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Validation program for the use of 'Rapid Test Kits' to safeguard and grow the WA Shellfish Industry

A single-laboratory validation study

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23/03/2022

FRDC Project No **2018-107**

ISBN 978-0-646-85809-8

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2022

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In submitting this report, the researcher has agreed to FRDC publishing this material in its edited form.

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Acknowledgements

The authors would like to acknowledge the assistance of Neogen® Australia for providing constructive comments regarding the use of the Reveal® 2.0 for ASP test kits and the AccuScan Pro reader. Neogen® Australia was given the opportunity to comment on the report manuscript. We also thank Tracey Stamp and Scott Whiddon from the Western Australian Department of Health for their comments on early drafts of this report and the other reviewers who also provided comment and valuable insight.

We would like to thank Glenn Dibben from Harvest Road Oceans for providing the fresh shellfish used for this study and Deborah Gardner from the Department of Primary Industries and Regional Development, Western Australia for assisting with the experimental procedures.

We are very grateful for the seed funding for this project provided by the Department of Primary Industries and Regional Development, Western Australia and the Department of Health, Western Australia.

Executive Summary

Naturally occurring marine biotoxins are produced by certain species of marine phytoplankton. When consumed by filter-feeding bivalve molluscs (shellfish), the toxins within the phytoplankton cells can become concentrated to levels that are harmful to humans who may, in turn, consume the shellfish.

There are four main types of poisoning which may occur if contaminated shellfish are consumed by humans: Paralytic Shellfish Poisoning (PSP), Diarrhetic Shellfish Poisoning (DSP), Neurotoxic Shellfish Poisoning (NSP) and Amnesic Shellfish Poisoning (ASP).

Phytoplankton species known to produce the biotoxins responsible for each of these types of poisoning are known to occur in Western Australian (WA) coastal and estuarine waters. The toxins responsible for PSP, DSP and NSP have also been detected in shellfish flesh collected from several regions in Western Australia when the causative phytoplankton species have been detected at significant cell densities. To date however, although the phytoplankton taxa responsible for its production are likely to occur at significant cell densities throughout Western Australia, there have been no detections of the toxin responsible for ASP from shellfish flesh samples collected anywhere in the state.

The production and sale of bivalve molluscs for human consumption in WA is regulated by the Department of Health (DoH) through implementation of the Western Australian Quality Assurance Program (WASQAP). Testing for the presence of phytoplankton taxa known to potentially produce marine biotoxins and for the marine biotoxins themselves is a mandatory requirement under the WASQAP.

The WASQAP operates on a model of surveillance followed by confirmation. The phytoplankton assemblages in the vicinity of shellfish aquaculture leases are regularly monitored for the presence of species known to be potential producers of biotoxins. If phytoplankton species known to be potential producers of biotoxins are detected at cell densities above the prescribed threshold levels, samples of shellfish flesh which were collected at the same time and location as the phytoplankton samples are sent for analysis to confirm the presence and concentration of biotoxins. Typically, when phytoplankton species known to be potential producers of biotoxins are detected at cell densities above the prescribed threshold levels, the impacted shellfish harvesting areas are closed pending the results of the toxin analysis and subsequent phytoplankton testing.

Identification of the phytoplankton species known to be potential producers of biotoxins responsible for PSP, DSP and NSP is relatively unambiguous with the biotoxins almost always being detected when the phytoplankton cell densities of the target species are at or above WASQAP threshold levels. The biotoxin responsible for ASP is Domoic Acid (DA) and this is produced by several, but not all, species within the diatom genus *Pseudo-nitzschia*.

Identification of *Pseudo-nitzschia* to species level is difficult and requires specialised microscopic techniques and/or genetic analysis and neither of these are undertaken as part of the routine WASQAP surveillance. Instead, it is the presence of the genus *Pseudo-nitzschia* which is reported for the WASQAP. For reporting purposes, the genus *Pseudo-nitzschia* is typically divided into two arbitrary (non-taxonomic) groups based on cell width. The first of these, *Pseudo-nitzschia* "delicatissima" group contains all taxa with a cell with of less than 3 μm with those taxa with a cell width of greater than 3 μm being placed in the *Pseudo-nitzschia* "seriata" group. These groups typically have different cell density Alert Levels with that for the "seriata" group usually set at a lower cell density as this group contains more of the potentially toxic taxa, including some of the most toxic taxa, and because their greater size means a greater cell biovolume containing, potentially, a greater amount of toxin.

As such, when the cell density for either group within this genus exceeds the WASQAP threshold level, it is never known if the *Pseudo-nitzschia* species present are known producers of DA, non-toxic strains of *Pseudo-nitzschia* species known to produce DA or non-toxin producing *Pseudo-nitzschia* species. The assumption is however, that the species present are potential producers of DA.

Although known toxin producing species of *Pseudo-nitzschia* are known to occur throughout Western Australia, there are no records of Domoic Acid ever being recorded.

A prolonged period of high cell densities of *Pseudo-nitzschia* spp. which occurred in Cockburn Sound between June and July 2017, during which there were no detections of DA in shellfish flesh, but which resulted in a lengthy closure of the shellfish harvest area which almost crippled the grower was the catalyst to discussions between the DoH the Department of Primary Industries and Regional Development (DPIRD) and the shellfish growers regarding what the DPIRD could do to assist industry in this space.

In addition to the *Pseudo-nitzschia* bloom itself, several other factors were identified as contributing to the lengthy closure of the shellfish harvesting area including the lack of capacity to undertake biotoxin testing in WA using either methods approved for the regulatory testing of biotoxins such as Liquid Chromatography with tandem mass spectrometry (LC-MS/MS) analysis or contemporary methods for the rapid detection of marine biotoxins - rapid biotoxin test kits. Had some information been available at the time of this *Pseudo-nitzschia* bloom from rapid biotoxin test kits to indicate a negative biotoxin result, the DoH acknowledged that the closure of the shellfish harvesting areas throughout the event would have been greatly reduced.

The validation and subsequent use of a rapid biotoxin test kit for DA (consistent with DoH requirements) has the potential to reduce such losses to industry due to harvest area closures and disposal of stock-in-hand and assist growth of the shellfish aquaculture industry within WA. This is consistent with the State Government's approach of providing strong support for aquaculture development proposals throughout the State.

The purpose of this study is not to propose the regulatory use of rapid biotoxin test kits but rather their use to better inform management decisions by both the growers and the regulators. This is 100% in agreement with the direction which the Australian Shellfish Quality Assurance Advisory Committee (ASQAAC) is taking Australia wide hence the already finalised validation studies on Paralytic Shellfish Poisoning (PSP) toxins and Diarrhetic Shellfish Poisoning (DSP) toxins using rapid biotoxin test kits.

We used the certified reference material CRM-ASP-Mus-d, obtained from the National Research Council, Canada-Institute for Marine Biosciences (NRC- IMB), as a source of spiked shellfish flesh. CRM-ASP-Mus-d consists of a thermally stabilised homogenised liquid slurry of whole mussel tissue (*Mytilis edulis*). The mussel slurry comprised a combination of DA-free mussel tissue and mussel tissue naturally contaminated with DA such that the final DA concentration was 49 μg/g ± 3 μg/g. We combined the certified reference material (CRM) with locally harvested mussel flesh (*Mytilis edulis*), which had been determined to be free of DA, to provide several spiked shellfish flesh samples at various DA concentrations ranging from 0 μg/g to 24 μg/g (4 μg/g above the regulatory limit).

We analysed these samples for the presence of DA using the Neogen® Reveal® 2.0 for ASP rapid biotoxin test kit, a single-step, lateral flow immuno-chromatographic assay, coupled with the Neogen® Accuscan® Pro test strip reader. The strip reader removes any ambiguity and subjectivity associated with test strips being read by the analysts conducting the tests.

We assessed kit performance according to the following parameters:

- Accuracy/Trueness – a measure of the agreement between the test result and the result obtained by an accredited analytical laboratory using LC-MS/MS. In other words, this is a measure of the trueness of the positive and negative test results obtained for each individual test conducted.
- Repeatability – a measure of the agreement of the replicate tests carried out on the same sample on the same occasion by the same analyst (in this case 4 replicate tests per shellfish extract).
- Reproducibility - this is typically a measure of agreement between tests carried out by different laboratories. In this case however, reproducibility is interpreted as follows:
    - Reproducibility (Method) – this is a measure of the reproducibility of the method by which 6 shellfish extracts are prepared from each of the spiked (or un-spiked) homogenates.
    - Reproducibility (Kits) – this is a measure of the agreement between test kits by which tests are conducted using 4 different test kits on each of the 6 shellfish extracts.

Apart from a very small number of non-conforming negative results which we discuss in detail, this test kit performed extremely well with respect to each of the parameters above.

In terms of the practicality and cost of using these test kits to industry, we have determined that they are sufficiently easy enough to use by growers on site with an initial setup cost of less than $5,500 (ex GST) including the cost of the new Raptor Solo® reader which replaces the Accuscan® Pro. Each test kit costs approximately $700 (ex GST) with each kit capable of conducting 24 individual tests making the cost per test approximately $30 (ex GST).

Although not a part of the present study, a validation study on the Neogen® Reveal® 2.0 for ASP test kits by Cabellero *et al*. (2013), assessed the kits using different shellfish matrices (oysters, clams and mussels), assessed the impact of some common potential interfering compounds (glutamic acid, glutamine and saxitoxin) on kit performance, assessed the kit performance using incubation times between 80% and 120% of the recommended incubation time and assessed the repeatability of test results between different analysts. Test kit results were as expected for all parameters assessed.

The results of this study, and the validation study by Cabellero *et al*. (2013), indicate that the Neogen® Reveal® 2.0 for ASP test kits are a very reliable qualitative test kit for the detection of Domoic Acid in shellfish flesh. We would recommend that these test kits be approved for use with respect to management decisions for both the Western Australian and the Australian shellfish aquaculture industries.

Keywords: Biotoxins, harmful algal blooms, rapid test kits, LC-MS, domoic acid, amnesic shellfish toxins, *Pseudo-nitzschia*

Validation program for the use of 'Rapid Test Kits' to safeguard and grow the WA Shellfish Industry: a single-laboratory validation study

Project Number: 2018-107

1. Introduction

Domoic Acid (DA) is a neurotoxin produced by some marine algae, in particular diatoms of the genus *Pseudo-nitzschia*. Domoic Acid is a water-soluble amino acid and is a structural analogue of two common neurotransmitters in vertebrates, glutamic acid (glutamate) and aspartic acid (aspartate). Glutamate is the most abundant neurotransmitter in vertebrates and is reported to be the neurotransmitter at 40% of all synapses in the brain (Alexander, 2009).

Domoic Acid is the causative agent for Amnesic Shellfish Poisoning (ASP), the name given to a syndrome provoked by the consumption of shellfish (and occasionally other seafood) contaminated with DA. Amnesic Shellfish Poisoning was first identified in 1987 after more than one hundred people in Canada began exhibiting symptoms now associated with ASP within 24 hours of consuming shellfish (mussels) contaminated with DA which had been harvested off Prince Edward Island (Tubaro, Sosa and Hungerford, 2012).

Shellfish (mussels, oysters, clams etc.) feed by pumping and passing large quantities of water across the surface of their gills where they not only extract oxygen from the water but also trap small food particles, the majority of which is phytoplankton. Shellfish can filter thousands of litres of water per day consuming most of the phytoplankton contained within it. When diatoms such as *Pseudo-nitzschia* are present in the phytoplankton, even at relatively low densities, they can be consumed by the shellfish in large quantities and the toxins within the diatoms concentrated within the tissues (particularly the gut) of the shellfish. These toxins need only reach concentrations of around 20 parts per million (20 μg/g of shellfish flesh) to be harmful to humans if ingested.

Domoic Acid is a potent neurotoxin with oral exposures of just a few milligrams per kilogram of body weight eliciting gastrointestinal effects in humans with slightly higher doses causing neurological symptoms. Gastrointestinal symptoms can occur within 24 hours of ingestion and include vomiting, nausea, diarrhea, abdominal cramps, and haemorrhagic gastritis. Neurological symptoms can develop within hours or days after exposure and include headaches, dizziness, disorientation, vision disturbances, loss of short-term memory, motor weakness, seizures, profuse respiratory secretions, hiccups, unstable blood pressure, abnormal heart rhythms and coma.

Exposure to very high concentrations of DA has proven to be fatal in people with existing risk factors such as old age and poor kidney function. Exposure to low concentrations, around 1% of the concentration known to cause neurological effects, can cause permanent kidney damage. Domoic Acid is a very stable compound and is heat resistant; cooking and/or freezing contaminated shellfish does not decrease its toxicity. There is no known antidote to Domoic Acid poisoning (Ramsdell, 2007; Pulido, 2008).

2. Background

2.1. Regulatory Background

Food businesses in Western Australia involved in the commercial harvest of bivalve molluscan shellfish are regulated by the Western Australian Department of Health (DoH). The commercial bivalve molluscan shellfish industry in Western Australia is regulated according to the conditions outlined in the Western Australian Shellfish Quality Assurance Program (WASQAP) and associated documents. The current relevant documents at the time of writing this report are:

- Western Australian Shellfish Quality Assurance Program (WASQAP). Industry Manual/User Guide. Version 7 (2020)
- Marine Biotoxin Monitoring and Management Plan (MBMMP). Western Australian Shellfish Quality Assurance Program. Version 2 (2020)

These documents can be obtained from the DoH website (www.health.wa.gov.au).

Legislation and Standards applicable to the WASQAP are as follows:

- Food Act 2008 (WA)
- Food Regulations 2009 (WA)
- Australia New Zealand Food Standards Code
- Australian Shellfish Quality Assurance Program: Export Standards 2004 edition
- Export Control (Fish and Fish Products) Orders 2005 as amended

The WASQAP and its associated documents follow the guidance of the Australian Shellfish Quality Assurance Advisory Committee (ASQAAC) and the Australian Shellfish Quality Assurance Program (ASQAP) Operations Manual 2019 and, unless otherwise stated, are consistent with these.

As a part of the WASQAP, all commercially licenced shellfish growers and harvesters in Western Australia are required to undertake routine analyses of water samples for the detection of phytoplankton taxa known to be potential producers of shellfish toxins as well as samples of shellfish flesh for direct toxin analysis. Shellfish flesh samples are collected at the same time as the water samples and either frozen pending the analysis of phytoplankton or sent directly for analysis if taken as part of a routine biotoxin analysis.

Phytoplankton taxa known to occur in Western Australian waters and targeted by the WASQAP are listed in the MBMMP. The MBMMP has a two-tiered alert level system based on the recorded cell density (recorded as cells L^{-1}) for each of the target taxa. Each of the WASQAP target phytoplankton taxa has a "general" Alert Level and an Alert Level to initiate shellfish flesh testing.

If the reported cell density of a potentially toxic phytoplankton taxon exceeds the "general" Alert Level but not the Alert Level to initiate flesh testing, the laboratory responsible for the analysis must immediately notify the regulator (the DoH), and the affected food business will be required to collect another water sample to be submitted for testing (a flesh sample is also collected and frozen as above).

If potentially toxic phytoplankton taxa are reported at cell densities that exceed the Alert Level to initiate flesh testing, the laboratory responsible for the analysis must immediately notify the regulator and the affected food business must then arrange for the chilled/frozen sample of shellfish to be tested for biotoxins.

This procedure as well as procedures relating to the closure and re-opening of commercial shellfish harvest areas are outlined in the MBMMP. Both the WASQAP and the MBMMP are regularly reviewed and updated every two to three years. Because this study was prompted by events which occurred in 2017, any regulatory criteria and/or actions refer to that manuals that were in effect at that time as follows:

- Western Australian Shellfish Quality Assurance Program (WASQAP). Industry Manual/User Guide. Version 6 (2017)
- Marine Biotoxin Monitoring and Management Plan (MBMMP). Western Australian Shellfish Quality Assurance Program. Version 1 (2016)

Both these documents are now obsolete. The 2016 edition of the MBMMP (Version 1) has been appended to this report as Appendix 1, the 2017 edition of the of the WASQAP Manual (Version 6) was not a public document and cannot be included as an appendix to this report.

2.2. Pseudo-nitzschia spp.

Pseudo-nitzschia is a genus of marine diatom which contains approximately sixty known species. Around half of these are known to be producers of DA. Of those approximately thirty species known to be or suspected to be potentially toxic, thirteen are known to be present in Australian waters.

Most of the potentially toxic phytoplankton taxa targeted in the WASQAP (and other Australian State programs) can be readily identified to species level using standard light microscopy techniques by suitably experienced algal taxonomists. Several taxa however, namely species of the dinoflagellate genus *Alexandrium* and species of the diatom genus *Pseudo-nitzschia*, require the use of specialised microscopic techniques and/or genetic studies as well as highly skilled algal taxonomists and technicians.

In the case of *Alexandrium* species, most laboratories responsible for the surveillance of potentially toxic phytoplankton can undertake the specialised microscopic techniques (staining of the cells using an ultra-violet light sensitive fluorochrome) and have microscopes equipped with an ultra-violet light source as well as the required level of expertise to identify this taxon to species level. However, even then, the morphological features of the individual cells which must be observed to provide an accurate identification to species level cannot always be observed, especially when the cell density is low and only one or a few cells may be recorded in each sample. For this reason, in addition to the fact that about half of the thirty-three species of *Alexandrium* are known producers of PSP toxins (most of which are known or suspected to occur in Australian waters) any detection of *Alexandrium* (even if it is identified to genus level only) is considered as a potentially toxic taxon by most shellfish surveillance programs.

In the case of *Pseudo-nitzschia* species, very few laboratories responsible for the surveillance of potentially toxic phytoplankton have the required microscopic resources (a scanning electron microscope (SEM)) to accurately this taxon to species level. Less than half of the thirty or so *Pseudo-nitzschia* species known or suspected to be producers of DA are known to be present in Australian waters. With no readily available and economic means of identifying members of this genus to species level, this presents a problem to the shellfish surveillance programs. This is further confounded by toxin production in known toxin producers to not be obligatory (they may only produce toxins under certain conditions) or strain-specific (only certain strains of a given species might produce toxins). It is for this reason that shellfish surveillance programs accept the identification of this taxon to genus level only.

As is the case for all shellfish quality assurance programs in Australia and most, if not all, similar programs in other countries, the genus *Pseudo-nitzschia* has been divided into two arbitrary (non-taxonomic) groups based on cell width. The first of these, *Pseudo-nitzschia* "delicatissima" group contains all taxa with a cell with of less than 3 μm with those taxa with a cell width of greater than 3 μm being placed in the *Pseudo-nitzschia* "seriata" group. These groups typically have different cell density Alert Levels with that for the "seriata" group usually set at a lower cell density as this group contains more of the potentially toxic taxa, including some of the most toxic taxa, and because their greater size means a greater cell biovolume containing, potentially, a greater amount of toxin.

Whilst Domoic Acid has been detected in shellfish flesh in eastern Australia during periods of high *Pseudo-nitzschia* cell density, there have been no detections of Domoic Acid in shellfish flesh samples from Western Australia since biotoxin testing started despite semi-regular exceedances of the WASQAP/MBMMP *Pseudo-nitzschia* Alert Levels.

The uncertainty associated with *Pseudo-nitzschia* spp. in Western Australia, particularly during periods where cell densities are above MBMMP threshold values has historically, and justifiably so, resulted in a precautionary approach being adopted by the Western Australian Department of Health.

2.3. "The Incident"

The following is a brief description of a period of prolonged high *Pseudo-nitzschia* spp. cell densities in Cockburn Sound between mid-June 2017 and late-July 2017 which caused significant financial and reputational loss to the local bivalve shellfish (mussel) industry, and which was the impetus for this study.

With respect to the prolonged nature of the *Pseudo-nitzschia* spp. "bloom", this was a particularly unusual event which had not occurred since the monitoring of commercial shellfish harvest areas began during the mid- to late- 1990s.

The circumstances of this incident and the events which transpired during it resulted in somewhat of a "perfect storm" resulting in considerable impact to the local bivalve shellfish (mussel) industry. In brief, these included:

- The prolonged nature of the "bloom".
- The lack of knowledge relating to *Pseudo-nitzschia* species known to occur in the south-west of Western Australia, particularly with respect to known toxin-producing taxa.
- The lack of any historical context or data which would better inform the decision-making processes of the Western Australian Department of Health.
    - Although no positive Domoic Acid toxin results have been recorded during periods of high *Pseudo-nitzschia* spp. cell density since the monitoring of commercial shellfish harvest areas began in Western Australia, the underlying reasons for this are unknown and the status quo could change at any time.
- The lack of a Western Australian laboratory capable of NATA Accredited testing of shellfish flesh for shellfish toxins and the subsequent requirement to send samples to a laboratory on the east coast of Australia.
- Delays (up to 8 days) in obtaining toxin results for flesh samples submitted for analysis which, in some cases, were not obtained until 1 day before the next water sampling for phytoplankton was scheduled.
    - These delays in obtaining results from the toxin analyses were due to one or more of the following factors:
        - Heavy workload of the receiving laboratory.
        - Machinery breakdown of the receiving laboratory.
        - Communication of erroneous results by the receiving laboratory.

The following discussion relates to WASQAP Alert Levels which were current at the time of the incident, these were the 2017 edition of the WASQAP Industry Manual/User Guide and the 2016 edition of the MBMMP (see Appendix 1). With respect to *Pseudo-nitzschia* spp, the Alert Levels in these documents were as follows:

	Alert Level	**Alert Level to initiate flesh testing**
***Pseudo-nitzschia* "delicatissima" group**	500,000 cells L^{-1}	500,000 cells L^{-1}
***Pseudo-nitzschia* "seriata" group**	50,000 cells L^{-1}	50,000 cells L^{-1}

The Alert Levels for *Pseudo-nitzschia* spp. have been amended several times since these versions.

Cell densities of *Pseudo-nitzschia* "seriata" group above the WASQAP Alert Level to initiate flesh testing were first reported from samples collected on June 12th, 2017. This exceedance was recorded at the Southern Flats (SF) site, the recorded cell density was 98,400 cells L^{-1}, an increase from 35,500 cells L^{-1} on the previous sampling occasion (May 22nd, 2017). Recorded cell density for this taxon at the SF site remained above the Alert Level until at least July 17th, 2017 (35 days) with values decreasing significantly to less than Alert Level densities by July 24th, 2017. (Figure 1).

At the Kwinana Grain Terminal (KGT) site, cell densities of *Pseudo-nitzschia* "seriata" group were first reported above the WASQAP Alert Level to initiate flesh testing June 25th, 2017 (58,800 cells L^{-1}) and again on July 10th, 2017 (124,000 cells L^{-1}). It is likely that cell densities above the Alert Level persisted throughout the period between these two occasions (although water samples were not collected from this site on June 29th and July 5th) (Figure 1).

The peak of the *Pseudo-nitzschia* "seriata" group "bloom" occurred on July 10th, 2017, at both locations with recorded cells densities of 438,800 cells L^{-1} and 124,000 cells L^{-1} for SF and KGT respectively (Figure 1).

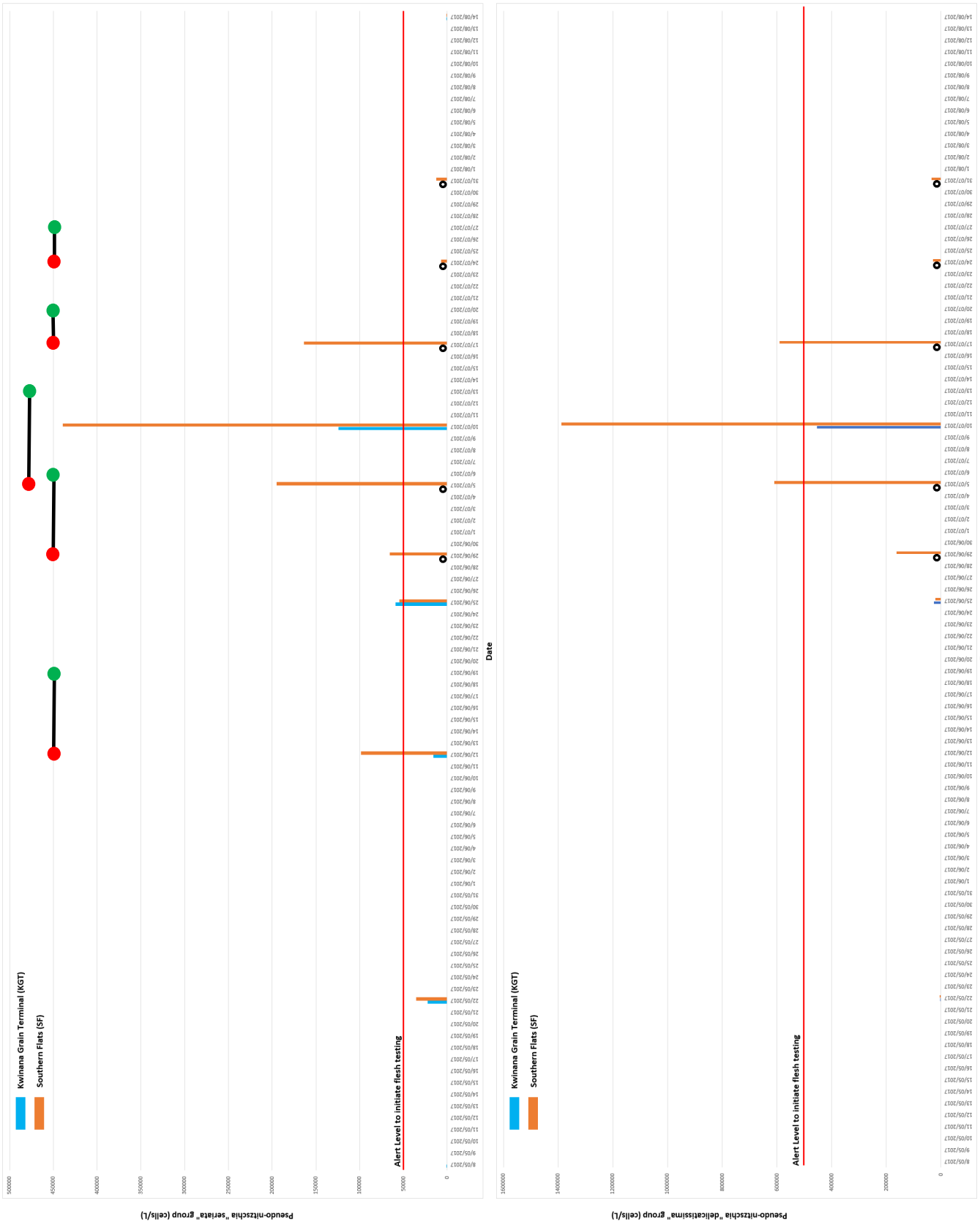

Figure 1: *Time series of* Pseudo-nitzschia *"seriata" group (top) and* Pseudo-nitzschia *"delicatissima" group (bottom) cell density for the Kwinana Grain Terminal (KGT) and Southern Flats (SF) bivalve shellfish harvest areas between May 8th, 2017, and August 14th, 2017. Note that the vertical axis (cell density) scales for both plots are different to reflect the respective cell density Alert Levels to trigger flesh testing (red line). The black circles on the horizontal axis indicate occasions where the KGT site was not sampled. The red dots above the plots indicate when samples of shellfish flesh were sent to the analytical laboratory for biotoxin analysis with the green dots indicating when the results of biotoxin analysis were received.*

Cell densities of *Pseudo-nitzschia* "delicatissima" group above the WASQAP Alert Level to initiate flesh testing were first reported from samples collected on July 5th, 2017. This exceedance was recorded at the SF site, the recorded cell density was 609,600 cells L^{-1}, an increase from 162,800 cells L^{-1} on the previous sampling occasion (June 29th, 2017). Recorded cell density for this taxon at the SF site remained above the Alert Level until at least July 17th, 2017 (12 days) with values decreasing significantly to less than Alert Level densities by July 24th, 2017. (Figure 1).

At the KGT site, cell densities of *Pseudo-nitzschia* "delicatissima" group did not exceed the WASQAP Alert Level to initiate flesh testing although the recorded cell density on July 10th, 2017, was very close to the Alert Level with 453,200 cells L^{-1} recorded. Water samples were not collected from this site on either occasion prior to or after this date (Figure 1).

The peak of the *Pseudo-nitzschia* "delicatissima" group "bloom" at the SF site occurred on July 10th, 2017, with a recorded cells density of 1,388,000 cells L^{-1} (Figure 1).

Dates when shellfish flesh samples were sent for toxin analysis and the dates which toxin results were received are indicated in Figure 1 by red and green dots respectively.

During this "incident" the Cockburn Sound bivalve molluscan shellfish harvesting sites (KGT and SF) were closed from June 14th until June 19th (5 days) and from June 29th until July 17th (18 days) although the grower decided to remain closed for an additional 7 days until July 24th (Glenn Dibben, personal communication).

This "incident" resulted in the grower disposing of 750 kg of cool room stock on June 14th and 2,000 kg of harvested stock on June 16th. In addition to the lost stock, the grower suffered a considerable loss of confidence with respect to their local customers (retailers) and the local market in general due to the inability to supply product and the perception that their product may be tainted (Glen Dibben, personal communication).

With respect to local market confidence and perception of the local product, it is possible, if not likely, that situations such as this may have implications for bivalve molluscan shellfish growers/harvesters elsewhere in the state and, potentially, for other seafood industries within Western Australia.

This incident prompted discussions between the Department of Health, the Department of Primary Industries and Regional Development (DPIRD), shellfish growers/harvesters throughout Western Australia and other stakeholders regarding the potential for rapid qualitative shellfish toxin test kits to be used for the detection of Domoic Acid in shellfish flesh. The DoH agreed to support a study to validate the use of these test kits.

2.4. Framework for the use of qualitative rapid test kits in Western Australia

The first version of the Western Australian MBMMP (2016, Section 2.4) (Appendix 1) states that rapid shellfish toxin test kits may be used for the initial toxin analysis of shellfish flesh samples following a cell density exceedance (for any of the listed potentially toxic phytoplankton taxa) of the WASQAP Alert Level to initiate flesh testing (on the condition that the analysis is undertaken by a NATA Accredited Laboratory). This is undertaken on the proviso that, if any toxin is detected by the rapid test kit(s), flesh samples must be sent for quantitative analyses and the harvesting area may be required to cease harvesting pending the results of the quantitative toxin analyses.

The section 2.3.2 of the second version of the Western Australian MBMMP (2020) (available online, see section 2.1) states that appropriately validated qualitative marine biotoxin screening methods can be used in the following situations:

a) to determine if a quantitative method should be undertaken on a sample from a closed area for re-opening purposes (i.e., to test the first of two samples collected to re-open areas); and
b) for routine testing of harvest areas in the open status when risk is considered low.

This is an ongoing process with advice and information being obtained from the Australian Shellfish Quality Assurance Advisory Committee (ASQAAC), Western Australian Government agencies (e.g., DPIRD), relevant experts, ongoing research and the Western Australian bivalve molluscan shellfish growers and harvesters,

3. Objectives

The objectives of this study are to:

- Verify the use of 'Rapid Biotoxin Test Kits' to enable testing for Amnesic Shellfish Poisoning (ASP) under WA conditions.
- Establish a capability (similar to that in other states) with regard to the requirements of the WASQAP which would include analytical laboratories, shellfish growers and government agencies.
- Work with the DoH and other stakeholders to recommend the use of such kits and update respective WASQAP management plans and guidance material accordingly.

4. Test Kits

The test kits chosen for this validation study were the Reveal® 2.0 for ASP test kits produced by Neogen® (Figure 2). These kits were chosen primarily because the qualitative test kits for PSP produced by this manufacturer had already undergone a successful single-laboratory validation for use in Australia by a research team in Tasmania using the flesh of several species of bivalve mollusc (including mussels) (Turnbull *et. al*., 2017) but also because several successful validations of these test kits for ASP have already been undertaken in Europe also on the flesh of a variety of species of bivalve mollusc - pacific oysters (*Crassostrea gigas*), cherrystone clams (*Mercenaria mercenaria*) and "common" mussels (*Mytilus edulis*) (Caballero *et. al*., 2013). This, coupled with their ease of use and portability, made them a stand-out candidate for this small study.

Figure 2: *Reveal® 2.0 for ASP test kit contents and the Accuscan Pro reader and test strips.*

The initial scope of this project also included assessing and validating a rapid quantitative method for the determination of Domoic Acid concentration in shellfish flesh. The test kits chosen for this component of the project were the Biosense® Enzyme Linked Immunosorbent Assay (ELISA) – based ASP test kits. This test kit was chosen because it had already been subjected to comprehensive validation studies, using the flesh of several species of bivalve mollusc (including mussels), and is approved AOAC® Official Method℠ 2006.02 (AOAC INTERNATIONAL, 2006). As such, this test kit also has regulatory approval for use as a means of quantifying DA concentration in shellfish flesh in several jurisdictions world-wide.

However, after conducting the first few tests using this method and obtaining variable results, and after discussions with industry representatives regarding potential reasons why this method may not be suitable, it was decided to not complete the testing using these test kits and instead undertake some additional testing using the Neogen test kits using the Certified reference material (CRM-ASP-Mus-d) which was set aside for the quantitative testing. The additional tests discussed were to investigate whether the Neogen test kits could be "forced" to provide positive readings at DA concentrations below the regulatory limit, this rationale behind this will be discussed later.

The main concern regarding the ELISA test kits discussed with industry representatives was the equipment cost of setting up to undertake these tests and the cost of the tests themselves. There are two recommended plate layouts for the Biosense® ELISA ASP tests. The first is a four-strip layout and the second is an eight-strip layout. Each kit comes with eight microplate strips each with twelve wells. The microplate strips are contained in two sealed pouches, each pouch containing four strips. Once a pouch is open, the microplate strips must be used or discarded. The four-strip plate layout allows for up to twelve tests to be conducted on a single occasion whereas the eight-strip layout allows for up to thirty-six tests to be conducted on a single occasion.

It is unlikely, at least for the foreseeable future, that the WA shellfish Industry will be requiring any more than a few samples, perhaps between one and four, to be analysed on any given occasion.

Assuming that four tests are required per occasion, each test kit can accommodate two batches of four tests (using the four-plate layout). Given that each test kit costs approximately $750.00 (ex GST), this would equate to approximately $95.00 per test (for eight tests) excluding the cost of consumables, chemicals, and labour. If the required tests are fewer, the cost per test increases such that the cost per test for three tests per occasion (six tests total) would be approximately $125.00 per test, the cost for two tests per occasion (four tests in total) would be approximately $190.00 per test and the cost of a single test per occasion (two tests in total) would be approximately $375.00 per test.

The requirement for sensitive, expensive, and generally non-portable equipment (e.g., microplate spectrophotometer and software, blender/homogeniser, benchtop centrifuge, vortex mixer, analytical balance, micro pipettors and volumetric glassware) requiring an initial investment of around $20,000 (ex GST) and relatively skilled analysts to operate it and conduct the tests was considered prohibitive and not appropriate for a shellfish grower/harvester to undertake the testing themselves.

This would mean that the testing would need to be undertaken by a third-party, an accredited laboratory. The anticipated low volume of testing required, at least in the near future, would most likely render this a costly undertaking.

There is still the advantage to industry of a potential quick turn-around time for results using this method which could potentially offset the cost of analysis but, after undertaking some analyses using this method and discussing the method with industry representatives, it does not appear to be viable for a laboratory to undertake this analysis on an ad-hoc basis (particularly for small volumes of samples). Given the nature of the equipment required and the level of laboratory skill required to undertake these analyses, it is also not likely to be viable for individual shellfish growers to conduct these analyses themselves as mentioned above.

Although the obtained results are qualitative rather than quantitative, none of these outlined problems exist for the Neogen® ASP test kits. We may re-visit the ELISA quantitative test kits at another time if it seems likely that they may be approved for regulatory use within Australia and if there is still an industry need.

5. Methodology

The following description of the method is taken directly from various Neogen sources including (Caballero *et. al*., 2013) (Figure 3).

5.1. Principle

Reveal® 2.0 for ASP is a single-step, lateral flow immuno-chromatographic assay based on the principle of competitive immunoassay. The following brief description of the method principle is taken from (Caballero *et. al*., 2013) as well as directly from the Neogen® website or provided literature with the test kit.

Following a simple distilled water extraction of DA from homogenised shellfish tissue, the extract is then diluted in running buffer. The dipstick-format Reveal device (test strip) is then placed into the diluted extract.

The extract is wicked through a reagent zone containing antibodies specific for DA conjugated to colloidal gold particles. If DA is present, it will be captured by the labelled antibody. Migration of the sample continues through a membrane, which contains a zone of DA conjugated to a protein carrier. This zone captures any unbound antibody-gold conjugate, resulting in a visible line. With increasing amounts of DA 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 DA in the sample. The test device also incorporates a control conjugate, which binds to a second line. The control line will form regardless of the amount of DA present in the sample, ensuring that the test device is functioning properly. Results are analysed as positive or negative using the Neogen® AccuScan® Pro Reader.

Reveal® 2.0 for ASP is intended for the qualitative screening of shellfish for DA, by producing a positive result with samples containing 20 µg/g or above. The test kit is designed for use by quality control personnel and other personnel familiar with handling shellfish possibly contaminated by DA toxins.

5.2. Reveal® 2.0 ASP Method

Figure 3 presents a pictorial guide of the Reveal® 2.0 for ASP extraction and analytical procedure.

5.2.1. Materials Provided

Reveal® 2.0 ASP (Neogen® item 9560) Test Kit containing:

- 24 Reveal 2.0 ASP lateral flow test strips
- 24 wells
- 24 vials of ASP buffer
- 25 extraction bags
- 48 disposable 100µl pipettes

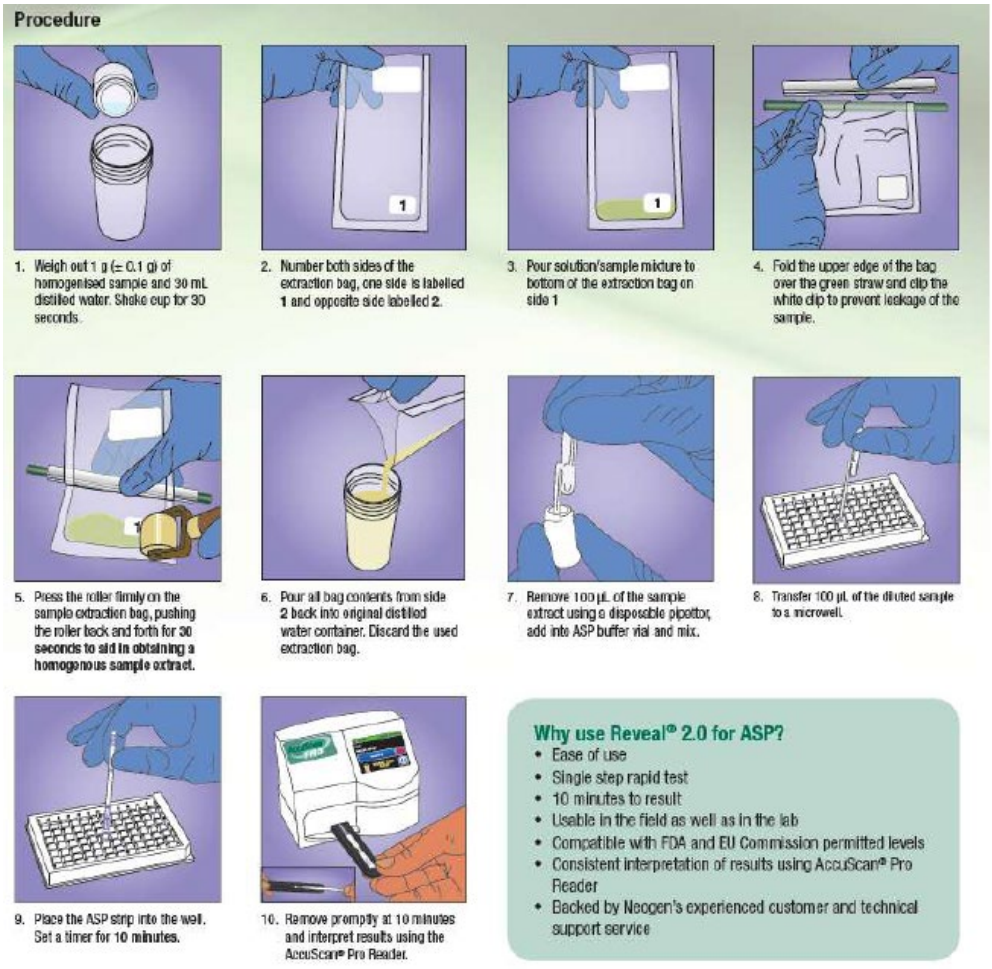

Figure 3: *Reveal® 2.0 for ASP pictorial instruction guide.*

5.2.2. Materials Recommended but not provided

Marine biotoxins starter kit (Neogen® item 9563) containing:

- Microwell holder
- 1 roller
- 1 bag clip (white clip with green straw)

Distilled water

Sample collection cups with lids (Neogen items 9428, 9428B)

Blender (Neogen® items 9493, 9477 or 9495)

Scale capable of weighing 0.5 – 400g ±0.1g (Neogen® item 9427)

Timer (Neogen® item 9452)

Graduated cylinder, 50 ml (Neogen item 9367) or bottle-top dispenser (Neogen® item 9448)

AccuScan® Pro reader (Neogen® item 9565)

All the above recommended items were used in this study with the following exceptions:

- Pipettes – A 100 µl manual pipettor was used rather than the supplied plastic pipettes
- Blender – A NutriBullet Rx Blender was used instead of the Neogen® product
- Scale – A Sartorius Entris 224 balance (220 g x 0.1 mg) was used instead of the Neogen® product
- Timer – A generic timer was used instead of the Neogen® product
- Graduated Cylinder – Quality, glass graduated cylinders were used instead of the Neogen® product

- Although not recommended, a Vortex Mixer (Thermofisher) was used whenever mixing or agitation was required.

5.2.3. Precautions

- The test strips must remain inside the stay-dry tube before use.
- Do not use kit contents beyond 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 solutions for each sample.

5.2.4. Storage Requirements

- Store Reveal® 2.0 ASP kit components at controlled room temperature (18-30°C, 64-86°F). Do not freeze.
- Test strips should remain in their original sample tubes until use to maintain shelf-life and ensure optimal performance.

5.2.5. AccuScan® Pro Reader Set up

- Enter the lot specific QR code by selecting the QR code icon on the reader. Place the QR code into the cartridge and insert the cartridge into the reader.

5.2.6. Sample Preparation and Extraction

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

- Obtain a representative sample. Shell the samples.
- Thoroughly rinse the samples with distilled or deionized water and allow any excess water to drain.
- Homogenise (e.g., blend, puree) the shellfish in a high-speed blender.
    - NOTE: A good homogenate is essential in order to obtain an accurate result.
- Weigh 1 g (± 0.05 g) of homogenised sample in a sample cup.
- Pour 30 mL (± 0.5 mL) of distilled water into sample cup containing the sample and secure the lid.
    - Shake the sample cup vigorously by hand for 30 seconds, until all shellfish tissue is in solution (a cloudy appearance or bubbles may form, which does not affect the running of the test).
    - Number both sides of an extraction bag using a marker, so that there is a side labelled “1” and the other side labelled “2”. Pour solution/sample mixture into the side labelled “1.”
        - NOTE: The extraction bag contains a mesh filter which allows for partial filtration of the sample. All samples/solutions should only be added to the side labelled “1”.

- 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.
- 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.
- Slide out the green straw and remove the white bag clip.
- Pour all the bag contents from side "2" back into the original sample cup (there may be small pieces of shellfish remaining on side "1" of the bag). Discard the used extraction bag.
- Cap and shake the sample cup vigorously by hand for 30 seconds (a cloudy appearance or bubbles may form, which does not affect the running of the test).
- Remove 100 µl of the sample extract using a disposable pipette* provided (or alternatively by use of a standard pipette) and add into an ASP buffer vial.
    - *To use the disposable pipettes, firmly press the top bulb of the pipette, insert the tip into the solution; slowly release the top bulb to draw up the sample extract. Excess volume (e.g., more than 100 µl) will overflow into the lower bulb, ensuring 100 µl is ready to dispense. Press the top bulb firmly and release slowly to dispense. Discard the used pipette.

5.2.7. Test Procedure

- Remove the appropriate number of microwells and place into the microwell holder.
- Shake the ASP buffer vial (containing diluted sample) vigorously by hand for 30 seconds.
- Immediately transfer 100 µl of diluted sample into each microwell using a new disposable pipette
- Remove the required number of test strips from the lateral flow device container and immediately close the container tightly.
- Place the ASP test strips with the sample end down (Neogen® logo on top) into the microwells.
- Allow the strip to develop in the microwell for 10 minutes.
- Immediately remove the test strip and read using the AccuScan® Pro reader (as described below)

5.2.8. Reading Test Results

- Test strips should be read within 1 minute of completion of the 10-minute incubation. Refer to AccuScan® Pro Reader Set Up for test selection and set up information.
- Fully insert the Reveal® 2.0 ASP test strip into the black cartridge adapter with the sample end first and results facing out.
- Insert the cartridge with test strip side up in the AccuScan® Pro. The reader will automatically begin analysing the cartridge. CAUTION: Removing cartridge prior to completion can result in invalid readings.
- The AccuScan® Pro reader will analyse the test strip and results will be displayed and stored in the reader.
- The reader will report positive with a result of 20 µg/g DA or greater. Any result of less than 20 µg/g will be reported as negative.

Notes:

- Ensure device is fully inserted into cartridge.
- The strips must be read using the Neogen AccuScan® Pro reader.

5.3. Experimental Procedure

In addition to assessing the reliability of these qualitative test kits to deliver accurate positive or negative results, the experimental procedure was designed to test the following parameters:

- **Accuracy/Trueness** – this is a measure of the agreement between the test result and the result obtained by the analytical laboratory, Symbio Laboratories, Sydney, Australia (Symbio). In other words, this is a measure of the trueness of the positive and negative test results obtained for each individual test conducted. This is measured across Tests 3 to 8 (Table 1).
- **Repeatability** – this is a measure of the agreement of the replicate tests carried out on the same sample on the same occasion by the same analyst (in this case 4 replicate tests per shellfish extract). This is measured across Tests 3 to 8 (Table 1).
- **Reproducibility** - this is typically a measure of agreement between tests carried out by different laboratories. In this case however, reproducibility is interpreted as follows:
    - **Reproducibility (Method)** – this is a measure of the reproducibility of the method by which 6 shellfish extracts are prepared from each of the spiked (or un-spiked) homogenates. This is measured across Tests 3 to 8 (Table 1).
    - **Reproducibility (Kits)** – this is a measure of the agreement between test kits by which tests are conducted using 4 different test kits on each of the 6 shellfish extracts. This is measured in Test #4 only (Table 1).

Approximately 1,000 g of fresh blue mussels (*Mytilus edulis*) were obtained from a commercial shellfish growing area in Cockburn Sound, Western Australia.

The shellfish were shucked, and the removed flesh was rinsed with deionised water and then allowed to drain over a sink. Approximately 500 g of the shellfish flesh was then homogenised into a puree using a NutriBullet Rx Blender. This was transferred to a clean plastic container and frozen (-40°C).

An additional 100 g of non-homogenised shellfish flesh was placed into clean sample cup and frozen (-40°C). This was sent to Symbio for the determination of DA concentration (for verification that there was no DA present in the shellfish flesh to be used for the study). This was referred to Test #1 (Table 1).

After verification that the shellfish flesh to be used contained no DA, the bulk homogenised shellfish sample was thawed and split between ten clean sample cups with approximately 50 g of shellfish homogenate per cup.

Where required, the homogenised shellfish samples were spiked with a certified reference material (CRM) obtained from the National Research Council, Canada- Institute for Marine Biosciences. This CRM (CRM-ASP-Mus-d) is a mussel tissue matrix containing DA (and some of its isomers, e.g., epi-DA). It is intended to be used to test the accuracy of entire analytical methods or to assist in the development of new analytical methods. CRM-ASP-Mus-d consists of a thermally stabilised homogenised liquid slurry of whole mussel tissue (*Mytilis edulis*). The mussel slurry comprised a combination of DA-free mussel tissue and mussel tissue naturally contaminated with DA such that the final DA concentration was 49 µg/g ± 3 µg/g. The CRM-ASP-Mus-d Certificate of Analysis can be obtained from the National Research Council, Canada website (https://nrc.canada.ca/en/certifications-evaluations-standards/certified-reference-materials/list/74/pdf/asp-mus-d-en.pdf).

Two of the homogenised shellfish samples were frozen and kept as either un-spiked controls or "spare" samples. Seven of the remaining eight homogenised shellfish samples were spiked with CRM-ASP-Mus-d according to the formula below to yield DA concentrations of 24 µg/g, 20 µg/g (x five preparations), 15 µg/g and 10 µg/g. The eighth homogenised shellfish sample was left unspiked.

The amount of CRM required for each preparation was determined using the following formula:

$$CRM = \frac{rw \times rc}{C}$$

where: CRM = the required weight of CRM required per preparation

rw = the required final weight of spiked flesh per preparation (g)
rc = the required final DA concentration of the spiked flesh preparation (µg/g)
C = the DA concentration of the CRM (49 µg/g ± 3 µg/g)

The amount of un-spiked shellfish homogenate required per preparation is obtained by subtracting the required amount of CRM (g) from the required final weight of spiked flesh per preparation (rw).

The CRM homogenate was provided in individual vials each containing 4.0 g ± 0.5 g.

The required amounts of both the shellfish homogenate and the CRM were weighed as accurately as possible into a clean sample cup and thoroughly mixed by placing the cup into a Vortex Mixer set to high speed for five minutes. The preparation weights for the spiked homogenates are recorded in Table 1 below.

Weighing out the exact required amounts of shellfish homogenate and CRM is virtually impossible given that one needs to be added to other. Spiked homogenates were prepared by first estimating the weight of CRM required for each preparation and then calculating the number of vials of CRM required (assuming 3.5 g of CRM per vial). The number of required vials was rounded up to the nearest whole number.

For each preparation, the slurry from each of the required CRM vials was emptied into a previously weighed sample cup. After all the required CRM was added, the sample cup was weighed, and the weight of CRM added was determined. From this, the required weight of homogenised shellfish for each preparation was determined as above.

This was added to the sample cup whilst still on the balance to an amount as close as possible to that required whilst attempting not to exceed the required amount.

One of the spiked shellfish homogenates prepared at 20 µg/g was divided into five 5 g subsamples, the remaining homogenate being frozen (-40°C). These 5 g subsamples were sent to Symbio for analysis of DA concentration. The purpose of this was to test the homogeneity of subsamples taken from the same spiked shellfish homogenate. This was referred to Test #2 (Table 1).

A 5 g subsample from each of the remaining spiked shellfish homogenates was sent to Symbio for analysis of DA concentration so that this could be verified against the desired DA concentration for each homogenate. A 5 g subsample from the unspiked homogenate was also sent to Symbio for analysis of DA concentration.

Details of the preparation of homogenates for each test are presented in Table 1 and a summary of the purpose of each test is presented below Table 1. The experimental setup for Test 3 and Tests 5 to 9 is presented in Figure 4. The experimental setup for Test 4 is presented in Figure 5.

The four separate preparations of spiked homogenate at a DA concentration of 20 µg/g were to be used for Tests 2 to 6 (Table 1). The reason that four separate spiked homogenates were prepared at this concentration is that 20 µg/g is the Regulatory Limit (RL) for DA in shellfish flesh and, as such, this was the most critical preparation and we wanted to maximise the likelihood of at least one preparation being at the RL. Given the uncertainties associated with preparation of the CRM material (±6%), the quantitative measurement of toxin concentration by Symbio Laboratories (±15%) and that associated with the preparation of the spiked homogenates (unquantified), of all the prepared concentrations, this was the one we needed to get as close to the desired toxin concentration as possible or at least prepare several batches to allow for some variation. If any of these preparations were deemed to have been unsuitable, they would not have been used.

Based on the results of Symbio analyses, the measured DA concentration of these preparations were 18 µg/g to 22 µg/g for Test #2 and 20 µg/g, 23 µg/g and 21 µg/g for Test 4, Test 5, and Test 6 respectively (Table 1). Any unused homogenate, spiked or unspiked, was frozen (-40°C).

Table 1: *Tests and spiked homogenate preparations together with calculated and actual DA concentrations obtained. Note: the ± percentage for the calculated DA concentration takes into account the measurement uncertainty (MU) of the CRM only (49 µg/g ± 3 µg/g = 49 µg/g ± 6%) and that for the Symbio results has a measurement uncertainty of ± 15% (personal communication from Symbio).*

	DA Concentration	Required Weights for Preparation			Actual Weights Added to Preparation			DA Concentration					
TEST	Required	Spiked Homogenate	CRM	Homogenised Flesh	CRM	Homogenised Flesh	Spiked Homogenate	Calculated	Range (± 6%)		Measured (Symbio)	Range (± 15%)	
TEST 1	100 g of non-homogenised shellfish flesh										<1	NA	
TEST 2	20	70	28.5714	41.4286	29.2187	37.0993	66.318	21.6	20.3	22.9	18	15.3	20.7
											19	16.15	21.85
											20	17	23
											22	18.7	25.3
											20	17	23
TEST 3	24	50	24.4898	25.5102	25.2984	26.1221	51.4205	24.1	22.7	25.6	24	20.4	27.6
TEST 4	20	50	20.4082	29.5918	21.3525	31.093	52.4455	19.9	18.8	21.1	20	17	23
TEST 5	20	50	20.4082	29.5918	22.3309	32.1744	54.5053	20.1	18.9	21.3	23	19.55	26.45
TEST 6	20	50	20.4082	29.5918	21.8617	31.2277	53.0894	20.2	19.0	21.4	21	17.85	24.15
TEST 7	15	50	15.3061	34.6939	18.502	41.4752	59.9772	15.1	14.2	16.0	15	12.75	17.25
TEST 8	10	50	10.2041	39.7959	10.9354	42.4752	53.4106	10.0	9.4	10.6	10	8.5	11.5
TEST 9	0	50 g of homogenised shellfish flesh									<1	NA	
TEST 10	NA	8 g of CRM homogenate									51	43.35	58.65

The purpose of each of the Test conducted was as follows:

- **TEST #1**: A 100 g sample of freshly collected shellfish flesh provided to Symbio Laboratories.
    - **The purpose of this test was to determine if the wild-harvested shellfish supplied contained any DA.**

- **TEST #2**: Five 5 g samples of spiked shellfish homogenate (all obtained from the same 20 µg/g preparation) sent to Symbio Laboratories.
    - **The purpose of this test was to assess the homogeneity of the spiked shellfish homogenates and determine whether any sub-sampling errors were likely to occur.**

- **TEST #3**: A 50 g sample of shellfish homogenate spiked to 24 µg/g (actual = 24 µg/g ± 15%) for testing with Neogen test kits.
    - Six shellfish extracts prepared from the same spiked homogenate.
        - Four replicate tests conducted using each shellfish extract (see Figure 2).
    - **The purpose of this test was to assess the accuracy/trueness and repeatability of the test kits at the given toxin concentration.**
    - **The reproducibility, as it relates to the preparation method, was also assessed with the preparation of six shellfish extracts.**

- **TEST #4**: A 50 g sample of shellfish homogenate spiked to 20 µg/g (actual = 20 µg/g ± 15%) for testing with Neogen test kits.
    - Six shellfish extracts prepared from the same spiked homogenate.
        - Four replicate tests conducted using each shellfish extract for each of four test kits (see Figure 3).
    - **The purpose of this test was to assess the accuracy/trueness and repeatability of the test kits at the given toxin concentration.**
    - **The reproducibility, as it relates to the preparation method, was also assessed with the preparation of six shellfish extracts.**
    - **The reproducibility, as it relates to results obtained for the same extracts by different test kits, was also assessed with the use of four different test kits per prepared extract.**

- **TEST #5**: A 50 g sample of shellfish homogenate spiked to 20 µg/g (actual = 23 µg/g ± 15%) for testing with Neogen test kits.
    - Six shellfish extracts prepared from the same spiked homogenate.
        - Four replicate tests conducted using each shellfish extract (see Figure 2).
    - **The purpose of this test was to assess the accuracy/trueness and repeatability of the test kits at the given toxin concentration.**
    - **The reproducibility, as it relates to the preparation method, was also assessed with the preparation of six shellfish extracts.**

- **TEST #6**: A 50 g sample of shellfish homogenate spiked to 20 µg/g (actual = 21 µg/g ± 15%) for testing with Neogen test kits.
    - Six shellfish extracts prepared from the same spiked homogenate.
        - Four replicate tests conducted using each shellfish extract (see Figure 2).
    - **The purpose of this test was to assess the accuracy/trueness and repeatability of the test kits at the given toxin concentration.**
    - **The reproducibility, as it relates to the preparation method, was also assessed with the preparation of six shellfish extracts.**

- **TEST #7**: A 50 g sample of shellfish homogenate spiked to 15 µg/g (actual = 15 µg/g ± 15%) for testing with Neogen test kits.
    - Six shellfish extracts prepared from the same spiked homogenate.
        - Four replicate tests conducted using each shellfish extract (see Figure 2).
    - **The purpose of this test was to assess the accuracy/trueness and repeatability of the test kits at the given toxin concentration.**
    - **The reproducibility, as it relates to the preparation method, was also assessed with the preparation of six shellfish extracts.**

- **TEST #8**: A 50 g sample of shellfish homogenate spiked to 10 µg/g (actual = 10 µg/g ± 15%) for testing with Neogen test kits.
    - Six shellfish extracts prepared from the same spiked homogenate.
        - Four replicate tests conducted using each shellfish extract (see Figure 2).
    - **The purpose of this test was to assess the accuracy/trueness and repeatability of the test kits at the given toxin concentration.**
    - **The reproducibility, as it relates to the preparation method, was also assessed with the preparation of six shellfish extracts.**

- **TEST #9**: A 50 g sample of unspiked shellfish homogenate for testing with Neogen test kits.
    - Six shellfish extracts prepared from the same spiked homogenate.
        - Four replicate tests conducted using each shellfish extract (see Figure 2).
    - **The purpose of this test was to assess the accuracy/trueness and repeatability of the test kits at the given toxin concentration.**
    - **The reproducibility, as it relates to the preparation method, was also assessed with the preparation of six shellfish extracts.**

- **TEST #10**: An 8 g sample of CRM homogenate provided to Symbio Laboratories to verify the DA concentration of the CRM (49 µg/g ± 6%, actual = 51 µg/g ± 15%).
    - **The purpose of this test was to verify the concentration of the CRM homogenate.**

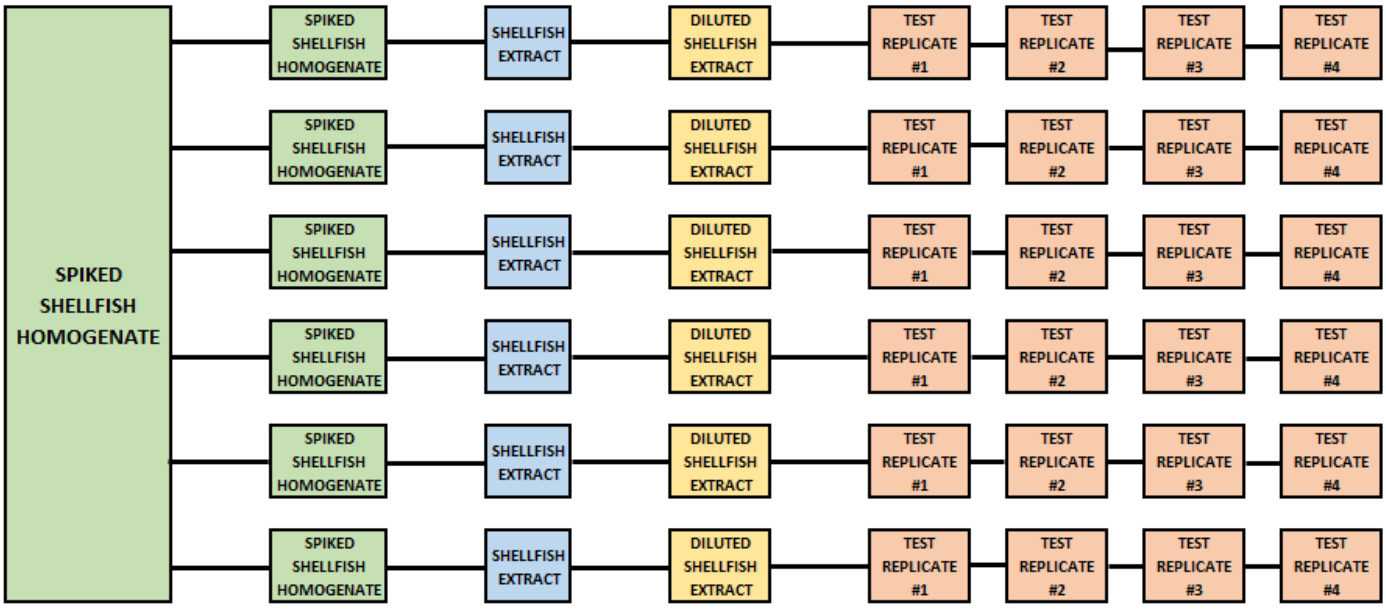

Figure 4: *Experimental Setup used for Test #3, Test #5, Test #6, Test #7, Test #8, and Test #9. Six 1 g samples of the spiked shellfish homogenate were used to prepare six replicate shellfish extracts (and subsequently diluted shellfish extracts). Four replicate tests were conducted on each of the diluted shellfish extracts.*

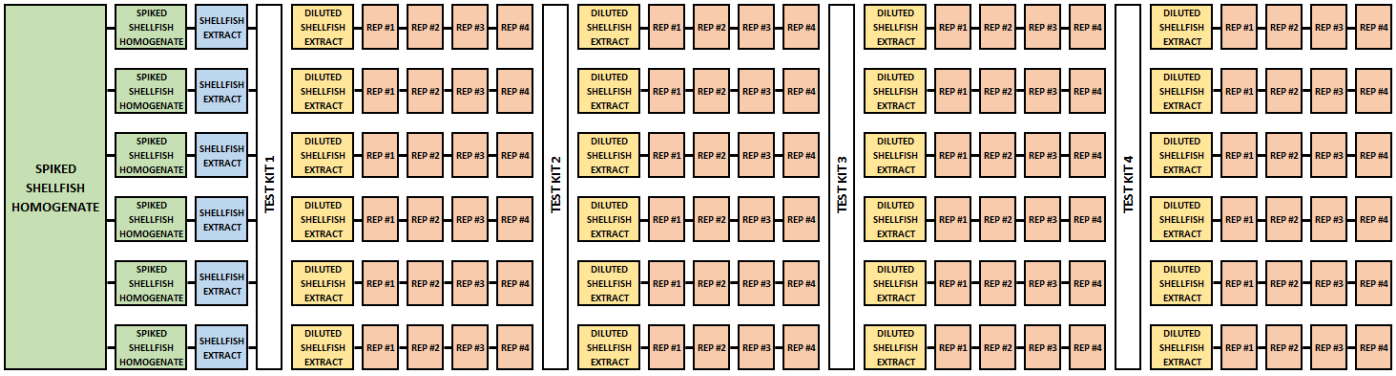

Figure 5: *Experimental Setup used for Test #4. Six 1 g samples of the spiked shellfish homogenate were used to prepare six replicate shellfish extracts (and subsequently diluted shellfish extracts). Four replicate tests were conducted on each of the diluted shellfish extracts – this was repeated across 4 separate test kits.*

5.3.1. Additional (unplanned) testing.

After the initial round of testing as described above, preliminary results were discussed with stakeholders and some members of the shellfish aquaculture industry were interested in whether these test kits could be used to test for a DA concentration at less than the regulatory limit of 20 µg/g, preferably at half of the regulatory limit (10 µg/g). The rationale behind this request was that, if these test kits were required to be used to provide management guidance due to an exceedance of cell density threshold values for *Pseudo-nitzschia* spp., additional confidence would be obtained if a reliable negative result could be achieved for a DA concentration less than the regulatory limit.

Although these test kits are designed specifically to provide a positive result at DA concentrations greater than or equal to the RL and a negative result for DA concentrations below the RL, the possibility that these test kits could be used to detect DA at lower concentrations was discussed with representatives of Neogen® (Daniel Speed, personal communication).

The response from Neogen® was to reiterate the intended use of these test kits and the positive/negative threshold value of 20 µg/g. We proposed the possibility that the test kits could be "forced" to provide a positive result at half of the RL (10 µg/g) if, during the preparation of the shellfish extract (see Section 5.2.6 above), twice the amount of shellfish flesh was used (2 g instead of 1 g) per 30 ml of distilled water or half the amount of distilled water was used (15 ml) with the recommended amount of shellfish flesh (1 g). Neogen® indicated that this had not been considered by them and could not be endorsed by them, it would be considered an "off-label" use of their product. They did indicate, however, that they would be interested in any results should we decide to investigate this option.

We decided to undertake preliminary testing to determine if the "forcing" of a positive result at a DA concentration of half of the RL (10 µg/g) was possible using the modified methods discussed above. For this we used some of the remaining spiked shellfish homogenate from Test 8 (spiked at 10 µg/g). Test 8.1 was conducted as per all other tests with the exception that 2.0 g of shellfish flesh (± 0.1 g) was used instead of 1.0 g (± 0.05 g), Test 8.2 was conducted as per all other tests with the exception that 15 ml of deionised water (± 0.25 ml) was used instead of 30 ml (± 0.5 ml).

We stress that these tests were preliminary and not a part of the original experimental design. These tests will not contribute to any discussion regarding the validation of these qualitative test kits as is the purpose of this study. We acknowledge that the impact of altering the ratios of shellfish homogenate, buffer solution and deionised water may impact the chemistry of the diluted shellfish extract (e.g., the pH) and comprise the test strip reaction. Similarly, we acknowledge that, for the preparation using twice the amount of shellfish extract, there may be increased interference or the introduction of matrix interference which did not exist with the other "normal" preparations. None of these considerations, or any others, were tested but would need to be should the possibility of "forcing" these test kits to yield a positive at half of the DA RL be something that the shellfish industry wishes to peruse.

6. Results

Results for each Test are presented in Table 2. Results for Test 1, Test 2 and Test 10 have already been presented in Table 1.

6.1. TEST 1

TEST 1 was a 100 g sample of freshly collected shellfish flesh provided to Symbio Laboratories to determine if the wild-harvested shellfish supplied contained any DA. The results obtained from Symbio indicated a DA concentration of <1 µg/g.

Based on this, it was concluded that the harvested shellfish flesh did not contain any DA and that this material was suitable for use in this study.

6.2. TEST 2

The purpose of Test 2 was to determine if sub-samples could reliably be taken from a prepared spiked shellfish homogenate and be representative of the spiked DA concentration. In other words, this was a test of homogeneity for the prepared spiked homogenates.

Five 5 g samples were taken from a single spiked homogenate (spiked to be 20 µg/g – refer to Table 1) and sent to Symbio for analysis. The results obtained were variable but, considering the 15% measurement uncertainty of Symbio results for this analysis, all were within the intended range of the spiked homogenate.

Based on these results, we concluded that the mixing of the prepared spiked shellfish homogenates was sufficient to ensure homogeneity and that sub-samples could be reliably taken.

Table 2: *Qualitative test results for Tests 3 to 9. The prepared DA concentration (µg/g) for each test is indicated (DA concentration as measured by Symbio Laboratories is presented in parentheses). The weight of spiked shellfish homogenate used per 30 ml of deionised water (15 ml of deionised water used for Test 8.2) for each shellfish extract is also indicated. Qualitative test results are indicated by either "+" for a positive result for DA or "-" for a negative result for DA.*

	DOMOIC ACID CONCENTRATION	SHELLFISH EXTRACT		RESULT			
		NUMBER	WEIGHT	REP 1	REP 2	REP 3	REP 4
TEST 3	24 (24)	1	1.0123	+	+	+	+
		2	1.0213	+	+	+	+
		3	1.0431	+	+	+	+
		4	1.0511	+	+	+	+
		5	1.0145	+	+	+	+
		6	1.0231	+	+	+	+

	DOMOIC ACID CONCENTRATION	SHELLFISH EXTRACT		RESULT				RESULT (KIT 2)				RESULT (KIT 3)				RESULT (KIT 4)			
		NUMBER	WEIGHT	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
TEST 4	20 (20)	1	1.0488	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
		2	1.0270	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		3	1.0376	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		4	1.0399	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		5	1.0100	-	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-
		6	1.0464	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

	DOMOIC ACID CONCENTRATION	SHELLFISH EXTRACT		RESULT			
		NUMBER	WEIGHT	REP 1	REP 2	REP 3	REP 4
TEST 5	20 (23)	1	1.0101	+	+	+	+
		2	1.0330	+	+	+	+
		3	1.0414	+	+	+	+
		4	0.9963	+	+	+	+
		5	1.0439	+	+	+	+
		6	1.0487	+	+	+	-

	DOMOIC ACID CONCENTRATION	SHELLFISH EXTRACT		RESULT			
		NUMBER	WEIGHT	REP 1	REP 2	REP 3	REP 4
TEST 6	20 (21)	1	0.9968	+	+	+	+
		2	1.0347	+	+	+	+
		3	1.0342	+	+	+	+
		4	1.0500	+	+	+	+
		5	1.0003	+	+	+	+
		6	0.9962	+	+	+	+

	DOMOIC ACID CONCENTRATION	SHELLFISH EXTRACT		RESULT			
		NUMBER	WEIGHT	REP 1	REP 2	REP 3	REP 4
TEST 7	15 (15)	1	1.0174	-	-	-	-
		2	1.0357	-	-	-	-
		3	0.9967	-	-	-	-
		4	1.0352	-	-	-	-
		5	1.0344	-	-	-	-
		6	1.0421	-	-	-	-

	DOMOIC ACID CONCENTRATION	SHELLFISH EXTRACT		RESULT			
		NUMBER	WEIGHT	REP 1	REP 2	REP 3	REP 4
TEST 8	10 (10)	1	2.0642	-	-	-	-
		2	2.0807	-	-	-	-
		3	2.0842	-	-	-	-
		4	2.0910	-	-	-	-
		5	2.0719	-	-	-	-
		6	2.0777	-	-	-	-

	DOMOIC ACID CONCENTRATION	SHELLFISH EXTRACT		RESULT			
		NUMBER	WEIGHT	REP 1	REP 2	REP 3	REP 4
TEST 8.1	10 (10)	1	2.0642	+	+	+	+
		2	2.0807	+	+	+	+
		3	2.0842	+	+	+	+
		4	2.0910	+	+	+	+
		5	2.0719	+	+	+	+
		6	2.0777	+	+	+	+

	DOMOIC ACID CONCENTRATION	SHELLFISH EXTRACT		RESULT			
		NUMBER	WEIGHT	REP 1	REP 2	REP 3	REP 4
TEST 8.2	10 (10)	1	1.0262	+	+	+	+
		2	1.0500	+	+	+	+
		3	1.0076	+	+	+	+
		4	0.9891	+	+	+	+
		5	1.0390	+	+	+	+
		6	1.0184	+	+	+	+

	DOMOIC ACID CONCENTRATION	SHELLFISH EXTRACT		RESULT			
		NUMBER	WEIGHT	REP 1	REP 2	REP 3	REP 4
TEST 9	0 (<1)	1	1.0262	-	-	-	-
		2	1.0500	-	-	-	-
		3	1.0076	-	-	-	-
		4	0.9891	-	-	-	-
		5	1.0390	-	-	-	-
		6	1.0184	-	-	-	-

6.3. TESTS 3 - 9

Tests 3 to 9 (Table 2) were designed to test the accuracy/trueness of the test results obtained by the Neogen test kits at DA concentrations of 24 µg/g (Test 3), 20 µg/g (Tests 4, 5 and 6), 15 µg/g (Test 7), 10 µg/g (Test 8) and 0 µg/g (Test 9).

The reproducibility of the method was also assessed by preparing six shellfish extracts from the same spiked shellfish homogenate for each concentration and the repeatability of the test procedure was assessed by preparing four replicate tests per shellfish extract.

6.3.1. Accuracy/Trueness

Accuracy/Trueness (henceforth referred to as trueness) is a measure of the agreement between the test result and the result obtained by the analytical laboratory (Symbio). In other words, this is a measure of the trueness of the positive and negative test results obtained for each individual test conducted.

6.3.1.1 Tests 3 – 6

Test 3 assessed the trueness of the Neogen test kits at a DA concentration of 24 µg/g, 20% greater than that which is expected to return a positive result (20 µg/g).

A total of 24 tests were conducted comprising 4 replicate tests for each of 6 shellfish extracts. All 24 tests returned a positive result representing a 100% result for trueness, at a DA concentration of 24 µg/g (Table 2).

Tests 4, 5 and 6 were intended to assess the trueness of the Neogen test kits at a DA concentration equal to that which is expected to return a positive result (20 µg/g).

For Test 4, a total of 96 tests were conducted comprising 16 replicate tests for each of 6 shellfish extracts. The 16 replicate tests for each shellfish extract were split between 4 different test kits (4 replicates per extract per kit). The purpose of testing the shellfish extracts using 4 different test kits was to assess the reproducibility of results between test kits. This homogenate, with a DA concentration of 20 µg/g as measured by LC-MS/MS analysis, was chosen for this extended test because 20 µg/g is the upper level of the range of DA concentrations (17.5 µg/g - 20 µg/g) for which the Reveal 2.0 ASP method will signal “partial” results - a mix of negative and positive results (although the majority would be expected to be positive) (Caballero *et. al*., 2013).

Of the 96 tests conducted, 89 returned a positive result (Table 2).

Replicate 4 from shellfish extract 1 for test kit 3 returned a negative result (Table 2). It was noted at the time of undertaking these tests that the first attempt to insert the test strip into the cartridge took more effort than was typical and the strip had to be firmly pushed into place. When the cartridge was inserted into the reader an error message was displayed - “Test failed (code 43): Multiple control lines found”. The cartridge was removed from the reader and the test strip was removed from the cartridge and examined. To the naked eye, there did not appear to be multiple control lines on the strip, and it did not appear to be damaged. The strip was re-inserted into the cartridge (with no difficulty) and the cartridge was inserted into the reader. A negative result was returned.

The difficulty sometimes encountered whilst inserting test strips into the cartridge (for no apparent reason) and the resulting error message when the test strip is read is a known issue with this method and test strip reader (Neogen Corporation, personal communication). This will be discussed further in Section 6.7 and Section 7.1.

Given this, rather than be considered a non-conforming result, this individual result is considered to be an erroneous result.

All 4 replicates for shellfish extract 5 for test kit 1 and replicates 3 and 4 for shellfish extract 5 for test kit 4 returned negative results (Table 2). Given that all 4 of the negative results for test kit 1 and the 2 negative results for test kit 4 were from the same shellfish extract, these are considered non-conformances.

It is likely that the DA concentration of shellfish extract 5 fell within the uncertainty range of these test kits (the range where "partial" results are expected). This will be discussed further in Section 7.1.

Ignoring the single erroneous result recorded for test kit 3, 89 out of 95 tests returned a positive result, representing a 93.7% result for trueness, at a DA concentration of 20 µg/g (Table 2).

For Test 5, a total of 24 tests were conducted comprising 4 replicate tests for each of 6 shellfish extracts. Of these 24 tests, 23 returned a positive result (Table 2).

Replicate test 4 from shellfish extract 6 returned a negative result. The same test strip issue as described above for test 4 was encountered for this test. For this reason, this test result will also be considered erroneous.

Ignoring this erroneous result from replicate 4 of shellfish extract 1, these results indicate a 100% result for trueness across 23 tests representing a 100% result for trueness, at a DA concentration of 20 µg/g.

For Test 6, a total of 24 tests were conducted comprising 4 replicate tests for each of 6 shellfish extracts. All 24 tests returned a positive result (Table 2), a 100% result for trueness at a DA concentration of 20 µg/g.

For all non-erroneous tests conducted at a DA concentration of 20 µg/g (Tests 4 to 6, 143 tests), 6 returned non-conforming negative results representing a 95.8% result for trueness.

For all non-erroneous tests conducted at a DA concentration equal to or greater than that for which the Reveal® 2.0 ASP method will signal a positive result (20 µg/g) (Tests 3 to 6, 167 tests), the trueness was 96.4% (161 out of 167 tests returning a positive result).

6.3.1.2 Tests 7 – 9

All 72 tests conducted at a DA concentration of 15 µg/g or less returned a negative result (Tests 8.1 and 8.2 were not included in this) (Table 2). This indicates 100% trueness for all tests (72 out of 72 tests returning a negative result).

6.3.2. Repeatability

Repeatability is a measure of the agreement of the replicate tests carried out on the same sample on the same occasion by the same analyst (in this case 4 replicate tests per shellfish extract per test kit).

Seventy-two sets of 4 replicate tests were conducted across tests 3 to 9 (Table 2). Of these, 69 showed 100% agreement between all 4 replicates. Two of the replicate sets which did not show 100% agreement were those which included results for one of the four replicate tests considered to be erroneous as described above. Ignoring these erroneous results, both these replicate sets showed 100% agreement.

Therefore, 71 out of the 72 replicate sets showed 100% agreement.

As described above, replicates 3 and 4 from shellfish extract 5 for test kit 4 (as part of test 4) returned negative results with replicates 1 and 2 returning positive results (Table 2). These are considered to be non-conformances most likely due to the DA concentration of shellfish extract 5 being within the uncertainty range of these test kits.

These 4 replicates were all taken from the same shellfish extract. It is only during the test procedure where the diluted shellfish extract is added to each of 4 microwells (Section 5.2.7, third bullet point) that these are treated as separate samples to be tested. Whilst we acknowledge that errors in methodology are always possible and should not be ruled out, we are confident that; 1) the same amount (100 µl) of diluted shellfish extract was added to each of the 4 microwells; 2) the test strips used were in good condition and not damaged; 3) the test strips were placed correctly into the microwells and incubated for the correct length of time; 4) the test strips were correctly inserted into the test strip cartridge and analysed by the reader; and 5) all 4 test strips were analysed within the recommended 1 minute window.

The manufacturers of these test kits have undertaken a thorough review of the sample preparation process and, whilst they do consider that it is possible to easily "miss-prep the testing samples" (Neogen Corporation, personal communication), we have no reason to believe, or evidence to suggest, that an error in methodology was responsible for the lack of agreement between the replicate tests. In our opinion, this lends additional support to the hypothesis that the DA concentration of the shellfish extract used was "borderline" with respect to the sensitivity of the test kits.

6.3.3. Reproducibility (Method)

As it relates to the method by which the shellfish extracts are prepared, reproducibility is a measure of the agreement between tests carried out on different extracts prepared from the same shellfish homogenate and tested using the same test kit. Total (100%) reproducibility is achieved when the same test result is obtained for all 24 tests (4 replicates x 6 shellfish extracts) for the same test kit at a given DA concentration.

In total, across all 9 tests, 54 shellfish extracts were prepared. Although only 6 shellfish extracts were prepared for Test 4, for this measure, this test can be considered 4 tests in one. Considering this, there were 54 shellfish extracts prepared across 12 tests (Table 2).

Agreement between shellfish extracts was 100% for all tests except Tests 4.1 and 4.4 (the single erroneous negative test results for Test 4.3 and Test 5 have been ignored as discussed above).

As has already been stated, the negative results returned for Tests 4.1 and 4.4 (shellfish extract 5 in both tests) are considered non-conformances. Taking this into account, there was 100% agreement with respect to reproducibility of method for 83.3% of tests (10 out of 12 tests).

6.3.4. Reproducibility (Kits)

Reproducibility with respect to test kits is a measure of the agreement between different test kits by which tests are conducted using 4 different test kits on each of the 6 shellfish extracts. This is measured in Test 4 only.

Again, ignoring the single erroneous negative result returned for Test 4.3, there was agreement between all test kits for 5 (83.3%) out of the 6 prepared shellfish extracts.

The DA concentration of shellfish extract 5, as has been discussed, is likely to have been within the uncertainty range of these test kits (the range where "partial" results are expected). There was agreement with respect to this shellfish extract for Test 4.2 and 4.3 but there were some negative results returned for Test 4.1 and 4.4. This suggests that, at least with respect to shellfish extracts with "borderline" DA concentrations, there may be some variability of test results between kits. This may be confounded by small variations in the volume of the supplied vials of ASP buffer which have a dispensing tolerance of 1.25 g – 1.35 g (~ 1.3 g ± 5%). Vials with a volume of ASP buffer at the upper end of the dispensing tolerance will result in a more dilute shellfish extract than those prepared with a vial of ASP buffer at the lower end of the dispensing tolerance (Neogen Corporation, personal communication).

6.4. TEST 10

Test 10 was designed to assess the Domoic Acid concentration of the CRM.

The CRM used (CRM-ASP-Mus-d) has a certified Domoic Acid concentration of 49 µg/g ± 3 µg/g. Results of analysis by Symbio Laboratories reported a Domoic Acid concentration of 51 ug/g. Although higher than the certified concentration, the result is acceptable taking into account the uncertainties associated with both the CRM (± 6%) and the Symbio result (± 15%).

6.5. TESTS 8.1 and 8.2

As outlined in Section 4.3.1, although the test results from these tests were used in conjunction with the other tests to assess the parameters in Section 5.3, tests 8.1 and 8.2 were not a part of the initial design of this study. These tests were an afterthought after discussions with stakeholders and some members of the shellfish aquaculture industry to determine if the "forcing" of a positive result at a DA concentration of half of the RL (10 µg/g) was possible using a modified method.

Test 8.1 was designed to test whether or not the Neogen test kits could be "forced" to report a positive result at a Domoic Acid concentration of half of the regulatory limit (10 µg/g) by using twice the recommended weight of shellfish flesh (spiked shellfish homogenate) in the preparation of the shellfish extract.

Test 8.2 was designed to test whether or not the Neogen test kits could be "forced" to report a positive result at a Domoic Acid concentration of half of the regulatory limit (10 µg/g) by using half the recommended volume of deionised water in the preparation of the shellfish extract.

Albeit preliminary in nature, the results of both these tests suggest that this may be possible, with all 48 tests returning a positive result. This has been discussed in Section 5.3.1.

6.6. Probability of Detection

Probability of Detection (POD) refers to a statistical model used in the validation of qualitative methods and produces a graphical representation of the response curve for the method being validated (Wehling *et. al.*, 2011).

Probability of Detection itself is quite a simple metric being the ratio of the number of positive tests to the total number of tests for, in this case, a given toxin concentration as follows:

$$\boldsymbol{POD = \frac{x}{N}}$$

where: x = the number of positive test results

N = the number of tests conducted

POD values for the tests conducted in this study are presented in Table 3.

A POD curve is the graphical representation of the POD (y axis) plotted against the toxin concentration (x axis). A POD curve for the tests conducted in this study are presented in Figure 6. The 95% confidence intervals for the POD are also plotted on the POD curve. The 95% confidence intervals for the POD were calculated according to Wehling *et. al*., (2011) and are presented in Table 3.

The data presented in Table 3 and the resulting POD curve presented in Figure 6 have been calculated assuming that the unexpected negative result obtained for Test 5 was indeed an erroneous result rather than a non-conformance as has been discussed. For comparison, Table 3 and Figure 6 have been reproduced in Appendix 2 and Appendix 3 respectively, with the erroneous negative result included.

Table 3: *Data for all tests conducted with the calculated POD, LCL and UCL.*

DA Concentration (ug/g)	Number of samples tested (N)	Number of positive tests (x)	Probability of Detection (POD)	95% Confidence Interval	
				Lower Confidence Limit (LCL)	Upper Confidence Limit (UCL)
0	24	0	0	0	0.13797748
10	24	0	0	0	0.13797748
15	24	0	0	0	0.13797748
20	96	89	0.927083333	0.857067924	0.964232838
21	24	24	1	0.86202252	1
23	23	23	1	0.86202252	1
24	24	24	1	0.86202252	1

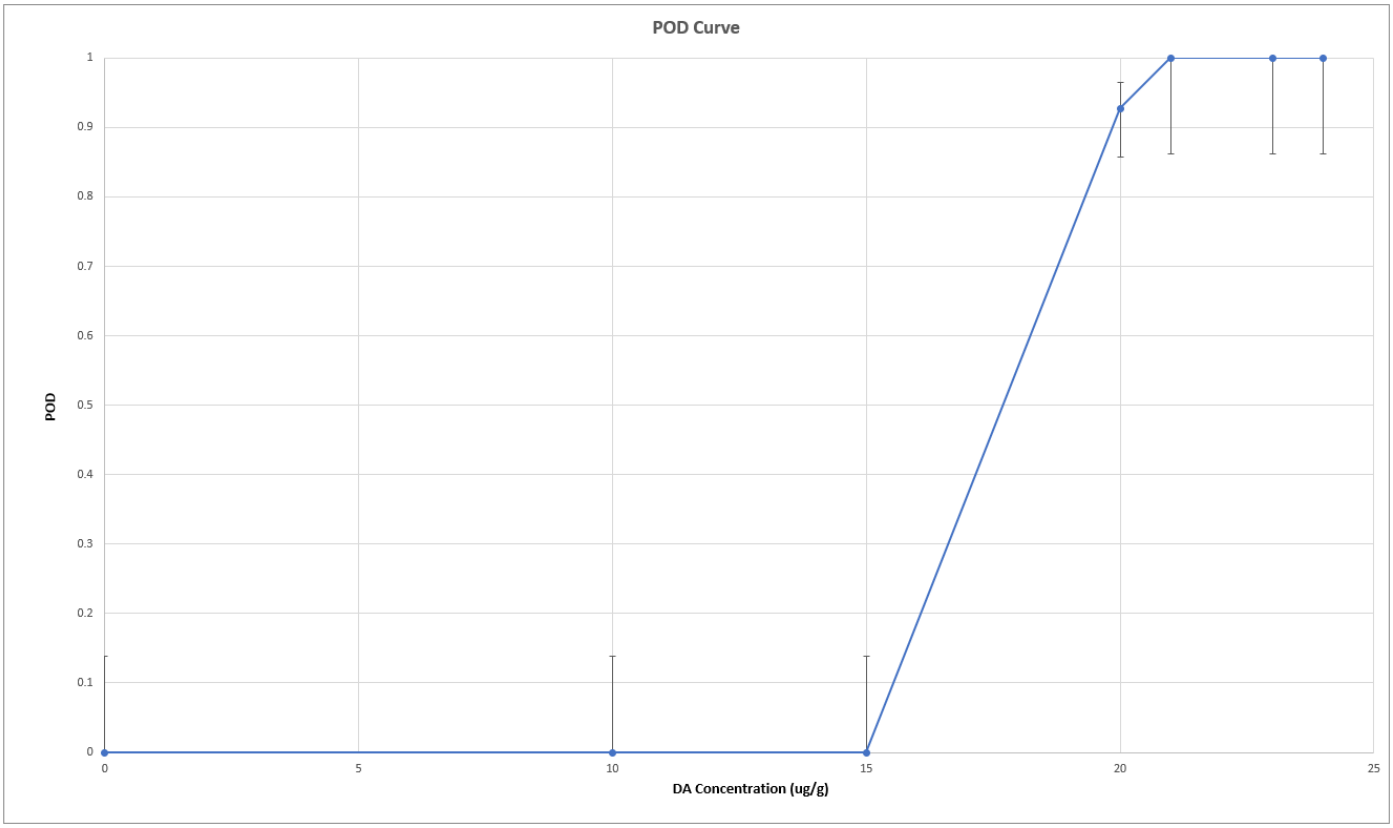

Figure 6: *Probability of Detection curve with the 95% Confidence Intervals represented by error bars.*

6.7. Comments on methodology (post-study)

Having conducted the study and prepared and analysed a significant number of samples, we found the methodology to be very simple and we encountered no difficulties or problems in following or undertaking the procedure.

The only issues we did encounter were with the reading of test results. Although Caballero *et. al*. (2013) demonstrated that the recommended 1-minute post incubation time frame for the reading of test strips did not have to be adhered to (see Section 7.3), we wanted to conduct our testing within the parameters set out in the kit instructions. This meant that we had to carefully manage and stagger our tests. We decided to attempt to read no more than 4 test strips in one minute which was (just) achievable and, fortunately, coincided with the number of replicates we were conducting per test. This is more of a consideration worth taking into account than an actual problem with the process, but it is nonetheless worth mentioning if the kit instructions are to be followed to the letter.

The second issue however is more significant. The process of inserting the test strips into the cartridge adapter can be a bit "fiddly", particularly if the user is under pressure to be within the 1-minute window for analysis. We found that, even when significant care is taken, the strips can sometimes take a bit of "force" to insert into the cartridge.

In both cases, the strip reader recorded an "error" message when the cartridge was inserted for analysis. The cartridge was immediately removed, and the test strip was removed from the cartridge and inserted again with the cartridge once again being inserted into the reader. There were no error messages recorded but, in each case, the strip reader gave a negative result.

Upon removal from the reader and the cartridge, the test strips were carefully examined for any evidence of damage and none could be found. We feel that, although in each case there was no evidence of damage to the strips, this may have resulted in the erroneous readings by the scanner.

This issue has been acknowledged by the product manufacturer (personal communication) and they have since released a new version of the reader, the AccuScan Raptor® Solo, for which both the incubation and reading of test strips occurs within the reader itself using a different type of cartridge to hold the strips and eliminates the problem above.

As this error pertains to the single negative results obtained for Test 4.3 (Section 5.4.3) and Test 5 (Section 5.5), as has been discussed previously, these will be considered to be erroneous results and will be excluded from any interpretation.

7. Discussion

7.1. Test Performance

Test 4.1 (Section 5.4.1, Table 3) yielded 4 unexpected negative results for all of the replicate test conducted on the diluted shellfish extract prepared from shellfish extract 5. Two unexpected negative results were also recorded for replicates prepared using the same shellfish extract analysed with a different test kit (Test 4.4, Section 5.4.4, Table 3).

As has been discussed previously, it is most likely that the concentration of DA within this shellfish extract fell within the uncertainty range of these test kits, a range where either positive or negative results could be obtained for a given test with the probability of a positive result increasing as the DA concentration nears 20 µg /g. Caballero *et. al*. (2013) state that the Reveal 2.0 ASP was designed to signal 100% negative at 0 µg /g and 10 µg /g, partials at 17.5 µg /g (the majority will signal as positive) and 100% positives at 20 µg /g DA and above.

Shellfish extract 5 was prepared using 1.0100 g of spiked homogenate, this was the smallest amount of homogenate used to prepare any of the six shellfish extracts by between 2% and 4%. Although the amount of shellfish homogenate used to prepare the shellfish extract was within the range specified by the Neogen method (1.0 g ± 0.05 g), it is likley that, taking into account the ± 6% uncertainty of the CRM used, the DA concentration in this extract was less than 20 µg/g and within the range of DA concentrations expected to yield "partial" results as above.

Comments provided by the manufacturer (Fiona Campbell, personal communication) also suggest that the volume of buffer solution in the tubes provided with the kits (dispensing tolerance = 1.25 g to 1.35 g) may have been greater in in Kit 1 and Kit 4 (to a lesser extent in Kit 4) which would have resulted in an increased dilution of shellfish extract 5.

The POD Curve for this study (Figure 6) shows a very steep increase in the POD for DA concentrations between 15 µg/g and 20 µg/g. Reading off the curve, it appears that the POD expected for a DA concentration of 17.5 µg/g (the concentration above which "partial" results can be expected as above) is less than 50% (~45%). Although not indicated on the POD curve, the 95% confidence interval at a DA concentration 17.5 µg/g would most likely be around ±15% suggesting an LCL POD 38.2% and an UCL POD of 51.2%. A POD of 50% or slightly more would be supportive of the results for shellfish extract 5 with 10 out of 16 tests returning positive results.

Although this may seem to be a significant issue, it is within the realm of the specifications of these test kits. Domoic Acid concentrations in wild shellfish samples analysed as part of biotoxin management programs which are between 17.5 µg/g and 20 µg/g will always be somewhat problematic with respect to these test kits but also with respect to laboratory analysis obtained via Liquid Chromatography coupled with Mass Spectrometry (LC-MS/MS analysis). LC-MS/MS analyses for DA have an inherent uncertainty of ±15% (Symbio Laboratories, personal communication) which means that a result reported as 20 µg/g using LC-MS/MS analysis could actually be as low as 17 µg/g or as high as 23 µg/g and a result of 17.4 µg/g could be as high as 20 µg/g.

What is certain, based on the results of this study, is that negative results will consistently be obtained for shellfish flesh samples with DA concentrations of 15 µg/g or less and positive results will consistently be obtained for DA concentrations of 24 µg/g or above.

There were also unexpected negative results returned for two other tests – replicate 4 from shellfish extract 1 in Test 4.3 (Section 5.4.3, Table 3) and replicate 4 from shellfish extract 6 in Test 5 (Section 5.5, Table 4). This has previously

been discussed in Section 6.7 and these negative results are considered to be erroneous results due to a known issue involving the insertion of test strips into the test strip cartridge rather than non-conformances.

Tests 8.1 and 8.2 were both designed to determine whether or not the Neogen ASP test kits could be "forced" to return a positive result for a Domoic Acid concentration half that of the regulatory limit (20 µg/g) by effectively preparing a shellfish extract which is twice the concentration of what is normally prepared with respect to the amount of shellfish flesh used. This "twice concentrated" shellfish extract was prepared by either doubling the amount of shellfish flesh used (Test 8.1) or halving the amount of deionised water used (Test 8.2) in the preparation of the shellfish extract.

Although the results of these two tests indicated that this "forcing" was possible, at this stage we consider the results to be preliminary and would like to conduct a more extensive testing regime in order to be more confident. At the time of writing this report, the shellfish industry has not been supportive of further testing. This was discussed previously in Section 5.3.1.

7.2. Replication of Tests

The results of this study demonstrate a high degree of confidence that these kits are fit for purpose. The very limited number of erroneous results were due to a known problem with the AccuScan Pro reader and should be resolved with the new and improved Raptor® Solo together with the slightly modified method.

We used test replication in this study and we feel that the reliability of these test kits is such that there is no need to use replication in the routine use of these test kits. However, replication is a valuable practice to adopt since it can identify any potential inconsistencies or errors associated with the reading of test strips if and when they occur (as was the case in this study).

The decision whether or not to use replication should come down to the individual user (or organisation) as it does increase the cost per test.

7.3. Useability of test kits

The Neogen Reveal® 2.0 for ASP test kits were found to be very easy to use. The equipment and consumables required to conduct the tests are either supplied with the test kits or are easily purchased from a reputable supplier of scientific equipment and consumables. The skill level required to use these test kits is very basic such that these test kits could readily be used on site although care does need to be taken to ensure that electronic equipment (e.g. balances) and other critical equipment (e.g. manual pipettors) are properly maintained and all consumables used are clean and fit for purpose.

The new Raptor® Solo and the slightly modified procedure makes these test kits even simpler to use.

7.4. Affordability

At the time of writing this report, the cost of the Neogen Reveal® 2.0 for ASP test kits was approximately $700 (ex GST). Each Test kit is capable of conducting 24 individual tests making the cost per test approximately $30 (ex GST).

The total time required to perform a single test is around 30 minutes (including shucking of shellfish and incubation of test strips). This needs to be taken into account if testing is not conducted in-house but the associated cost would not be high.

The cost of the new Raptor Solo reader is about $4,000 (ex GST) and is a necessary purchase.

Other equipment and consumables required to be purchased is as follows (prices are ex GST):

- Electronic Balance (capable of weighing 0.5 – 400 g ± 0.1 g) From ~ $800
- Blender/Homogeniser From ~ $200

- Marine biotoxins starter kit (Neogen item 9563) ~ $150
- Consumables (for ~ 200 tests), glassware etc. ~ $300

Other equipment which is recommended but not required is as follows (prices are ex GST):

- Variable or fixed volume manual pipettor capable of delivering 100 µL From ~ $150
- Vortex mixer and suitable holder(s) From ~ $500

Taking into account a set-up cost for equipment of around $5,800 (ex GST), the cost per test of $30 (ex GST) and around $5 (ex GST) of consumables per test, the total cost per test, amortised over 200 tests, would be about $65 (ex GST) assuming that a shellfish grower/harvester were to be undertaking the analysis themselves.

However, if these test kits were to be used by an independent laboratory on behalf of the shellfish aquaculture industry, the cost per test, based on a single test, could be anywhere between $100 (ex GST) and $200 (ex GST) depending upon the hourly charge out rate of the laboratory. In calculating this, we have assumed that 30 minutes is required to conduct a single test (including setup and clean-up), it is likely that the cost per test would reduce with a greater number of tests to be conducted on a given occasion.

The shelf life of the test kits is typically 12 months from the date of manufacture but Neogen Australia orders test kits in batches so it is likely that the shelf life of test kits purchased could be considerably less than 12 months. This needs to be taken into account when planning a testing regime as unused tests or test kits would be discarded once they reach the end of their shelf life.

The AccuScan reader (AccuScan Pro or AccuScan Raptor® Solo) will not read test strips from kits that have expired and whilst they can be forced to do so, this is not recommended.

8. What wasn't considered in this study

This study was designed to be very specific to meet the immediate requirements of the Western Australian shellfish aquaculture industry. As such there were several things that were not investigated that may have been a part of a larger, broader study designed to incorporate some of the following considerations.

All of these however, have been given consideration in another comprehensive single-laboratory validation study conducted by Neogen (Caballero *et. al*., 2013).

8.1. Shellfish Matrix

Caballero *et. al*. (2013) tested the accuracy of the test kits at Domoic Acid concentrations of 0 ug/g (e.g. un-spiked), 10 µg/g, 17.5 µg/g and 20 µg/g using pacific oysters (*Crassostrea gigas*), cherrystone clams (*Mercenaria mercenaria*) and "common" mussels (*Mytilus edulis*).

For oysters, accuracy of the assay for 0 µg/g, 10 µg/g, and 20 µg/g DA was 100%. All tests at 10 µg/g DA were negative, and all tests at 17.5 µg/g and 20 µg/g DA were positive. There were no false-positive results on un-spiked control samples.

For clams, accuracy of the assay for 0 µg/g, 10 µg/g, and 20 µg/g DA was 100%. All tests at 10 µg/g DA were negative. Four of twenty tests at 17.5 µg/g DA were negative and all 20 µg/g DA tests were positive. There were no false positive results on un-spiked control samples.

For mussels, accuracy of the assay for 0 µg/g, 10 µg/g, and 20 µg/g DA was 100%. All tests at 17.5 µg/g and 20 µg/g DA were positive. There were no false-positive results on un-spiked control samples.

Caballero *et. al*. (2013) conclude that, after excluding data from the 17.5 µg/g DA spike (presumably for the clam assay), overall accuracy of the Reveal 2.0 ASP test was 100%.

These results indicate very good agreement between different shellfish matrices.

8.2. Interfering Compounds

Caballero *et. al*. (2013) tested for the following interfering compounds at various concentrations – Oakadaic Acid (10 ug/g), Glutamic Acid (100 µg/g), Glutamine (100 µg/g) and Saxitoxin (5 µg/g). These were added to spiked shellfish homogenates (oysters, clams, and mussels) each at Domoic Acid concentrations of 0 µg/g, 10 µg/g, and 20 µg/g.

There was no evidence of interference by okadaic acid, glutamic acid, glutamine, or saxitoxin on assay performance in any of the three shellfish matrices. All tests produced expected results at Domoic Acid concentrations of 0 µg/g, 10 µg/g, and 20 µg/g.

8.3. Ruggedness

Caballero *et. al*. (2013) tested for effect of test strip incubation times on the test results for preparations using oysters, clams, and mussels at Domoic Acid concentrations of 0 µg/g, 10 µg/g, and 20 µg/g. Test strips were read after incubation times of 8 minutes, 10 minutes (the recommended incubation time) and 12 minutes.

All test results were as expected regardless of the incubation time.

Whilst this suggests that incubation time is not critical (within reason), we recommend that it should always be adhered to as closely as possible.

8.4. Robustness

Caballero *et. al*. (2013) tested for robustness of the testing procedure by having tests on the spiked homogenates (10 µg/g, 15 µg/g 17.5 µg/g and 20 µg/g) prepared from CRM-ASP-Mus-d (as used in this study) conducted by 3 different analysts on each of two consecutive days. Three replicate samples were analysed per test.

All test results were as expected with the exception of a single false negative recorded by one analyst for a sample spiked at 17.5 µg/g on the first day of analysis. All other samples spiked at 17.5 µg/g (17 across both days for all three analysts) recorded positive results.

In addition to this, the spiked homogenates were also tested using HPLC (testing laboratory not indicated). The HPLC results correlated well with expected concentrations of Domoic Acid but there was up to a 9.7% difference.

8.5. Comparison with Reference Method

Whilst the prepared shellfish homogenates for this study were sent to a laboratory for testing using the relevant reference method (LC-MS/MS analysis) this was more to confirm that the homogenates were prepared correctly and were within range of Domoic Acid concentrations required. However, there was very good agreement between the prepared concentrations, the results of LC-MS/MS analysis and the qualitative test kit results.

Caballero *et. al*. (2013) took this a step further and specifically tested the agreement between the results of the lateral flow qualitative test kits and the results of the relevant reference method (HPLC and LC-UV). To do this, they used naturally contaminated mussels and clams which had been analysed for Domoic Acid concentration using HPLC and LC-UV and tested four replicates of each batch using the qualitative test kits.

When compared to the reference method all results for the qualitative test kits were as would be expected. All samples containing between 1.9 µg/g and 12.6 µg/g of Domoic Acid gave negative results, all samples containing

between 28.2 µg/g and 30.6 µg/g of Domoic Acid gave positive results, and all samples containing between 16 µg/g and 17.6 µg/g of Domoic Acid gave a mix of positive and negative results.

These results indicate very good agreement with the reference method but again illustrate the importance of taking into account uncertainties when interpreting results as discussed in Section 6.1.

9. Conclusions

The results of this study indicate that the Neogen Reveal® 2.0 for ASP test kits are a very reliable qualitative test kit for the detection of Domoic Acid in shellfish (mussel) flesh. We would recommend that these test kits be approved for use with respect to management decisions for both the Western Australian and the Australian shellfish aquaculture industries.

Qualitative test kit results showed 100% agreement with the prepared Domoic Acid concentrations (as confirmed by LSMSMS analysis) in homogenates spiked at 24 µg/g, 15 µg/g, 10 µg/g and un-spiked homogenates.

Qualitative test kit results showed very good agreement with the prepared Domoic Acid concentrations (as confirmed by LC-MS/MS analysis) in homogenates spiked at 20 µg/g with 138 out of 144 (96%) tests recording the expected positive result.

The six non-conforming negative results obtained have been discussed and highlight the importance of taking into account the measurement uncertainty of analytical tests conducted to determine the Domoic Acid concentration in shellfish flesh.

Several factors were not considered in this study and have been addressed in Section 7.0. The main factor not considered in this study is how these test kits perform on different shellfish matrices but the results of Caballero *et. al*. (2013) discussed in Section 8.0 demonstrate that these kits are equally effective on at least three different shellfish matrices (mussels, oysters and clams).

The "forcing" of the Neogen qualitative test kits to return a positive result at a Domoic Acid concentration less than the regulatory limit also seems possible and reliable based on a preliminary analysis. This may be pursued further with the cooperation of Neogen Corporation if it is considered necessary by the shellfish aquaculture industry.

The assessment of Enzyme Linked Immunosorbent Assay (ELISA) quantitative test kits for this study was abandoned for several reasons (see Section 3.0) but may be re-visited at a later date if it seems likely that they may be approved for regulatory use within Australia and if there is still an industry need.

10. Implications

We have shown that the Neogen Reveal® 2.0 for ASP test kits are a very reliable qualitative test kit for the detection of Domoic Acid in shellfish (mussel) flesh.

The implications of this for the Western Australian shellfish industry is that the use of these test kits can be incorporated into the Western Australian Marine Biotoxin Monitoring and Management Plan (a supplementary document to the Western Australian Shellfish Quality Assurance Program) which would allow for their use in the following situations:

a) to determine if a quantitative method should be undertaken on a sample from a closed area for re-opening purposes (i.e., to test the first of two samples collected to re-open areas); and
b) for routine testing of harvest areas in the open status when risk is considered low.

In the first of these situations, where an area has been closed due to the biotoxin concentration in shellfish flesh being above the maximum level, at least two successive shellfish tissue samples taken a week apart showing that the concentrations of biotoxin in the bivalve shellfish tissue are below the maximum level must be obtained before that

area can be re-opened. In addition to this the cell density of potentially toxic phytoplankton present in the water must be below threshold levels and showing a decreasing trend.

The conditions regarding the concentration of potentially toxic phytoplankton present in the water can be tested relatively quickly if not by routine sample collection, then by the collection of additional water samples as required. However, with no current capacity in Western Australia to undertake biotoxin testing using LC-MS/MS analysis, shellfish tissue samples must be sent interstate for biotoxin testing and as outlined in section 2.3, there can be a considerable amount of time before results are obtained. In addition to this, the only tool that the shellfish grower has available to determine when to collect shellfish tissue samples for biotoxin analysis for re-opening is the cell density data for the potentially toxic phytoplankton present in the water samples.

Biotoxin management decisions regarding when to begin collecting shellfish tissue samples for biotoxin analysis for re-opening will be much better informed using rapid test kits to give an indication of biotoxin concentrations in shellfish flesh in conjunction with data on the cell density of potentially toxic phytoplankton.

In the second of these situations, where an area is considered low risk, qualitative biotoxin results from rapid test kits will be able to be used to maintain an "open" status. This is the most significant implication for the Western Australian shellfish industry particularly relating to the circumstances that prompted this study. Had these test kits been available for use at the time of the prolonged *Pseudo-nitzschia* "bloom" in Cockburn Sound during June and July 2017, and assuming this area was classified as low risk with respect to ASP toxins, the impact on the grower with respect to loss of stock, loss of income, prolonged closures and reputation loss would have been nowhere near as great.

11. Recommendations

We recommend that the use of these Neogen Reveal® 2.0 for ASP test kits be incorporated into the Western Australian Marine Biotoxin Monitoring and Management Plan for the qualitative detection of Domoic Acid in shellfish flesh as per the conditions of use already outlined in the WA MBMMP.

We acknowledge that this validation study was conducted using a single shellfish species (*Mytilus edulis*) and, although other validation studies for the same test kits on other shellfish species (Section 8.1) indicate very good agreement between different shellfish species, it may be necessary to conduct similar validation studies using other shellfish species grown or harvested in Western Australia. This could also be expanded to shellfish species grown and/or harvested Australia wide.

12. Further Development

It is hoped that the outcome of this study will be that the Neogen Reveal® 2.0 for ASP test kits be incorporated into the Western Australian Marine Biotoxin Monitoring and Management Plan for the qualitative detection of Domoic Acid in shellfish flesh as per the conditions of use already outlined in the WA MBMMP.

It may be that, given the limitations of this study, these test kits may only be "approved" to be used for the testing of mussel (Mytilus edulis) tissue. As indicated above, additional validations may be required for other shellfish species.

If there is interest from both the shellfish aquaculture industry and the regulators (primarily the DoH) to pursue the "forcing" of these test kits to return a positive result at a Domoic Acid concentration less than the regulatory limit, then this can also be investigated taking into account the potential issues already discussed in Section 5.3.1.

13. Extension and Adoption

The outcome of this study, in the form of the final report, will be communicated to the Western Australian Department of Health and Department of Primary Industries and Regional Development and presented to the Australian Shellfish Quality Assurance Advisory Committee at the 2022 Annual General Meeting. Any outcomes regarding the use of these test kits in Western Australia will then be relayed to the industry and relevant stakeholders.

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15. Appendix 1 - Marine Biotoxin Monitoring and Management Plan (MBMMP). Version 1 (2016)

Government of Western Australia
Department of Health

Marine Biotoxin Monitoring and Management Plan 2016

WESTERN AUSTRALIA SHELLFISH QUALITY ASSURANCE PROGRAM

Marine Biotoxin Monitoring and Management Plan 2016

WASQAP 2016

Prepared by the WA Department of Health

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Version Control

Version	Date	Author	Reason	Sections
Draft Marine Biotoxin Monitoring and Management Plan 2015	May 2015	WASQAP	Updating Biotoxin Management Plan from WASQAP Manual 2011 following Biotoxin Risk Assessment carried out in 2014	All
Draft Marine Biotoxin Monitoring and Management Plan 2015	July 2015	WASQAP	Amendments following consultation	All
Marine Biotoxin Monitoring and Management Plan 2016	May 2016	WASQAP	Updated plan following outcome of Department of Agriculture and Water Resources Review. Annual Review	All

Next Review June 2017

Contents

Marine Biotoxin Monitoring and Management Plan 2016
WASQAP 2016

1.0 Introduction

This document should be read in conjunction with the WASQAP Operations Manual and Australian Shellfish Quality Assurance Program (ASQAP) Manual (2016).

1.1 Purpose

This marine biotoxin monitoring and management plan (MBMMP) has been developed under the WASQAP to ensure regular industry monitoring within shellfish growing areas to gain a better understanding of the risk level and to mitigate the risk of contaminated shellfish. The management plan takes into account the inherent risk, the cost of managing the risk, whilst considering the legislative and financial burden on seafood producing businesses.

1.2 Scope:

This MBMMP is designed for the aquaculture and commercial shellfish harvesting industry, particularly bivalve molluscs harvested from the following areas:

- Cockburn Sound - Southern Flats and Kwinana Grain Terminal
- Mistaken Island
- Oyster Harbour
- Shark Bay

1.3 Background References to MBMMP:

This MBMMP is based on information provided in Part B of the Cawthron report (No. 645) entitled Australian Marine Biotoxin Management Plan for Shellfish Farming and the Report prepared by Centre of Excellence for Science, Seafood and Health (CESSH) in 2014 entitled “Review the Tasmanian paralytic shellfish toxin (PST) event and Safefish recommendations to determine an interim risk management approach for WA”.

CESHH report acknowledged that whilst filter feeding bivalve shellfish species (e.g. mussels, oysters) have a high capacity to accrue biotoxins, the report concluded that there is a low putative biotoxin risk in Western Australia's commercially harvested areas. Additionally, following the routine biotoxin sampling carried out as part of the biotoxin review during 2015/2016 only one sample was found to be on the regulatory limit. Therefore the sampling frequencies for phytoplankton and biotoxin testing have been set accordingly (i.e. twice monthly for phytoplankton testing and once a month for biotoxin testing).

It is acknowledged that the use of flesh testing is the cornerstone of the regulatory approach, and whilst phytoplankton provides a support role, (particularly in the early identification of impending blooms) together they provide a good risk management tool.

1.4 Roles and Administrative Responsibilities:

Roles and administrative responsibilities for the MBMMP are the same as for the WASQAP. When an algal bloom occurs in the vicinity of lease areas, additional management support may be provided by the Department of Water (DOW).

1.5 Growing Areas and Sampling Sites:

Harvest areas and sampling sites are shown in the WASQAP Operations Manual (section 2 details sampling locations for shellfish harvesting areas in WA). Phytoplankton sampling protocols and procedures for sample collection and dispatch to the analytical laboratory are also detailed in the Manual.

2.0 Monitoring & Sampling Procedure (This procedure applies to routine sampling and sampling undertaken in the event of an exceedance).

All sampling is performed in accordance with section 4 of WASQAP.

2.1 Biotoxin events are notoriously unpredictable, even when the best routine monitoring program is actively implemented it is possible that new biotoxin events and challenges will arise. Therefore aside from the mandatory sampling

programs, environmental factors should also be considered as useful indicators to a pending food safety biotoxin event. Such clues can be drawn from, but should not be limited to, fish kills, meteorological data, pollution spills (especially those involving nutrients) and obvious blooms (which may be noted on the WASQAP Sampling Program Information Sheet).

Phytoplankton sampling should be undertaken frequently and regularly, with the frequency remaining constant throughout the year, as potentially harmful species can occur at any time of the year (Cawthron Report No.646, Nov 2001). Therefore, phytoplankton water samples are collected bimonthly throughout the year as per the sampling program for growing areas. However, sampling is less frequent for Mistaken Island harvesting area as traditionally it is only harvested from a few months a year. For seasonal start-up two phytoplankton samples and one biotoxin sample is taken prior to the commencement of harvesting.

However, the frequency of phytoplankton sampling may increase in response to results of the regular monitoring program. Biotoxin flesh samples are taken when phytoplankton alert levels have been triggered (refer to Table 1). In addition to this, flesh samples for biotoxin screening for Amnesic Shellfish Poisoning / Diarrhetic Shellfish Poisoning / Paralytic Shellfish Poisoning (ASP/DSP/PSP) are routinely tested for every month. While potential Neurotoxic Shellfish Toxins (NST) producers have been identified occasionally they have never been detected at significant levels. Therefore, NSP toxins are currently not routinely tested for in WA, however the NSP risk in WA waters is currently being further assessed.

2.2 A water sample is taken by industry representatives and sent to the Laboratory for phytoplankton analysis for target species enumeration. If a phytoplankton sample is not received within one week of its scheduled date the businesses operating in the harvesting area should consider voluntary cessation of harvesting (unless prior arrangements have been made with DOH Food Unit).

Water samples are analysed within 72 hours of arrival at the laboratory. At the same time water samples are taken, a flesh sample is also collected and stored chilled at 5°C or frozen.

2.3 If no potentially toxic species are identified at levels that exceed the alert levels in Table 1 the sample is frozen and held in frozen storage for 6 weeks.

2.4 If potentially toxic species are identified at levels that exceed the alert levels ('to initiate flesh testing' in Table 1) the analyst will immediately notify (by phone and/or email foodsafety@health.wa.gov.au) the DOH Food Unit. Additionally notification containing the specified subject heading is also to be sent to the following email address: algalblooms@health.wa.gov.au

Subject: Algal Bloom Shellfish Hazard

Importance: High

2.5 If the phytoplankton count reaches the alert level to 'initiate flesh testing' for the particular species, the food business must arrange for the chilled/frozen sample of shellfish to be screened for biotoxins. The initial toxin analyses may be undertaken using the appropriate Jellett rapid kit, provided the testing is undertaken by a NATA accredited laboratory. If any toxin at all is detected then samples must be sent for quantitative analyses and the harvesting area may cease harvesting. (Additionally another water and flesh sample will be taken (seven days after the original sample was taken) and the water sample submitted for analysis and the flesh sample stored for possible biotoxin testing). The food business may decide to voluntary cease harvesting pending the biotoxin results (in line with Safefish Report 2013 recommendations 'regulatory decisions should be made on flesh results. Phytoplankton should be used to trigger further sampling". (Refer to Figures 1 and 2). If biotoxins are detected the sample must be submitted for biotoxin confirmation analysis.

2.6 If the phytoplankton alert levels are exceeded but are not at the level to 'initiate flesh testing', the phytoplankton Laboratory is to notify the DOH Food Unit and food business. An additional water and shellfish sample must be collected as soon as practicable and sent to the phytoplankton Laboratory for analysis.

If the subsequent sample shows that the phytoplankton levels for the particular phytoplankton species exceed the ‘alert levels to initiate flesh testing’, the frozen sample of shellfish (second sample) should be screened for biotoxins, and depending on the results undergo biotoxin confirmation. The food business may decide to voluntary cease harvesting pending the biotoxin results. (The initial toxin analyses may be undertaken using the appropriate Jellett rapid kit, provided the testing is undertaken by a NATA accredited laboratory. If any toxin at all is detected then samples must be sent for quantitative analyses and the harvesting area may cease harvesting. Refer to figure 1

2.7 If algal biotoxins are determined to be present at levels which exceed the maximum permitted concentrations specified in the Australia New Zealand Food Standards Code (*the Code*) Standard 1.4.1 Contaminants and Natural Toxicants (Table 2), the food business must close the harvesting area. Where a harvesting area is not closed the Department of Health would consider the provisions contained within its Compliance and Enforcement Policy to ensure no harvesting takes place. The protocols detailed in the WASQAP for surveillance, communication, media release and product recall will be carried out. The size and extent of the closure may be determined in liaison with DOW and Department of Fisheries (DOF).

The levels documented in Table 1 relate to discrete or composite samples. These levels were developed based on the WA biotoxin risk assessment and consideration of levels used internationally and in various states of Australia. They should be revised as further monitoring and research is undertaken that supports a change. The Laboratory remains vigilant for the wider spectrum of potentially toxic species and any novel species.

Table 1: Summarises the phytoplankton levels (in cells/L) that trigger management action.

Micro-algae species	Type of Toxin	Alert Level (refer to flow chart for actions)	Alert level to initiate flesh testing (cells/L)
***Alexandrium catenella*[1]**	PSP	100	200
***Alexandrium minutum*[1]**	PSP	100	200
***Alexandrium ostenfeldii*[1]**	PSP	100	200
***Alexandrium tamarense*[1]**	PSP	100	200
Gymnodinium catenatum	PSP	500	1,000 mussels 2,000 (other shellfish)
Dinophysis acuminata	DSP	1,000	1,000
Dinophysis acuta	DSP	500	1,000
Dinophysis caudata	DSP	500	1,000
Dinophysis fortii	DSP	500	1,000
Prorocentrum lima	DSP	500	500
***Pseudo-nitzschia* seriata group (*P.multiseries* and *P.australis*)[2]**	ASP	50,000	50,000
***Pseudo-nitzschia* delicatissima group[2]**	ASP	500,000	500,000
Karenia brevis	NSP	500	1,000
Karenia/Karlodinium/ Gymnodinium group[3]	NSP	100,000	250,000

N.B. The cell levels within each toxin group are cumulative. (For example 600 cells/L of both D.acuta and D. fortii would mean a total count of 1200 cells/L exceeding the critical level to initiate flesh testing. Example 2, Prorocentrum lima 400 cells/L and 150 cells/L Dinophysis acuminate would exceed the critical level to initiate flesh testing. Whereas 400 cells/L Dinophysis acuminata and 150 cells/L Dinophysis acuta would **not** trigger testing.

[1] *Alexandrium* species may be difficult to identify when numbers are low. If any doubt exists, they should be treated as potentially toxic.

[2]Species within the *Pseudo-nitzschia* groups are difficult to identify. The toxic species of most concern in each group are listed for those laboratories that have capacity to identify these algae to species level. Otherwise all algae within these groups should be considered potentially toxic. The *Pseudo-nitzschia seriata* group includes *P. australis, P. pungens* and *P. multiseries.* The *Pseudo-nitzschia delicatissima* group includes *P. turgidula, P. fraudulenta, P. delicatissima, P. pseudodelicatissima* and *P. multistriata*.

[3]The *Karenia/Karlodinium/Gymnodium* group includes *Karenia bidigitata, Karenia brevisulcata, Karenia mikimotoi, Karenia papilionacea, Karenia selliformis, Karlodinium micrum* and *Gymnodinium impudicum*. If there is evidence of fish kills near the growing area, NST testing should be considered.

A formal closure of a harvesting area may also be invoked after the reporting of cases of human illness consistent with the case definition for PSP, NSP, DSP, and/or ASP that have resulted from the consumption of shellfish from a particular area. Additionally, a formal closure may be invoked if the DOH Food Unit determines a closure is necessary for other reasons (e.g. toxins present in neighbouring areas, reporting of a potentially toxic phytoplankton species not previously reported from the harvest area etc.).

A summary of the WASQAP biotoxin detection and action process relevant to commercially produced shellfish is displayed below:

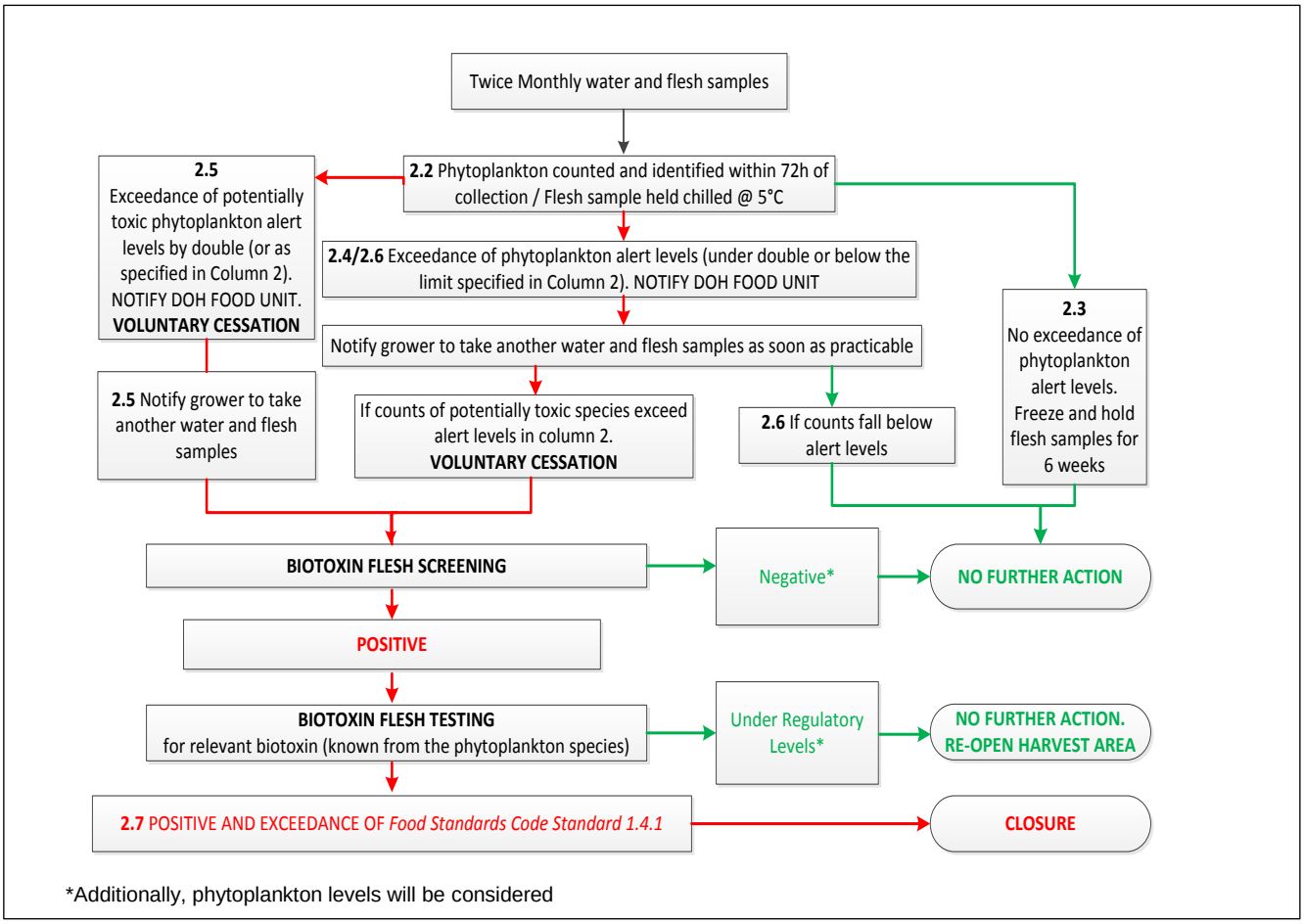

Figure 1 WASQAP marine biotoxin monitoring and management procedures. ***Note – further steps are required after an exceedance of the Australia New Zealand Food Standards Code Standard 1.4.1, refer to figure 2.***

Table 2 Marine Biotoxin Regulatory Closure Levels

A harvest area must be closed for the harvesting of shellfish when toxins in shellfish are found to be above the levels prescribed in the Australian and New Zealand Food Standards Code, Contaminants and Natural Toxicants Standard 1.4.1 as detailed below.

Analysis	Frequency	Maximum Level
Paralytic Shellfish Toxin (PST) (saxitoxins equivalent) High Performance Liquid Chromatography (HPLC)	Phytoplankton over trigger levels or routine biotoxin testing	0.8mg mg/kg saxitoxin equivalent
Amnesic Shellfish Toxin (AST) (domoic acid equivalent) Liquid Chromatography coupled with Mass Spectrometry (LCMSMS analysis)	Phytoplankton over trigger levels or routine biotoxin testing	20mg/kg Domoic acid equivalent
Neurotoxic Shellfish Poisoning (NSP toxins*)	Phytoplankton over trigger levels	200 MU/kg
Diarrhetic Shellfish Toxin (DST) (okadoic acid equivalent) (LCMSMS)	Phytoplankton over trigger levels or routine biotoxin testing	0.2 mg/kg Okadaic acid equivalent
YTX YESSOTOXINS (LCMSMS) **AZP Azaspiracids** (LCMSMS)		YTX is not regulated in Australia and although it is toxic to mice when applied intraperitoneally, its oral toxicity is questionable (Cawthron Institute, 2001).

NB: DSP toxins include okadaic acid, Dinophysis toxins (DTX1, DTX2, DTX3), Pectenotoxins PTX, PTX2.It does not include (PTX2-sa), yessotoxins, gymnodimine or azaspiracid.

*NSP toxins may now also be measured using chemical methodology (LCMS/MS). However, no mg/kg equivalence value or guidance is provided within the ANZFSC for this method. The US Food and Drug Authority acknowledge that 0.8 mg/kg brevetoxin-2 is equivalent to 200MU/kg.

3.0 Harvesting Area Re-Opening Criteria

The re-opening of a harvesting area following a biotoxin closure event shall only occur on the basis of bivalve shellfish meat test results (confirmed full profile of biotoxins). Phytoplankton results may be used to qualify meat testing requirements (refer to Figure 2).
If biotoxin tests on at least two successive meat samples taken a week apart show that the concentrations of biotoxin in the bivalve shellfish tissue are below the maximum level (ML) in the Code;

- AND water samples collected during the same period show levels of toxic algae at or below the alert levels; AND
- the algal levels are not increasing in number,

then re-opening of the harvest area may occur.

Should there be a toxin event in a harvest area then each individual shellfish species harvested shall be sampled. This will assist in determining the food safety risk of each species.

However, in the event that two consecutive shellfish meat samples taken a week apart are found to comply with the ML but phytoplankton samples collected during the same period show levels of toxic phytoplankton above the alert levels, re-opening may only occur after a 3rd consecutive compliant meat test result - which may be taken a further 48 hours or more after the second bivalve shellfish meat sample.

Following the re-opening of a harvest area the sampling requirements will be at a minimum weekly bivalve shellfish and phytoplankton samples for at least 2 weeks.

Harvest Area Re-opening Procedure

All those notified of the closure will be notified of the re-opening by the appropriate means. This will be initially by telephone followed by email. (Refer to opening procedure in WASQAP Operations Manual).

IF A SHELLFISH HARVESTING AREA IS CLOSED FOR OTHER REASONS BESIDES PHYTOPLANKTON/BIOTOXIN EXCEEDANCES (I.E. HIGH BACTERIOLOGICAL COUNTS) THE FOOD BUSINESSES SHOULD CONTINUE TO COLLECT PHYTOPLANKTON AND FLESH SAMPLES.

A summary of the WASQAP biotoxin detection and action process relevant to commercially produced shellfish is displayed below.

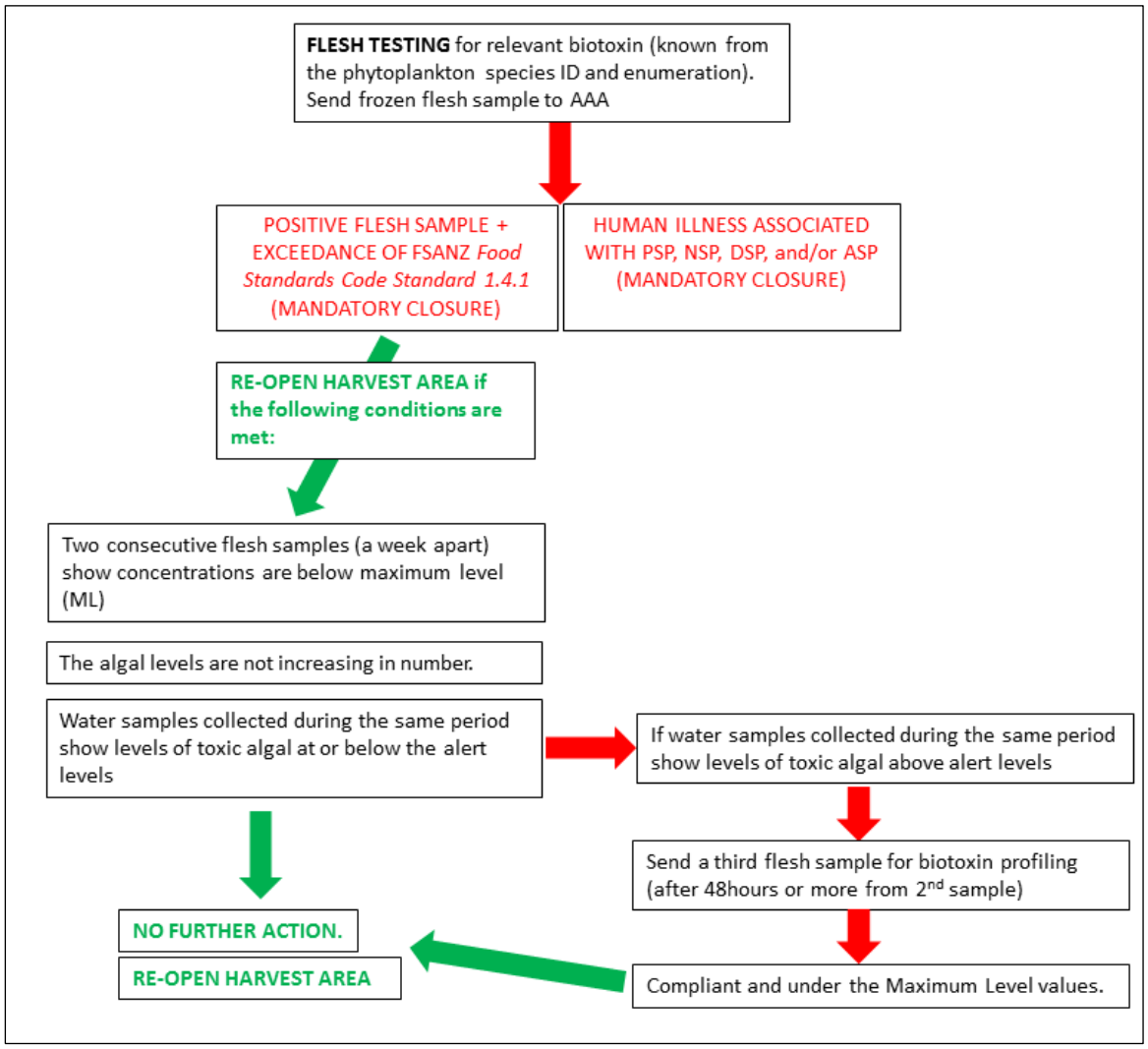

Figure 2: **Procedures** for re-opening commercial harvesting areas after experiencing an exceedance of the FSC standard 1.4.1 for biotoxins in the flesh of shellfish.

4.0 Recreational Shellfish Samples

It should be noted that recreational wild-capture of shellfish is not within the scope of WASQAP. It is impossible to guarantee the safety of eating wild shellfish without having a comprehensive monitoring program that tests the waterway concerned for harmful microorganisms and toxins. Such programs are extremely expensive and difficult to undertake. The DOH therefore recommends only eating shellfish harvested commercially under strict monitoring programs.

5.0 Review

The MBMMP will be reviewed to reflect changes in further monitoring and research in phytoplankton and biotoxins levels. Additionally it will be reviewed on scientific knowledge that supports a change.

Appendix 1- Phytoplankton species

Some name changes have occurred since original publication of the Cawthron report. These have been included in the list below and the list will be updated as new information is provided on toxigenic genera. The IOC (UNESCO) has a comprehensive and regularly updated list of harmful microalgae http://www.marinespecies.org/hab/. (Cawthron Report No 645 and NSW Shellfish Program Marine Biotoxin Management Plan 2014)

Category A – Species known to be present in Australian waters and proven to produce toxins either in Australia or internationally:

Alexandrium catenella (saxitoxin and derivatives)
Alexandrium minutum (saxitoxin and derivatives)
Alexandrium ostenfeldii (saxitoxin and derivatives, also produces spirolides in Canada)
Alexandrium tamarense (saxitoxin and derivatives, also has non-toxic strains)
Dinophysis acuminata (pectenotoxin, okadaic acid?, dinophysis toxins? and diol esters?)
Dinophysis acuta (pectenotoxin, okadaic acid?, dinophysis toxins? and diol esters?)
Dinophysis caudata (pectenotoxin, okadaic acid?, dinophysis toxins? and diol esters?)
Dinophysis fortii (pectenotoxin, okadaic acid?, dinophysis toxins? and diol esters?)
Dinophysis hastata (okadaic acid?, dinophysis toxins? and diol esters?)
Dinophysis mitra (okadaic acid?, dinophysis toxins? and diol esters?)
Dinophysis rotundata (okadaic acid?, dinophysis toxins? and diol esters?)
Dinophysis tripos (some strains produce okadaic acid, dinophysis toxins and diol esters)
Gymnodinium catenatum (saxitoxin and derivatives)
Gymnodinium cf breve (Karenia cf brevis) (brevetoxins)
Prorocentrum lima (okadaic acid?, dinophysis toxins? and diol esters?)
Pseudonitzschia australis (domoic acid)
Pseudonitzschia delicatissima (domoic acid)HNTA
Pseudonitzschia fraudulenta (domoic acid) HNTA
Pseudonitzschia multiseries (domoic acid)
Pseudonitzschia pseudodelicatissima (domoic acid) HNTA
Pseudonitzschia pungens (usually non-toxic, but toxic strains produce high concentrations of domoic acid per cell)
Pseudonitzschia turgidula (domoic acid)
Pyrodinium bahamense var. compressum (in tropical habitats) (saxitoxin and derivatives)
Note: HNTA Historically non-toxic in Australia

Category B – Potential toxin producing species (ie toxicity untested/unclear) known to be present in Australian coastal waters:

Alexandrium pseudogonyaulax (possible STX and derivatives, goniodomin)
Chattonella marina/antiqua (possible brevetoxins)
Fibrocapsa japonica (possible brevetoxins)
Heterosigma akashiwo (possible brevetoxins)
Pseudonitzschia cuspidata (possible domoic acid)
Pseudonitzschia heimii (possible domoic acid, non-toxic in New Zealand)
Pseudonitzschia lineola (possible domoic acid)
Pseudonitzschia multistriata (possible domoic acid, non-toxic in New Zealand)
Pseudonitzschia subfraudulenta (possible domoic acid)
Pseudonitzschia subpacifica (possible domoic acid)

Category C – Other potential toxin producing species world-wide that may be present in Australian waters:

Alexandrium angustitabulatum (possible saxitoxin and derivatives, identified in New Zealand waters)
Alexandrium acatenella (possible saxitoxin and derivatives)
Alexandrium cohorticula (possible saxitoxin and derivatives)
Alexandrium fraterculus (possible saxitoxin and derivatives)
Alexandrium fundyense (possible saxitoxin and derivatives)
Alexandrium lusitanicum (possible saxitoxin and derivatives)
Alexandrium tamiyavanichi (possible saxitoxin and derivatives)
Coolia monotis (produces cooliatoxin)
Dinophysis norvegica (Major DSP producer in Europe)
Gymnodinium aureaolum (possible brevetoxins)
Gymnodinium bidigitatum ((possible brevetoxins) found in New Zealand waters)
Gymnodinium galatheanum (Karlodinium micrum) (possible brevetoxins)
Gymnodinium impudicum (possible brevetoxins)
Gymnodinium mikimotoi (Karenia mikimoto) (possible brevetoxins)
Gymnodinium papillionaceceum Karenia papillonacea) (possible brevetoxins)
Gymnodinium pulchellum (Takayama pulchella) (possible brevetoxins)
Gymnodinium selliforme (Karenia selliformis) (gymnodimine, found in New Zealand waters)
Lingulodinium polyedra (yessotoxin producer in Japan)
Nitzschia navis-varingica (domoic acid was recently confirmed for an isolate from brackish Vietnamese waters)
Ostreopsis siamensis (produces palytoxin)
Pfiesteria piscicida Not possible to identify with routine monitoring. Culturing and immunolabelling required
Prorocentrum concavum (okadaic acid?, dinophysis toxins? and diol esters?)
Prorocentrum elegans (okadaic acid?, dinophysis toxins? and diol esters?)
Prorocentrum hoffmannianum (okadaic acid?, dinophysis toxins? and diol esters?)
Prorocentrum maculosum (produces prorocentrolides)
Prorocentrum minimum (Prorocentrum cordatum) (The toxin linked to this organism (185 fatalities in Japan) has not yet been elucidated, and the role of P. minimum is still in question)
Protoceratium reticulatum (yessotoxin producer in New Zealand)
(? Indicates this toxin has not been confirmed at the time of this report as being produced by Australian strains of this species)
Gonyaulax spinifera (possible yessotoxin)
Pseudonitzschia calliantha (domoic acid)
Numerous Karenia species have recently been described. Toxicity and applicability to the Australian program require more investigation.

Appendix 2 - Toxic Shellfish Poisoning Case Definitions

Paralytic Shellfish Poisoning (PSP)

Causative toxins: Saxitoxins (STX's), Gonyautoxins (GTXs) and C toxins (CTXs)

STXs have been recorded from Tasmania, Victoria, South Australia and New South Wales.

Microalgal sources: Gymnodinium catenatum, Alexandrium species (including A. minutum, A. catenella, A. tamarense, A. fundyense, A. ostenfeldii, plus others), Pyrodinium bahamense var. compressum, also freshwater species such as Anabaena spp., and Microcystis spp.

Symptoms:
•STXs block nerve conduction, manifesting as respiratory distress due to partial paralysis of the muscles necessary for breathing.
• Mild neurological symptoms encompass tingling or numbness around the lips or in fingers and toes (paraesthesias), sensations of floating or weightlessness (dysaesthesias), or gastrointestinal upset (nausea, vomiting, diarrhoea, gut pains).
• More severe poisoning may present with functional weakness (impaired grip strength, staggering gait), difficulty breathing and signs of acute respiratory insufficiency, e.g. cyanosis of the lips or fingernails.
• Severe STX intoxication can cause catastrophic acute respiratory failure and death by asphyxiation

Clinical Case Definition: The following neurological symptoms occurring within 12 hours of consuming shellfish:

- neurosensory;
- paraesthesia, i.e. numbness or tingling around the mouth, face or extremities;
- and one of the following neuromotor/neurocerebellar symptoms:
    - weakness such as trouble rising from seat or bed
    - difficulty in swallowing
    - difficulty in breathing
    - paralysis
    - clumsiness
    - unsteady walking
    - dizziness/vertigo
    - slurred/unclear speech
    - double vision

Amnesic Shellfish Poisoning (ASP)

Causative toxins: Domoic acid (DA) is a neurotoxin produced by a group of marine microalgae known as diatoms.

Microalgal sources: In Australia the known causative diatoms are from the *Pseudo-nitzschia seriata* group (*P. multiseries* and *P. australis*) and the *P. delicatissima* group.

No reports of illness attributable to DA poisoning have been received in Australia.

Symptoms:
• Mild intoxication may involve only gastro-intestinal upset (nausea, vomiting, diarrhoea, gut pains).
• Symptoms of neuro-intoxication include headache, convulsive seizures, myoclonus (involuntary, irregular muscle contractions), cognitive impairment and disorientation, anterograde amnesia (inability to lay down new memories following neurological damage), respiratory difficulty and coma.

Clinical Case Definition: Vomiting or diarrhoea or abdominal cramps within 24 hours of consuming shellfish;

- and no other probable cause identified by microbiological examination of a faecal specimen from the case or microbiological testing of left-over food;
- and/or one or more of the following neurological signs/symptoms occurring within 48 hours of consuming shellfish:

  - confusion
  - memory loss
  - disorientation
  - seizure
  - coma

Diarrhetic Shellfish Poisoning (DSP)

Causative toxins: Okadaic acid (OA), Dinophysistoxins (DTXs), Pectenotoxins (PTXs), Yessotoxins (YTXs) and Azaspiracids (AZAs).

NB. Pectenotoxin, an unrelated lipophilic toxin that is often detected with OA, is included in DST's for regulatory purposes in Australia, but there is some controversy over its toxicity to humans. Azaspiracids are not yet confirmed to be in this group.

Microalgal sources: DSTs are produced by marine microalgae known as dinoflagellates. In Australia the known causative species are *Dinophysis acuminata*, *D. acuta*, *D. caudata*, *D. fortii* and *Prorocentrum lima*. DST producing species are found in all states in Australia at various levels.

Symptoms:
• Nausea, diarrhoea, vomiting, abdominal pain and headache are the characteristic symptoms. The symptoms usually start between 30 minutes to a few hours after consumption.
• Usually resolves by three days following consumption of contaminated shellfish.
No fatalities have been reported.
• May present a risk of dehydration requiring fluid and electrolyte replenishment, particularly in young children or the elderly.

• *Okadaic acid* is a potent tumour promoter, which raises concerns about the possibility of harmful effects from chronic, low-dose exposure. Such exposures are difficult to measure, so the concerns of public health agencies are currently directed toward concentrations of OA in shellfish that cause acute gastro-intestinal illness.

There is no epidemiological evidence of human health effects from yessotoxin. However it is lethal to mice when administered intraperitoneally, and causes damage to heart muscles and livers in mice. Azaspiracids cause vomiting and diarrhoea in humans. In animal tests, these toxins have caused neurotoxic effects and severe damage to the intestine, spleen and liver tissues. The microalgal source is currently unconfirmed.

Clinical Case Definition: Vomiting or diarrhoea occurring within 24 hours of consuming shellfish and no other probable cause identified by microbiological examination of a faecal specimen from the case or microbiological testing of leftover food.

Neurotoxic Shellfish Poisoning (NSP)

Causative toxins: Brevetoxins (BTX's)

Microalgal sources: Karenia brevis (=Gymnodinium breve), K. cf brevis (=Gymnodinium cf breve), plus potentially K. papilionacea (=Gymnodinium papilionaceum), K. mikimotoi (=Gymnodinium mikimotoi) and similar species; Chattonella species, Heterosigma akashiwo and Fibrocapsa japonica.

Symptoms: The symptoms occur within 3-5 hours and are chills, headache, diarrhoea, muscle weakness, joint pain, nausea and vomiting. There can be altered perceptions between hot and cold, difficulty in breathing, double vision, trouble in walking and swallowing.

Clinical Case Definition: Two or more of the following neurological symptoms occurring within 24 hours of consuming shellfish:

- neurosensory:
  - paraesthesia, i.e. numbness or tingling around the mouth, face or extremities
  - alternation of temperature sensations such as a prickly feeling on the skin during a bath/shower or exposure to sun, or difficulty distinguishing hot or cold objects
- neuromotor/neurocerebellar:
  - weakness such as trouble rising from seat or bed
  - difficulty in swallowing
  - difficulty in breathing
  - paralysis
  - clumsiness
  - unsteady walking
  - dizziness/vertigo
  - slurred/unclear speech
  - double vision

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16. Appendix 2 – Alternate representation of Table 3 from Section 5.6

The following is an alternate representation of Table 3 from Section 5.6 with the unexpected negative result obtained for Test 5 included.

Table 4: *Data for all tests conducted with the calculated POD, LCL and UCL.*

DA Concentration (ug/g)	Number of samples tested (N)	Number of positive tests (x)	Probability of Detection (POD)	95% Confidence Interval	
				Lower Confidence Limit (LCL)	Upper Confidence Limit (UCL)
0	24	0	0	0	0.13797748
10	24	0	0	0	0.13797748
15	24	0	0	0	0.13797748
20	96	89	0.927083333	0.857067924	0.964232838
21	24	24	1	0.86202252	1
23	24	23	1	0.86202252	0.964232838
24	24	24	1	0.86202252	1

17. Appendix 3 – Alternate representation of Figure 6 from Section 5.6

The following is an alternate representation of Figure 6 from Section 5.6 with the unexpected negative result obtained for Test 5 included.

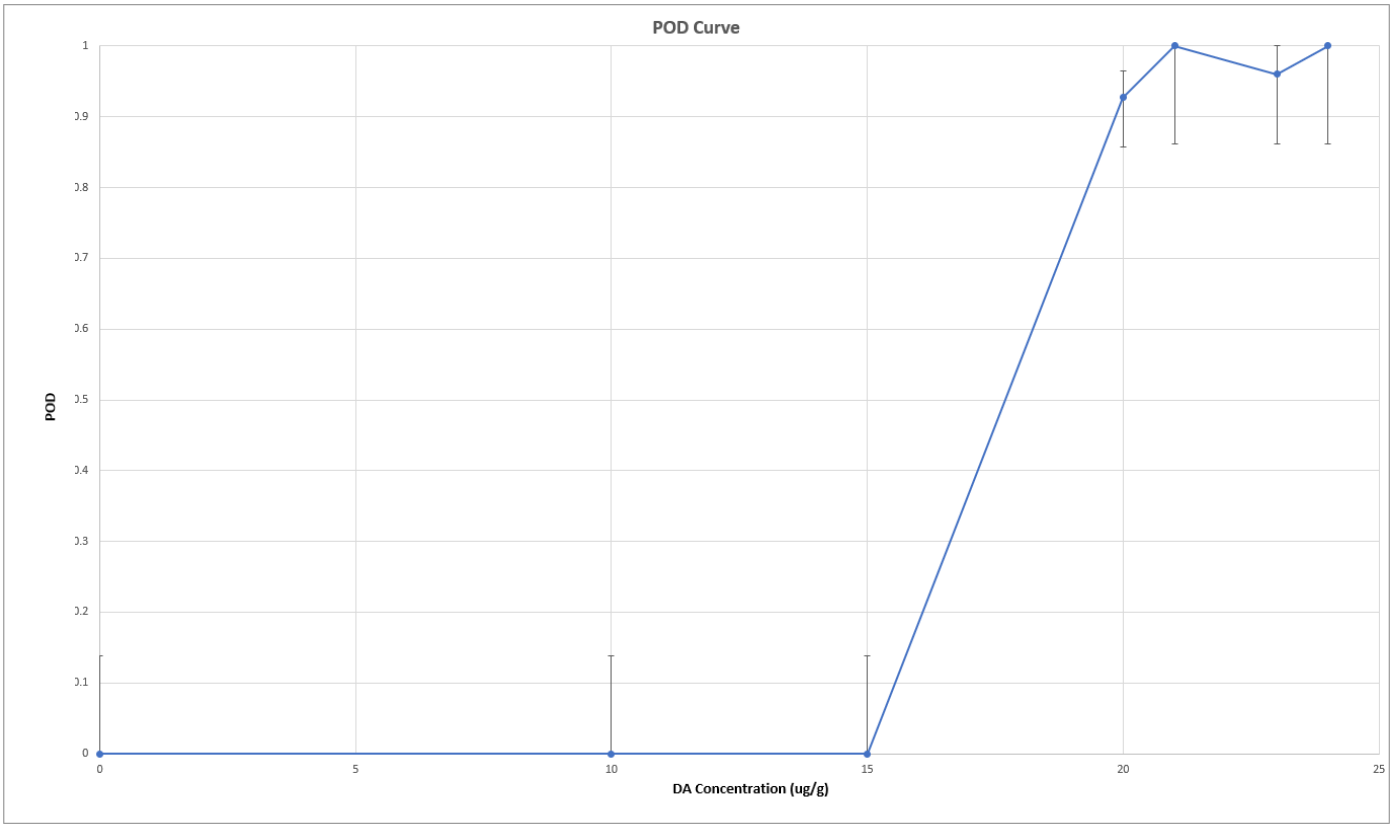

Figure 7: *Probability of Detection curve with the 95% Confidence Intervals represented by error bars.*