Proposal Subject Rapid Screening Method for ASP

Specific NSSP Guide Reference NSSP Guidance Documents, Chapter II

CONSTITUTION BY-LAWS and PROCEDURES of the INTERSTATE SHELLFISH SANITATION CONFERENCE
PROCEDURE XVI. PROCEDURE FOR ACCEPTANCE AND APPROVAL OF ANALYTICAL METHODS FOR THE NSSP
And:
NATIONAL SHELLFISH SANITATION PROGRAM
2003 MODEL ORDINANCE

III. LABORATORY
@.02 Methods.

C. Biotoxin. Methods for the 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.

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:

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.

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.

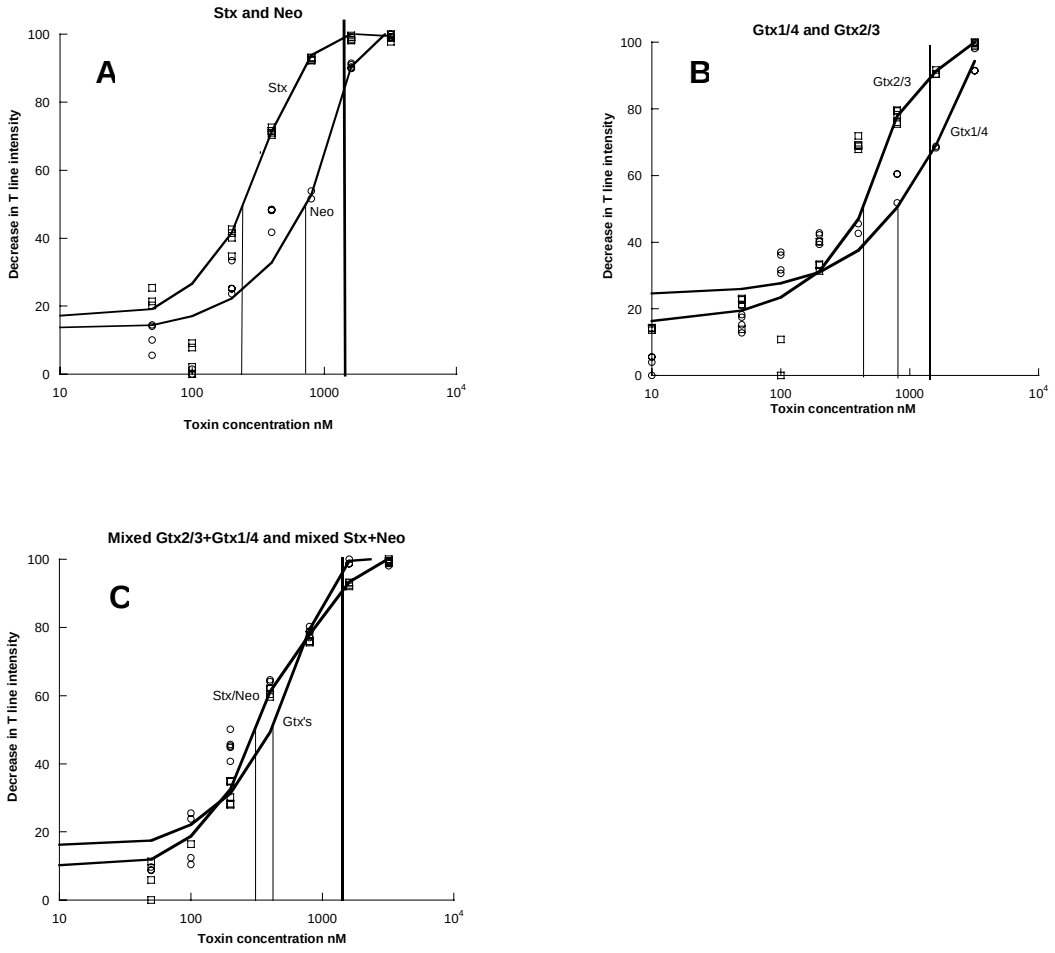

Figure 1.
Non-toxic mussel homogenate was extracted by the AOAC method into an equal volume of 0.1 M HCl. Samples were spiked with NRC certified toxin standards to 3200 nM. Dilution series were prepared by mixing with non-toxic extract. The extracts containing different toxin concentrations were then 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.

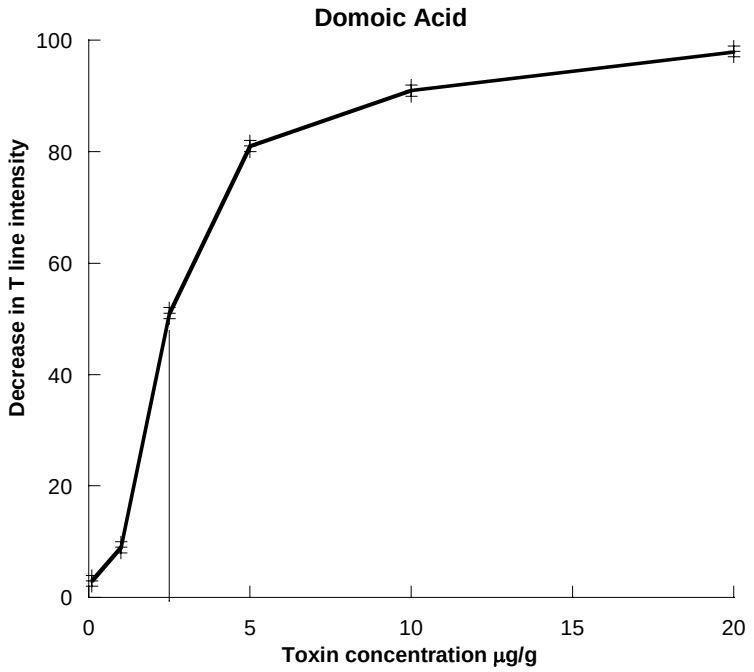

Figure 2.
Non-toxic mussel homogenate was extracted into four volumes of 50% methanol a sample spiked with domoic acid to 20 µg/g homogenate. Serial dilutions were made with non-toxic extract and mixed with ASP running buffer solution. A sample (100 µl) of each solution was applied to each test strip.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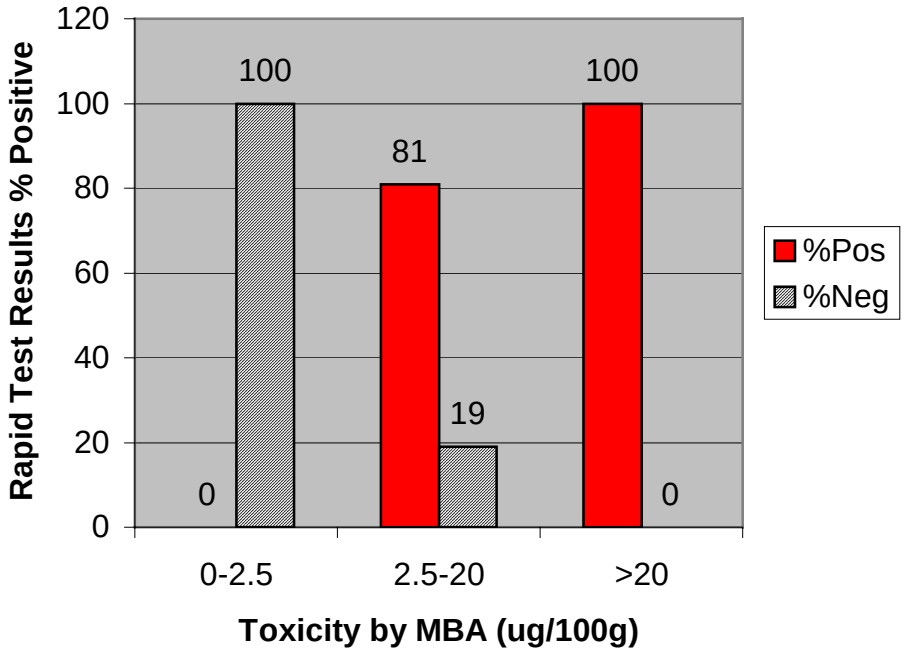
Detection of ASP in Shellfish Tissue
from UK



Tissue Types from UK
(n= 111)