—[a]
20 December 1977	43	40	50	50	43
18 January 1978	100	5	86	56	77
28 March 1978	10	6	100	71	100
18 April 1978	0	100	0	43	57
19 May 1978	0	44	64	75	43
21 July 1978	40	33	80	100	100
6 September 1978	22	17	0	0	95
31 October 1978	45	44	17	—	100

[a] —, No data.

the ≥99.9% confidence level; critical $r = 0.372$; $n - 3 = 45$) (Table 3). The temperature at which significant false-positive results begin to be observed may be related to changes in the composition of the bacterial population (14).

Based on the results of this study, it is concluded that, in the past, total coliform MPN (confirmed test) results for cold, estuarine water samples (i.e., <15°C) were subject to error, and reported values may have been higher than was, in fact, the case. It is recommended that the total coliform MPN evaluation of estuarine water, shellfish, and sediment samples include the completed test whenever the temperature of the water falls below 15°C.

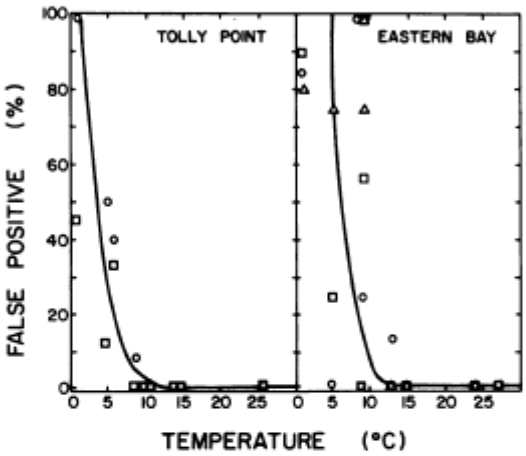

FIG. 1. *Effect of temperature on occurrence of false-positive confirmed MPN tests. Oysters (○), bottom water (□), and sediment (△) were sampled and processed as described in the text. Sample dates are listed in Table 1.*

TABLE 3. *Composite correlation matrix of salinity, temperature, and microbiological parameters*[a]

Parameter	Salinity (‰)	Temp (°C)	Log TVC	Presumptive FP%[b]	Confirmed FP%
Salinity	1.000				
Temperature	0.224	1.000			
Log TVC	0.376[c]	0.206	1.000		
Presumptive FP%	−0.014	−0.104	0.106	1.000	
Confirmed FP%	−0.125	−0.593[d]	0.193	0.068	1.000

[a] Results recorded for each sample, station, and date were combined, and the combined results were correlated.

[b] FP%, False-positive result, percentage of occurrence.

[c] Significant at the 0.05 level, critical $r = 0.367$.

[d] Significant at the 0.01 level, critical $r = 0.372$.

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Closing the Door on the Fecal Coliform Assay

The fecal coliform assay, the results of which have led to numerous misinterpretations over the years, may have outlived its usefulness

Michael P. Doyle and Marilyn C. Erickson

Since its inception in 1904, the fecal coliform assay has been used to assess the presence of fecal contamination in water and foods. Assays to detect *Escherichia coli*, a more specific indicator of fecal contamination, were previously not as popular due to the longer time period for detection required (five days) and their complexity. Recent advances in the detection of *E. coli*, however, have eliminated these impediments and detection occurs within 24 hours or less. Many limitations and complications have been associated with the fecal coliform assay, thereby raising questions about its continued appropriateness and usefulness in food and water testing. The microbiology literature is replete with reports of studies that correlate results of fecal coliform levels with the presence of *E. coli* including several recent examples that advocate the fecal coliform test as an acceptable indicator in manure composts and foods. However, the value of the fecal coliform assay as an indicator of fecal contamination is negated when bacteria of nonfecal origin are the principal microbes detected by the assay.

Historically, the definition of fecal coliforms has been based on methods used for their detection. Specifically, fecal coliforms are gram-negative bacilli, not sporulated, oxidase-negative, optional aerobic or anaerobic, able to multiply in the presence of bile salts or other surface agents that have equivalent properties, and are able to ferment lactose with acid and gas production in 48 h at the temperature of 44 ± 0.5°C. Several genera of bacteria that are common contaminants of nonfecal sources (e.g., plant materials and pulp or paper mill effluents) meet this definition. Examples include *Klebsiella*, *Enterobacter*, and *Citrobacter* species. Moreover, these bacteria which are false-positive indicators of fecal contamination can grow under appropriate conditions in nonfecal niches such as water, food, and waste. The International Commission on Microbiological Specifications for Foods in its evaluation of this issue reported the term fecal coliforms has arisen from attempts to find rapid, dependable methods for establishing the presence of *E. coli* and closely related variants without the need to purify cultures. Species of *Enterobacteriaceae* other than *E. coli* are associated with plants and do not indicate fecal contamination, yet they are identified as fecal coliforms by the fecal coliform assay. Hence, *E. coli* is the only valid index organism for the monitoring of foods containing fresh vegetables.

To reduce the possibility of false-positive results, a confirmatory test for *E. coli* is recommended. In spite of this precaution, there have been several instances where fecal coliform results have been incorrectly interpreted. One of the most sensational situations occurred in 1995 when the U.S. news media reported that high populations of fecal coliforms in restaurant-brewed tea indicated the presence of feces in tea. The dominant fecal coliforms identified were *Klebsiella pneumoniae* and some *Enterobacter* spp., but no *E. coli*. Although there was ample evidence of fecal coliform contamination of iced tea served in restaurants (e.g., 64% of samples at fecal coliform of >1,100 MPN/ml), there had been no history of outbreaks of illnesses resulting from consumption of iced tea.

Another instance where fecal coliform data have been inappropriately interpreted involved two Canadian recalls of sprouts where high levels of fecal coliforms were later identified to be *K. pneumoniae*. In the health hazard alert accompanying these recalls, a warning was issued that this organism could cause gastrointestinal illness in humans. While this bacterial strain is an opportunistic pathogen outside the intestinal tract causing respiratory and urinary tract infections, gastrointestinal illness rarely occurs. Hence, the overly cautious warning was likely due to the association of this bacterium with the fecal organism group.

A quick perusal of the Internet including both

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governmental and academic sites revealed information is being provided that fails to address the possibility that bacteria testing positive in the fecal coliform assay may originate from nonfecal sources. For example, a U.S. Environmental Protection Agency (EPA) page listing drinking water contaminants and their maximum contaminant levels stipulates that "fecal coliforms and *E. coli* only come from human and animal fecal waste." To the contrary, as noted above, there is a preponderance of data indicating that fecal coliforms do not only originate from fecal waste. Similarly, the Kentucky Division of Water site indicates that fecal coliform bacteria "are associated only with the fecal material of warm-blooded animals" and the Food Safety Authority of Ireland site reports that "faecal coliforms found in water are a direct indication that the water has been contaminated with animal or human effluent." Collegiate and K-12 academic sites also provide similar misleading information. Unfortunately, these generalizations can lead to misinterpretation of results by those who do not have a complete understanding of the fecal coliform assay and the subtleties associated with interpreting the results of such assays.

Concerns regarding the inappropriate interpretation of results of the fecal coliform assay and its limited usefulness as an indicator of fecal contamination are not new. They have surfaced several times over the past decade. When the issue of fecal coliforms in tea made media headlines, it was suggested that the fecal coliform assay be reevaluated for its usefulness in food testing. The following year, two commentaries published in *ASM News* opined that the fecal coliform term should be excluded from microbiology. This was further supported by investigators of a study comparing *E. coli*, total coliform, and fecal coliform populations as indicators of wastewater treatment efficiency, who concluded that *E. coli*-based effluent and stream standards (not fecal coliform standards) should be developed to protect public health. A subsequent review of the suitability of the coliform group as an indicator of microbial water safety led other investigators to recommend elimination of the fecal coliform assay. This proposal was further corroborated by studies revealing that only 50% of fecal coliform colonies enumerated as fecal coliforms in foods were identified as *E. coli*.

In the past few years, several changes in monitoring protocols have already been initiated by national and international regulatory agencies. In the European community as well as in Australia and New Zealand, the "fecal coliforms" term has been replaced by what is considered a more appropriate descriptor of this class of microorganisms, "thermotolerant coliforms". Both WHO's Guidelines for Drinking Water Quality and the Australian Drinking Water Guidelines, however, continue to advocate that thermotolerant coliform measurements are an acceptable alternative to *E. coli* measurements. While this change in terminology reduces the likelihood that positive results may be interpreted as meaning the presence of fecal contamination, it does not eliminate the possibility that nonfecal coliforms may be present and give positive results.

In 1986, the U.S. EPA published a document that encouraged states to use *E. coli* or enterococci as the basis of their water quality criteria to protect fresh recreational waters and to use enterococci as the basis for water quality criteria in marine waters. While these guidelines have been criticized, a systematic review and meta-analysis of data reaffirmed these recommendations. More specifically, this analysis revealed that *E. coli* was a more consistent predictor of gastrointestinal illness than other bacterial indicators in fresh water. Despite these recommendations, state and local authorities have been slow to respond in adopting these guidelines. To address some of the advantages and impediments to implementation of these guidelines, costs for the three bacterial indicators were surveyed in the Tacoma/Seattle region and were found to be fairly comparable and thus not a limiting factor. In contrast, an inherent weakness cited by the Washington State Department of Ecology was that using enterococci as an indicator organism in marine waters would complicate efforts to model data obtained from freshwater sources in which *E. coli* was monitored. Another weakness is the continuing requirement by the Food and Drug Administration to use fecal coliforms as an indicator microorganism in shellfish marketed across state borders. Despite this requirement, no significant relationship has been observed between levels of *E. coli* and enterococci and non-*E. coli* fecal coliforms in oysters. Consequently, the continued use of fecal coliforms as an indicator in shellfish would likely hinder widespread acceptance of more appropriate indicators. Moreover, in a National Academies of Science (NAS) report to evaluate candidate indicator organisms and/or indicator approaches, the committee was adverse to abandoning the current indicator microbes until new and better methods are developed and validated. While the NAS Committee foresaw the advent of increasingly sophisticated and powerful molecular biology techniques that would provide new opportunities for the development of improved assays for indicator microbes, we contend that immediate replacement of the fecal coliform assay with an *E. coli* assay would apply the best science available to providing public health protection.

In conclusion, physicians and public health officials have repeatedly misinterpreted results of the fecal coliform assay when applied to food, beverage, or water samples. To prevent future occurrences, the fecal coliform assay should at a minimum be redefined to specifically qualify that it is not a reliable indicator of either *E. coli* or the presence of fecal contamination. An even better alternative would be to eliminate the fecal coliform assay as an indicator of fecal contamination of foods, beverages, and water. The *E. coli* assay is a more reliable indicator of fecal contamination, although not absolute, and could serve as a replacement for the fecal coliform assay.

Literature citations and relevant references which provide the basis for this commentary can be found in the online version of this article.

Proposal for Consideration at the Interstate Shellfish Sanitation Conference 2011 Biennial Meeting	☒ **Growing Area** ☐ **Harvesting/Handling/Distribution** ☐ **Administrative**
Name of Submitter:	Mercuria Cumbo
Affiliation:	Maine Department of Marine Resources
Address:	Lamoine Water Quality Lab 22 Coaling Station Rd. Lamoine, ME 04605
Phone: **Fax:** **Email:**	207 667-5654 207 664-0592 mercuria.cumbo@maine.gov
Proposal Subject:	Total Coliform Method for Shellfish Dealer Process Water using the membrane filtration techniques with mEndo LES agar
Specific NSSP Guide Reference:	2009 NSSP Section IV Guidance Documents Chapter II Growing Areas .10 Approved NSSP Laboratory Tests - Type I and Type II Microbiological Methods, UV treated Seawater
Text of Proposal/ Requested Action	Accept Total Coliform Method for Shellfish Dealer Process Water using the membrane filtration techniques with mEndo LES agar as an alternative method to the APHA MPN method for the presence/absence of total coliforms in UV treated seawater. Single Laboratory Validation Study Results and Method approval application attached.
Public Health Significance:	This method produces results in 24 hours and is a less labor intensive method for laboratories. This more rapid test method would allow operators of facilities who provide disinfected process water for shellfish in wet storage and depuration operations the ability to know they have a problem and take the required remediation action on a more timely basis. It would reduce the workload for the laboratory performing the testing.
Cost Information (if available):	This alternative test should be less costly to the laboratories.

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	Total Coliform Method for Shellfish Dealer Process Water using the membrane filtration techniques with mEndo LES agar
Name of the Method Developer	Mercuria Cumbo and Cathy L. Vining
Developer Contact Information	Maine Department of Marine Resources Lamoine Water Quality Laboratory 22 Coaling Station Rd. Lamoine, ME 04605 207-667-5654 Mercuria.cumbo@maine.gov Cathy.l.vining@maine.gov

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.	Y	Alternative method which is more rapid than current NSSP approved method. Less labor intensive for laboratory
2. What is the intended purpose of the method?	Y	Shellfish dealer disinfected process water
3. Is there an acknowledged need for this method in the NSSP?	Y	NSSP requires testing of disinfected process water
4. What type of method? i.e. chemical, molecular, culture, etc.	Y	Microbiological culture
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title	Y	Total Coliform Method for Shellfish Dealer Process Water using the membrane filtration technique with mEndo LES agar
Method Scope	Y	Presence/absence of total coliform in disinfected shellfish process water
References	Y	See attached document
Principle	Y	See attached document
Any Proprietary Aspects	Y	none
Equipment Required	Y	Membrane filtration apparatus
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	NA	Performance criteria previously established for this method.
2 Measurement Uncertainty	NA	Performance criteria previously established for this method.
3 Precision Characteristics (repeatability and reproducibility)	NA	Performance criteria previously established for this method.
4 Recovery	NA	Performance criteria previously established for this method.
5. Specificity	NA	Performance criteria previously established for this method.
6. Working and Linear Ranges	NA	Performance criteria previously established for this method.
7 Limit of Detection	NA	Performance criteria previously established for this method.
8 Limit of Quantitation / Sensitivity	NA	Performance criteria previously established for this method.
9. Ruggedness	NA	Performance criteria previously established for this method.
10. Matrix Effects	NA	Performance criteria previously established for this method.
11. Comparability (if intended as a substitute for an established method accepted by the NSSP)	Y	See attached document. Method is comparable for presence/absence with the NSSP approved APHA MPN Lactose Broth/Brilliant green Bile Broth Total Coliform method.
D. Other Information		
1. Cost of the Method		Comparable to approved method
2. Special Technical Skills Required to Perform the Method		Comparable to approved method
3. Special Equipment Required and Associated Cost		Membrane filtration apparatus
4. Abbreviations and Acronyms Defined		
5. Details of Turn Around Times (time involved to complete the method)		22 - 24 hours
6. Provide Brief Overview of the Quality Systems Used in the Lab		Study performed in two Maine State Shellfish Sanitation program laboratories which have quality assurance plans, have been evaluated and found to conform with the requirements of the NSSP for microbiological laboratories.
Submitters Signature **Mercuria Cumbo**	Date: **June 3, 2011**	
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.

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

Application from Maine State Department of Marine Resources Single Laboratory Validation Study in support of acceptance of an alternative method for determining the presence/absence of total coliforms in disinfected shellfish process water.

Section A. Justification for New Method

Name of the New Method:	Total Coliform Method for Shellfish Dealer Process Water using the membrane filtration technique with mEndo LES agar
Specify the Type of Method:	microbiological, membrane filtration
Name of Method Developer:	Mercuria Cumbo and Cathy L. Vining
Developer Contact Information	Maine Department of Marine Resources Lamoine Water Quality Lab 22 Coaling Station Rd. Lamoine, ME 04605 Mercuria.Cumbo@maine.gov Cathy.L.Vining@maine.gov
Date of Submission:	June 3, 2011

Introduction:

This single laboratory validation study was conducted at both of the Maine Department of Marine Resources (MEDMR) Water Quality Laboratories, the laboratories that support the MEDMR growing area classification program. The Laboratories are referred to as Lamoine and Boothbay in this report. The study was carried out in each laboratory separately using disinfected recirculating wet storage process water from five facilities who submit samples on a weekly basis to the MEDMR laboratories for testing. The Lamoine study analyzed three of the facilities and the Boothbay study analyzed two of the facilities. The study was conducted over a one year period and represents all seasons.

The results of the study indicate that the MF method using mEndo LES agar is a viable alternative procedure for the APHA MPN as a presence/absence test for total coliforms in disinfected shellfish process water.

Purpose and Intended Use of the Method:

Where disinfection is applied to process waters in wet storage and depuration facilities, the NSSP Program Guide for the Control of Molluscan Shellfish requires that the disinfection system produces process water with no detectable coliforms using an NSSP approved method. We are proposing the use of the Total coliform membrane filtration method using mEndo LES agar for the presence or absence of total coliforms in disinfected shellfish process water as an alternative to the APHA multiple tube fermentation MPN total coliform method.

Need for the New Method in the NSSP, Noting Any Relationships to Existing Methods:

Currently there is one NSSP approved method; APHA multiple tube fermentation MPN method for total coliforms. This method requires two media and up to five days to complete. On the contrary the membrane filtration (MF) method is read in 22 to 24 hours. This method is comparable to the MPN method but has the advantage of providing sample results more quickly. When there are problems with the disinfection or process water system causing the presence of coliforms, the quicker analysis turnaround would allow the operator to take action on a timelier basis. The MF method requires less media and generally is less labor intensive for the laboratory.

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

There are two limitations with membrane filtration methods. Turbidity in the samples can hinder filtration and the presence of high levels of non coliform bacteria can suppress the growth of coliforms. Neither of these limitations should be applicable to disinfected process water. Successful disinfection of process water requires that turbidity be eliminated. Shellfish process water is generally filtered before it goes to the disinfection process. The disinfection process should reduce the levels of all bacteria in the process water. Neither turbid process water samples nor non-coliform bacteria were encountered in any of the process waters analyzed for this validation study.

Other Comments:

This method has been in use for more than 40 years and is published in Standard Methods for the Examination of Water and Wastewater. It has been approved by EPA for use with potable water (for compliance with the US Drinking water program) and environmental waters including marine waters. The purpose of this validation study was to determine its comparability with the NSSP approved method for determining the presence/absence of total coliforms in disinfected shellfish process water. The membrane filtration technique as a microbiological tool for bacterial identification has a precedent in the NSSP with the MF method for fecal coliforms.

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

Section B. Method Documentation

Total Coliform Method in Shellfish Dealer Process Water using the Membrane Filtration Techniques with mEndo LES Agar

Method Scope.

This method is a standard widely used method with a long history. It first appeared in the Twelfth Edition of Standard Methods for the Examination of Water and Wastewater in 1965 for fresh water applications. Since this time its uses have widened. It is an EPA approved method for the Safe Drinking Water Program and EPA approved for analyzing environmental waters including marine waters for Total Coliforms. The FDA has adopted this method for testing bottled water as an indication of insanitation or possible contamination. Within the US Safe Drinking Water Program the total coliform standard is no detectable coliforms and generally uses a presence/absence reporting format. Disinfected shellfish process waters must meet this standard. The purpose of this validation study was to determine the method's applicability to determine the presence/absence of total coliforms in disinfected shellfish process water. Specifically, the study was conducted on five different recirculating wet storage facilities in Maine. Each facility has a different process water system providing filtration followed by UV disinfection.

References:

American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Water and Wastewater*, 20th Edition. APHA/AWWA/WEF, Washington, D.C. 9222B.

U.S. Environmental Protection Agency. 1978. *Microbiological Methods for Monitoring the Environment, Water and Wastes*. EPA/600/8/78/017. EPA, Washington, D.C. Part III Section B.

U.S. Food and Drug Administration (FDA).1995.*Bacteriological Analytical Manual*. U.S. FDA, 8th Edition, AOAC, Arlington,VA. Chapter 4 Section III.

Principle:

The membrane filtration method using mEndo LES agar provides a direct count of bacteria in processed water based on the development of colonies on the surface of the membrane filter. A quantity of water is filtered using a vacuum pump through the membrane which retains the bacteria. After filtration the membrane containing the bacterial cells is placed on mEndo agar, a selective and differential medium, incubated at 35°C for 24 hours. Following incubation, red colonies with or without metallic sheen are counted with the aid of a fluorescent lamp and stereo dissecting microscope. One volume of 100ml sample is used. Counting of colonies is not necessary; any presence of total coliforms is unacceptable.

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

Analytes/Measurands:

Total coliform

Proprietary Aspects:

None

Method:

Sample Collection and Preservation:

1. Process water shall be collected in a labeled sterile bottle or sample bag large enough to contain 110 ml of sample. Sample container must be filled to allow an air space for allow shaking of the sample.
2. Paperwork must accompany the sample which identifies the sample collector, sample location, date and time of collection.
3. Sample shall be placed in a cooler with ice or ice packs to maintain the cooler temperature between 0 and 10°C during transport.
4. Samples are placed in the refrigerator when received at the laboratory.
5. Samples are analyzed as soon as possible and not longer than 30 hours from time of collection.

Equipment:

Reagent grade water
Sterile 1 liter media bottle w/ magnetic stir bar
Top loading balance
Petri dishes, sterile, plastic, 15 x 60 mm w/ loose lids
Membrane filtration units (filter base and funnel), sterilized
Ultraviolet unit for sanitization of filter funnel between filtrations
Filter manifold or filtering funnel
Carboy (vacuum capable) or Erlenmeyer vacuum flask to collect filtered waste water
Vacuum pump
Nalgene Autoclavable Low Boy Carboy, 8 liter and dispensing tubing, or autoclavable squirt bottle
Incubator maintained at 35 ± 5°C
Refrigerator maintained at 0-4°C
Timer for timing UV sterilization
Membrane filters, sterile, white, grid marked, 47 mm diameter with 0.45 ± 0.02 µm pore size
Forceps, straight or curved, with smooth tips to handle filters without damage
Alcohol burner
95% ethanol, methanol or isopropanol in small wide-mouth container for flaming forceps
Hand tally or electronic counting device
Stereo dissecting microscope with a cool white fluorescent lamp
Inoculation loops, 10 ul, sterile, disposable

Micropipetter (20-200ul)
Sterile Micropipetter tips
Quality control bacteria
Escherichia coli culture 10^{-7} dilution for QC (EC) or *Enterobacter aerogenes* culture 10^{-7} dilution for QC (EA)
Staphylococcus aureus culture 10^{-2} dilution for QC (SA)

Media and Reagents:
mEndo Agar LES
95%ethanol (not denatured)
Phosphate Buffered Saline (PBS)

1. Determine how many plates will be needed for testing. Each sample requires 1 plate plus start, end and 3 bacteria QC plates.
2. Use commercially available mEndo LES agar. Agar is used at the rate of 51 grams of mEndo LES agar in 1 liter of reagent grade water containing 20 ml of 95% ethanol (not denatured). Weigh appropriate amount of agar for volume of agar needed.
3. To prepare the media, heat slowly while stirring; boil for 1 minute in a large flask or sterile 1 liter bottle with cap which will help ensure complete boil time without boiling over. Do not autoclave.
4. Pour 5 to 7 grams mEndo agar into 60 mm Petri dishes. Weigh plates and record. If any plates fall below 5 grams they must be discarded.
5. Maximum storage time for plates is two weeks - preferably plates should be prepared shortly before use. Store prepared plates in the refrigerator in the dark.
6. Prepare phosphate buffered saline either in carboy or squeeze bottle. Autoclave appropriate amount of time for the volume.

Procedure:

1. Check bulbs in the UV sterilizer to insure that lights are working. Use appropriate eye protection to view UV bulbs.
2. Place sterile filter base unit(s) on filtering flask or filter manifold.
3. Use sterile forceps to aseptically place a sterile membrane filter on the filter base, grid side up. Filter forceps are sterilized by dipping into the 95% alcohol and flaming in the alcohol burner.
4. Begin series with a blank QC plates first by adding 20-40 ml of sterile PBS to the funnel with filter. Start filtration. Rinse sides of funnel at least twice with 20-30 ml of sterile PBS.
5. Use sterile forceps to aseptically remove the membrane filter from the filter base and roll it onto the mEndo Agar to avoid the formation of bubbles between the membrane and the agar surface. Place the filter grid side up on the media. Reseat the membrane if bubbles occur. Close the dish, invert.
6. In between filtering place the funnel(s) and base(s) in the UV sterilization unit for 2 minutes.
7. Repeat the above process for samples.

8. Process water samples must be shaken 25 times in a 12" arc in 7 seconds and 100 ml quantity is filtered.
9. Start filtration and rinse funnel sides with 20 to 30 ml of PBS twice. When sample is completely through the filter, turn off the vacuum pump and remove filter with sterile forceps (alcohol dip and flamed), then placing onto the appropriately labeled mEndo plate.
10. Remember to sterilize forceps between use by dipping in alcohol and flaming.
11. Finish with bacteria QC controls by adding 20-40 ml of PBS to a funnel for each of the controls. Aseptically add 100 µl of 10^{-7} EC or EA solution to one funnel. This should give a count of 10-20 bacteria per plate. Inoculate a second funnel with 10 µl loopful of SA containing 20-40 ml PBS for a negative control. Rinse, filter, then place filters on labeled mEndo plates.
12. Incubate all plates at 35 ± 0.5°C for 22 - 24 hours.
13. Read plates under a stereo dissecting microscope. All red colonies with or without a metallic sheen are counted.
14. Any number of colonies shall be reported out as Positive for total coliforms in 100 ml of sample.

Quality Control:

Quality control measures are all those required in the NSSP microbiology laboratory checklist for the lab in general and specifically for the membrane filtration method. A membrane filtration procedure currently is approved and quality control requirements are established in the checklist.

Validation Data:

This method, membrane filtration using mEndo LES agar, is a standard method that has been employed for testing coliforms for more than 40 years. Performance criteria have previously been established for use for drinking water and environmental fresh and marine waters. The purpose of this study was to determine whether it would be acceptable as an alternative to the current NSSP approved method for determining the presence or absence of total coliforms in disinfected shellfish process water.

Two studies were conducted, one in each of the MEDMR Water Quality Laboratories. In this report the Laboratories are referred to as Lamoine and Boothbay. The data was analyzed for each laboratory separately.

Disinfected recirculated shellfish process water from five Maine facilities was used for the two studies; three facilities submit samples on a weekly basis for total coliform testing to Lamoine and two facilities submit samples to Boothbay. The process waters are normally absent of total coliforms, so it was necessary to spike the samples. For each round of testing an unspiked sample was tested by both methods. All unspiked samples were negative for total coliforms. The study was conducted over a period of a year, so all seasons are represented. To determine comparability testing was performed by both methods. For each round of testing, process water from one facility was spiked and analyzed. The samples were either spiked with *Escherichia*

coli, ATTC 11775 or *Enterobacter aerogenes,* ATTC 13048. Five to six aliquots of each process water sample were spiked at levels to provide determinate numbers of bacteria. Any dilutions that produced indeterminate results for either method was deleted from the computations. The spiked samples were analyzed in triplicate by both the APHA MPN method and MF method using mEndo LES agar. Ten rounds of testing were performed in Lamoine with 156 data points for each method. Boothbay completed 11 rounds with 150 data points for each method. One hundred (100) ml of sample was analyzed for each replicate. MPN were divided between 20 tubes with 5 ml per tube. The range for the MPN is <1 to >60 MPN/100ml. The range for the MF test is <1 to >80 CFU/100 ml.

Comparability :

For each laboratory the replicate data for the individual dilutions was averaged. The two methods were plotted against each other and a linear regression computed. All computations were performed on the log 10 transformation. The result is provided in the Figure 1 and 2. All of the data is presented in Table 1 and 2.

The linear regression line for the Lamoine method comparison is $y = 0.9687x - 0.0195$. Since we are looking at a presence/absence condition, the area of concern when comparing the method is 0. The linear regression was computed with the MPN as the x value and MF as the y value. The y intercept of the equation for the Lamoine data is -0.0195, statistically less than 0. The linear regression for Boothbay is $y = 0.97x + 0.027$. The y intercept of the Boothbay data is 0.027, essentially 0.

Figure 1 Lamoine Method Comparison

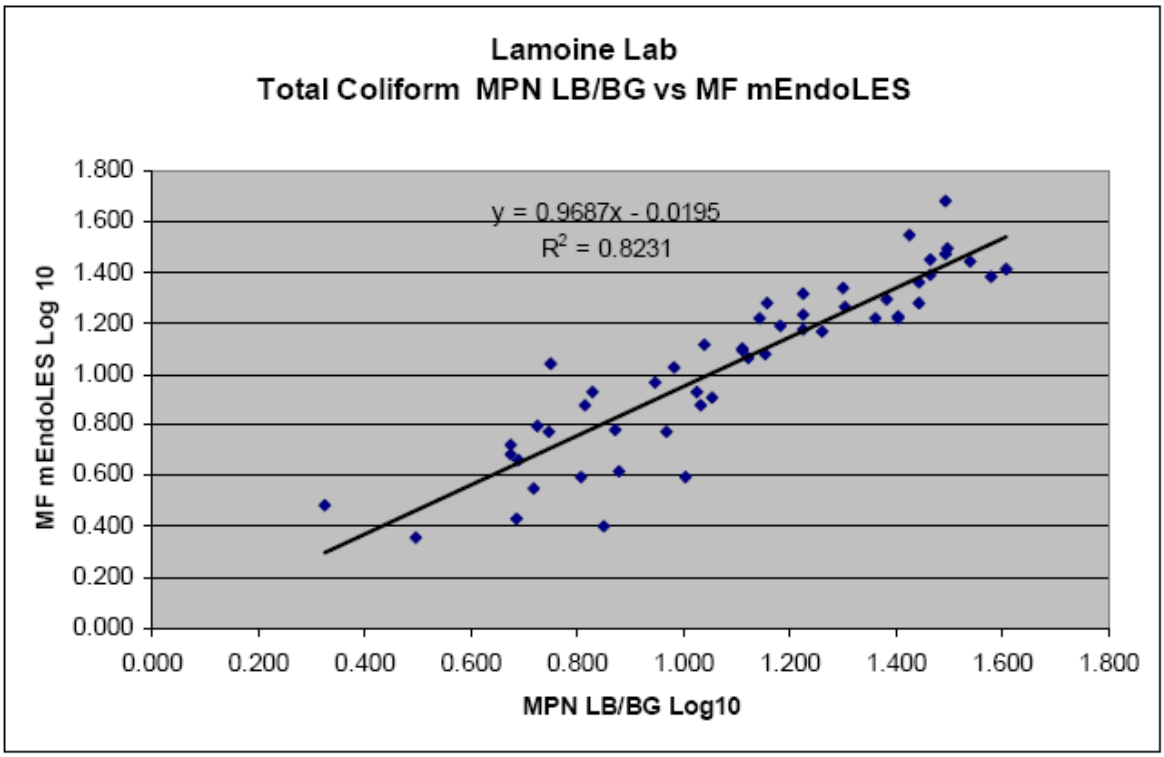
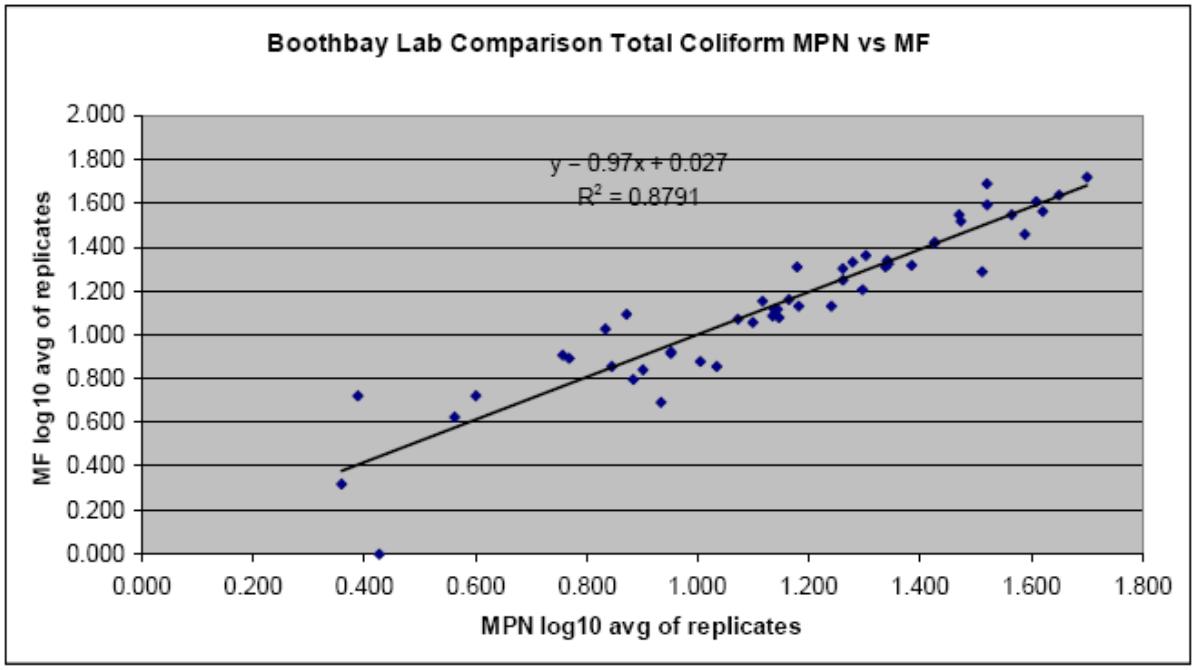


Figure 2. Boothbay Method Comparison



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

Table 1 Lamoine method comparison data

DMR Water Quality Laboratory											
Lamoine, ME											
MF mENDO vs. MPN LB/BG Process Water Comparison											
				MPN LB/BG				MF mEndo LES			
Spiking Bacteria	Dealer	Date	Sample	Rep1	Rep2	Rep3	Geomean	Rep1	Rep2	Rep3	Geomean
Enterobacter aerogenes	TBR	9/21/2010	1	21	38	38	31	52	47	46	48
	TBR		2	21	38	38	31	24	28	39	30
	TBR		3	10	21	14	14	17	21	19	19
	TBR		4	12	16	12	13	12	13	10	12
	TBR		5	4.5	12	3.3	6	15	11	8	11
	TBR		6	8.6	8.6	5.8	8	9	4	2	4
	RDR	10/26/2010	1	28	32	24	28	25	25	19	23
	RDR		2	28	21	14	20	18	22	16	19
	RDR		3	21	8.6	12	13	13	11	13	12
	RDR		4	8.6	8.6	14	10	6	2	5	4
	RDR		5	2.1	5.8	8.6	5	3	7	7	5
	RDR		6	4.5	2.1	1	2	2	7	2	3
	MER	11/2/2010	1	28	28	18	24	24	19	17	20
	MER		2	21	24	12	18	16	13	15	15
	MER		3	12	10	10	11	10	12	5	8
	MER		4	5.8	7.1	10	7	7	4	8	6
	MER		5	10	4.5	5.8	6	4	5	3	4
	RDR	11/30/2010	2	60	32	28	38	31	23	20	24
	RDR		3	24	28	18	23	14	17	19	17
	RDR		4	14	21	12	15	18	17	12	15
	RDR		5	14	8.6	12	11	8	6	11	8
	RDR		6	5.8	4.5	4.5	5	4	3	8	5
	MER	12/7/2010	1	46	28	24	31	33	32	29	31
	MER		2	24	32	32	29	21	25	28	24
	MER		3	12	16	14	14	12	22	17	16
	MER		4	8.6	12	8.6	10	12	9	11	11
	MER		5	5.8	5.8	4.5	5	8	5	6	6
	MER		6	8.6	7.1	5.8	7	2	4	2	3
Escherichia coli	RDR	1/18/2011	4	38	38	46	40	23	24	32	26
	RDR		5	32	28	18	25	18	17	15	17
	RDR		6	14	24	14	17	14	21	17	17
	RDR	2/2/2011	2	21	28	32	27	34	34	38	35
	RDR		3	14	14	24	17	24	14	27	21
	RDR		4	12	10	18	13	14	18	8	13
	RDR		5	8.6	5.8	14	9	11	9	8	9
	RDR		6	7.1	8.6	4.5	7	6	8	9	8
	MER	2/8/2011	1	28	32	46	35	33	25	26	28
	MER		2	21	18	21	20	24	26	17	22

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

DMR Water Quality Laboratory

Lamoine, ME

MF mENDO vs. MPN LB/BG Process Water Comparison

				MPN LB/BG				MF mEndo LES			
Spiking Bacteria	Dealer	Date	Sample	Rep1	Rep2	Rep3	Geomean	Rep1	Rep2	Rep3	Geomean
	MER		3	21	14	16	17	14	17	14	15
	MER		4	16	7.1	7.1	9	7	5	6	6
	MER		5	3.3	4.5	7.1	5	4	7	4	5
	MER		6	4.5	2.1	12	5	5	1	4	3
	RDR		2	32	24	32	29	21	34	32	28
	RDR		3	28	18	32	25	16	14	22	17
	RDR	3/8/2011	4	16	5.8	14	11	20	8	14	13
	RDR		5	12.0	12.0	8.6	11	8	6	9	8
	RDR		6	4.5	4.5	9	6	7	6	5	6
	MER		2	28	32	24	28	18	19	20	19
	MER		3	14	24	8.6	14	14	14	9	12
	MER	4/12/2011	4	12	12.0	2.1	7	10	5	12	8
	MER		5	4.5	4.5	7.1	5	3	5	3	4
	MER		6	3.3	2.1	4.5	3	3	2	2	2

Table 2 Boothbay method comparison data

DMR Boothbay Water Quality Lab

MF mEndo LES vs. MPN LB/BG Process Water Comparison

				MPN LB/BG				MF mEndo LES counts			
Spiking Bacteria	Dealer	Date	Sample	Rep1	Rep2	Rep3	Geomean	Rep 1	Rep 2	Rep 3	Geomean
Escherichia coli	CHS		1	60	46	46	50.3	52	54	51	52.3
	CHS		2	24	14	32	22.1	20	24	19	20.9
	CHS	7/6/2010	3	14.01	14	13.99	14.0	14	12	10	11.9
	CHS		4	7.1	5.8	4.5	5.7	11	6	8	8.1
	CHS		1	38	38	46	40.5	36	44	42	40.5
	CHS		2	28	32	38	32.4	14	22	23	19.2
	CHS	7/19/2010	3	10	4.5	7.1	6.8	12	11	9	10.6
	CHS		4	3.3	3.3	5.8	4.0	6	2	12	5.2
	CHS		5	1	5.8	3.3	2.7	1.01	1	1	1.0
	SMF		1	38	46	21	33.2	54	49	44	48.8
	SMF	8/30/2010	2	24	24	18	21.8	26	20	16	20.3
	SMF		3	9	10	14	10.8	9	10	4	7.1
	SMF		4	2.1	3.3	7.1	3.7	5	3	5	4.2
	CHS	9/13/2010	1	28	38	46	36.6	32	46	30	35.3
	CHS		2	18	16	24	19.0	23	24	18	21.5

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DMR Boothbay Water Quality Lab

MF mEndo LES vs. MPN LB/BG Process Water Comparison

				MPN LB/BG				MF mEndo LES counts			
Spiking Bacteria	Dealer	Date	Sample	Rep1	Rep2	Rep3	Geomean	Rep 1	Rep 2	Rep 3	Geomean
	CHS		3	16	12	16	14.5	20	11	14	14.5
	CHS		4	10	14	14	12.5	10	12	12	11.3
	CHS		5	4.5	10	10	7.7	6	10	4	6.2
	SMF		1	24	24	46	29.8	31	36	32	32.9
	SMF		2	21	32	21	24.2	22	14	29	20.7
	SMF	9/27/2010	3	10	12	21	13.6	16	14	8	12.1
	SMF		4	5.8	10	7.1	7.4	12	12	13	12.3
	SMF		5	10	4.5	4.5	5.9	12	5	8	7.8
	CHS		1	28	46	28	33.0	43	33	41	38.7
	CHS		2	18	28	21	22.0	29	15	24	21.9
	CHS	1/10/2011	3	18	14	14	15.2	14	12	15	13.6
	CHS		4	16	14	10	13.1	16	13	14	14.3
	CHS		5	7.1	7.1	10	8.0	12	4	7	7.0
	CHS		1	60	32	46	44.5	43	50	38	43.4
	CHS		2	24	28	28	26.6	30	27	23	26.5
	CHS	1/31/2011	3	24	18	18	19.8	21	11	18	16.1
	CHS		4	14	12	16	13.9	13	21	8	13.0
	CHS		5	12	7.1	12	10.1	5	12	7	7.5
Enterobacter Aerogenes	SMF		1	38	24	28	29.4	35	39	32	35.2
	SMF		2	16	21	18	18.2	25	19	17	20.1
	SMF	8/2/10	3	10	7.1	10	8.9	12	7	7	8.4
	SMF		4	2.1	3.3	2.1	2.4	6	8	3	5.2
	SMF		5	3	4	1	2.3	3	1	3	2.1
	CHS		2	32	18	14	20.1	21	21	28	23.1
	CHS	8/16/2010	3	10	10	7.1	8.9	6	9	10	8.1
	CHS		4	12	3.3	8.6	7.0	4	9	10	7.1
	SMF		2	38	60	32	41.8	38	34	38	36.6
	SMF	10/26/2010	3	28	24	28	26.6	29	29	22	26.4
	SMF		4	16	18	12	15.1	20	19	22	20.3
	SMF		5	12	12	18	13.7	13	14	12	13.0
	CHS	11/29/2010	1	60	21	46	38.7	18	33	39	28.5
	CHS		2	18	21	16	18.2	15	17	22	17.8
	CHS		3	21	18	14	17.4	12	19	11	13.6
	CHS		4	8.6	16	12	11.8	17	7	14	11.9

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

DMR Boothbay Water Quality Lab											
MF mEndo LES vs. MPN LB/BG Process Water Comparison											
				MPN LB/BG				MF mEndo LES counts			
Spiking Bacteria	Dealer	Date	Sample	Rep1	Rep2	Rep3	Geomean	Rep 1	Rep 2	Rep 3	Geomean
	CHS		5	8.6	8.59	8.61	8.6	8	3	5	4.9

Summary of Results:

The membrane filtration method using mEndo LES agar for total coliform is a published standard method in use for over forty years. It is approved for use with drinking water (potable) and environmental water, fresh and marine. The performance criteria for this test has been previously established, therefore there was no need to complete performance criteria for this single laboratory validation study. The purpose of the study was to determine its comparability with the NSSP approved method.

As a presence/absence test the area of concern is zero (0). From the linear regression when the MPN is 0, at Lamoine the MF method is less than 0 (statistically) and at Boothbay it is 0.027, essentially 0.

This study indicates that the MF method is a viable alternative procedure for the APHA MPN as a presence/absence test for total coliforms in disinfected shellfish process water.

Proposal for Consideration at the Interstate Shellfish Sanitation Conference 2011 Biennial Meeting	☒ **Growing Area** ☐ **Harvesting/Handling/Distribution** ☐ **Administrative**
Name of Submitter:	Mississippi Department of Marine Resources
Affiliation:	Mississippi State Government
Address:	1141 Bayview Avenue Biloxi MS 39530
Phone: **Fax:** **Email:**	228-374-5000 228-374-5220 dale.diaz@dmr.ms.gov
Proposal Subject:	Addition to the Requirements for the Authority during a suspected shellfish related outbreak
Specific NSSP Guide Reference:	2009 NSSP Section II Model Ordinance Chapter II @.01 Outbreaks of Shellfish-Related Illness Paragraph E, Section (1).
Text of Proposal/ Requested Action	E. When the investigation outlined in §.02B. cannot be completed within 24 hours, the Authority shall: (1) Follow the closure procedures. ~~outlined in § .01C; and if the investigation does not indicate a growing area problem, the area shall be immediately reopened and product recall terminated.~~
Public Health Significance:	N/A
Cost Information (if available):	Not available.
Research Needs:	There is a need of one type of post harvest processing technology that could be used as a determining factor that when applied as a process to recondition a batch of recalled oysters whether shucked, shellstock and post harvest processed oysters, all the rest of the oyster related pathogens causing foodborne illnesses are deemed clean. It is patterned after the analysis of water using fecal coliform as an identifier of the presence of pathogens in the water. In any oyster recall, dealers and processors often experience financially devastating product recalls and experience the loss of their investments on the product. The number of oyster dealers had decreased over the years for various reasons. Those remaining are finding it difficult to cope without alternatives to destruction of product.
Estimated Cost:	Not available at this time.
Proposed Source of Funding/Support:	Not available at this time.
Time Frame Anticipated:	Not available at this time.

Proposal for Consideration at the Interstate Shellfish Sanitation Conference 2011 Biennial Meeting	☒ **Growing Area** ☐ **Harvesting/Handling/Distribution** ☐ **Administrative**
Name of Submitter:	Mississippi Department of Marine Resources
Affiliation:	Mississippi State Government
Address:	1141 Bayview Avenue Biloxi MS 39530
Phone: **Fax:** **Email:**	228-374-5000 228-374-5220 dale.diaz@dmr.ms.gov
Proposal Subject:	Addition to the Requirements for the Authority during a suspected shellfish related outbreak
Specific NSSP Guide Reference:	2009 NSSP Section II Model Ordinance Chapter II @.01 Outbreaks of Shellfish-Related Illness Paragraph C.
Text of Proposal/ Requested Action	C. When the investigation outlined in §.02B. ~~does not~~ indicates a growing area problem: ~~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.
Public Health Significance:	The basis for this addition is to allow the Authority time to determine if suspected shellfish related outbreak is due to growing area problems or problems associated with the location where the shellfish were served. It would be expected that if the suspected outbreak were growing area related, illnesses would be seen at more than one location. It is difficult to determine the actual cause within 24 hours when faced with illness reported from a single location.
Cost Information (if available):	None
Research Needs:	There is a need of one type of post harvest processing technology that could be used as a determining factor that when applied as a process to recondition a batch of recalled oysters whether shucked, shellstock and post harvest processed oysters, all the rest of the oyster related pathogens causing foodborne illnesses are deemed clean. It is patterned after the analysis of water using fecal coliform as an identifier of the presence of pathogens in the water. In any oyster recall, dealers and processors often experience financially devastating product recalls and experience the loss of their investments on the product. The number of oyster dealers had decreased over the years for various reasons. Those remaining are finding it difficult to cope without alternatives to destruction of product.

Estimated Cost:	Not available at this time.
Proposed Source of Funding/Support:	Not available at this time.
Time Frame Anticipated:	Not available at this time.

Proposal for Consideration at the Interstate Shellfish Sanitation Conference 2011 Biennial Meeting	☒ **Growing Area** ☐ **Harvesting/Handling/Distribution** ☐ **Administrative**
Name of Submitter:	Mississippi Department of Marine Resources
Affiliation:	Mississippi State Government
Address:	1141 Bayview Avenue Biloxi MS 39530
Phone: **Fax:** **Email:**	228-374-5000 228-374-5220 dale.diaz@dmr.ms.gov
Proposal Subject:	Addition of the Requirements for the Authority During a Suspected Oyster Related Outbreak of *Norovirus*
Specific NSSP Guide Reference:	Section II Model Ordinance Chapter II. Risk Assessment and Risk Management @.01 Outbreaks of Shellfish Related Illness
Key Words:	*Norovirus*
Text of Proposal/ Requested Action:	@.01 Outbreaks of Shellfish-Related Illness. 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 for 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* 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* cause 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 (N*orovirus)* (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):	

Proposal for Consideration at the Interstate Shellfish Sanitation Conference 2011 Biennial Meeting	☒ **Growing Area** ☐ **Harvesting/Handling/Distribution** ☐ **Administrative**
Name of Submitter:	Mississippi Department of Marine Resources
Affiliation:	Mississippi State Government
Address:	1141 Bayview Avenue Biloxi MS 39530
Phone: **Fax:** **Email:**	228-374-5000 228-374-5220 dale.diaz@dmr.ms.gov
Proposal Subject:	Addition to the Requirements for the Authority During a Suspected Shellfish Related Outbreak
Specific NSSP Guide Reference:	2009 NSSP Section II Model Ordinance Chapter II @.01 Outbreaks of Shellfish-Related Illness J.
Key Words:	Reconditioning
Text of Proposal/ Requested Action:	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 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 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. J. Whenever the Molluscan shellfish products are deemed to be contaminated with a pathogen that would subject it to a recall, reconditioning of the product will be permitted as an alternative to control the hazard. Any such reconditioning process that is used must be validated to reduce the level of the pathogen in question to a level which is not reasonably likely to cause illness or alter the product to a form that is intended to be cooked. ~~I~~K. The Authority shall assess annually Vibrio parahaemolyticus illnesses associated with the consumption of molluscan shellfish. The assessment will include a record of all V. parahaemolyticus shellfish-associated illnesses reported within the state and from receiving states, the numbers of illnesses per event, and actions taken by the Authority in response to the illnesses.
Public Health Significance:	
Cost Information (if available):	

Proposal for Consideration at the Interstate Shellfish Sanitation Conference 2011 Biennial Meeting	☒	**Growing Area**
	☐	**Harvesting/Handling/Distribution**
	☐	**Administrative**

Name of Submitter:	Debbie Rouse John M. Hickey Eric M. Hickey John Mullen Joseph Migliore Darcie Couture US Food and Drug Administration (FDA)
Affiliation:	Debbie Rouse-Delaware Department of Natural Resources John M. Hickey-Massachusetts Department of Marine Fisheries Eric M. Hickey-Massachusetts Deparment of Public Health John Mullen-Rhode Island Department of Health Joseph Migliore-Rhode Island Department of Environmental Management Darcie Couture-Maine Department of Marine Resources US Food and Drug Administration (FDA)
Address:	See SSCA Contacts on ISSC Website: http://www.issc.org/Contacts/Default.aspx
Phone: **Fax:** **Email:**	See SSCA Contacts on ISSC Website: http://www.issc.org/Contacts/Default.aspx See SSCA Contacts on ISSC Website: http://www.issc.org/Contacts/Default.aspx See SSCA Contacts on ISSC Website: http://www.issc.org/Contacts/Default.aspx
Proposal Subject:	Control of Marine Biotoxins
Specific NSSP Guide Reference:	Section II Model Ordinance Chapter IV. Shellstock Growing Areas @. 04. Marine Biotoxin Control D. Controlled Harvest From Closed Federal Waters Section IV Guidance Documents Chapter II. Growing Areas .03 Example of Protocol for Onboard Screening and Dockside Testing for PSP in Closed Federal Waters
Key Words:	PSP; Federal Waters; Onboard Screening; Dockside Samples
Text of Proposal/ Requested Action:	Chapter IV Shellfish Growing Areas @.04 Marine Biotoxin Control. Insert new item A. (5) (5) Prior to allowing the landing of shellfish harvested from waters closed due to periodic toxic algal blooms associated with PSP, and where routine monitoring of saxitoxin levels is not conducted, the State Authority in the landing State in cooperation with appropriate Federal agencies shall develop agreements or memorandums of understanding between the Authority and individual shellfish harvesters or individual shellfish dealers. The agreements or memorandums of understanding shall provide strict safety assurances. At a minimum agreements or memorandums of understanding shall include provisions for: (a) harvest permit requirements. (b) training for individuals conducting onboard toxicity screening using NSSP methods. (c) vessel monitoring; (d) identification of shellfish for each harvesting trip to include: (i) Vessel name and owner (ii) Captain's name (iii) Person conducting onboard screening tests

(iv) Port of departure name and date
(v) Port of landing name and date
(vi) Latitude and longitude coordinates of designated harvest area
(vii) Onboard screening test results
(viii)Volume and species of shellfish harvested
(ix) Intended processing facility name, address and certification number
(x) Captain's signature and date

(e) Pre-harvested sampling that includes a minimum of five (5) samples from the intended harvest area be tested for saxitoxins. Harvesting shall not be permitted if any of the pre-harvested samples contain saxitoxin levels in excess of 44ug/l00g.
(f) Submittal of onboard screening homogenates and test results to the authority in the state of landing.
(g) The collection and saxitoxin level testing of a minimum of seven (7) dockside samples. The SSCA may require more samples based on the size of the vessel and the volume of shellfish harvested.
(h) Holding and providing separation until dockside samples verifiy that saxitoxin levels are below 80ug/100g.
(i) Disposal of shellfish should dockside test results exceed 80ug /100g.
(j) Notification prior to unloading.
(k) Unloading schedule.
(l) Access for Dockside Sampling.
(m) Record Keeping.
(n) Early Warning/Alert System

NOTE: The plan may include other requirements, as deemed necessary by the authority in the state of landing, to ensure adequate public health protection under the NSSP.

Insert new Additional Guidance reference at Model Ordinance Chapter IV@ .04. A. (5) as follows:

Additional Guidance – Section IV Guidance Documents Chapter II. Growing Areas .03 Protocol for the Landing of Shellfish from Federally Closed Waters due to PSP

Add new guidance to Section IV. Guidance Documents, Chapter II. Growing Areas .03 and re-number Section IV. Guidance Documents, Chapter II. Growing Areas .03 through .15 as .04 through .16.

Protocol for the Landing of Shellfish from Federally Closed Waters due to PSP

When the harvest of molluscan shellfish is closed in Federal Waters due to Paralytic Shellfish Poison (PSP), exceptions to the prohibitions may be authorized provided the Authority in the State of landing in cooperation with appropriate Federal agencies shall develop agreements or memorandums of understanding between the Authority and individual shellfish harvesters or individual shellfish dealers. This guidance provides descriptions of the specific information to be included in the protocol.

A. Harvest Permit Requirements

The Authority in the landing state will only allow the landing of shellfish from

federal waters closed due to PSP from vessels in possession of an appropriate Exempted Fishing Permit (EFP) issued by the National Marine Fisheries Service (NMFS). The NMFS shall receive concurrence from the SSCA in the State of landing.

B. Training

The Authority shall ensure that all shipboard persons conducting onboard sampling have been trained by a National Shellfish Sanitation Program (NSSP) Laboratory Evaluation Officer (LEO) or a US Food and Drug Administration (FDA) marine biotoxin expert to conduct onboard PSP screening using a NSSP recognized method(s).

C. Vessel Monitoring

The Authority shall ensure that the harvesting location(s) of each landing vessel has been appropriately monitored. This requirement may be met by the vessel participating in the Federal Vessel Monitoring System (VMS).

D. Identification of Shellfish

Prior to landing each vessel shall provide the Authority with a record identifying each lot of shellfish as follows: For each harvesting trip the Captain or Mate shall record the following information on a "Harvest Record." Electronic logging of this information may be permitted provided it is made available to the authorized individual at dockside.

1. Vessel name and Federal Fishing Permit number
2. Name and telephone number of the vessel Captain and vessel owner
3. Date(s) of harvest
4. Number of lots and volume of catch per lot or number of containers per lot
5. Location(s) of harvest (GPS coordinates or latitude/longitude coordinates in degrees:minutes:seconds)
6. Identification of each harvest lot, including cage tag numbers for surfclams and ocean quahogs, and container numbers or identification codes for other shellfish species.
7. Location (GPS coordinates or latitude/longitude coordinates in degrees:minutes:seconds) of each PSP screening sample
8. Results of each PSP screening test. Screening test kits for each sample shall be submitted to the authorized authority along with the "Harvest Record" as stated in Section D.
9. Destination(s) and purchaser(s) of each lot and amount of each lot to each destination

The Captain or Mate shall sign the "Harvest Record." The "Harvest Record" shall be checked by the individual authorized to sample the harvested shellfish. Failure to provide complete and accurate information will result in revocation or suspension of the NMFS EFP and rejection of the entire lot(s) of harvested shellfish. Four (4) copies of the "Harvest Record" shall be prepared. One (1) copy shall remain with the vessel, one (1) copy shall be provided to the SSCA in the state of landing, one (1) copy shall accompany the catch to the

processing firm(s), and one (1) copy shall be retained by the laboratory authorized to conduct lot sample analyses.

CONTAINER LABELING:

Each container of shellfish shall be clearly labeled with the following NSSP required information at the time of harvest:

1. For surfclams and ocean quahogs existing NMFS tagging requirements
2. For all other molluscan shellfish (including Stimpson clams also known as Arctic surfclams) using Tyvek tags:
   a. Vessel name
   b. Type and quantity of shellfish
   c. Date of harvest
   d. Harvest lot area defined by GPS coordinates or latitude/longitude coordinates in degrees:minutes:seconds

E. Pre-Harvest Sampling

Prior to commercial harvesting of molluscan shellfish, a minimum of five (5) screening samples shall be collected within each area of intended harvest (lot area) and tested for PSP toxins in accordance with a NSSP recognized screening method. Each screening sample shall be collected during a separate and distinct gear tow. Screening sample tows shall be conducted in a manner that evenly distributes the five (5) samples throughout the intended harvest area for each area of intended harvest (see Section H.). Only shipboard officials trained in the use of the designated NSSP screening method may conduct these tests. Each of the five (5) samples must test negative for PSP toxins. A positive result from any one (1) sample shall render the "lot area" unacceptable for harvest. The harvest vessel captain shall immediately report all positive screening test results, by telephone, to the SSCA within the intended state of landing and the NMFS. The Captain should also notify other permitted harvest vessels of the positive screening test and advise them to avoid the questionable area. For each screening test, positive and negative, the remaining sample material (homogenate) shall be maintained under refrigeration. Test kits, positive and negative, shall accompany the remaining sample homogenates to the certified laboratory. Confirmatory testing shall be performed on homogenate from each positive screening test using a NSSP recognized test method. Upon request by the SSCA in the state of landing, confirmatory testing of homogenate from negative screening tests shall be conducted using a NSSP recognized test method.

Each screening sample shall be comprised of at least twelve (12) whole animals with the exception of mussels and "whole" or "roe-on" scallops. For mussels each sample shall be comprised of thirty (30) animals. For "whole" scallops each sample shall be comprised of twenty (20) scallop viscera and gonads. For "roe-on" scallops each sample shall be comprised of twenty (20) scallop gonads.

F. Submittal of Onboard Screening Homogenates and Test Results

All screening results shall be recorded on the "Harvest Record" as stipulated in Section D. of this Protocol. Test kits used to screen each lot shall accompany

the "Harvest Record". Upon landing of the harvest vessel, the "Harvest Record" and accompanying test kits shall be provided to the individual (state shellfish official, FDA official, NMFS official) authorized to sample the harvested shellfish as described in Section G. of this Protocol.

G. Dockside Sampling

After dockside samples are collected, molluscan shellfish may be processed while awaiting PSP analytical results. Each lot must be identified and segregated during storage while awaiting dockside sample test results. Under no circumstances will product be released from the processor prior to receiving satisfactory paralytic shellfish toxin test results.

The dockside sampling protocol for molluscan shellfish shall be as follows:

1. For each lot of molluscan shellfish, a minimum of seven (7) composite samples, each comprised of at least twelve (12) whole animals, shall be taken at random by the individual authorized to sample, with the following exceptions:
   a. For each lot of mussels, a minimum of seven (7) composite samples, each comprised of at least thirty (30) whole animals, shall be taken at random by the individual authorized to sample.
   b. For each lot of "whole" scallops, a minimum of seven (7) composite samples, each comprised of twenty (20) scallop viscera and gonads, shall be taken at random by the individual authorized to sample.
   c. For each lot of "roe-on" scallops, a minimum of seven (7) composite samples, each comprised of twenty (20) scallop gonads, shall be taken at random by the individual authorized to sample.
2. Shellfish samples collected in accordance with G.1 shall be tested for the presence of paralytic shellfish toxins using NSSP recognized methods.
3. Laboratory test results for each lot of shellfish shall be forwarded to the SSCA in the state in which the shellfish is being held prior to the product being released by the SSCA.

H. Holding and Lot Separation

A harvest lot is defined as all molluscan shellfish harvested during a single period of uninterrupted harvest activity within a geographic area not to exceed three (3) square miles. Once harvesting has ceased and the harvest vessel moves to another location, regardless of the distance, a new harvest lot will be established. Any harvest vessel containing more than one lot shall clearly mark and segregate each lot while at sea, during off loading, and during transportation to a processing facility. Prior to harvesting in Federal waters, each harvest vessel shall submit to the NMFS a written onboard lot segregation plan. The SSCA in the intended state of landing and the FDA Regional Shellfish Specialist must approve the proposed lot segregation plan.

I. Disposal of Shellfish

If test results of any one (1) of the seven (7) samples collected in accordance with G.1 equal or exceed 80ug of paralytic shellfish toxins/100g of shellfish

tissue (n=7, c=0), the entire lot must be discarded or destroyed at the cost of the harvester under the supervision of the SSCA in accordance with state laws and regulations except when:

A lot of "whole" or "roe-on" scallops equals or exceeds 80ug paralytic shellfish toxins/100g of tissue, the adductor muscle may be shucked from the viscera and/or gonad and marketed. The remaining materials (viscera and/or gonad) must be discarded or destroyed under supervision of the SSCA in accordance with state laws and regulations.

Confirmatory PSP analyses shall be according to NSSP recognized methods and shall be conducted by laboratories certified in accordance with NSSP guidelines. Private laboratories may be used if certified by a Federal or state shellfish Laboratory Evaluation Officer (LEO) in accordance with NSSP guidelines.

J. Notification Prior to Unloading

Prior to the issuance of an EFP, the harvester shall be responsible for notifying the SSCA in the state of landing and in a manner approved by the SSCA that molluscan shellfish is being harvested for delivery to the intended receiving processor.

Each vessel shall give at least twelve (12) hours notice to the individual authorized to sample prior to unloading shellfish. Notice of less than twelve (12) hours may be approved by the authorized individual at his/her discretion. SSCAs may approve industry sampling and sample transport to the NSSP certified testing laboratory in accordance with the practices and procedures used by the SSCA under the NSSP. Such procedures may be approved by the SSCA only when sample collection and sample transport training is provided by the SSCA.

Shellfish from a federally closed harvest area must be kept separate and not sold until so authorized by the SSCA.

Failure to comply with the provisions of this Protocol will result in the suspension or revocation of the vessel's EFP.

K. Unloading Schedule

Unloading shall take place between 7:00 A.M. and 5:00 P.M. Monday through Friday, unless otherwise mutually agreed upon by the individual authorized to sample, the processing plant manager, the harvest vessel captain, and the SSCA in the state of landing, sample testing, and processing.

L. Access for Dockside Sampling

Individuals authorized to sample shall be provided access to the catch of shellfish.

M. Record Keeping

	Record keeping requirements shall be as follows: 1. The vessel shall maintain Harvest Records for at least one (1) year. 2. The processor(s) shall maintain Harvest Records for at least one (1) year or two (2) years if the product is frozen. 3. The SSCA in the State of landing shall retain Harvest Records for at least two (2) years. N. Early Warning/Alert System PSP sample data acquired as a result of onboard screening and dockside testing shall be transmitted to a central data register to be maintained by the FDA. These data, both screening and confirmatory, shall be transmitted to the FDA by the NSSP certified laboratory conducting PSP analyses of the sampled lot(s) within one week of the completion of the PSP analyses. The data provided shall include the following: 1. shellfish species 2. harvest location name and coordinates (GPS or latitude/longitude) 3. harvest date 4. onboard screening test method, date, and results 5. laboratory test date and test results Results of all samples having acceptable levels of paralytic shellfish toxins (<80ug/100g) shall immediately be reported to the SSCA in the state of landing. If the results of any one (1) sample equal or exceed 80ug/100g the testing laboratory shall immediately notify the FDA Regional Shellfish Specialist, the SSCA, and the processor by telephone. The FDA shall notify the NMFS. The NMFS shall notify permitted harvesters to advise them to cease fishing in the affected area(s). NOTE: Due to the resources necessary to meet the requirements of this Protocol, State Shellfish Control Authorities (SSCAs) may find it necessary to require industry to fund associated costs. These costs may include sample collection, screening, transportation, analysis, inspection, enforcement, and other related expenses.
Public Health Significance:	The surf clam and ocean quahog fishery is one of the largest shellfish fisheries in the U.S. producing up to 130 million pounds of meats per year, generating about $75 million ex-vessel per year. Atlantic surf clams and ocean quahogs are found in the North Atlantic from North Carolina to the Gulf of St. Lawrence. The surf clam and ocean quahog fisheries in the U.S. are managed by the National Marine Fisheries Services (NMFS) in accordance with a management plan prepared by the Mid Atlantic Fishery Management Council under an individual transferable quota system implemented in 1990. The management plan includes requirements for trip announcements, landings time andport, and each vessel is equipped with a Vessel Monitoring System (VMS). The VMS allows the regulators to identified, tracked locations of harvest within 100 feet and steaming speed, for every clam vessel authorized to operate in federal waters. Allocations are issued to quota holders each year in the form specifically identified

	tags that must be attached to containers of surf clams or ocean quahogs. Ownership of the tags and harvest activities are closely monitored by NOAA Fisheries. Surf clams and ocean quahogs are processed for use in strips, soups, chowders, and sauces.Although surf clams and ocean quahogs are not consumed raw they are shipped alive in interstate commerce and are subject to NSSP regulation. Thirteen processing facilities are located in six states: MA, RI, NJ, DE, MD, and VA. A fleet of approximately 40 vessels land their catch in five states; MA, RI, NJ, MD, and NY. Because the U.S. FDA does not have the resources necessaryt to routinely monitor the Northwest Atlantic Ocean where Alexandrium blooms responsible for PSP have historically occurred, waters of the Northwestern Atlantic west of 69.00° W Longitude have been closed since 1990. In 2005 federal waters east of 69.00° W Longitude and north of 40.00° N Latitude were also closed in response to an unprecedented toxic algal bloom (PSP) that occurred throughout the Northewest Atlantic Ocean, affecting state and federal waters. Much of this area remains closed today to the harvest of all molluscan shellfish, all of the area remains closed to the harvest of whole and roe-on scallops. These areas combined represent approximately 50% of the total surf and ocean quahog resource along the Atlantic coast. The result has been increased pressure on the remaining resource and economic loss to the fishery and its affiliated land based components. Beginning in 2008, a pilot program was initiated to evaluate the Onboard Screening and Dockside Testing Protocol (Protocol), outlined in this ISSC Proposal and developed by FDA, NMFS, EPA, North and Mid Atlantic State shellfish authorities, and representatives of the Atlantic Fishery Management Council. The purpose of the pilot, which was given ISSC Executive Board concurrence, was to test the effectiveness of the Protocol for ensuring the safe harvest of shellfish harvested from Federal waters closed because of the historical occurrence of significant PSP episodes. Harvesting was conducted under an Experimental Fishing Permit issued to a single vessel by NMFS. Four States participated in the Pilot including NJ, DE, RI, and MA. Under the Pilot, shellfish are tested at sea to ensure that harvest levels do not exceed 44ug PSP/100g meat. Once landed the shellfish is again tested using the traditional Mouse Bioassay (MBA) and only permitted to leave the processing facility for entry into the commercial market when all samples have demonstrated PSP levels compliant with NSSP requirements. To date there have been over 70 sucessful harvest trips to offshore Federal waters on Georges Bank, accounting for the safe landing of approximately 330,000 bushels of clams. The Pilot has demonstrated the efficacy of the Protocol in all regards. Adoption of this Proposal by the ISSC will pave the way for additional vessels, operating under NMFS permit in accordance with Protocol requirements, to safely harvest from offshore Federal waters closed as a result of histoicall episodes of toxic PSP blooms.
Cost Information (if available):	

Proposal for Consideration at the Interstate Shellfish Sanitation Conference 2011 Biennial Meeting	☒ **Growing Area** ☐ **Harvesting/Handling/Distribution** ☐ **Administrative**
Name of Submitter:	ISSC Executive Office/US Food and Drug Administration
Affiliation:	ISSC Executive Office/US Food and Drug Administration
Address:	209-2 Dawson Road Columbia, SC 29223 5100 Paint Branch Parkway College Park, MD 20740
Phone: **Fax:** **Email:**	803-788-7559/240-402-1410 803-788-7576/240-402-2601 issc@issc.org paul.distefano@fda.hhs.gov
Proposal Subject:	Recall Notification
Specific NSSP Guide Reference:	2009 NSSP Section II Model Ordinance Chapter II Risk Assessment and Risk Management @ .01 Outbreaks of Shellfish Related Illness I.
Text of Proposal/ Requested Action	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, ISSC and the Authorities in other states involved in the recall.
Public Health Significance:	Presently shellfish recalls are not listed on the USFDA website. In an effort to assure public notification of shellfish recalls, which would be consistent with other food programs, FDA is asking ISSC to include recalls on the ISSC website. FDA will provide a link in its website to the ISSC website for shellfish recalls. Should this option not be acceptable to ISSC, FDA will include notification on the FDA website.
Cost Information (if available):	