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Risk from Diarrhetic Shellfish Toxins and *Dinophysis* to the Australian Shellfish Industry

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Executive Summary

Marine biotoxins produced by harmful algal blooms (HABs) can cause damage to shellfish aquaculture industries worldwide. In Australia, significant shellfish contamination events have occurred due to several different HABs producing different biotoxins. Paralytic Shellfish Toxins (PSTs), produced by *Alexandrium catenella*, have caused a significant impact on the Tasmanian economy since 2012, with the damage from one event (2012/13) estimated at $23 million.

Australian aquaculture industries are keen to adopt efficient, fast and cost-effective management tools for biotoxins and the phytoplankton producing them. While Diarrhetic Shellfish Toxins (DSTs) produced by species of *Dinophysis* are a growing concern, there is still no clear identification of the DST toxin profiles present in Australian shellfish, nor assessment of laboratory capabilities to detect these toxins. Moreover, there has been no comparison of the efficacy of commercially available rapid test kits on Australian shellfish. There is also a need for the development of a rapid onsite test for the presence of DST producing microalgae, so that harvest management can become simpler, faster and with fewer closures.

With this in mind, the present study aimed to generate new knowledge about DSTs in Australian shellfish by:

- Conducting an initial assessment of DST profiles present in Australian shellfish and assess laboratory capabilities to detect these toxins;
- Generating knowledge about commercially available DST test kits and rapid molecular techniques for toxin and species detection;
- Comparing the efficacy of DST toxin detecting kits across oysters, mussels and pipis;
- Developing a DST qPCR assay for species detection for onsite farm use;
- Providing cost versus benefit analysis of improved testing of DSTs in Tasmanian shellfish.

We first examined DSTs in spiked and naturally contaminated shellfish - Sydney Rock Oysters (*Saccostrea glomerata*), Pacific Oysters (*Magallana gigas/Crassostrea gigas*), Blue Mussels (*Mytilus galloprovincialis*) and Pipis (*Plebidonax deltoides/Donax deltoides*), using LC-MS/MS (Liquid Chromatography—tandem Mass Spectrometry) and LCMS (Liquid Chromatography—Mass Spectrometry) in 4 laboratories, and 5 rapid test kits. The rapid test kits included three quantitative ELISA (Enzyme-Linked Immunosorbent Assay) kits by Beacon™, Eurofins/Abraxis™ and EuroProxima™; a quantitative PP2A (Protein Phosphatase Inhibition Assay) kit by Eurofins/Abraxis™, and a qualitative LFA (Lateral Flow Assay) kit by Neogen™.

We found all toxins in all species could be recovered by all laboratories using LC-MS/MS and LC-MS, however, DST recovery at low and mid-level concentrations (<0.1 mg/kg) was variable (0-150%), while recovery at high-level concentrations (>0.86 mg/kg) was higher (60-262%). While no clear differences were observed between shellfish, all kits delivered an unacceptably high (25-100%) level of falsely compliant results for spiked samples (ie. sample spiked above the regulatory limit but resulted in a concentration below the regulatory limit). The Neogen and the PP2A kits performed satisfactorily for naturally contaminated pipis (0%, 5% falsely compliant, respectively). Correlations between spiked DSTs and quantitative methods was highest for LC-MS (r^2 = 0.92) and the PP2A kit (r^2 = 0.78). Overall, our results do not support the use of any DST rapid test kit as a stand-alone quality assurance measure at this time.

We then developed a rapid and quantitative polymerase chain reaction (qPCR) assay to detect species belonging to the genus *Dinophysis* in environmental samples. This assay had no cross-reactivity to other closely related species, and an assay efficiency of 91.5% for *D. acuminata*, 91.3% for *D. fortii*, 92.4% for *D. caudata*, and 97.9% for gene fragment based serial dilutions. This novel assay was then evaluated for its potential to detect *Dinophysis* in environmental samples. The assay was successful in the early detection of a bloom of *D. acuminata* in the Manning River on 9/2/2019 (~7,441 cell L^{-1}), compared to microscopy counts of 5,300 cell L^{-1} on 17/2/2019.

A cost-benefit analysis of rapid detection of Diarrhetic Shellfish Toxins (DSTs) was subsequently undertaken. This was a case study of the Pacific Oysters (*Magallana gigas/Crassostrea gigas*) industry in Tasmania with the following aims:

- to estimate the reduction/annual savings in monitoring costs for regulatory authorities by implementing rapid diagnostic testing for DSTs; and
- to calculate the reduction in commercial loss and economic impact from potentially harmful DST blooms in Tasmania following the introduction of the rapid diagnostic testing.

The analysis considered three hypothetical scenarios for implementation of DST rapid testing:

1. Implement Neogen DST rapid kit testing on-farm in Tasmania, replacing confirmatory DST testing in low risk areas in 3 out of 4 weeks;

2: Implement Neogen DST rapid kit testing in laboratory in Tasmania, replacing confirmatory DST testing in low risk areas in 3 out of 4 weeks;

3. Implement qPCR testing on-farm in Tasmania, replacing confirmatory DST testing in low risk areas in 3 out of 4 weeks.

Results revealed that, while the qPCR or Neogen technology both offer cost advantages when compared with the business as usual practice (BAU), it was not possible to calculate the exact extent of this without further work to validate (including number of samples, Quality Assurance and National Association of Testing Authorities accreditation) the two alternative testing technologies.

All scenarios considered, however, represented a net cost saving over 10 years when compared with the current practice of weekly LC MS tests for DSTs conducted by a laboratory service provider (BAU). The highest savings occurred under Scenario 2, in which the Neogen technology is centralised in the laboratory and spread across all 24 Tasmanian growing areas.

A full validation study covering each of the major testing methods examined (Neogen rapid test, qPCR test) is recommended as an important area of further research. Furthermore, the scenarios considered in this analysis were for domestic supply only, in compliance with potential use under the ASQAP programme. Further considerations would be needed for use in any export programme.

Keywords Biotoxins, harmful algal blooms, qPCR, rapid test kits, LC-MS, okadaic acid, diarrhetic shellfish toxins, *Dinophysis*

1. Introduction

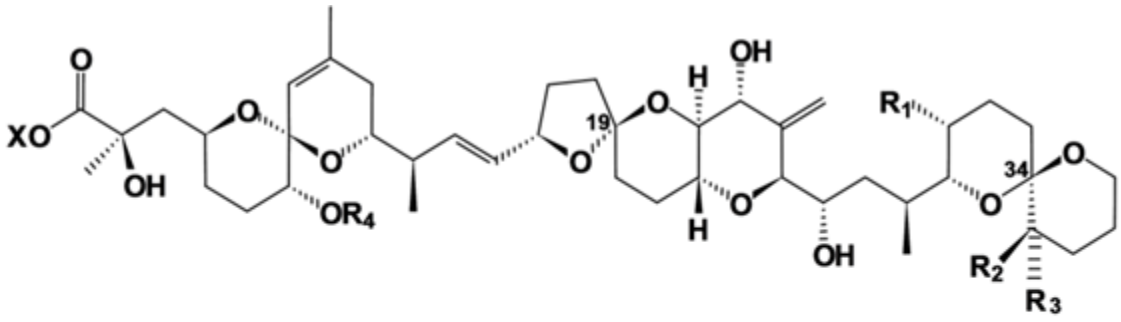
1.1 Background

Marine biotoxins are chemical compounds produced by certain microalgae, most notably dinoflagellates and diatoms. These contaminants can bioaccumulate in fish, crabs, lobster, abalone or filter-feeding bivalves (shellfish) and cause poisoning to seafood consumers. Approximately 60,000 human intoxications occur per year worldwide, with an overall mortality of ~1.5% (Kantiani et al., 2010)). As well as seafood related illnesses, marine biotoxin contamination can lead to damaged public perceptions of seafood, direct economic losses and a restriction in the growth of the shellfish industry.

Diarrhetic Shellfish Toxins (DSTs) are produced by the dinoflagellates *Dinophysis* and less commonly *Prorocentrum* and, via the food chain, can bioaccumulate and cause Diarrhetic Shellfish Poisoning (DSP). DSP is a gastrointestinal disorder caused by the human consumption of seafood (mainly shellfish) contaminated with the marine phycotoxin okadaic acid (OA) and its derivatives, the dinophysistoxins (DTXs). While symptoms are dose dependent and include diarrhoea, nausea, vomiting and abdominal pain, it is considered that OA and DTXs are potent inhibitors of certain protein phosphatases and may also promote tumour/cancer formation (Lee et al., 2016), although the impact of chronic exposure to DSTs is still not well known.

1.2 Diarrhetic Shellfish Toxins (DSTs)

Diarrhetic shellfish toxins are a group of heat stable, polyether toxins including okadaic acid (OA) and its isomer 19-epi-okadaic acid; the OA congeners dinophysistoxin -1 (DTX-1) and dinophysistoxin-2 (DTX-2); and the 7-acyl derivatives of OA, DTX-1 and DTX-2 that are collectively known as DTX-3 (Macleod et al., 2015) (Fig. 1). Altogether they are referred to as the OA group toxins or the 'okadaates' (OAs).



Name	R1	R2	R3	R4	C-19*	C-34*
Okadaic acid (OA)	CH_3	H	H	H	S	S
Dinophysistoxin-1 DTX1	CH_3	CH_3	H	H	S	R
Dinophysistoxin-2 DTX2	H	H	CH_3	H	S	R

* Relative stereochemistry. OA, X = H; Methyl okadaate, X = CH_3; OA diol esters, X = C_4 to C_{10} unsatured diols

Figure 1. Chemical structure of okadaic acid and its congeners (OAs) (Sourced: (Reguera et al., 2014)).

Another group of toxins, the pectenotoxins (PTX), are also produced by *Dinophysis* spp. Pectenotoxins (PTXs) are not currently included in Codex Standard for Live and Raw Bivalve Molluscs (Codex, 2015), and therefore not included in this study. Several other jurisdictions such as Canada, Chile, European Union do regulate for PTX (but not PTX-2sa), but the European Food Safety Authority has issued an opinion to deregulate PTX. Furthermore, DSP regulation in Australia is governed by Food Standards

Australia New Zealand with a maximum regulatory limit (ML) of 0.2 mg OA eq/kg (FSANZ, 2017), while most international standards including the Codex Standard, state a ML of 0.16 mg OA eq/kg (Codex, 2015).

1.3 DST Producing Microalgae

Species belonging to the genus *Dinophysis* Ehrenberg (and more rarely benthic *Prorocentrum)* are the most problematic DST producers worldwide. Being cosmopolitan, this genus has over 100 species represented worldwide, ten of which (*Dinophysis acuminata*, *Dinophysis acuta*, *Dinophysis caudata*, *Dinophysis fortii*, *Dinophysis infundibulum*, *Dinophysis miles*, *Dinophysis norvegica*, *Dinophysis ovum*, *Dinophysis sacculus* and *Dinophysis tripos*) have been unambiguously found to be toxic, producing DSTs (okadaic acid and dinophysistoxins) even at low cell densities (<10^3 cells L^{-1}) (Reguera et al., 2014; Reguera et al., 2012; Simoes et al., 2015) (Fig. 2).

DSP was first described after a large toxin event occurred in Japan in 1976 (Yasumoto et al., 1980; Yasumoto et al., 1978), whereby many people became sick after eating scallops (*Patinopecten yessoensis*). This contamination was linked to toxins produced by *D. fortii*. Following this event, further toxic episodes occurred in Japan, Spain and France, with several thousands of cases of human poisonings occurring over the 1970s and 1980s, and leading to the development of many regional monitoring programs. This monitoring has seen a gradual increase in reported DSP episodes in countries including Chile, Argentina, Mexico, the east coast of North America, Scandinavia, Ireland, Great Britain, Spain, Portugal, Italy, Greece, India, Thailand, Australia and New Zealand (Lembeye et al., 1993; Taylor et al., 2013; Whyte et al., 2014; Yasumoto et al., 1978)

Figure 2. Global distribution of known DSP outbreaks (January 2016). Source: Patrizio A. Diaz and Beatriz Reguera, Instituto Español Oceanografico (in (Lassus et al., 2016).

Despite its importance in relation to human health, *Dinophysis* life history, toxicity, genetic diversity, and population heterogeneity were poorly understood until very recently due to the inability to successfully maintain laboratory cultures (Nishitani et al., 2003; Sampayo, 1993). Furthermore, because of their typically low cell density in the water column, *Dinophysis* have often escaped detection by standard quantitative methods (Reguera et al., 2012). In 2006 however, using a mixotrophic culture approach, *Dinophysis* was successfully grown in the presence of its prey, the phototrophic ciliate *Mesodinium rubrum* and chryptophyte *Teleaulax* spp. (Park et al., 2006). Since this breakthrough, worldwide efforts to investigate this genus have increased rapidly, with new insights now available into their toxicity, nutrition, population dynamics and polymorphic life cycle (Reguera et al., 2012).

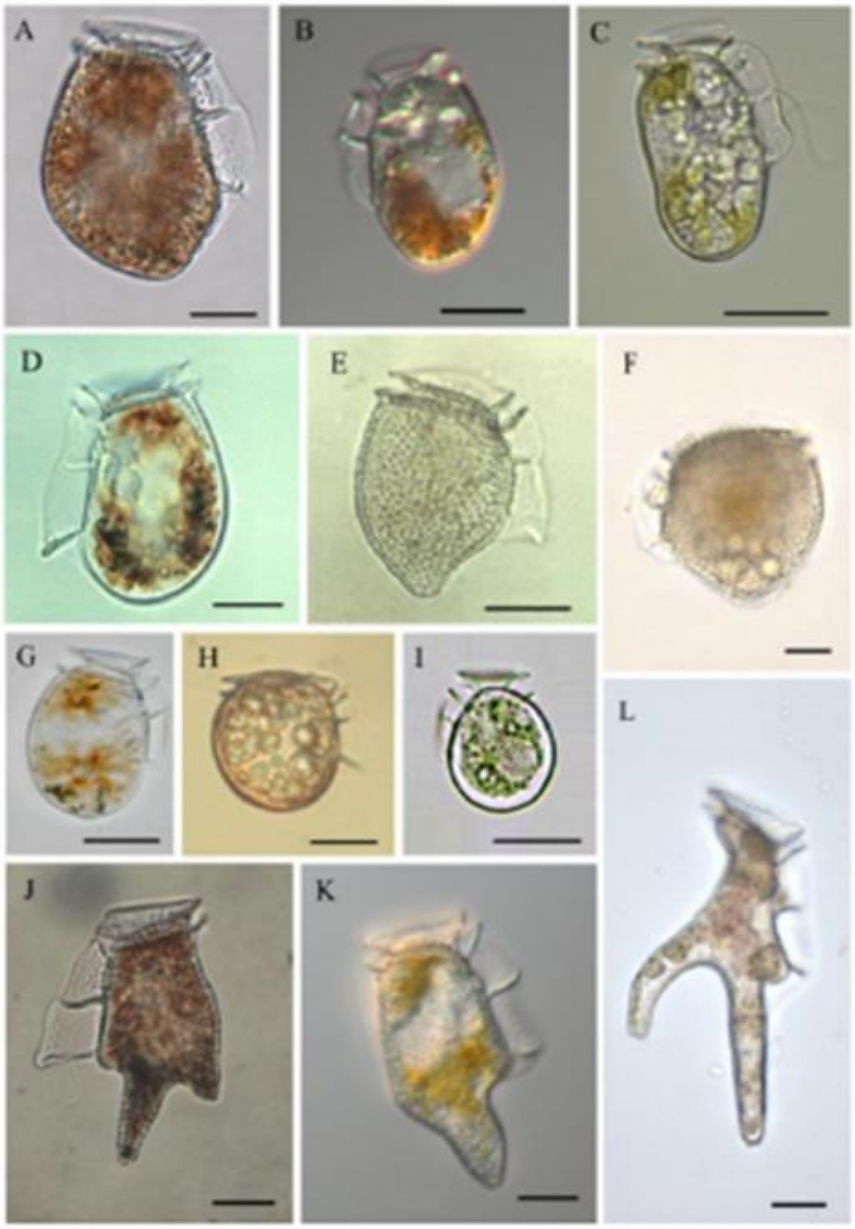


Figure 3. Micrographs of known toxin-containing *Dinophysis* and *Phalacroma* species. (A) *D. acuta*; (B) *D. acuminata*; (C) *D. sacculus*; (D) *D. fortii*; (E) *D. norvegica*; (F) *Phalacroma mitra*; (G) *D. ovum* (perhaps same species as *D. acuminata* (Ha Park et al. 2019); (H) *P. rotundatum*; (I) *D. infundibula*; (J) *D. tripos*; (K) *D. caudata*; and (L) *D. miles*. All live/fixed specimens from the Galician Rías (Northwest Spain) except H, which is from the Gullmar Fjord (Sweden), and F and L, tropical specimens courtesy of J. Larsen. Scale bar = 20 µm. Sourced: (Reguera et al., 2014).

1.4 DSP events in Australia

Dinophysis is common in Australian waters, with 36 species reported (Ajani et al., 2011; Hallegraeff and Lucas, 1988; McCarthy, 2013). Toxic species include *D. acuminata*, *D. acuta*, *D. caudata*, *D. fortii*, *D. norvegica*, and *D. tripos*. There have been three serious human DSP poisoning events in Australia. The first episode was caused by contamination of Pipis (*Plebidonax deltoides*) in New South Wales in 1997 (NSW) by *D. acuminata* (Quaine et al., 1997). One hundred and two people were affected and 56 cases of gastroenteritis reported. A second episode occurred again in NSW in March 1998, this time

with 20 cases of DSP poisoning reported (Madigan et al., 2006). The final event occurred in Queensland in March 2000, in which an elderly woman became seriously ill after eating local Pipis (Burgess and Shaw, 2001). While no human fatalities from DSP are known globally, DSTs continue to be a major food safety challenge for the shellfish industry.

1.4.1 DSTs in New South Wales

In 2013, a synthesis of harmful phytoplankton species in oyster growing estuaries of NSW identified *Dinophysis* as one of three potentially high-risk genera for biotoxin events (others being *Alexandrium* and *Pseudo-nitzschia*) (Ajani et al., 2013). The study found the NSW Food Authority's regulatory "Phytoplankton Action Limit" (PAL) which triggers shellfish flesh sampling (defined as 500 'total *Dinophysis*' cells L^{-1}) (NSW Food Authority, 2017) was exceeded in 136 samples across 31 estuaries over a 5 year period. It was concluded from this meta-analysis that blooms of *Dinophysis* posed a potential threat to this AUD$32M (farm gate value) per annum industry (Trenaman et al., 2014).

Since the commencement of routine biotoxin monitoring from classified NSW shellfish aquaculture areas in 2005 (predominately *Saccostrea glomerata* with some *Crassostrea gigas*, *Ostrea angasi* and *Mytilus edulis*), there have been 29 positive test results for the presence of DSTs recorded (<1%) (NSW Food Authority, unpublished data) with no human illnesses reported to date. Typically, higher concentrations of toxins associated with *Dinophysis* spp. have been reported in wild harvest pipis (*Plebidonax deltoides*) with a maximum biotoxin concentration of 0.4 mg kg^{-1} OA reported in October 2013 (Farrell et al., 2015). DSTs continue to be a major food safety challenge for the NSW pipi industry, with up to 40% of pipis in an end-product market survey between 2015 - 2017 returning positive results for DST, and two market place samples (1%) containing DST above the regulatory limit (Farrell et al., 2018).

1.4.2 DSTs in Victoria

Routine biotoxin monitoring commenced in Victoria in 2016. Prior to that, biotoxin testing was only initiated following the exceedance of a phytoplankton trigger level (J. Mercer pers comm.). While there have been a number of exceedances in *D. acuminata* cell numbers since this time, there have only been two events were DSTs levels were above the regulatory limit. These were in blue mussels from Port Phillip Bay in August 2011 (max. 0.23 mg OA eq/kg), and in pipis from East Gippsland in June 2017 (OA concentration unavailable).

1.4.3 DSTs in Tasmania

Routine biotoxin monitoring for shellfish in Tasmania commenced in September 2013 (TSQAP). Prior to that time, all DST biotoxin tests were conducted in response to phytoplankton trigger level exceedances. Species tested for biotoxins include *Katelysia scalarina* (Cockle), *Magallana gigas* (Pacific Oyster), *Mytilus galloprovincialis* (Blue Mussel) and *Venerupis largillierti* (Clam) across twenty-eight growing areas. Since the inception of routine monitoring, one regulatory exceedance occurred in 2012 (0.324 mg OA eq/kg) which resulted in the closure of two growing areas (Eaglehawk Bay, Garfish Bay Dart Island), and two exceedances in 2016: 0.278 mg OA eq/kg for Garfish Bay/Dart Island and Eaglehawk Bay; and (max) 0.56 mg OA eq/kg for Spring Bay.

1.4.4 DSTs in South Australia

Biotoxin data is available from 2002 with sampling historically done throughout the summer months. Okadaic acid is the only positive DST detected and *D. acuminata* is the main species identified (C. Wilkinson pers. comm.). Positive detections have occurred on many occasions since 2002, with 19 detections exceeding the regulatory limit, and a maximum of 0.51 mg OA eq/kg reported on 10-Jun-2014 in cockles from the Coorong.

Biotoxin testing for export areas (only) now occurs monthly for the following areas/species: Port Lincoln (mussels) this consists of Lower Eyre, Boston Bay, Bickers Island and Proper Bay; Port Douglas (oysters); Coorong (pips). All other areas are tested monthly using a rapid test (Neogen) and all extra testing (LC-MS and rapid tests), are done only if phytoplankton cell densities are elevated.

1.4.5 DSTs in Western Australia

Routine monitoring for DSTs in Western Australia began in 2015 (J. Cosgrove, A. Charles pers. comm.). Positive detections have occurred for mussels (4 in 2015, 9 in 2016, 8 in 2017) with maximum detection of 0.2 mg OA eq/kg. Prior to routine monitoring, 22 other positive DSTs have been recorded from mussels, blue mussels and "shellfish", with a maximum reported concentration of 1.99 mg OA eq/kg in blue mussels from Wilsons Inlet in 2005.

1.4.6 Summary of DSTs in Australian shellfish

Positive DST detections continue to occur periodically in Australian shellfish although these events remain largely unstudied. Using the official analytical method of LC-MS/MS, shellfish data spanning 2012 to 2017 from four Australian states (Tasmania, Victoria, Tasmania, South Australia and Western Australia) showed that 53 (0.65%) shellfish samples out of 8156 analysed exceeded the domestic regulatory limit. Exceedances were more common in cockles/pipis, clams, and mussels than oysters and scallops (4.9, 1.1, 1.1, 0.03 and 0% of samples analysed respectively). Of those that exceeded this threshold, OA was the most commonly detected toxin analogue, with only one sample containing DTX-1, and no samples containing DTX-2 (unpublished data).

1.5 Detection methods for DSTs

Detection methods for DSTs using liquid chromatography with tandem mass spectrometry (LC-MS/MS) (McNabb et al., 2005; Quilliam et al., 1995) and implemented as part of seafood safety programs, are considered the "gold standard" across the globe. These methods replaced the mouse bioassay (MBA; AOAC 959.08) which was previously the most commonly used laboratory analysis tool (e.g. (Christian and Luckas, 2008)). However, the development of more rapid, cost effective (on farm) testing methods for the presence of DSTs would potentially make harvest management simpler, faster and result in fewer closures. Three types of rapid test kits for the detection of DSTs are currently commercially available. These include an antibody based enzyme-linked immunosorbent assay (ELISA) test; a functional protein phosphatase inhibition activity (PPIA) assay; and a lateral flow analysis (LFA) rapid test. ELISA assays involve an antigen immobilized on a (micro) plate which are then complexed with an anti-body that is linked to a reporter enzyme. These assays were first developed in the 1960s and 1970s for primarily medical diagnosis purposes (Lequin, 2006). Detection is accomplished by assessing the conjugated enzyme activity via incubation with a substrate to produce a quantifiable product. Functional PPIA assays quantify okadaic acid (OA) and DST analogues including DTX-1, DTX-2 and DTX3 by colorimetric phosphatase inhibition, based on the reversible inhibition of protein phosphatase type 2A (PP2A) by the toxin, and the resulting fluorescence derived from enzymatic hydrolysis of the substrate. A lateral flow test involves the shellfish extract transported across a reagent zone in which OA specific antibodies are combined with coloured particles. If toxin is present, it is captured by the particle-antibody complex, and as its concentration increases, the intensity of the test "line" decreases (Jawaid et al., 2015).

In 2010 Dubois et al. published the first paper on the development and suitability of the ELISA method for the detection of marine toxins in shellfish. Three RTKs were developed a result of this study - one for domoic acid, one for okadaic acid and one for saxitoxin. A comparison across assay techniques was then undertaken, whereby cell counts, LC-MS, the newly developed Abraxis ELISA and PP2A Okatests were compared. Samples of blue mussels (*Mytilus edulis*) were collected fortnightly over a bloom event and ~40 individuals were homogenised and toxins extracted from a 1 g subsample as per the

manufacturer's protocols (methanol extraction). The protocol was further modified using an extra hydrolysis step to quantify the total DSP toxin content including esters and DTX-3. The ELISA showed matrix effects on hydrolysed samples, which had both high and low levels of toxins, while the PP2A performed well in detecting both high and low concentrations of DSP toxins in mussel samples. While the Okatest was recommended in preference to the ELISA, it was concluded to be a specific assay (could not detect other regulated DSP toxins) and therefore could not replace LC-MS/MS, but could be used as a product testing technique.

In 2012, a single lab validation study of the PP2A kit compared to the mouse bioassay and LC-MS was undertaken (Smienk et al., 2012), followed by an interlaboratory study using this same kit (Smienk et al., 2013). Samples of mussels, scallops, clams and cockles were analysed as blind duplicates (naturally contaminated except for one that was partially spiked), and blanks were distributed to sixteen laboratories over eleven countries. Results determined the OkaTest as a suitable test for quantitative determination of the OA toxin group and it was recommended as 'complimentary' to the reference method (LC-MS) for ongoing monitoring (EU approved as such).

Also in 2013, after many people became ill in Puget Sound (USA) after eating recreationally harvested mussels, a study was undertaken to examine the possibility of a RTK being used as an early warning/preharvest tool (Eberhart et al., 2013). Ten to twenty individuals of naturally contaminated blue mussels, geoducks, clams (two species) and pacific oysters were both hydrolysed and non-hydrolysed at two dilutions and examined using three RTKs – the lateral flow (Jellett/Scotia), ELISA (Abraxis) and PPIA (Okatest) kits, and compared to LC-MS. In summary, the Jellett kit gave false negatives, the ELISA showed low cross reactivity to DTX-1, while PP2A was reported at the most promising.

In 2015, a study reported on the development and validation of a LFA test kit (Neogen), a qualitative test strip/reader for the OA group toxins in shellfish (Jawaid et al., 2015). This validation method tested 2g spiked (OA, DTX-1, DTX-2 and DTX-3 with hydrolysis procedure) and naturally contaminated shellfish (mussels, scallops, oysters, and clams; n=72) and compared the results to LC-MS. No matrix effects, false compliant results or false noncompliant results at <50% MPL (maximum permitted level) were observed, suggesting this novel method was reliable.

In 2016, a further study compared four test kits for DSTs in shellfish from Argentina and again compared these results to LC-MS ((Turner and Goya, 2016). Kits included two qualitative lateral flow kits (Scotia and Neogen), and the quantitative PPIA kit (OkaTest) and ELISA kit (Max Signal – no longer commercially available). The specificity was reported as good for all kits, with no false positives (that is, all samples found to contain <16 mg OA eq/kg (LOQ) as determined by the regulatory LC-MS/MS testing method returned negative RTK results). Again in 2016, Johnson et al. tested four RTKs on naturally contaminated shellfish from Great Britain (Johnson et al., 2016). The quantitative PP2A (OkaTest) was the only test to show the complete absence of false compliant results (mussel samples containing OA-group toxins above the ML of 0.16 mg OA eq/kg), showed a fair correlation to LC-MS but an overall overestimation of sample toxicity with some indication of matrix effect, most notably in oysters. The quantitative ELISA (MaxSignal) gave a reasonable correlation with LC-MS, no evidence of overestimation, accurate at low concentrations and only one falsely compliant result. The two lateral flow assays (Neogen and Scotia) were observed to show high agreement with LC-MS and no indications of false positives (containing low or non-detectable levels of toxins as determined by LC-MS/MS), although both returned one false negative.

In a comprehensive review of field methods for detection of marine biotoxins in shellfish, McLeod et al. (Macleod et al., 2015) concluded that the ELISAs and LFAs had poor reactivity to the DSP congener DTX-2 and can give false negative results when high levels of DTX-3 are present (and the hydrolysis step is not undertaken to release ester forms). LFAs were also found to give some falsely complaint

results when DSP was below the ML, but this was dependent on the toxin profile, geographic region and shellfish species involved.

There are currently five commercially available rapid test kits for the detection of DSP in shellfish. These include three quantitative ELISA kits by Beacon™, Eurofins/Abraxis™ and EuroProxima™; a quantitative PP2A kit by Eurofins/Abraxis™, and a qualitative LFA kit by Neogen™.

1.6 Other rapid methods including molecular techniques for DST and species detection

Another rapid ELISA based method developed in recent years is the colloidal gold immunoassay (Ling et al., 2017; Wang et al., 2017). This "ic-ELISA" test, or 'indirect competitive test' is under development for simultaneously detecting both OA and tetrodotoxin in seafood. While results are promising from this work, there is no availability of these kits on the commercial market to date.

Finally, molecular genetic methods to detect and enumerate harmful algal species such as quantitative Polymerase Chain Reaction (qPCR) are growing in popularity. Real-time PCR is a laboratory technique, which can detect, characterize and quantify nucleic acids. A PCR instrument combines the functions of a thermal cycler and a fluorimeter, enabling targeted DNA (in this case that belonging to *Dinophysis*) to be labelled using a fluorescent binding dye, and then amplified using heat cycles. After each cycle the amplified DNA molecule is measured, so that the final fluorescence signal is proportional to the amount of replicated DNA. From this final measure, the quantity of the original DNA input can be calculated.

The qPCR method is sensitive, specific and lower in cost that many other methods (Penna and Galluzzi, 2013), and has been successfully developed and validated for the detection of multiple toxin producing species (Erdner et al., 2010; Galluzzi et al., 2010; Galluzzi et al., 2004; Godhe et al., 2008; Godhe et al., 2001; Hosoi-Tanabe and Sako, 2005; McLennan et al., 2021)). To date, there has been only one published example of the development of a real-time qPCR assay for the detection of *Dinophysis* (from Irish coastal waters) (Kavanagh et al., 2010). In this study, the highly conserved, large ribosomal sub-unit (LSU) D1-D2 target region was amplified and used to delineated *D. acuta* and *D. acuminata* based on melt-peak temperature.

2. Objectives

The objectives of this study are to:

1. Generate knowledge about commercial DST test kits and rapid molecular techniques (such as qPCR) for DST toxin and species detection (see Introduction)

2. Identify DST profiles present in Australian shellfish (see Introduction) and assess laboratory capabilities to detect these toxins

3. Compare the efficacy of commercially available toxin detecting kits using relevant sample matrices

4. Develop a quantitative PCR assay for *Dinophysis* species detection for potential onsite farm use

5. Provide cost versus benefit analysis of improved testing of DSTs in Tasmanian shellfish (Appendix 1)

6. Conduct a workshop to train shellfish industry members in the use of the rapid method of qPCR for *Dinophysis* detection in environmental samples, and seek their advice and feedback on how to best move forward (see Extension)

3. Methods

3.1 Interlaboratory comparison for LC-MS/MS

3.1.1 Shellfish preparation

Sample preparation was based on the standard operating procedure for the determination of lipophilic marine biotoxins in molluscs by LC-MS/MS and LC-MS. Specifically, raw samples of Sydney Rock Oysters (*S. glomerata*), Pacific Oysters (*M. gigas/C. gigas*), Blue Mussels (*M. galloprovincialis*) and Pipis (*P. deltoides/D. deltoides*) were sourced from the Sydney Fish Markets on 6/6/2019. From here on, these matrices are referred to as SRO, PO, MUS and PIPI, respectively. These were stored at 4-8 ℃ and transported immediately to the laboratory for processing. All shellfish were washed thoroughly with fresh water, shucked (if necessary) and tissue removed. Stock material of each species was made by pooling the tissue of 3-6 individuals (for each spike treatment) of that species, homogenising and spiking with fixed volumes of relevant standards (see below) and homogenising again. Subsamples of this species-specific tissue homogenate were then accurately weighed (~3g) and aliquoted into 5 mL polypropylene Bacto sample jars (Model No. SCP5014UU) and frozen at -20℃ until they were dispatched to contract laboratories for toxin determination by LC-MS/MS and LC-MS.

3.1.2 Standard reference materials

Certified reference materials (CRMs) were purchased from the National Research Council Canada (NRC) for shellfish spiking and quality control testing. These included: i) CRM DSP-Mus-c which is a thermally sterilized homogenate (4.0 ± 0.75 g) of mussel tissue (*M. edulis*) and the dinoflagellate *P. lima*, with toxin levels of okadaic acid (OA), dinophysistoxin-1 (DTX-1) and dinophysistoxin-2 (DTX-2) at 1.07 ± 0.08 µg/g, 1.07 ± 0.11 µg/g and 0.86 ± 0.08 µg/g, respectively (positive control); ii) CRM-OA-d which contained ~0.5 mL of a solution of OA in methanol at a concentration of 8.4 ± 0.4 µg/mL; iii) CRM-DTX-1-b which contained ~0.5 mL of a solution of dinophysistoxin 1 (DTX-1) in methanol at a concentration of 7.8 ± 0.5 µg/mL; and iv) CRM-DTX-2-b which contained ~0.5 mL of a solution of dinophysistoxin-2 (DTX-2) in methanol at a concentration of 3.8 ± 0.2 µg/mL.

3.1.3 Spiking of shellfish matrices

A subsample (3g) of each pooled, species-specific matrix (SRO, PO, MUS and PIPI) was first analysed by LC-MS at Laboratory 3 (see below) to ensure each matrix contained no DSTs before the experiment began (limit of detection (LOD) = 0.006-0.007 mg/kg for analogues OA, DTX-1 and DTX-2) (Appendix 1).

Spiking of each species- specific homogenate with a range of DST concentrations then followed for both LC-MS/MS and LC-MS. These concentrations were chosen based on the capability of most laboratories to achieve a limit of reporting (LOR) of ~0.01 mg/kg (Table 1, Appendix 1). In brief, one batch of each matrix was spiked with OA (@ 7.2 µl/3g, which is equivalent to 2 x LOR (0.02 mg/kg); the second one with DTX-1(@ 14.0 µl/3g, which is 4 x LOR (0.04 mg/kg), and the third with DTX-2 (@ 8 µl/3g, which is equivalent to the LOR (0.01 mg/kg). While increasing the spiking concentration of this latter analogue would provide a more rigorous comparison of the laboratories capabilities, our decision to spike DTX-2 at the LOR was based on cost and the infrequency of this analogue identified in Australian shellfish to date. A ~3g aliquot of each of these species-specific homogenates was then sent to each laboratory to test their LOR and any matrix effect (Table 1).

Next, a second species-specific homogenate was spiked with a combination of all three toxins: 35 µl/3g OA for SRO and PO which is 10 x LOR (0.1 mg/kg) or 7.2 µl/3g OA for MUS and PIPI which is equivalent to 2 x LOR (0.02 mg/kg); 17.6 µl/3g DTX-1 which is 5 x LOR (0.05 mg/kg) into all shellfish species; and 16 µg/3g DTX-2 which is 2 x LOR (0.02 mg/kg) again into all shellfish species. These combination-spiked

samples were then aliquoted (~3g) and sent to each laboratory to test toxin profile detection capability and also any matrix effect (Table 1).

Furthermore, to test the reproducibility/repeatability of each laboratory, a third batch of the SRO homogenate was spiked with OA (@ 7.2 µl/3g which is equivalent to 2 x LOR (0.02 mg/kg) and three replicate aliquots of this stock material (3g) were dispatched to each laboratory. Finally, one sample (~3g) of the CRM DSP-Mus-c was sent to each laboratory as a positive control. In total, 19 samples (randomly numbered 1-19), were dis-patched frozen to each of four laboratories (Table 1).

Table 1. List of Australian shellfish samples, toxin volume of CRM added per 3g of homogenised shellfish tissue, and OA equivalent concentrations (shaded) dispatched to each laboratory for DST determination using LC-MS.

	DST Spiking Volumes				
Matrix	OA only	DTX-1 only	DTX-2 only	OA/DTX-1/DTX-2	Total
Sydney Rock Oysters	7.2 µl/3g (3)*	14 µl/3g	8 µl/3g	35, 17.6, 16 µl/3g	6
Pacific Oyster	7.2 µl/3g	14 µl/3g	8 µl/3g	35, 17.6, 16 µl/3g	4
Mussel	7.2 µl/3g	14 µl/3g	8 µl/3g	7.2, 17.6, 16 µl/3g	4
Pipi	7.2 µl/3g	14 µl/3g	8 µl/3g	7.2, 17.6, 16 µl/3g	4
Concentration mg/kg	0.02 mg	0.04 mg	0.01	0.02 or 0.1#, 0.05, 0.02	
Positive Control (CRM DSP-Mus-c)	-	-	-	-	1
Total Samples					n=19

*n=3 for reproducibility/repeatability; # 0.02 mg/kg for mussel and pipi; 0.1 mg/kg for Sydney Rock Oysters and Pacific Oyster

3.1.4 LC-MS/MS toxin determination

Four commercial and/or government analytical laboratories with experience in conducting LC-MS/MS and LC-MS of marine biotoxins in shellfish were engaged to determine DSTs in spiked shellfish, identified only as Laboratories 1-4. The aim of this part of the study was to determine an inter-laboratory comparison of standardised samples, in order to obtain a baseline result using currently mandated seafood safety procedures in Australia (ASQACC, 2016). The LC-MS/MS and LC-MS methods engaged by each of the laboratories, their limits of detection and limits of reporting/quantification are shown in Appendix 1. No recovery corrections were applied to the final results reported from any of the labs.

3.2 Rapid test kit comparison

3.2.1 Shellfish preparation

Raw samples of SRO, PO, MUS and PIPI (same species as above), were sourced from the Sydney Fish Markets on 29/4/2020. These were stored at 4-8 °C and transported immediately to the University of Technology Sydney laboratory for processing. Again, all shellfish were washed thoroughly with fresh water, shucked and tissue removed. Bulk material of each species was then made by pooling the tissue of individuals of that species up to 90 g, homogenising and separating into 3 batches for downstream processing. The first batch served as unspiked controls and were first examined by LC-MS at Laboratory 3 (see above) to ensure each matrix was clear of toxins before the experiment began. The second batch was spiked with CRM-OA-d at ~12 µl/g (0.1 OA eq. mg/kg) which is half the ML, and the third batch was spiked at ~24 µl/g which is equal to the ML. Once prepared all batches were returned to the freezer (-20°C) until further processing.

Additionally, during Oct/Nov 2019, DSTs were detected in wild harvest Pipis from Sydney Fish Markets (~400 mg/kg), and a recall was immediately actioned. A batch of these naturally contaminated Pipis were obtained and prepared as positive controls: Sample 4A - 14/11/19 Stockton 4-6 km; 4B - 7/11/19 Stockton 4km; 4C - 31/10/19 Stockton 2-4kms; and 4D – Sydney Fish Market Stockton recall Nov 2019. Once the OA toxin con-centration was determined using LC-MS for these environmentally contaminated samples, samples with toxin level closest to the regulatory level (0.2 mg OA eq/kg) were chosen, and 10 replicates of these positive controls were ran on each kit to test the reliability/repeatability of each kit.

A subsample (3g) of each pooled, species-specific matrix was first examined by LC-MS (Laboratory 3) to ensure each matrix was clear of toxins before the experiment began (unspiked controls). All remaining batches (spiked and positive controls) were then subsampled and prepared according to the rapid test kit protocols for each kit or for LC-MS analysis. Duplicate samples of each treatment/shellfish were tested using both LC-MS and the five test kits.

3.2.2 Rapid test kits

A list of DST rapid test kits screened, their method details including their limit of quantification or working range, amount of tissue required, cost, time for analysis etc. are summarised in Table 2.

3.2.2.1 Qualitative test

3.2.2.1.1 Neogen LFA

Neogen Reveal 2.1 DSP Test strips (Lot: 9561-49, Neogen Corporation, Scotland, UK) and DSP hydrolysis packs (Lot: 9555-09) were stored at room temperature until experiments began. Each shellfish sample (2g) was defrosted to room temperature (20-25°C), then transferred to the extraction bag provided before being homogenised with 8 mL analytical grade methanol (Sigma-Aldrich, Sydney, Australia). Sample extract was then poured from each extraction bag (from opposite side of mesh divider) into a 15 mL falcon tube, prior to filtration using a 0.45 µm sterile Minisart® syringe filter into another clean 15 mL tube. Eighty µL of filtered extract was then transferred to a clean glass vial, followed by 100 µL of 2.5 M NaOH, before being capped tightly and mixed using a vortex on full speed for 30 secs. The sample vial was then transferred to a heater block set at 76 °C for 40 mins, after which time the sample was cooled on ice. At room temperature, 100 µL of 2.5 M HCl was added to the sample extract, mixed by hand for 30 secs, before 100 µL transferred into a DSP buffer A vial (provided). The sample was again vigorously mixed, before 100 µL was transferred to a microwell plate. A DSP strip was then placed into the microwell plate for 15 mins before being immediately placed into the AccuScan® PRO 2.0 scanner for result interpretation.

3.2.2.2 Quantitative tests

3.2.2.2.1 Abraxis PP2A

The Eurofins/Abraxis Okadaic Acid (PP2A) Microtiter Plate kit (Product No. 520025, Lot No. 19/1259, Eurofins Abraxis, Warminster, USA) was stored at 4°C prior to use. Upon opening, the solutions were prepared as per manufacturer's protocols and allowed to reach room temperature before analysis began. Each shellfish subsample (5g) was de-frosted and 25 mL methanol (Sigma-Aldrich, Sydney, Australia) added before homogenisation in a tube shaker for 2 mins. Sample was then centrifuged at 2000g for 10 min at 4°C and 640 µL of the methanolic extract removed and transferred to a clean 15 mL falcon tube. The extract was then mixed with 100 µL of 2.5 N NaOH, sealed and placed in a water bath at 76 ± 2oC for 40 mins. After removal from the water bath, 80 µL of 2.5 N HCl was added to each sample, followed by 20mL buffer solution.

For the test protocol, a volume of 50 µL of each OA standard (provided at 0.5, 0.8, 1.2, 1.8, and 2.8 nM) and each shellfish sample were added to the 96 well-plate provided. To each of these wells, 70 µL phosphatase solution was added. The plate was then tapped gently to ensure mixing, before being covered with parafilm and incubated for 20 min at 30 ± 2°C. Immediately after this incubation period, 90 µL of chromogenic substrate was added to each well, and again, the plate was tapped gently to ensure mixing. The plate was then incubated (covered) for a further 30 min at 30°C ± 2°C, after which 70 µL of stop solution was added to each well. Absorbance was immediately read at 405 nm using a Tecan Infinite M1000 PRO plate reader.

For data analysis, a standard curve was obtained by plotting the absorbance values in a linear y-axis and the concentration of okadaic acid in a logarithmic x-axis. The OA concentration contained in the sample (Cs) was then calculated using the following equation:

$$x = EXP((y - b)/a), \quad (1)$$

where x was the OA concentration in the sample (Cs) and y the absorbance of the sample. The concentration of DSTs in tissue (Ct) was then determined as:

$$Ct\ (mg/kg) = ((Cs\ (nM) \times FD \times MW\ (g/mol) \times Ve\ (L))/Mt\ (g))/1000$$

where Ct: DST concentration in tissue expressed as equivalents of OA; Cs: toxins concentration in sample; FD: Methanolic extract dilution factor (i.e. 640 µL/20 mL → x 31.25); MW: Okadaic acid molecular weight = 805; Ve: Methanolic extract volume (0.025L); Mt: Tissue weight (5g).

3.2.2.2.2 Beacon ELISA

The Beacon Okadaic Acid (ELISA) Plate kit (Cat. No. 20-0184, Lot No. 6289J, Beacon Analytical Systems Inc., Sako, USA) was stored at 4 °C and all reagents brought to room temperature before use. Each shellfish sample (1 g) was defrosted and 2 mL 80% methanol (Sigma-Aldrich, Sydney, Australia)/water added, before homogenisation and transfer to a clean 15 ml falcon tube. A further 8 mL of 80% methanol/water was then added, before vortexing for 5 min followed by centrifugation at 3000 rpm for 5 mins. The supernatant was then filtered into a clean 15 mL tube through a 0.45 µm sterile Minisart® syringe filter and the extract diluted 1:50 into 10% methanol/10mM PBS (Sigma-Aldrich, Sydney, Australia) (ie. 40 µL of filtered extract into 1.96 mL of 10% methanol/10mM PBS).

For the test procedure, 50 µL of enzyme conjugate was added into each test well, followed by 100 µL of each OA calibrator (provided at 0, 0.2, 0.5,1,2 and 5 µg/L) or shellfish sample, and 50 µL of antibody. Wells were then mixed for 30 sec using gentle shaking, followed by incubation at room temperature for 30 min. The content of the well plates were then decanted, and well plates washed four times using Milli-Q water, and inverting the plate onto absorbent paper between each wash. After the final wash, 100 µL of substrate was added to each well, before incubation for 30 min at room temperature. Finally, 100 µL of stop solution was added to each well and absorbance read at 450 nm using the Tecan Infinite M1000 PRO plate reader.

For quantitative interpretation of the absorbance readings, a standard curve was then constructed by plotting the absorbance of the calibrators (standards) on the y-axis versus the concentration of okadaic acid in a logarithmic x-axis. The OA concentration (ppb) contained in the sample (Cs) was then calculated using equation (1) above. Finally, to obtain the final DST (mg/kg) in each sample, a factor of x500 to account for the dilution during shellfish extraction step was applied.

Table 2. List of DST rapid test kits available, their method details and requirements (NR=not reported; ND=not detected). Note: LC-MS Cost ~$300 per sample and ~2 hours for analysis. *AU$1 has been added to the cost of each sample for consumables.

Kit No./Name	1. Neogen	2. Abraxis PP2A	3. Beacon ELISA	4. Abraxis ELISA	5. EuroProxima ELISA
Method	Lateral Flow Assay (LFA) – single sample	Protein Phosphatase Inhibition (PPI) - 96 well plate	ELISA 96 well plate	ELISA 96 well plate	ELISA 96 well plate
Qualitative or Quantitative	Qualitative	Quantitative	Quantitative	Quantitative	Quantitative
Analogues and Cross reactivity	OA (100%), DTX-1 (89%), DTX-2 (47%) & DTX-3	OA (1.2nM), DTX-1 (1.6nM), DTX-2 (1.2nM), DTX3	OA (100%), DTX-1 (120%), DTX-2 (20%)	OA (100%), DTX-1 (50%), DTX-2 (50%)	OA (100%), DTX-1 (78 DTX-2 (2.6%)
Limit of Quantification or Working Range	0.08 mg/kg [23]	0.06 to 0.35 mg/kg	0.1 mg/kg	0.1 - 5.0 mg/kg	0.04 mg/kg
Standards included	no	0.4, 0.6, 1.0, 1.5 and 2.3 µg/L	0, 0.2, 0.5,1,2, 5 µg/L	0, 0.1, 0.2, 0.5, 1, 2, 5 µg/L	0, 0.2, 0.5, 1.0, 2, 5, 10 µ
Hydrolysis step	yes	yes	no	yes	no
Amount of tissue required	2g	5g	1g	1g	1g
Samples per kit	24	~35-40 samples	~35-40 samples	~40 samples	~35-40 samples
Cost per kit (AU$)	$974.50	$1277	$849	$848	$999
Cost per sample* (AU$)	$42	$33	$22	$22	$26
Scanner (AU$)	$4000				
Reported False Positives	No false positives compared to ND by LC-MS [36]	14% positive compared to ND by LC-MS [36]	NR	Some false positives [34]	NR
Time for Analysis	~ 1.5 hours	~ 3 hours	~ 3 hours	~ 4 hours	~ 3 hours

3.2.2.2.3 Abraxis ELISA

The Eurofins/Abraxis Okadaic Acid (DSP) ELISA, Microtiter Plate (Product No. 520021, Lot No. 19/1178, Eurofins Abraxis, Warminster, USA) was stored at 4°C and brought to room temperature before use. All solutions were prepared as per manufacturer's protocols. Each shellfish subsample (1 g) was defrosted and 6 mL methanol (Sig-ma-Aldrich, Sydney, Australia)/ Milli-Q water (80/20) added, before homogenisation for 2 min. Each sample was then centrifuged for 10 min at 3000g and the supernatant transferred to a clean 15 mL falcon tube. A further 2 mL methanol/ Milli-Q was added to the shellfish residue, the sample centrifuged again for 10 min at 3000g, and the supernatant added to the first portion. The final volume was brought up to 10 mL with methanol/ Mil-li-Q, before filtration into a clean 15 mL tube through a 0.45 µm sterile Minisart® syringe filter. For the hydrolysis step, 500 µL of each sample extract was added to a 2 mL glass vi-al, and 100 µL of 1.25 N NaOH added. The sample was then vortexed for 15-20 sec before incubation on a heat block at 80 °C for 40 min. Each sample was then cooled and 100 µL of 1.25 N HCl added and vortexed for 15-20 sec. Finally, 10 µL of the hydrolysed extract was mixed with 990 µL of 1 x sample diluent (1:100 dilution) in a 2 mL glass vial with cap and vortexed again.

For the assay procedure, a volume of 100 µL of each OA standard (provided at 0, 0.1, 0.2, 0.5, 1, 2, 5 ppb) and shellfish sample was added to each strip well and placed into the well plate provided. To each of these, 50 µL of enzyme conjugate and 50 µL of antibody solution was added. The plate was then covered with parafilm, rotated carefully to mix and left to incubate for 60 min at room temperature, after which the covering removed and the contents decanted by inverting the plate onto paper towel. Each well was then thoroughly washed three times using the diluted wash buffer (~25 µL for each wash/each well), blotting after each step. Following the final washing step, 150 µL of substrate solution was added to each well, before covering with parafilm, rotating gently to mix, and incubating at room temperature for 30 mins. Finally, 100 µL of stop solution was added to each well plate prior to immediate absorbance reading at 450 nm using the Tecan Infinite M1000 PRO plate reader.

Kit performance was evaluated by calculating %B/Bo for each standard by dividing the absorbance value for each standard by the Zero standard mean absorbance. A standard curve was then constructed by plotting the %B/Bo for each standard on the y-axis versus the concentration of okadaic acid in a logarithmic x-axis. The OA concentration (ppb) contained in the sample (Cs) was then calculated using equation (1) above. Finally, to ac-count for hydrolysis sample extraction, hydrolysis and dilutions during the hydrolysis step, all results were multiplied x 1,400 to obtain the DSP concentration (ppb) before con-version to mg/kg.

3.2.2.2.4 EuroProxima ELISA

The EuroProxima Okadaic Acid ELISA (Catalogue No. 5191OKA, Lot No. UN6635, Arnhem, Netherlands) was stored at 4°C before use and subsequently brought to room temperature before use. Reagents were prepared as specified in the manufacturer's proto-col. To begin, 1 mL of water was added to each 1 g of shellfish, the sample vortexed for 1 min, and a further 2 mL of 100% methanol (Sigma-Aldrich, Sydney, Australia) added. The sample was again vortexed for 1 min followed by centrifugation at 2000g for 10 min. The clear supernatant was then filtered using a 0.45 µm sterile Minisart® syringe filter into a clean 15 mL falcon tube and the sample subsequently diluted 1:50 with the sample dilution buffer provided.

For the assay procedure, 100 µL of the zero standard (0 ng/mL) was pipetted into the first well, and 50 µL thereafter of each OA standard (provided at 0, 0.2, 0.5, 1.0, 2.0, 5.0 10.0 ng/mL) and shellfish samples into the 96 well-plate provided. Following on, 25 µL of enzyme conjugate and 25 µL of antibody was added to each well, except A1. The plate was then sealed with parafilm and gently shaken for 1 min before incubation at room temperature for 30 min. Parafilm was subsequently

removed, the well contents discarded onto absorbent paper, and all wells washed three times with rinsing buffer. After the final rinse, 100 µL of substrate solution was added to each well, mixed thoroughly and left to incubate for 15 min in the dark prior to 100 mL of stop solution being added. Absorbance was read at 450 nm using the Tecan Infinite M1000 PRO plate reader.

For data interpretation, the mean optical density (OD) value of the wells A1 and A2 were subtracted from the individual OD reading from each of the standards and samples. The OD values of the six standards and samples are then divided by the OD value of the zero standard (well no. B1) and multiplied by 100. The zero standard is then equal to 100% (maximum OD) and the other OD values are % of the maximal OD. A calibration curve was then constructed with the values (% maximal OD) plotted on the y-axis versus the concentration of okadaic acid (ng/mL) in a logarithmic x-axis. The OA concentration (ng/mL) contained in the sample (Cs) was then calculated using equation (1) above, but this time where x was the OA concentration in the sample (Cs) and y the % max OD of the sample. Finally, to obtain OA equivalents in the final shellfish, a factor of x 200 (and /1000) was applied.

3.3 Data assessment for LC-MS/MS and rapid test kit comparison

Toxin recovery from samples analysed using LC-MS/MS were assessed in four ways:

1. Where sample replication was available, mean (± SD) toxin recoveries were calculated and compared to the spiked concentration and LOR, and finally compared across laboratories.

2. To determine each analogue recovery using LC-MS/MS, toxin results from each shellfish species were compared to spiked toxin concentration, and then compared across laboratories.

3. For shellfish that were spiked with a combination of OA analogues, results were compared to both spiked concentration and the ML (0.2 mg/kg OA), as well as across laboratories.

4. Finally, the recovery of toxins in certified reference material CRM (DSP-Mus-c) were compared across laboratories.

To examine the performance of the rapid test kits, firstly we assessed the performance of the qualitative Neogen kit by comparison to the spiked toxin concentration in each sample (% false positives/% false negatives). Secondly, the performance and recovery of all quantitative methods (including LC-MS) were compared (% overestimated; % underestimated; % recovery; Pearson's correlation using Excel 2016) to the spiked concentration of each sample. For those samples spiked at, or above, the ML adopted by the Food Standards Australia New Zealand (0.2 OA mg/kg), we also determined if they were "falsely compliant" or "falsely non-complaint" with the ML. These terms refer to the comparison of the results obtained to the maximum regulatory limit. For example, if a sample was spiked above the regulatory limit and resulted in a concentration below the regulatory limit, it was referred to as "falsely compliant". Conversely, if a sample was spiked below the regulatory limit and returned a concentration above the regulatory limit, it was referred to as "falsely non-compliant". Thirdly, a comparison across species-specific matrices was undertaken to assess the suitability of rapid test kits across a range of shellfish species. Finally, the reliability or repeatability of each kit was assessed (defined as the standard deviation of the mean, Excel 2016) from the replicate positive controls (naturally contaminated Pipi samples) across all quantitative kits.

3.4 Development of a *Dinophysis* qPCR assay

3.4.1 Isolation of clonal strains and maintenance of cultures

For the development of a *Dinophysis* qPCR assay, *Dinophysis* DNA was required. As *Dinophysis* strains do not exist in Australia, we sourced this DNA from cultures grown in Japan (S. Nagai, Japan Fisheries

Research and Education Agency, National Research Institute of Fisheries Science). The method used to grow *Dinophysis* spp. was as follows:

Mesodinium rubrum and *Teleaulax amphioxeia* were isolated from Inokushi Bay (131°89'E, 32°79'N) in Oita Prefecture, Japan, at the end of February 2007 (Nagai et al., 2008). *M. rubrum* culture was maintained by mixing 50 mL of the culture grown until the late logarithmic growth phase (ca. 6×10^3 cells mL^{-1}) with 100 mL of modified f/2 medium (Guillard, 1975) of 250 mL capacity polycarbonate Erlenmeyer flasks (Corning, NY, USA). The culture medium was prepared with 1/3 nitrate, 1/3 phosphate, 1/3 metals, and 1/10 vitamins of the f/2 medium, plus any enrichment from the natural seawater collected from Tokyo Bay (35.3460 N, 139.6570 E). Salinity was adjusted to 30 practical salinity units (psu). Transfers were made once a week, with the addition of 25–100 µL of *T. amphioxeia* culture (containing $0.5–2.0 \times 10^4$ cells). The *M. rubrum* culture was maintained at a temperature of 18 °C under a photon irradiance of 100 µmol m^{-2} s^{-1} provided by cool-white fluorescent lamps, with a 12:12-h light: dark cycle. The *T. amphioxeia* culture was maintained by reinoculating 0.3 mL of the culture ($7.0–8.0 \times 10^4$ cells mL^{-1}) into 150 mL of the modified f/2 medium of 250 mL capacity polycarbonate Erlenmeyer flasks (Corning) under the same conditions as those used for *M. rubrum*.

D. caudata cells were isolated by micropipetting from a seawater sample collected from Nagasaki, Japan (32.8088 N, 129.7708 E) in 2013 and incubated in individual wells of a 48-well microplate (Iwaki, Japan). Similarly, *D. acuminata* cells were isolated from Mombetsu, Hokkaido, Japan (44.3368 N, 143.3808 E) in 2017, and *D. fortii* cells were isolated from the Saroma Lake, Hokkaido, Japan (44.1405 N, 143.8009 E) in 2015 and incubated in individual wells of a 48-well microplate (Iwaki), respectively. Each cell was grown in 1.0 mL of the culture medium containing ca. 1.0×10^3 cells of the marine ciliate *M. rubrum* as the prey species. *Dinophysis* cells were incubated under the same conditions as those for the *M. rubrum* culture, except for *D. caudata* set at 25 °C. After one month of incubation, several strains were established in each species, and clonal strains of DA_MOM02 (*D. acuminata*), DC_NAG01 (*D. caudata*), and DF_SAL90 (*D. fortii*) were used for further experiments. Small aliquots (0.1 mL) of the established cultures in each species were inoculated into 2.9 mL of fresh *M. rubrum* culture (ca. 2×10^3 cells mL^{-1}, just after reinoculation for the maintenance without adding *Teleaulax* culture) in 12-well microplates, and they were incubated for three weeks under the same conditions as mentioned above.

For scale-up of the cultivation, 3 mL of *Dinophysis* cells (ca. 3×10^3 cells mL^{-1}) were inoculated into 150 mL of fresh *M. rubrum* culture (ca. 2×10^3 cells mL^{-1}, without adding *Teleaulax* culture) of 250 mL capacity polycarbonate Erlenmeyer flasks (Corning). Five flasks were prepared in each strain, and they were incubated for one month under the same conditions as those used for the maintenance culture. After mixing five flasks' cultures in each strain, 1mL of each culture was sampled in triplicate for cell counting and toxin analysis. *Dinophysis* cells were harvested using a nylon sieve (mesh size, 10 µm), washed with 50 mL of fresh culture medium, and inoculated into 2 mL of plastic tubes. The tubes were centrifuged at 14,000 rpm for 2 min, and the supernatant was removed by pipetting. Samples were kept at -80 °C until use.

3.4.2 Sequences of 5.8S rDNA with the ITS region

Genomic DNA was extracted from several cells of each species by 5% Chelex buffer (Nagai et al. 2012). PCR amplification was carried out on a thermal cycler (PC-808, ASTEC, Fukuoka, Japan) with a reaction mixture consisted of 1 µL template DNA, 1 µM each of ITS (5.8S rDNA with the ITS region) primer sets (Adachi et al. 1994), 0.2 mM of each dNTP, 1× PCR buffer, 1.5 mM Mg2+, 1U KOD-Plus-Ver.2 (TOYOBO, Osaka, Japan), and RNA free distilled H2O to bring up to 25 µL volume. The PCR cycling conditions were as follows: 2 min at 94 °C, 30 cycles at 94 °C for 15 sec, 56 °C for 30 sec, and 68 °C for 40 sec. Sequences of the target regions were obtained by the direct Sanger sequencing method using the Dynamic ET terminator cycle sequencing kit (GE Healthcare, Little Chalfont, UK) and a DNA sequencer

(ABI3730, Applied Biosystems). The sequences were aligned using MEGA version 10 (Kumar et al., 2018) and the consensus sequences were obtained for each species. The BLAST search was performed to confirm the availability of sequences of the same species on the GenBank. All newly obtained sequences were then deposited into the DDBJ databank.

3.4.3 DNA extraction for qPCR assay development

DNA was extracted from pellets corresponding to ~1.1 x 10^5, 1.2 x 10^5 and 4.3 x 10^5 cells of *D. acuminata, D. fortii* and *D. caudata* respectively, using the DNeasy 96 PowerSoil Pro QIAcube HT Kit (Qiagen, Hilden Germany). Minor modifications were made to the manufacturer's protocol during the extraction process eg centrifugation instead of a vacuum pump. Cells pellets were preserved at 4°C in Longmire buffer prior to the extraction process. The buffer was then heated at 65°C for 10 mins and cells were lysed using 0.7 mm garnet beads (Capella Science Pty Ltd) on a vortex adapter (Qiagen) at top speed for 10 mins. Six hundred and fifty µL of buffer CD3 (provided by manufacturer, Qiagen) was then added to the lysate and the mixture was added onto silica-based spin columns (provided by manufacturer, Qiagen). The liquid was removed through centrifugation and purified using ethanol-based buffers (as per manufacturer's protocol) and finally eluted in 80 µL of buffer C6 (provided by manufacturer). DNA from these samples were stored in -20°C until further analysis.

3.4.4 Toxin Determination

The samples were frozen at –30 °C until the toxins were extracted by solid-phase extraction (SPE). The SPE of toxins was carried out modified to a previous method (Suzuki et al. 1997, 1998, 2009). The 1 mL frozen and thawed samples were applied to the MonoSpin C18 centrifuge cartridge column (GL Science Inc., Tokyo, Japan) equilibrate with 0.5 mL each methanol and distilled water. The SPE column was washed with 0.5 mL distilled water, and the toxins were eluted with 0.1 mL methanol. The methanol elutes were directly analyzed by LC-MS/MS. LC-MS/MS analysis of the toxins was carried out according to a previous method (Suzuki et al., 2011). A Nexera-20XR series liquid chromatograph (Shimadzu, Kyoto, Japan) was coupled to a QTRAP 4500 mass spectrometer (SCIEX, MA, USA) of hybrid triple quadrupole/linear ion trap. Separations were performed on LC columns (internal diameter, 100 mm × 2.1 mm) packed with 1.9 µm Hypersil GOLD C8 (Thermo Fisher Scientific Inc., Waltham, MA, USA) and maintained at 30 °C. Eluent A was water, and eluent B was acetonitrile water (95:5), containing two mM ammonium formate and 50 mM formic acid. Toxins were eluted from the column with 50% B at a flow rate of 0.3 mL min^{-1}. Multiple reaction monitoring (MRM) LC-MS/MS analysis with negative-mode ionization was carried out using the target parent ions and the fragment ions in Q1 and Q3 for each toxin as follows: OA, m/z 803.5 > 255.1; DTX-1, m/z 817.5 > 255.1; PTX-2, m/z 857.5 > 137.0; PTX-1 and PTX-11, m/z 873.5 > 137.0; PTX-2 Seco acid (PTX-2 SA), m/z 875.5 > 137.0. The lowest detection limits of OA/DTX-1 and PTX-2 were 0.1 and 1.2 ng mL^{-1}. These levels are equivalent to 0.2 pg $cell^{-1}$ of OA (and DTX-1) and 2.4 pg $cell^{-1}$ of PTX-2, when 100 cells of the toxic plankton were analyzed using our LC-MS/MS method.

3.4.5 qPCR assay development

3.4.5.1 Primer design and specificity

In order to design a specific and efficient qPCR assay for *Dinophysis*, eighteen ITS1/5.8S/ITS2 rRNA sequences from nine *Dinophysis* species, were initially downloaded from GenBank (http://www.ncbi.nlm.nih.gov), aligned using ClustalW, and examined by eye for regions of similarity and differences. Due to the largely conserved ITS region across all sequences, primers were designed for genus level only detection and were based on *Dinophysis acuminata* in silico tool NCBI Primer-BLAST. Twenty sets of primers pairs ranging from 106 to 150 bp in length resulted. To determine which primer set would sufficiently amplify the DNA extracted from each of the *Dinophysis* cell pellets described above, qPCR assays were subsequently undertaken.

Each qPCR assay was conducted using triplicate 20 µL reactions containing 1 µL of DNA from template from each of the three *Dinophysis* species, 10 µL of iTaq Universal SYBR Green Supermix (Bio-Rad, CA, USA), 0.5 µL of each of the forward and reverse primers, and 8 µL of DNA nuclease-free water (Ambion®). The qPCR assay was performed on the Bio-Rad CFX96 Touch™ Real-Time PCR Detection System™ platform with the following thermal cycling program: 95 °C for 3 mins, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and finally a temperature gradient for melt curve construction at a resolution of 0.5 °C. A negative control using nuclease-free water instead of the template DNA to detect for contamination was also included in the test run. This, and all subsequent assays, were run in 96 well plates with a clear seal (Bio-Rad, CA, USA).

An evaluation of the cross-reactivity of the most appropriate primer set followed. This was first assessed in silico, by downloading and aligning ITS1/5.8S/ITS2 rRNA sequences from the closely related genera *Phalacroma* and *Ornithocercus* from GenBank (http://www.ncbi.nlm.nih.gov). The number of Single Nucleotide Polymorphisms (SNPs) were then identified in the binding sites of the Dacu_11F/Dacu_11R qPCR primers. Specificity was also then tested in the laboratory, using DNA from other available phytoplankton species. This included 12 dinoflagellates and 1 diatoms (Table 3). The qPCR assay protocols for this specificity testing remained identical to those outlined above.

3.4.5.2 qPCR assay efficiency

To evaluate the mean qPCR efficiency (or performance) of the novel *Dinophysis* specific assay, standard curves were established using both a cell-based calibration and a gene-based approach (Bustin et al., 2009). In order to do this, the DNA from all three *Dinophysis* species was five-fold serially diluted. Dilutions ranged from 700 to 0.07 cells/µL for *D. acuminata*, 1500 to 0.15 cells/µL for *D. fortii* and 5,350 to 0.54 cells/µL for *D. caudata*. For the gene-based calibration curve, a ten-fold dilution series was established using a synthetic gene fragment (gBlock® IDT, USA) which was 257 base pairs in length and based on the ITS region of *D. acuminata*. The molecular weight and the amount of gBlock was supplied by IDT, from which the exact copy number of the gene fragment per microliter was calculated (Conte et al., 2018)). Copy numbers used in the qPCR assay ranged from 3.8×10^7 to 3.8 gene copies/µL. All samples were amplified in triplicates as per the qPCR protocol outlined above. Standard curves were then established for all three species and the gene fragment assay using the sample quantification cycle (Cq) (y-axis) and the natural log of concentration (x-axis). The percentage efficiency of each reaction was then calculated by the equation:

$$E = -1+10(-1/\text{slope}),$$

and deemed to be satisfactory if the amplification efficiency was between 90 – 110% (Bustin et al., 2009). Finally, to determine the relationship between cell number of each species and gene copy number, the slope of the log-linear standard curve was used to solve for x (concentration) for both species and gblock equations and the resulting 'factor' antilogged to return a number of gene copies per cell for each of the three species. The quantification of this relationship was then used in the interpretation of qPCR assay results from environmental samples.

Table 3. Cross-reactivity of the selected qPCR Dacu_11F/Dacu_11R primer pair on *Dinophysis* spp. and other available phytoplankton species including strain code and location of strain isolation.

Template	Strain code (Accession No.)	Location of Isolation	ITS PCR amplification
Dinophysis acuminata	DA_MOM_02	Mombetsu, Hokkaido, Japan	+
Dinophysis fortii	DF_SAL_90	Saroma lake, Hokkaido, Japan	+
Dinophysis caudata	DC_NAG_01	Nagasaki, Japan	+
Alexandrium pacificum	HRP4-2	Hawkesbury River, Australia	-
Pseudo-nitzschia cuspidata	P_WAG170419_1	Wagonga Inlet, Australia	-
Coolia malayensis	MAB	Malabar, Australia	-
Heterocapsa ovata	SA20	Port Lincoln, South Australia	-
Gambierdiscus polynesiensis	CG14	Rarotonga, Cook Islands	-
Fukuyoa yasumotoi	OIRS230	Orpheus Island, Australia	-
Prorocentrum lima	SM43	Raine Island, Australia	-
Amphidinium "massartii"	CS259	Kirrimine Beach, Qld	-
Ostreopsis siamensis	HER24	Heron Island, Australia	-
Thecadinium kofoidii	THE	Gordons Bay, Australia	-

3.4.5.3 *Comparison of cell counts and qPCR assay for* Dinophysis *bloom dynamics*

3.4.5.3.1 Water sampling for eDNA and *Dinophysis* cell enumeration

Water samples (500 ml) were collected at approximately 2-weekly intervals from a depth of 0.5 m from the Manning River, NSW, for microscopic phytoplankton identification and enumeration in accordance with the NSW Marine Biotoxin Management Plan (NSW MBMP) and the Australian Shellfish Quality Assurance Program (ASQAP). Once collected, samples were immediately preserved with 1% Lugol's iodine solution, and returned to the laboratory for concentration using gravity-assisted membrane filtration. Detailed cell examination and counts were then performed using a Sedgewick Rafter counting chamber and a Zeiss Axiolab or Standard microscope equipped with phase contrast. Cells were identified to the closest possible taxon using light microscopy (maximum magnification × 1000), and cell counts to determine the abundance of individual *Dinophysis* species carried out with a minimum detection threshold of 50 cells L^{-1}.

As part of the Cooperative Research Centre for Food Agility's Oyster Transformation Project (www.foodagility.com/research/food-safety-in-the-oyster-industry), approximately weekly water samples were carried out at a second sampling site for environmental DNA (hereafter known as eDNA). This sampling program provided us with a unique opportunity to test our *Dinophysis* specific qPCR assay on environmental samples both before, during and after a *Dinophysis* bloom event, which was reported on 17 February 2019 at a maximum cell concentration of 5,300 cells L^{-1} of *D. acuminata.* Triplicate three-litre surface water samples (0.5 m) were collected weekly from this site using the water sampler described in Ruvindy et al. (Ruvindy et al., 2018). In brief, water samples were passed firstly through a 100 µm (pore size) nylon mesh and then a second 11 µm mesh. Mesh was then backwashed with filtered seawater to retain the phytoplankton. Finally using a syringe filter with an 8 µM filter (Merck), the sample was filtered one last time, and the filter place into a 5mL tube (Eppendorf) containing 2 mL Longmire buffer. Samples for eDNA were then stored at 4 °C until further downstream processing.

3.4.5.3.1 qPCR assay using eDNA for bloom dynamics

Filtered samples in 2 mL Longmire buffer were incubated at 65 °C for 10 mins and vortexed for 10 mins using Qiagen Vortex Genie 2 (at top speed) prior to eDNA extraction. eDNA was extracted using the QIAcube HT automated nucleic acid isolation system and the DNeasy 96 PowerSoil Pro QIAcube HT Kit (Qiagen). In summary, 1 mL of buffer was loaded onto the S-block (provided by manufacturer) followed by the addition of 650 µL of buffer CD3 (provided by manufacturer). The mixture was then added onto the QIAamp 96 plate and liquid was removed using a vacuum pump. eDNA was purified on a column using ethanol-based buffers (as per manufacturer's protocol) and eluted in 80 µL of buffer C6 (provided by manufacturer). eDNA samples were then stored in -20°C until further analysis. The eDNA extracts were 10-fold serially diluted ranging from 1.25×10^4 to $1.2^5 \times 100$ cells for each standard curve. The qPCR reactions to generate the cell-based calibration curve were performed in the same way as those used for gene-based calibration curve.

Triplicate eDNA samples and gene fragment serial dilution samples were prepared for qPCR analysis. For this final assay, the reaction volumes were 5 µL, comprising of 2.5 µL SYBR Green Mix (Bio-Rad), 1.1 µL nuclease free water, 0.2 µL of forward and reverse primer (0.5 µM final concentration) and 1 µL of eDNA template. Two negative controls were also run to detect for contamination. The plate was prepared with an epMotion®5075l Automated Liquid Handling System. The qPCR assay was performed using the BIORAD CFX384 Touch™ Real-Time PCR Detection System™ using the cycling conditions as described above.

4. Results

4.1 Interlaboratory comparison for LC-MS/MS

No toxins were detected in any of the four shellfish species matrices (SRO, PO, MUS, PIPI) screened before spiking began (see Methods). Of the triplicate SROs spiked with OA at 0.02 mg/kg, Laboratory 1 detected OA in all three samples (x = 0.01, SD ± 0.00, min <0.01, max 0.02 mg/kg), Laboratory 2 and 4 reported concentrations below the detection limit for all samples (< 0.01 mg/kg and <0.025 mg/kg respectively), and Laboratory 3 detected OA in all three samples (x = 0.013, SD ± 0.006, min 0.01, max 0.02 mg/kg). In summary, two out of the four laboratories detected OA at this low level, with recoveries be-tween ~50- 100% (Table 4).

Table 4. Results of LC-MS/MS and LC-MS for Sydney Rock Oysters (SRO) spiked with 0.02 mg/kg okadaic acid (no DTX-1 or DTX-2 added).

Replicate	Species Code	Analyte Code	Spike mg/kg	Lab 1 mg/kg	Lab 2 mg/kg	Lab 3 mg/kg	Lab 4 mg/kg
1	SRO	OA Free	0.02	0.01	<0.01	0.01	<0.025
	SRO	OA Total	0.02	0.01	<0.01	0.01	<0.025
2	SRO	OA Free	0.02	0.02	<0.01	0.01	<0.025
	SRO	OA Total	0.02	<0.01	<0.01	0.01	<0.025
3	SRO	OA Free	0.02	0.01	<0.01	0.02	<0.025
	SRO	OA Total	0.02	0.01	<0.01	0.02	<0.025

Note: Spike below limit of reporting for Laboratory 4

Of the four shellfish species spiked with OA at 0.02 mg/kg, Laboratory 1 detected this toxin in all four matrices (x = 0.013, SD ± 0.005, min 0.01, max 0.02 mg/kg), Laboratory 2 did not detect OA in SRO or PO, however it was detected in both MUS and PIPI (x = 0.015, SD ± 0.007; min < 0.01, max 0.02 mg/kg), and Laboratory 3 did not detect OA in PO or MUS, but detected it in SRO and PIPI (x = 0.015, SD ± 0.007; min < 0.01, max 0.02 mg/kg). Laboratory 4 did not detect OA at this concentration (less than detection limit < 0.025 mg/kg). Laboratory 4 however, did detect OA in one PIPI sample at 0.03 mg/kg (> spike concentration). In summary, OA was detected in all matrices at this concentration, although not all laboratories detected toxins in all four matrices. Recovery across all laboratories ranged from ~50 -150% (Table 5).

For the shellfish spiked with DTX-1 at 0.04 mg/kg, Laboratory 1 recovered this analogue in all matrices (x = 0.035, SD ± 0.006; min 0.03, max 0.05 mg/kg), with one PIPI sample returning a concentration of 0.01 OA mg/kg. Laboratory 2 detected DTX-1 in all matrices (x = 0.025, SD ± 0.006; min 0.02, max 0.03 mg/kg), also with a detection of OA in PIPI at 0.02 mg/kg. Laboratory 3 detected DTX-1 in all matrices (x = 0.025, SD ± 0.006; min 0.02, max 0.03 mg/kg), while Laboratory 4 did not detected this toxin in MUS (other matrices x = 0.026, min <0.025, max 0.04 mg/kg) (Table 6). In summary, DTX-1 was detected in all shellfish matrices at this concentration; however, one laboratory did not detect DTX-1 in MUS. The overall recovery of this analogue was ~50 -100% across laboratories with two detections of OA in PIPIs.

For all shellfish spiked with DTX-2 at 0.01 mg/kg, Laboratory 1 did not recover this analogue in SRO or PIPI, and was only detected it in PO and MUS (both at 0.01 mg/kg). No toxin at this concentration was recovered from either Laboratory 2 nor Laboratory 3, while Laboratory 4 was unable to detect this toxin (below the limit of reporting < 0.025 mg/kg) (Table 7). In summary DTX-2 was only detected in PO and MUS at this low con-centration, and only at one laboratory. Overall recovery was ~50 -100%.

Table 5. Results of LC-MS/MS and LC-MS for Australian shellfish - Sydney Rock Oysters (SRO), Pacific Oysters (PO), Blue Mussels (MUS) and Pipis (PIPI) spiked with 0.02 mg/kg okadaic acid (no DTX-1 or DTX-2 added)

Sample	Species Code	Analyte Code	Spike mg/kg	Lab 1 mg/kg	Lab 2 mg/kg	Lab 3 mg/kg	Lab 4 mg/kg
1	SRO	OA Free	0.02	0.01	<0.01	0.02	<0.025
	SRO	OA Total	0.02	0.01	<0.01	0.02	<0.025
2	PO	OA Free	0.02	0.02	<0.01	<0.01	<0.025
	PO	OA Total	0.02	0.02	<0.01	<0.01	<0.025
3	MUS	OA Free	0.02	0.02	0.01	<0.01	<0.025
	MUS	OA Total	0.02	0.01	0.01	<0.01	<0.025
4	PIPI	OA Free	0.02	0.01	<0.01	0.01	<0.025
	PIPI	OA Total	0.02	0.01	0.02	0.01	0.03

Note: Spike below limit of reporting for Laboratory 4

Table 6. Results of LC-MS/MS and LC-MS for Australian shellfish - Sydney Rock Oysters (SRO), Pacific Oysters (PO), Blue Mussels (MUS) and Pipis (PIPI) spiked with 0.04 mg/kg DTX-1 (no OA or DTX-2 added).

Sample	Species Code	Analyte Code	Spike mg/kg	Lab 1 mg/kg	Lab 2 mg/kg	Lab 3 mg/kg	Lab 4 mg/kg
1	SRO	DTX-1 Free	0.04	0.05	0.02	0.03	0.04
	SRO	DTX-1 Total	0.04	0.04	0.02	0.03	0.026
2	PO	DTX-1 Free	0.04	0.04	0.02	0.02	0.03
	PO	DTX-1 Total	0.04	0.04	0.02	0.02	<0.025
3	MUS	DTX-1 Free	0.04	0.04	0.03	0.02	<0.025
	MUS	DTX-1 Total	0.04	0.03	0.03	0.02	<0.025
4	PIPI	DTX-1 Free	0.04	0.05	0.02	0.03	0.031
	PIPI	DTX-1 Total	0.04	0.03	0.03	0.03	<0.025
	PIPI	OA Total	-	0.01	0.02	<0.01	<0.025

Table 7. Results of LC-MS/MS and LC-MS for Australian shellfish - Sydney Rock Oysters (SRO), Pacific Oysters (PO), Blue Mussels (MUS) and Pipis (PIPI) spiked with 0.01 mg/kg DTX-2 (no OA or DTX-1 added).

Sample	Species Code	Analyte Code	Spike mg/kg	Lab 1 mg/kg	Lab 2 mg/kg	Lab 3 mg/kg	Lab 4 mg/kg
1	SRO	DTX-2 Free	0.01	<0.01	<0.01	<0.01	<0.015
	SRO	DTX-2 Total	0.01	<0.01	<0.01	<0.01	<0.015
2	PO	DTX-2 Free	0.01	0.01	<0.01	<0.01	<0.015
	PO	DTX-2 Total	0.01	<0.01	<0.01	<0.01	<0.015
3	MUS	DTX-2 Free	0.01	0.01	<0.01	<0.01	<0.015
	MUS	DTX-2 Total	0.01	<0.01	<0.01	<0.01	<0.015
4	PIPI	DTX-2 Free	0.01	<0.01	<0.01	<0.01	<0.015
	PIPI	DTX-2 Total	0.01	<0.01	<0.01	<0.01	<0.015

Note: Spike below limit of reporting for Laboratory 4

When shellfish were spiked with all toxins (in varying concentrations between 2-10 x LOR depending on toxin analogue; see Methods), laboratory recovery of total toxin per sample for each laboratory was as follows – Laboratory 1: 53-75%; Laboratory 2: 35-88%; Laboratory 3: 13-41%; and Laboratory

4: 0-88% (Table 8). More specifically, all toxins were recovered in all matrices for Laboratory 1, with an individual toxin/sample recovery ranging from 40% - 200%, with the lowest matrix average recovery in SRO at 57% and the highest in PIPI at 103%. For Laboratory 2, DTX-2 was not detected in SRO or PO, while in-dividual toxin/sample recovery ranged from 40% - 400%, with the lowest matrix average recovery in SRO at 43%, and the highest in PIPI at 170%. For Laboratory 3, OA was not detected in MUS or PIPI, and DTX-2 was not detected in PIPI. The individual toxin/sample recovery ranged from 20% - 50%, with the lowest matrix average in PIPI at 40% and the highest in MUS at 47%. Finally for Laboratory 4, DTX-2 was not detected across all matrices and OA was not detected in MUS. Individual toxin/sample recovery ranged from 50% - 340% with the lowest matrix average in MUS at 50% and the highest in PIPI at 154%. Overall, most toxins were detected by all laboratories at these concentrations, individual recovery across all labs/matrices ranged from 0-88%, while the recovery across shellfish matrices varied.

Table 8. Results of LC-MS/MS and LC-MS for Australian shellfish - Sydney Rock Oysters (SRO), Pacific Oysters (PO), Blue Mussels (MUS) and Pipis (PIPI) spiked with a combination of DST analogues – OA 0.1 mg/kg; DTX-1 0.05 mg/kg; and DTX-2 0.02 mg/kg.

Sample	Species Code	Analyte Code	Spike mg/kg	Lab 1 mg/kg	Lab 2 mg/kg	Lab 3 mg/kg	Lab 4 mg/kg
1	SRO	DTX-1 Free	0.05	0.05	0.01	0.02	0.038
	SRO	DTX-1 Total	0.05	0.04	0.02	0.02	0.03
	SRO	DTX-2 Free	0.02	0.02	<0.01	0.01	<0.015
	SRO	DTX-2 Total	0.02	0.01	<0.01	0.01	<0.015
	SRO	OA Free	0.1	0.05	0.04	0.04	0.089
	SRO	OA Total	0.1	0.04	0.04	0.04	0.062
2	PO	DTX-1 Free	0.05	0.03	0.02	0.02	0.036
	PO	DTX-1 Total	0.05	0.03	0.03	0.02	0.029
	PO	DTX-2 Free	0.02	0.02	<0.01	0.01	<0.015
	PO	DTX-2 Total	0.02	0.02	<0.01	0.01	<0.015
	PO	OA Free	0.1	0.06	0.04	0.04	0.08
	PO	OA Total	0.1	0.04	0.05	0.04	0.067
3	MUS	DTX-1 Free	0.05	0.02	0.03	0.02	0.03
	MUS	DTX-1 Total	0.05	0.03	0.03	0.02	<0.025
	MUS	DTX-2 Free	0.02	0.01	0.01	0.01	<0.015
	MUS	DTX-2 Total	0.02	0.02	0.01	0.01	<0.015
	MUS	OA Free	0.01	0.01	0.01	<0.01	<0.025
	MUS	OA Total	0.01	0.01	<0.01	<0.01	<0.025
4	PIPI	DTX-1 Free	0.05	0.03	0.03	0.01	0.033
	PIPI	DTX-1 Total	0.05	0.03	0.03	0.01	0.036
	PIPI	DTX-2 Free	0.02	0.02	0.02	<0.01	<0.015
	PIPI	DTX-2 Total	0.02	0.01	<0.01	<0.01	<0.015
	PIPI	OA Free	0.01	0.02	0.01	<0.01	<0.025
	PIPI	OA Total	0.01	0.02	0.04	<0.01	0.034

Note: Spike of OA for MUS and PIPI below limit of reporting for Laboratory 4

In our final analysis to determine the recovery of CRM (OA/DTX-1/DTX-2), all laboratories detected all toxin analogues. Individual toxin recoveries ranged from 88 to 131% for Laboratory 1, 79-81% for Laboratory 2, 83 to 95% for Laboratory 3 and 101-262% for Laboratory 4 (Table 9). However, considering that these recoveries are the result of one sample per lab, they should be treated as indicative only

Table 9. Results of LC-MS/MS for Certified Reference Material CRM DSP-Mus-c.

Sample	Species Code	Analyte Code	Concentration mg/kg	Lab 1 mg/kg	Lab 2 mg/kg	Lab 3 mg/kg	Lab 4 mg/kg
1	+CONT	DTX-1 Free	1.07	1.4	0.87	0.91	1.1
	+CONT	DTX-1 Total	1.1*	1.4	1.04	2.31	1.3
	+CONT	DTX-2 Free	0.86	0.76	0.68	0.82	0.87 (0.522)
	+CONT	DTX-2 Total	2.2*	2.0	1.97	1.32	2.6 (1.56)
	+CONT	OA Free	1.07	1.1	0.85	0.89	2.8
	+CONT	OA Total	2.4*	2.2	2.29	1.79	5.0

*CRM are certified for free toxin; they report higher total toxin concentration post hydrolysis but these are not certified

4.2 Rapid test kit comparison.

4.2.1 Wild harvest Pipis

Prior to rapid test kit screening, OA, DTX-1 and DTX-2 analysis by LC-MS for wild harvest Pipis resulted in a OA toxin range of 0.1 to 0.3 mg/kg (Sample 4A - 0.1 mg/kg, 4B – 0.1 mg/kg, 4C – 0.2 mg/kg, and 4D – 0.3 mg/kg). After hydrolysis, no DTX-1 or DTX-2 was detected in any samples. Three batches comprising 10 replicates of each OA toxin concentration of 0.1, 0.2 and 0.3 mg/kg were subsequently screened using each rapid test kit.

4.2.2 LC-MS

Using LC-MS (Laboratory 3), all control shellfish samples (no toxin added) returned a 'not detected' result (Table 7A). For OA spiked samples, 43/46 (~93%) returned concentrations at, or slightly above, the spiked toxin concentrations 0.1 & 0.2 mg/kg (Tables 10A-B). The three samples (7%) that returned concentrations lower that the spiked concentration were all spiked Pipi samples: sample 22 reported 0.09 mg/kg when it was spiked with OA at 0.1 mg/kg; sample 23 reported 0.15 mg/kg when it was spiked with OA at 0.2 mg/kg; and finally, sample 24 reported 0.09 mg/kg when it was spiked with OA at 0.2 mg/kg (Tables 10A-B). The latter two of these samples were falsely compliant at the regulatory limit (7%, 2/28). A Pearson's correlation analysis between LC-MS results and the concentration of spiked toxin revealed a very strong relationship ($r^2 = 0.86$) (Fig. 1). Subsequently, this method returned a mean recovery of 106.5 %, meeting the criteria set out in the AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals (AOAC, 2002).

4.2.3 Rapid test kits

4.2.3.1 Qualitative test

4.2.3.1.1 Neogen LFA

The Neogen kit returned negative readings for the eight negative control samples across all species-specific shellfish matrices. However, 23 out of 46 samples (50%) of spiked samples (across all shellfish matrices) returned a negative result when they contained okadaic acid (Tables 10A-B). Within this group, 18% (5/28 samples again across all matrices) returned a false compliant result when they were spiked at, or above, the regulatory limit (=/>0.2 mg OA eq/kg), while no naturally contaminated Pipis returned falsely compliant results with this kit.

Table 10A. Results of LC-MS and rapid test kits for Okadaic Acid spiked into Australian shellfish (Sydney Rock Oysters [SRO], Pacific Oyster [PO], Blue Mussel [MUS] and Pipis [PIPI]). Note: Abraxis PP2A Working Range = 0.06 to 0.35 mg/kg; Beacon ELISA Limit of Quantification = 0.1 mg/kg; Abraxis ELISA Working Range = 0.1 – 5.0 mg/kg; Europroxima ELISA Limit of Quantification = 0.04 mg/kg.

Sample no. and shellfish matrix	OA mg/kg	LC-MS	Neogen	Abraxis PP2A	Beacon ELISA	Abraxis ELISA	Europroxima ELISA
Sample 1 (SRO)	-	ND	-	0.02	0.05	0.00	0.03
Sample 2 (SRO)	-	ND	-	0.07	0.06	0.03	0.01
Sample 3 (SRO)	0.1	0.12	-	0.05	0.12	0.00	0.04
Sample 4 (SRO)	0.1	0.13	-	0.02	0.11	0.01	0.19
Sample 5 (SRO)	0.2	0.23	+	0.17	0.18	0.01	0.08
Sample 6 (SRO)	0.2	0.23	-	0.05	0.24	0.01	0.09
Sample 7 (PO)	-	ND	-	0.07	0.07	0.00	0.08
Sample 8 (PO)	-	ND	-	0.03	0.05	0.00	0.02
Sample 9 (PO)	0.1	0.12	-	0.05	0.12	0.01	0.04
Sample 10 (PO)	0.1	0.17	-	0.11	0.18	0.03	0.04
Sample 11 (PO)	0.2	0.23	-	0.12	0.20	0.03	0.04
Sample 12 (PO)	0.2	0.23	-	0.21	0.20	0.03	0.07
Sample 13 (MUS)	-	ND	-	0.02	0.05	0.03	0.01
Sample 14 (MUS)	-	ND	-	0.03	0.06	0.02	0.02
Sample 15 (MUS)	0.1	0.19	-	0.16	0.12	0.01	0.09
Sample 16 (MUS)	0.1	0.17	-	0.06	0.10	0.01	0.02
Sample 17 (MUS)	0.2	0.23	+	0.16	0.21	0.01	0.11
Sample 18 (MUS)	0.2	0.23	-	0.08	0.19	0.02	0.04
Sample 19 (PIPI)	-	ND	-	0.04	0.09	0.01	0.02
Sample 20 (PIPI)	-	ND	-	0.02	0.09	0.01	0.01
Sample 21 (PIPI)	0.1	0.1	-	0.13	0.17	0.02	0.04
Sample 22 (PIPI)	0.1	0.09	-	0.05	0.17	0.04	0.02
Sample 23 (PIPI)	0.2	0.15	+	0.18	0.43	0.01	0.09
Sample 24 (PIPI)	0.2	0.09	-	0.13	0.43	0.01	0.06

ND=not detected (0.01 mg/kg detection limit)

4.2.3.2 Quantitative tests

4.2.3.2.1 Abraxis PP2A

The Abraxis PP2A returned 25% (2/8) false positive results, that is, they returned concentrations of toxin within the kit's working (range 0.06 to 0.35 mg/kg), when the samples contained no okadaic acid. Of those shellfish that were spiked, 29% (13/45) of samples returned values that were outside the working range (8 samples below 0.06 mg/kg and 5 samples above 0.35 mg/kg), with 27% (12/45) samples being underestimated and 44% (20/45) returning a concentration which was equal to, or greater than, the spiked toxin concentration (Tables 10A-B). When samples were spiked at, or above, the regulatory limit, the Abraxis PP2A returned 29% (8/28) falsely compliant results (Table 8). These results were for both spiked and naturally contaminated samples. A Pearson's correlation analysis between the Abraxis PP2A results and spiked toxin concentrations was significant at $r^2 = 0.72$ (Fig. 1). This kit returned a mean recovery of 92.2 %, again meeting the criteria set out in the AOAC Guidelines (AOAC, 2019)(Table 11).

Table 10B. Results of LC-MS and rapid test kits for Okadaic Acid in naturally contaminated Pipis [PIPI] Note: Abraxis PP2A Working Range = 0.06 to 0.35 mg/kg; Beacon ELISA Limit of Quantification = 0.1 mg/kg; Abraxis ELISA Working Range = 0.1 – 5.0 mg/kg; Europroxima ELISA Limit of Quantification = 0.04 mg/kg.

Sample no. and shellfish matrix	OA mg/kg	LC-MS	Neogen	Abraxis PP2A	Beacon ELISA	Abraxis ELISA	Europroxima ELISA
Sample 25 (PIPI)	0.1	0.1	-	0.04	0.07	0.02	0.03
Sample 26 (PIPI)	0.1	0.1	-	0.10	0.05	0.01	0.02
Sample 27 (PIPI)	0.1	0.1	-	0.04	0.06	0.07	0.02
Sample 28 (PIPI)	0.1	0.1	-	0.07	0.10	0.04	0.03
Sample 29 (PIPI)	0.1	0.1	-	0.07	0.06	0.03	0.03
Sample 30 (PIPI)	0.1	0.1	-	0.05	0.08	0.02	0.02
Sample 31 (PIPI)	0.1	0.1	-	0.15	0.06	0.02	0.02
Sample 32 (PIPI)	0.1	0.1	-	0.10	0.06	0.06	0.02
Sample 43 (PIPI)	0.1	0.1	-	0.06	0.10	0.18	0.03
Sample 44 (PIPI)	0.1	0.1	-	NS	0.08	0.17	0.02
Sample 33 (PIPI)	0.2	0.2	+	0.23	0.08	0.25	0.03
Sample 34 (PIPI)	0.2	0.2	+	0.22	0.13	0.14	0.02
Sample 35 (PIPI)	0.2	0.2	+	0.24	0.10	0.24	0.03
Sample 36 (PIPI)	0.2	0.2	+	0.16	0.11	0.17	0.02
Sample 37 (PIPI)	0.2	0.2	+	0.25	0.13	0.22	0.02
Sample 38 (PIPI)	0.2	0.2	+	0.25	0.04	0.14	0.04
Sample 39 (PIPI)	0.2	0.2	+	0.20	0.06	0.06	0.02
Sample 40 (PIPI)	0.2	0.2	+	0.27	0.05	0.16	0.01
Sample 41 (PIPI)	0.2	0.2	+	0.22	0.10	0.05	0.02
Sample 42 (PIPI)	0.2	0.2	+	0.23	0.11	0.02	0.02
Sample 45 (PIPI)	0.3	0.3	+	0.38	0.05	0.21	0.03
Sample 46 (PIPI)	0.3	0.3	+	0.39	0.06	0.19	0.02
Sample 47 (PIPI)	0.3	0.3	+	0.39	0.05	0.33	0.02
Sample 48 (PIPI)	0.3	0.3	+	0.36	0.09	2.05	0.03
Sample 49 (PIPI)	0.3	0.3	+	0.33	0.07	0.88	0.02
Sample 50 (PIPI)	0.3	0.3	+	0.36	0.10	0.11	0.03
Sample 51 (PIPI)	0.3	0.3	+	0.34	0.17	0.23	0.03
Sample 52 (PIPI)	0.3	0.3	+	0.34	0.06	0.24	0.03
Sample 53 (PIPI)	0.3	0.3	+	0.32	0.08	0.19	0.02
Sample 54 (PIPI)	0.3	0.3	+	0.25	0.05	0.17	0.06

NS=no sample

4.2.3.2.2 Beacon ELISA

With a limit of quantification reported as 0.1 mg/kg, the Beacon ELISA kit returned 0% (0/8) false positives and 43% (20/46) of spiked samples below the limit of quantification. Of the samples that were spiked (and results above the quantification limit), 22% (10/46) were underestimated, while 35% (16/46) were equal to, or greater than, the spiked toxin concentration (Tables 10A-B). When samples were spiked at/above the regulatory limit, or were naturally contaminated at/above the regulatory limit, the Beacon ELISA returned 79% (22/28) falsely compliant results (Table 11). A Pearson's correlation analysis between the Beacon ELISA kit test results and the spiked toxin concentrations was

Table 11. Summary of results comparing LC-MS (Laboratory 3) and five commercially available test kits to spiked Australian shellfish (results are across all species-specific shellfish matrices). Note: Abraxis PP2A Working Range (WR) = 0.06 to 0.35 mg/kg; Beacon ELISA Limit of Quantification (LOQ) = 0.1 mg/kg; Abraxis ELISA Working Range = 0.1 – 5.0 mg/kg; Europroxima ELISA Limit of Quantification = 0.04 mg/kg; ML = Maximum limit (=Regulatory Limit 0.2 eq OA mg/kg); Repeatability is defined as the standard deviation of the mean (see Methods).

	LC-MS	**Neogen**	**Abraxis PP2A**	**Beacon ELISA**	**Abraxis ELISA**	**Europroxima ELISA**
% False Positive (blank matrix)	0 (0/8)	0 (8/8)	25 (2/8)	0 (0/8)	0 (0/8)	13 (1/8)
% False Negative (spiked matrix)	0 (0/54)	50 (23/46)	-	-	-	-
% Results outside WR or LOQ	-	-	29 (13/45)	43 (20/46)	59 (27/46)	65 (30/46)
% Samples Underestimated	7 (3/46)	-	27 (12/45)	22 (10/46)	24 (11/46)	33 (15/46)
% Samples Equal or Overestimated	93 (43/46)	-	44 (20/45)	35 (16/46)	17 (8/46)	2 (1/46)
% Falsely Compliant with ML (overall)	**7 (2/28)**	**18 (5/28)**	**29 (8/28)**	**79 (22/28)**	**71 (20/28)**	**100 (28/28)**
% Falsely Compliant with ML (spiked)	**25 (2/8)**	**63 (5/8)**	**88 (7/8)**	**25 (2/8)**	**100 (8/8)**	**100 (8/8)**
% Falsely Compliant with ML (naturally contaminated)	**0 (0/20)**	**0 (0/20)**	**5 (1/20)**	**100 (20/20)**	**55 (11/20)**	**100 (20/20)**
% Falsely Non-compliant with ML	0 (54/54)	0 (54/54)	0 (53/53)	0 (54/54)	0 (54/54)	0 (54/54)
Mean (SD) Recovery %	106.5 (22.2)	-	92.2 (34.2)	77.7 (51.2)	66.2 (107.9)	26.7 (29.1)
Repeatability (0.1-0.3 eq OA mg/kg PIPI)	0.00	-	0.01	0.00-0.01	0.02-0.18	0.00
Coefficient of Determination (r^2)	0.86	-	0.72	0.05	0.08	0.01

extremely weak at $r^2 = 0.05$ (Fig. 1). This kit returned a mean recovery of 77 %, outside the criteria in the AOAC Guidelines (AOAC, 2019) (Table 11).

4.2.3.2.3 Abraxis ELISA

Similar to the Abraxis PP2A, the Abraxis ELISA reports a working range of 0.01 to 0.5 mg/kg. This kit returned 0% (0/8) false positives and 59% (27/46) of spiked samples below the working range. Of the samples that were spiked (and results within the working range), 24% (11/46) were underestimated and 17% (8/46) were equal to, or greater than, the spiked toxin concentration (Tables 10A-B). Again, when spiked or naturally contaminated at/above the regulatory limit, the Abraxis ELISA returned 71% (20/28) falsely compliant results (Table 11). A Pearson's correlation analysis between the Abraxis ELISA kit test results and the spiked toxin concentrations was weak at $r^2 = 0.08$ (Fig. 1). Subsequently, this kit returned a mean recovery of 66 %, well outside the criteria in the AOAC Guidelines (AOAC, 2019)(Table 11).

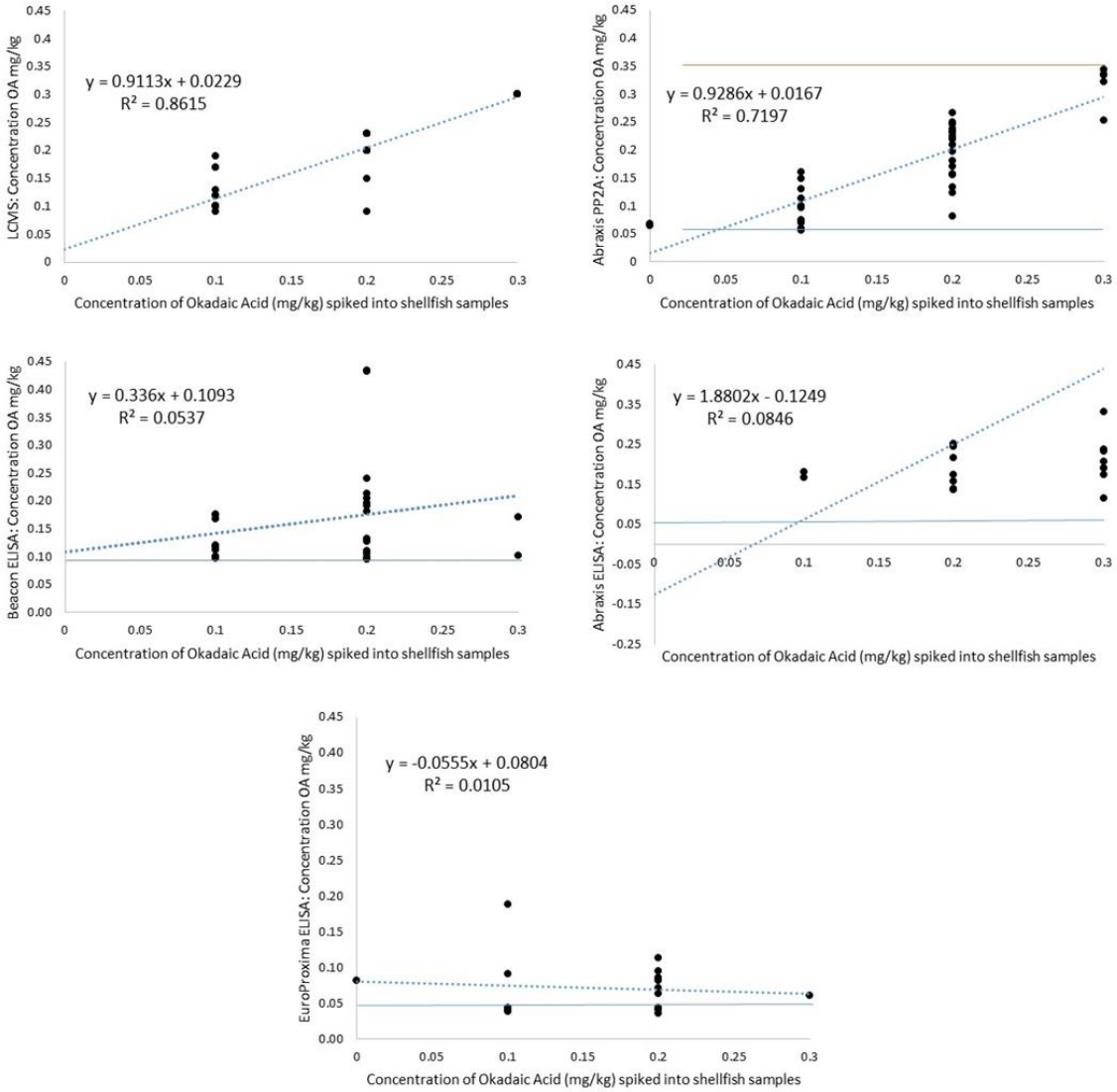


Figure 4. Linear regression plots showing relationship between spiked toxin concentration with both LC-MS and quantitative rapid test kits results in Australian shellfish samples calculated data within each method's working range. Blue lines represent lower working range and red line upper working range of method. Note: Abraxis PP2A Working Range (WR) = 0.06 to 0.35 mg/kg; Beacon ELISA Limit

of Quantification (LOQ) = 0.1 mg/kg; Abraxis ELISA Working Range = 0.1 – 5.0 mg/kg; Europroxima ELISA Limit of Quantification = 0.04 mg/kg.

4.2.3.2.4 EuroProxima ELISA

With a limit of quantification reported as 0.04 mg/kg, the EuroProxima ELISA kit re-turned 13% (1/8) false positives and 65% (30/46) of spiked samples returning results out-side the limit of quantification (<0.04 mg/kg). Of the samples that were spiked (and results reported were above the limit of quantification), 33% (15/46) were underestimated, while only 2% (1/46) were equal to, or greater than, the spiked toxin concentration (Tables 10A-B). When either spiked or naturally contaminated at, or above, the regulatory limit, the EuroProxima returned 100% (28/28) falsely compliant results (Table 8). A Pearson's correlation analysis between this rapid kit test and the spiked toxin concentrations was extremely weak at $r^2 = 0.01$ (Fig. 1). This kit returned a very low mean recovery of 26.7%, well outside the criteria set in the AOAC Guidelines (AOAC, 2019) (Table 11).

4.2.3.3 Repeatability of kits

The repeatability/reliability of all kits was high (standard deviations of the mean ranged from 0.00 to 0.01, with the lower the variation, the higher the reliability of the results). The only exception to this was the Abraxis ELISA kit. From the naturally contaminated Pipi batch with the highest toxin concentration (0.3 OA mg/kg), the repeatability of this kit was low at 0.02 (based on a relatively low number of samples however) (Table 11).

4.3 Development of a *Dinophysis* qPCR assay

4.3.1 *Dinophysis* species identification and enumeration.

The three strains were unequivocally identified as *D. acuminata* (strain DA_MOM_02), *D. fortii* (DF_SAL_90) and *D. caudata* (DC_NAG_01) (accession numbers: LC634028- LC634030).

4.3.2 Toxin Determination

Three toxin analogues (OA, DTX-1, PTX-2) were detected in all three Japanese strains tested, with the exception of DTX-1 in *D. caudata* (Table 12). Mean (±SE) OA pg/cell ranged from 0.01 (± 0.00) in *D. caudata*, to 1.3 (± 0.10) in *D. acuminata*, and to 13.21 (±1.54) in *D. fortii*. Mean DTX-1 was detected at 17.38 (±3.04) pg/cell in *D. fortii* and 23.90 (±3.31) pg/cell in *D. acuminata*. Mean PTX-2 ranged from 52.77 (±9.96) pg/cell in *D. caudata*, 63.19 (±1.42) pg/cell in *D. acuminata*, and to 185.93 (±27.66) pg/cell in *D. fortii* (Table 12).

4.3.3 qPCR assay development

4.3.3.1 Primer design and specificity

Dacu_11F/Dacu_11R primer pair, which comprised 133 bp from the ITS region of *Dinophysis*, was the only primer set which showed sufficient specificity to amplify all three *Dinophysis* species (Dacu_11F AAGCAAGCGGGAGCAAGTTT, Dacu_11R GCAGAAGGTTATGCTCATCGC). This primer pair amplified a single peak at approximately the same temperature (*D. acuminata* 80.5℃, D. fortii 80.5℃ and D. caudata 80.5-81℃), with an average Cq value of 15.29 for *D. acuminata*, 14.17 for *D. fortii* and 16.17 for *D. caudata*. This specificity was subsequently examined *in silico* against three species of *Ornithocerus* and two species of *Phalacroma (P. rapa, P.* cf. *rotundatum)*, which resulted in 8-10 SNPs in forward primer binding region and 4 (no sequence data available in this region for *Phalacroma* spp.) in the reverse primer-binding region of the Dacu_11F/Dacu_11R primer pair respectively. In addition, no cross-reactivity was observed in the laboratory against any other phytoplankton species tested (Table 13).

Table 12. Toxin analogues and their concentrations as determine for the three strains *Dinophysis acuminata*, *D. fortii* and *D. caudata* used for cell-based qPCR assay development.

Strain	Rep	Cells/mL	OA pg/cell	DTX1 pg/cell	PTX2 pg/cell
D. acuminata	1	2417	1.12	27.43	64.96
	2	2367	1.31	26.99	64.22
	3	2633	1.48	17.28	60.39
	Mean		1.30	23.90	63.19
	SD		0.10	3.31	1.42
D. fortii	1	800	15.63	17.63	203.75
	2	733	13.64	22.51	222.37
	3	1033	10.36	12.00	131.66
	Mean		13.21	17.38	185.93
	SD		1.54	3.04	27.66
D. caudata	1	3000	0.007	-	-
	2	3200	0.009	-	42.81
	3	2200	0.009	-	62.73
	Mean		0.01	-	52.77
	SD		0.00	-	9.96

Table 13. Cross-reactivity of the selected qPCR Dacu_11F/Dacu_11R primer pair on *Dinophysis* spp. and other available phytoplankton species including strain code and location of strain isolation.

Template	Strain code (Accession No.)	Location of Isolation	ITS PCR amplification
Dinophysis acuminata	DA_MOM_02	Mombetsu, Hokkaido, Japan	+
Dinophysis fortii	DF_SAL_90	Saroma lake, Hokkaido, Japan	+
Dinophysis caudata	DC_NAG_01	Nagasaki, Japan	+
Alexandrium pacificum	HRP4-2	Hawkesbury River, Australia	-
Pseudo-nitzschia cuspidata	P_WAG170419_1	Wagonga Inlet, Australia	-
Coolia malayensis	MAB	Malabar, Australia	-
Heterocapsa ovata	SA20	Port Lincoln, South Australia	-
Gambierdiscus polynesiensis	CG14	Rarotonga, Cook Islands	-
Fukuyoa yasumotoi	OIRS230	Orpheus Island, Australia	-
Prorocentrum lima	SM43	Raine Island, Australia	-
Amphidinium massartii	CS259		-
Ostreopsis siamensis	HER24	Heron Island, Australia	-
Thecadinium kofoidii	THE	Gordons Bay, Australia	-

4.3.3.2 qPCR assay efficiency

To test for primer efficiency, five-fold serially diluted cell-based curves were established for each species. The percentage efficiency of each reaction was determined to be 91.5% for *D. acuminata*, 91.3% for *D fortii*, and 92.4% for *D. caudata*, all which were deemed acceptable (Fig. 5A-C). The eight-

fold serially diluted gene fragment based curve also reported a suitable efficiency of 97.9% (ie. slope for Cq vs. gene copy number = -3.7) (Fig. 6).

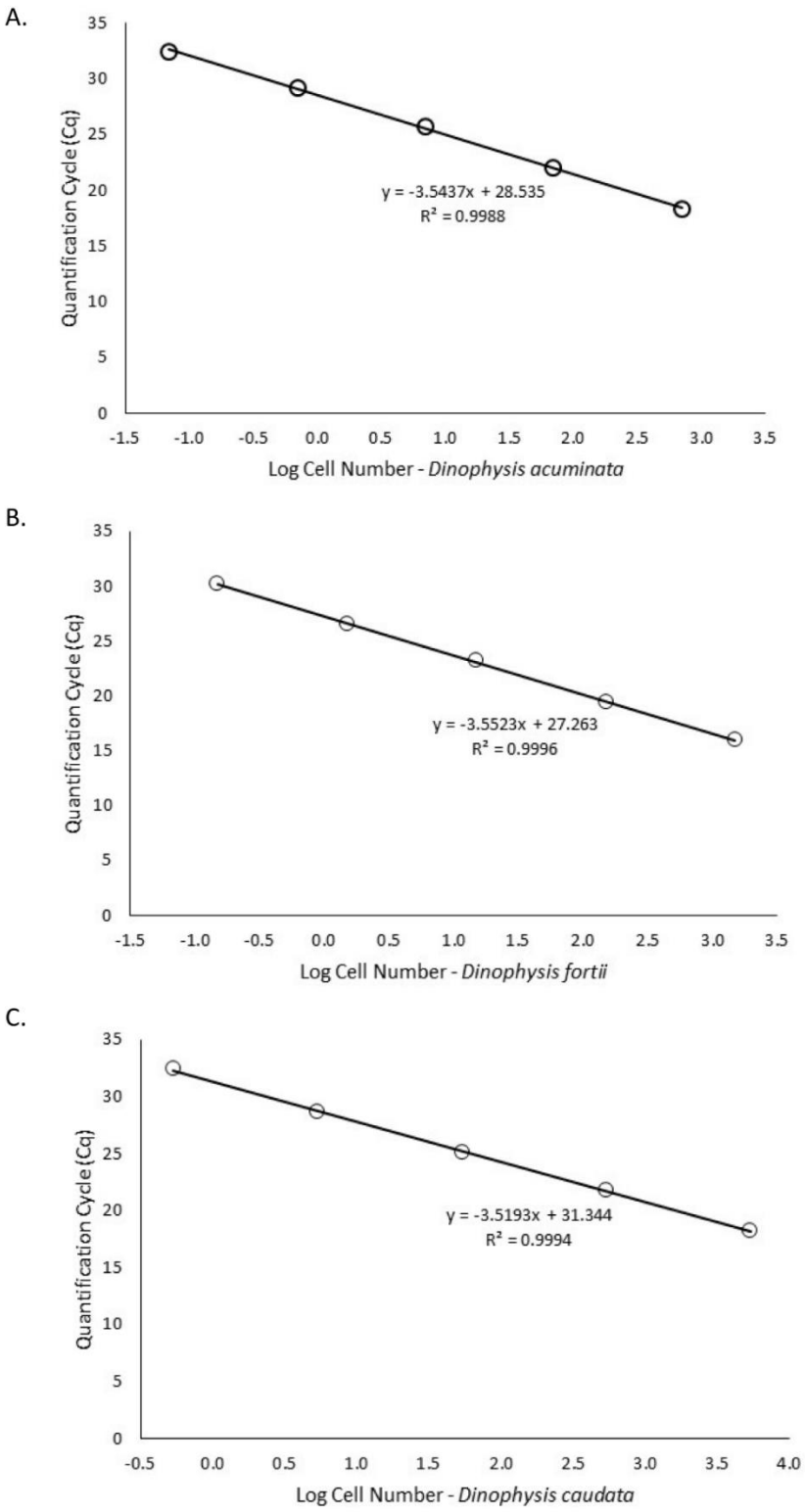


Figure 5A-C. Standard curves for Dacu_11F/Dacu_11R primer pair using cell-based serial dilutions of A. *Dinophysis acuminata*; B. *Dinophysis fortii*; and C. *Dinophysis caudata*.

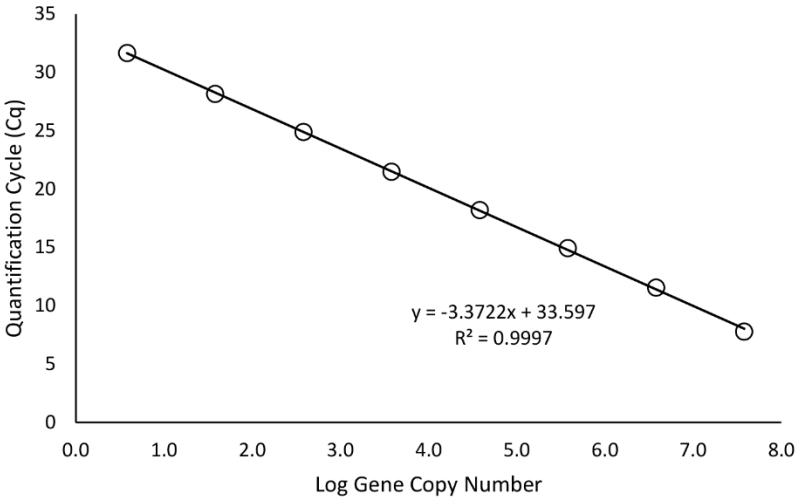


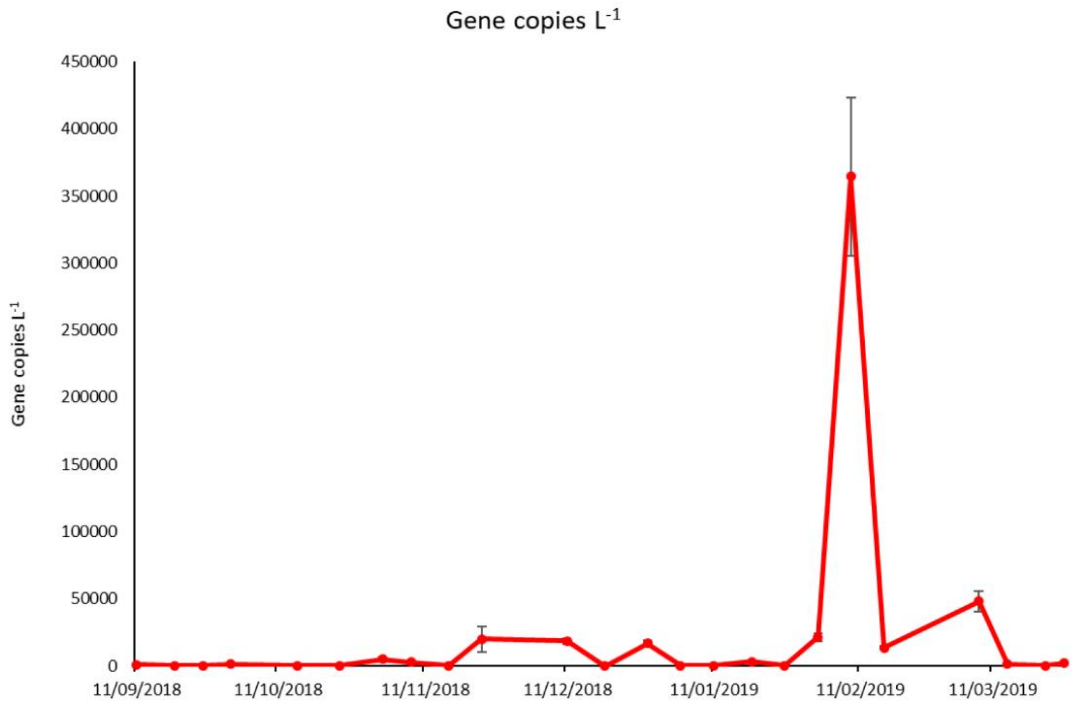
Figure 6. Standard curve for Dacu_11F/Dacu_11R primer pair using ITS gene fragment-based serial dilutions.

To determine the relationship between cell number of each species and the copy number of the ITS1/5.8S/ITS2 gene, the slope of the log-linear standard curve was solved for x (concentration) for all species and gblock equations. The resulting factors were x 49.1 for *D. acuminata*, x 114.3 for *D. fortii* and x 7.3 for *D. caudata*.

4.4 Evaluation of qPCR for *Dinophysis* bloom dynamics

To evaluate the effectiveness of the *Dinophysis* qPCR assay for the detection of *Dinophysis* in environmental samples, we compared microscopy based *D. acuminata* and *D. caudata* cell counts with eDNA samples collected from the Manning River across the same time period. Sixteen water samples collected from 10/9/2018 to 31/3/2019 showed *D. acuminata* peaked on 17/2/2019 at a cell concentration of 5,300 cells L^{-1}, while *D. caudata* reached a maximum cell concentration of 300 cells L^{-1} on 3/12/2018 (Fig. 7A). Using the *Dinophysis* assay developed in this study, we then screened twenty-four eDNA samples (in triplicate) across this similar time period (11/9/2018 to 26/3/2019) and successfully detected gene copies of *Dinophysis* in 62 out of 72 replicate samples (however being a genus only assay, we could not discriminate between species). Mean gene copy number peaked on 9/2/2019 and corresponded to 364,591 gene copies L^{-1} (Figure 7B). Assuming the bloom was dominated by *D. acuminata* (as reported by microscopy) at this time, we then used the x factor for *D. acuminata* (x 49) to determine the peak cell concentration of *D. acuminata* to be ~7,441 cells L^{-1}.

A.



B.

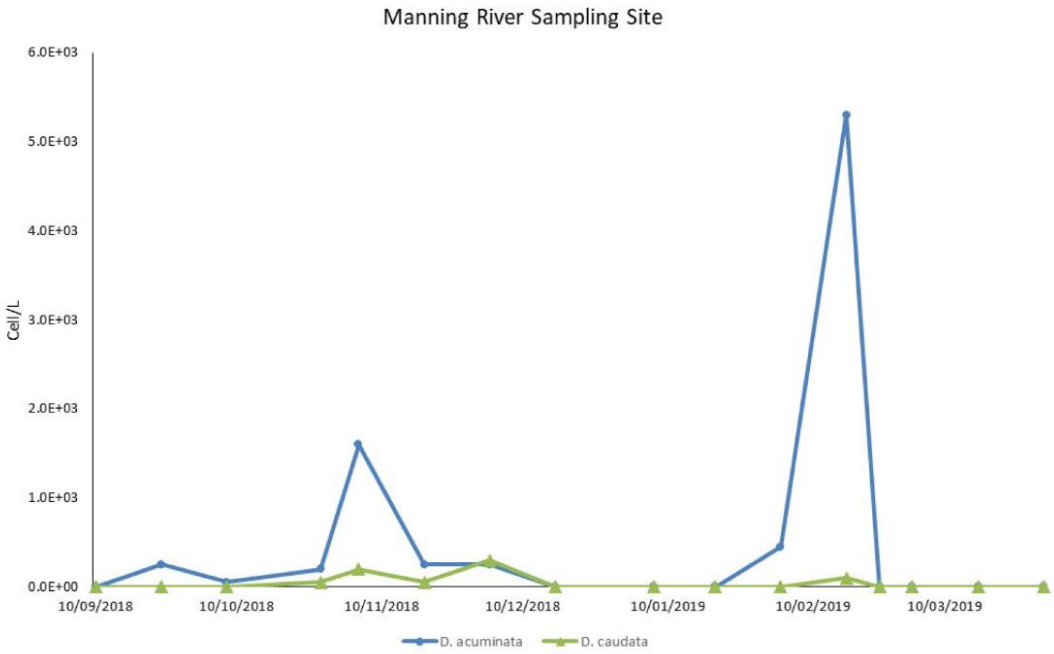


Figure 7A-B. Comparative quantification of *Dinophysis acuminata* and *D. caudata* (cell/L) using A. qPCR (for *Dinophysis* spp.) and B. Microscopy in Manning River, NSW.

5. Discussion

5.1 DSTs in Australia

Toxic *Dinophysis* blooms and their impacts remain one of the most problematic HABs worldwide, especially in the Mediterranean and European waters (Hallegraeff et al., 2021). Positive DST detections periodically occur in Australian shellfish, although these events remain largely un-studied (Farrell et al., 2018; Hallegraeff et al., 2021). Using the official analytical method of LC-MS/MS and LC-MS, shellfish data spanning 2012 to 2017 from four Australian states (Tasmania, Victoria, South Australia and Western Australia) showed that 53 (0.65%) shellfish samples out of 8156 analysed exceeded the domestic regulatory limit (0.2 mg OA eq/kg). Exceedances, across all samples combined, for cockles/pipis, clams, mussels, oysters and scallops were 4.9, 1.1, 1.1, 0.03 and 0% respectively. Of those that exceeded this threshold, OA was the most commonly detected toxin analogue, with only one sample containing DTX-1, and no samples containing DTX-2 (unpublished data).

5.2 LC-MS/MS (and LC-MS) Laboratory Comparison

In the present study, we spiked four different shellfish matrices (SRO, PO, MUS, PIPI) with fixed volumes of relevant, CRM to determine the ability of laboratories to quantify DSTs in shellfish using LC-MS/MS and LC-MS. We found that all spiked analogues, OA, DTX-1, DTX-2, were recovered in all shellfish species across all laboratories, but results were not consistent across all samples. For example, low and mid-concentration toxin recovery was variable both within and between laboratories (0-150%), while high concentration toxin recovery, which included CRM, was higher, between 60%-262%. Two false positives were reported in Pipi samples in which OA was detected at 0.01 and 0.02 mg/kg (Laboratory 1 and Laboratory 2, respectively), and one anomalously high concentration of 2.8 mg/kg was reported from CRM that was submitted at a concentration of 1.07 mg/kg (Table 6). These results need to be interpreted in light of each laboratory's measurement uncertainty (MU), which was reported as ~ 10 -26% dependent on the analogue detected (Appendix 1). Another issue that must be considered is the homogeneity of toxin within the shellfish, and how that may contribute to the variability in results, particularly at the low- to mid-level spiked concentrations. Finally, we cannot completely discount that there may have been some very low toxin concentrations in these samples which were not detected by the original LC-MS screening. Lab 3 in fact, had the highest level of detection (0.006-0.007 mg/kg for analogues OA, DTX-1 and ±DTX-2) across all the labs used in this study.

In a single laboratory validation study to detect and quantify six lipophilic toxins (azaspiracid, domoic acid, gymnodimine, okadaic acid, pectenotoxin and yessotoxin) in Greenshell mussel, Pacific Oyster, cockle and scallop roe, McNabb et al. (McNabb et al., 2005) reported mean OA recoveries between 92% (from a toxin concentration of 0.5-1.0 mg/kg) and 99% (from a toxin concentration of 0.05-0.10 mg/kg). Across all six toxins recoveries ranged from 71-99%. As discussed above, this variability was also apparent in our results, albeit in the converse way, whereby shellfish with a higher spiked toxin concentration generally re-ported a better recovery than those at lower concentrations. McNabb's study concluded that with some slight methodological adjustments (methanol-water @ 9 + 1; 18 mL for 2g of shellfish tissue), the LC-MS/MS method provides good precision/accuracy and high specificity, and is therefore suitable for the quantification of biotoxins in shellfish for regulatory purposes.

In another study to compare the mouse bioassay (MBA) to electrospray ionisation (ESI) LC-MS/MS for the quantification of lipophilic toxins in ~200 samples of shellfish (Suzuki and Quilliam, 2011), it was similarly concluded that LC-MS/MS was a powerful tool for both the identification and structure elucidation of many toxins including OA/DTX analogues, but also for the discovery of unknown toxin

analogues. Furthermore, studies have shown that LC-MS/MS demonstrates linearity, specificity, repeatability and reproducibility in shellfish samples collected from the environment (Schirone et al., 2018), and is able to resolve the toxin profiles of OA analogues in various *Dinophysis* species isolated from bloom samples (Uchida et al., 2018).

There are however, disadvantages to using LC-MS/MS and LC-MS for the detection of toxins in shellfish. LC-MS/MS (and LC-MS) is expensive, particularly for farmers in low risk areas who have a regulatory requirement to undertake marine biotoxin testing using LC-MS/MS at regular intervals (eg. weekly). The cost is also high for farmers in remote areas, where transport of samples to specialised laboratories is expensive. The LC-MS/MS and LC-MS method is also complex, requiring expert analyst training in dedicated laboratories for sophisticated instrument maintenance and performance. Time delays are an-other concern, whereby it can take between 2-7 days to obtain results from a contract laboratory, potentially causing a loss in harvest time and profits to shellfish farmers, and risk to consumers. Finally, high quality and expensive reference material is required to calibrate the method. Despite these disadvantages, and in the absence of a more reliable, sensitive and rapid test, there remains an international acceptance that LC-MS/MS and LC-MS continue to be the standard operating procedure (along with the MBA in many Latin American and Asian countries), for the determination of lipophilic marine biotoxins in molluscs (European Union, 2015).

5.3 Rapid test kits comparison

In the search for an inexpensive and reliable alternative method to LC-MS/MS or LC-MS, and that could be used for screening purposes to serve as an early warning for the shellfish industry, we compared five Rapid test kits against the LC-MS method. Fifty-five shellfish samples (24 spiked and 30 naturally contaminated pipis) were screened with four quantitative (Beacon, Abraxis and EuroProxima ELISA kits and the Abraxis PP2A kit) and one qualitative (Neogen LFA) rapid test kit to detect OA in Sydney Rock Oysters, Pacific Oysters, Blue Mussels and Pipis. Okadaic acid was the only DST analogue to be tested with these kits for multiple reasons: i) It has been the dominant analogue detected in Australian shellfish to date; ii) The cost of purchasing sufficient CRM for spiking all other analogues to detection levels is high; and iii) Rapid test kit results are reported as µg OA eq/kg, and a spike of varying DST analogues will not reveal individual analogue concentrations (noting the Neogen rapid test kit is qualitative only). Furthermore, each kit reports a level of cross reactivity to the various analogues, and while in most cases this is 100% for OA, it varies for DTX-1 and DTX-2 between kits. For example, if three samples were individually spiked with the same concentration of okadaic acid, DTX-1 and DTX-2, the concentration of okadaic acid from the Abraxis ELISA kit would read as double the concentrations of the other two compounds. This is because DTX-1 (50%) and DTX-2 (50%) only give half of the response that okadaic acid does with this technology.

With this in mind, all quantitative kits should theoretically provide a comparable concentration of OA to that obtained using the LC-MS method. Regression analyses showed the correlations between the ELISA Rapid test kits and LC-MS in our study were all very low (0.002-0.19), while the correlation between the PP2A Abraxis kit and LC-MS was moderate to high (0.78) (Fig. 4). Observed variations between these methods could not be attributed to matrix effects however, as no clear differences were observed between spiked samples across methods. Certain kits nonetheless, performed better on naturally contaminated samples (Pipis only) compared to spiked samples (Neogen and Abraxis PP2A). The reasons for this remain unclear, but support the assertion by Turner et al. (Turner et al., 2020) that validation studies need to include both relevant shellfish species and naturally contaminated shellfish samples, so that any rapid test kit performance is measured using local toxin profiles.

After the development of the first ELISA method by Dubois et al. (Dubois et al., 2010), a comparison across assay techniques was undertaken whereby cell counts, LC-MS/MS, the newly developed

Abraxis ELISA and PP2A Okatests were compare(Turner et al., 2020)d. Naturally contaminated samples of edible Blue Mussels (*Mytulis edulis*) were examined for total DST toxin content including esters and DTX-3. The ELISA showed matrix effects on hydrolysed samples, which had both high and low levels of toxins, while the PP2A adequately detected both low and high DST concentrations in mussel samples. While the Okatest was recommended in preference to the ELISA, it was concluded to be a specific assay (could not detect other regulated DSTs), and therefore could not replace LC-MS/MS or LC-MS. Subsequent to these findings, three further studies – a single laboratory validation and an interlaboratory study on the PP2A Okatest (Smienk et al., 2013; Smienk et al., 2012), and a comparison across three RTKs (the lateral flow (Jellett/Scotia), ELISA (Abraxis) and PPIA (Okatest) kits) (Eberhart et al., 2013), were undertaken. Considering issues such as an unacceptable number of false negatives (Jellett), and low cross-reactivity with DTX-1 (the dominant toxin profile in the shellfish tested) by the ELISA, Eberhart et al. concluded that the PP2A was the most promising kit on the market. It is these differences in toxin profiles, the inclusion (or not) of a hydrolysis step, and whether the shellfish tested is spiked or naturally contaminated, that prevents a direct comparison between these studies and the present study, although it highlights the issues that must be standardised in any future validation study.

In 2015, the development and validation of a new rapid test kit, the Neogen LFA, this time a qualitative test strip/reader for the OA group toxins in shellfish was reported (Jawaid et al., 2015). This validation method tested both spiked (OA, DTX-1, DTX-2 and DTX-3 with hydrolysis procedure) and naturally contaminated shellfish (mussels, scallops, oysters, and clams) and compared the results to LC-MS/MS. While our study showed only minor differences in shellfish matrices (low number of samples tested however) and zero falsely compliant results in naturally contaminated samples, Jawaid et al. showed no matrix effects, false compliant results or false noncompliant results at <50% MPL (maximum permitted level). Both Jawaid and the present study suggest this method, with some further work, may be an effective early warning tool for the shellfish industry. The results reported in this study, however, do not support the use of any DST rapid test kit as a stand-alone quality assurance measure at this time, and further research and development work is needed.

Since the development of the LFA technology, two additional studies generated rapid test kit comparisons (Johnson et al., 2016; Turner and Goya, 2016). The first study compared DSTs in shellfish from Argentina using two qualitative lateral flow kits (Scotia and Neogen), the quantitative PPIA kit (OkaTest), and the ELISA kit (Max Signal – no longer commercially available) and compared the results to LC-MS/MS. The specificity was reported as good for all kits, with no false compliant results against the ML of <16 mg OA eq/kg). The second study screened four RTKs, again on naturally contaminated shellfish, but this time from Great Britain. The quantitative PP2A (OkaTest) was the only test to show the complete absence of false negative results (i.e. mussel samples containing OA-group toxins above the ML of 0.16 mg OA eq/kg which returned negative results), showed a fair correlation to LC-MS/MS but with an overall overestimation of sample toxicity with some indication of matrix effect, particularly in oysters (Johnson et al., 2016). The quantitative ELISA (MaxSignal) gave a reasonable correlation with LC-MS/MS, no evidence of overestimation, accurate at low concentrations and only one false negative (as above, a mussel samples containing OA-group toxins above the ML of 0.16 mg OA eq/kg which returned a negative result). The two lateral flow assays (Neogen and Scotia) were observed to show high agreement with LC-MS/MS and no indications of false positives, although both returned one false negative (Johnson et al., 2016).

In the present study, all four quantitative kits showed varying levels of over/underestimation (many at the ML). Many results were outside the working range or limit of these kits. This ranged from 29% of samples using the Abraxis PP2A to 65% with the Euro Proxima ELISA (Table 11). Two kits also showed false positives from blank matrices (i.e. samples that did not contain toxins), being the Abraxis PP2A and EuroProxima ELISA at 25% and 13% respectively. All methods (quantitative and qualitative) delivered high levels (25% to 100%) of falsely compliant results for spiked samples. The Neogen and

Abraxis PP2A performed satisfactorily (0%, 5% falsely compliant at the regulatory limit or above, respectively) for naturally contaminated pipis. For quantitative tests, mean percent recovery ranged from 27% (EuroProxima ELISA) to 107% (LC-MS/MS), while only the LC-MS method and the Abraxis PP2A kit (92%) fell within the "acceptable recovery" range of 80-100% as set by the AOAC Guidelines (AOAC, 2019).

5.4 qPCR assay for *Dinophysis* detection

Despite the largely conserved ITS1/5.8S/ITS2 region across all *Dinophysis* spp. sequences examined, the difficulty in growing *Dinophysis* spp. in the laboratory, and the largely monospecific nature of *Dinophysis* blooms, we have successfully developed a rapid, sensitive and efficient quantitative real-time qPCR assay to detect *Dinophysis* in environmental samples. This novel assay quantified *Dinophysis* cells in a similar way to microscopic enumeration, but has a faster turnaround time (~2hrs) and does not require taxonomic expertise. For these reasons, we believe this assay will be a valuable early warning tool for HAB monitoring. The large variation in toxin content between strains, species and environmental conditions, suggests however, that this early warning technique would trigger further investigation into any *Dinophysis* bloom. Future work would also need to include the development and validation of a simplified and commercialised qPCR pipeline (eg. Phytoxigene™ DinoDTec) for the detection of *Dinophysis* spp. for on farm usage.

6. Benefit-cost analysis of rapid detection of Diarrhetic Shellfish Toxins (DSTs): case study of the Pacific Oysters (*Magallana gigas/Crassostrea gigas*) industry, Tasmania

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Executive Summary

The analysis presented in this report is intended to satisfy the requirements of Objective 5 of FRDC 2017-203:

- Conduct an economic impact analysis to estimate the reduction/annual savings in monitoring costs for regulatory authorities in Tasmania by implementing rapid diagnostic testing for DSTs.
- Calculate the reduction in commercial loss and economic impact from potentially harmful DST blooms in Tasmania following the introduction of the rapid diagnostic testing.

A summary of our findings for each component of Objective 5 follows.

Reduction/annual savings in monitoring costs for regulatory authorities in Tasmania by implementing rapid diagnostic testing for DSTs.

While we can say that the qPCR or Neogen technology both offer cost advantages when compared with the laboratory service provider (BAU) practice, we are unable to quantify the exact extent of this without further work to validate the two alternative testing technologies. All scenarios considered in this report would represent a net cost saving over 10 years when compared with the current practice of weekly LC-MS tests for DSTs conducted by a laboratory service provider. The highest savings in our analysis occur under Scenario 2, in which the Neogen technology is centralised in the laboratory and spread across all 24 growing areas.

However, we were not able to account for QA costs and potential NATA accreditation as these were difficult to ascertain without understanding the implementation standards for the test. The exact number of samples needed to obtain a result (Neogen rapid test, qPCR test) was also important for the projected savings under Scenarios 1 and 3. While our analysis assumed that one sample would be required in each of these scenarios, this may not be the case and the cost efficiency of both technologies increases substantially when fewer samples are needed to generate a result. A sensitivity analysis of our results with respect to the number of samples required to obtain a result is shown in Appendix D.

Therefore, a full validation study covering each of the major testing methods examined in this project (Neogen rapid test, qPCR test) is recommended as an important area of further research.

Reduction in commercial loss and economic impact from potentially harmful DST blooms in Tasmania following the introduction of the most appropriate diagnostic testing.

To be implemented under the ShellMAP programme, we expect that any new testing regime would need to be implemented at a frequency and scale that ensures the risk of contaminated product leaving a Tasmanian growing area continues to be negligible. Therefore, we would not estimate any change in the *expected* commercial loss and economic impact from potentially harmful DST blooms in Tasmania following the introduction of the most appropriate diagnostic testing considered under this project. This risk would continue to be negligible for Tasmanian growers.

The scenarios considered in this study are for domestic supply, in compliance with potential use under the ASQAP programme. Further considerations would be needed for use in any export programme.

Considerations for industry in interpreting these results

- This report highlights that a validation study is needed to determine how many samples should be used. However, it is not a recommendation to implement rapid testing technologies immediately.
- The actual savings in the case of centralising rapid testing at a laboratory (Scenario 2) are likely to be less than the numbers reported herein as some costs (e.g., Quality Assurance and National Association of Testing Authorities accreditation) were unable to be included in the model.
- The scenarios considered in this study are for domestic supply, in compliance with potential use under the ASQAP programme. The use of the kits to satisfy export market access is beyond the scope of this report, and further considerations would be needed for use in any export programme.

6.1 Introduction

Bivalve shellfish such as oysters, mussels, clams, pipis, and scallops are viewed as seafood delicacies. However, the consumption of shellfish may pose a public health risk if harvested outside of a comprehensive shellfish quality assurance program. Diarrhetic Shellfish Toxins (DSTs) are one such risk to human health posed by shellfish consumption. DSTs are generally produced by marine dinoflagellates of the genus *Dinophysis* and can bioaccumulate in shellfish under certain circumstances and subsequently cause human illness. There have been three major illness outbreaks of Diarrhetic Shellfish Poisoning (DSP) in Australia in the past two decades (Quaine et al., 1997, Madigan et al. 2006, Burgess and Shaw 2001).

Currently, Tasmanian seafood safety regulations require oysters to be collected and transported to analytical laboratories and tested for the presence of DSTs using a standard LC-MS method (i.e., Quilliam 1995). This testing is done through laboratory services provided by Analytical Services Tasmanian (St Johns Avenue, New Town) as part of the Shellfish Market Access Programme (ShellMAP), and requires weekly couriering of shellfish samples from 24 growing areas around the State to the laboratory for testing. However, it is not known whether other methods, such as rapid testing methods (qPCR and/or rapid test kits), may provide an economic advantage over the currently used LC-MS method.

This report outlines a cost versus benefit analysis in the case of three possible scenarios for the implementation of rapid testing of DSTs for the Pacific oyster industry in Tasmania. This analysis has been undertaken to meet Objective 5 of FRDC 2017-203, and as such aims to:

- Conduct an economic impact analysis to estimate the reduction/annual savings in monitoring costs for regulatory authorities in Tasmania by introducing rapid test kits in low risk periods for diagnostic testing for DSTs.
- Calculate the reduction in commercial loss and economic impact from potentially harmful DST blooms in Tasmania following the introduction of the rapid diagnostic testing.

The use of Pacific Oyster (*Magallana gigas/Crassostrea gigas*) from Tasmania for this case study followed discussion with the Research Advisory Committees and was based on the available data in this state, the prominence of this species in Tasmanian shellfish aquaculture production. This analysis can serve as an example for determining the benefits Australia-wide for a variety of shellfish industries.

The analysis in this report considers the three hypothetical scenarios for implementation of DST rapid testing for Pacific Oysters in Tasmania:

- Scenario 1: Implement Neogen DST rapid kit testing on-farm in Tasmania, replacing confirmatory DST testing in low risk areas in 3 out of 4 weeks.[1]
- Scenario 2: Implement Neogen DST rapid kit testing in laboratory in Tasmania, replacing confirmatory DST testing in low risk areas in 3 out of 4 weeks[1].
- Scenario 3: Implement qPCR testing on-farm in Tasmania, replacing confirmatory DST testing in low risk areas in 3 out of 4 weeks[1].

Because Tasmania is considered a low-risk area overall for DST, in each of these scenarios the implementation of the Neogen or qPCR testing would relate to all 24 growing areas of the State.

[1] The Australian Shellfish Quality Assurance Program requires a minimum of monthly sampling in low risk areas. This scenario is based on monthly analysis using the LC-MS method, supplemented by weekly sampling with the DST rapid test kits.

Reduction/annual savings in monitoring costs for regulatory authorities in Tasmania by introducing rapid diagnostic testing for DSTs

In this section we apply economic benefit versus cost analysis to investigate the reduction/annual savings in monitoring costs for regulatory authorities in Tasmania by introducing rapid diagnostic testing for DSTs. Our method of analysis estimates the costs and savings for each scenario against the current practice by industry and regulators, and uses these to calculate the Net Present Value of Savings accrued in each scenario over a ten-year time horizon. We calculate the savings to both growers and regulators collectively (which comprises the referent group for our analysis), and therefore report results for each scenario that represents a collective benefit to both groups. We assume a commercial basis for the discount rate used in our analysis (i.e., representative rate for business finance), and this reflects the industry's interest in the food safety programme in Tasmania.

6.2 Methods

This study applies economic cost versus benefit analysis to understand the benefits of rapid DST testing on-farms or in laboratories in the Tasmanian context. We apply our analysis to a case study of Pacific Oyster aquaculture in the State, both because of the availability of data for this sector, and because of its prominence in shellfish aquaculture in the State. Our analysis considers the change in costs for participants in the Pacific Oyster industry, and the current laboratory testing provider in Tasmania, under three possible scenarios. Each scenario is evaluated relative to the business-as-usual (BAU) case of weekly in-lab LC-MS testing provided by the laboratory service provider.

The analysis for each scenario in this report focuses on the following categories of benefits and costs:

- The reduction in LC-MS testing costs at the laboratory due to a proposed new testing regime,
- The upfront establishment costs, and ongoing costs of the proposed new testing regime (e.g., farmer time, test-related consumables, changes in transport cost),
- Any new or additional costs to the laboratory service provider of a proposed testing regime (e.g., incremental tech staff salaries, additional costs of consumables to the laboratory, and the cost of any capital equipment), and
- Validation and implementation costs of a new testing regime in order to meet regulatory requirements.

Further details on the BAU case are provided later in this section of the report. Non-market values such as existence values for on-farm testing, or perceived changes in public health outcomes, are outside the scope of this study.

Model assumptions

Core assumptions

Time horizon:	10 years	It is estimated that within ten years a new testing technology will likely have been developed for DSTs (i.e., that supersedes any of the approaches considered in this benefit-cost analysis).
Discount rate:	5% p.a.	The discount rate for this analysis has been assumed to 5% per annum, and which is intended to be representative of small business finance (Ash Norris, *per. comm.*).

Opportunity cost of grower time:	$79,445.60 per year	Based on Average Weekly Ordinary Time Earnings (https://www.abs.gov.au/statistics/labour/earnings-and-work-hours/average-weekly-earnings-australia/latest-release, access on 13 May 2021).
Cost of tech staff time:	$79,445.60 per year	Based on Average Weekly Ordinary Time Earnings (https://www.abs.gov.au/statistics/labour/earnings-and-work-hours/average-weekly-earnings-australia/latest-release, access on 13 May 2021).
Number of growing areas monitored in Tasmania:	24	Note: some growing areas have multiple species, meaning sometimes multiple tests, but for this study we have estimated costs only or Pacific Oyster.
Number of Neogen tests required to generate a result:	one (1)	A full validation study would be needed to reliably determine the number of samples required to generate a result. We have assumed one (1) sample is required per result in the case for both tests (Neogen, qPCR) for this analysis.
Number of qPCR tests required to generate a result:	one (1)	A full validation study would be needed to reliably determine the number of samples required to generate a result. We have assumed one (1) sample is required per result in the case for both tests (Neogen, qPCR) for this analysis.
Cost savings due to reduced LC-MS testing	Valued at the individual commercial prices for LC-MS DST testing in Tasmania	At present ShellMAP receives a bulk price for testing that does not change with the number of tests conducted. This is a confidential arrangement between ShellMAP and Analytical Services Tasmania and is unique across Australia. As we cannot assume what change in this pricing there would be for a reduced number of DST tests, we worked off the individual commercial prices for DST testing, making this case study applicable to all States

Neogen rapid test kit

Neogen rapid test capital equipment:

Raptor Solo Diagnostic Reader	$4,000
Heater Block	$500
Transfer Pipette 100-1000uL	$500
Vortex	$400

Neogen rapid test capital equipment – assumed service life:

Service life of Neogen test equipment – **under regular use**:	5 years	Within the 10-year time horizon we have assumed the Neogen rapid test capital equipment (i.e., Raptor Solo Diagnostic Reader, heater block, transfer pipette, and vortex) would turn over twice (i.e., once in every 5-year timespan).
Service life of Neogen test equipment – **under frequent use**:		In the case of frequent use in the laboratory setting in Scenario 2, we assume that 5 sets of testing equipment will be purchased every 5 years and used on-rotation over that period before being completely renewed. *(NB: some equipment may have longer or shorter replacement times, but complete renewal at regular intervals is considered good practice).*

Cost of consumables for the Neogen rapid test kit:

8mL x analytical grade methanol (4L @ $75)	$0.1500
0.1mL x NaOH (2.5M) (1kg salt @ $159)	$0.0019
0.1mL x HCl (2.5 M) (2.5L @ $200)	$0.0080
10mL x deionized water (10L @ $30)	$0.0300
6 x pipette tips (1000 @ $336)	$2.0160
1 x glass vial (100 @ $50)	$0.5000
2 x 10ml falcon tube (500 @ $500)	$2.0000
1 x Neogen test (24 samples @ $974.50)	$40.6042
Cost of consumables per sample ($)	**$45.31**

Time required to conduct test:

Estimated time required to conduct test	Estimated based on a minimum time requirement of 1 hour per test result, plus an expected test time of 1.5 hours per batch of 10 samples. (Note: this includes the time for homogenisation.)

qPCR test

qPCR test capital equipment[2]:

qPCR machine	$10,000
Sampler	$500
Transfer Pipette 100-1000uL	$500
Mini Centrifuge	$600
Vortex	$400

qPCR test capital equipment – assumed service life:

Service life of qPCR test equipment – **under regular use**:	10 years	This has been assumed based on advice from the University of Technology Sydney (UTS) research team responsible for developing the qPCR test for Dinophysis spp. in water/shellfish.

Cost of consumables for the qPCR test:

1 x PCR tubes (120 @ $360)	$3.0000
1 x Cell lysis tubes (50 @ $250)	$5.0000
1 x kit assay ($25 per sample)	$25.0000
3 x pipette tips (1000 @ $442)	$1.3260
1 x 8 micrometre filter paper (100 @ $72)	$0.7200
1 x Syringe (40 @ $26)	$0.6500
0.1[3] x Syringe filter holder (12 @ $320)	$2.6667
Cost of consumables per sample ($)	**$38.36**

Time required to conduct test:

Estimated time required to conduct test	Estimated based on a minimum time requirement of 1 hour per test result, plus an expected test time of 2 hours per batch of 10 samples. (Note: this includes the time for homogenisation.)

[2] In the case of the on-farm qPCR test in Scenario 3, we have assumed the existence of a laptop already for each of the testing stations in each growing area of the State.
[3] The syringe filter holder would be used at least 10 times.

The current practice in Tasmania – the business-as-usual (BAU) case

In Tasmania, weekly biotoxin testing is currently undertaken for shellfish farmed within 24 growing areas around the State. This testing is done as part of the ShellMAP programme, and currently implements the LC-MS method. Testing is for three major toxin groups: DSTs, Paralytic Shellfish Toxins (PSTs), Amnesic Shellfish Toxin (AST).

The current practice in Tasmania requires growers to courier weekly samples from each growing area to a laboratory in St Johns Avenue, New Town. In the case of Pacific Oysters, growers are required to use ice packs to store a sample of one dozen (12) oysters in a 'six-pack' esky and courier this to the laboratory (gross weight less than 2kgs). In most cases there is an approximately 24-hour turn-around time for test results.

The current price of laboratory[4] testing for DSTs in Tasmania has a tiered structure depending on the maximum turnaround time. At the time of this cost/benefit analysis, these costs were $380 for a 2- to 3-day turnaround; and $299 for a 5-day turnaround (Analytical Services Tasmania, *per comm.*). These prices include all analytes in the current suite (a total of 17, including domoic acid and the Total - and Free- forms of the DSTs).

No testing for DSTs is currently done on-farm in Tasmania.

[4] Note: at present ShellMAP receives a bulk price for testing that does not change with the number of tests conducted. This is a confidential arrangement between ShellMAP and Analytical Services Tasmania and is unique across Australia. As we cannot assume what change in this pricing there would be for a reduced number of DST tests, we worked off the individual commercial prices for DST testing, making this case study applicable to all States

Cost-benefit calculations

Scenario 1: Implement Neogen DST rapid kit testing on-farm in Tasmania, replacing confirmatory DST testing in low risk areas in 3 out of 4 weeks

Incremental LC-MS testing costs for the laboratory:	Weekly testing for DSTs continues for each of the 24 growing areas (as in BAU), but on-farm Neogen rapid test kit replaces the current LC-MS laboratory test in 3 out of every 4 weeks. Incremental LC-MS testing cost savings for the laboratory is therefore current average testing cost of $339.50[5] multiplied by 39 weeks (i.e. ¾ x 52 weeks) multiplied by 24 growing areas.
Incremental capital cost for on-farm testing to growers:	Neogen rapid test capital equipment costs applied in Year 0, and again at intervals according to the assumed service life the equipment. All testing equipment assumed to be renewed at the same time *(NB: some equipment may have longer or shorter replacement times, but complete renewal at regular intervals is considered good practice).* It is assumed that one set of testing equipment will be maintained at each growing area of the State.
Incremental transport costs for growers:	No change is assumed in grower transport requirements for sample to the laboratory, because a sample of 12 oysters is still required weekly by the laboratory for other shellfish toxins testing regardless of the frequency of DST testing.
On-going costs for on-farm testing to growers:	As in the case of '*incremental LC-MS testing costs for the laboratory*', this calculation assumes that one on-farm test result is needed in each growing area (24 areas) for 3 out of every 4 weeks of the year (39 weeks). This gives the total requirement of 24 x 39 = 936 independent on-farm test results per year. The number of samples required to get a test result (*in this case <u>one</u> sample*) is used to estimate the consumables cost per result, and the amount of grower time per result. The cost of grower time per result is then estimated using *Opportunity cost of grower time* assumption. The annual on-going costs to growers for implementing the on-farm testing technology is the cost of consumables plus cost of grower time per test result, multiplied by the total number of independent on-farm test results that are needed each year.
Validation and implementation costs:	Validation and implementation costs have been based on those costs incurred in the case of the PST rapid test technology (now implemented on farms in Tasmania). (*PASE project: Implementing PST screening test kits to the Bivalve Industry*).

[5] The lab service provider for LC-MS testing in Tasmania has tiered pricing depending on turnaround time. At the time of this analysis, these costs were $380 for a 2- to 3-day turnaround; and $299 for a 5-day turnaround (Analytical Services Tasmania, *per comm.*). These prices include all analytes in the current suite (a total of 17, including domoic acid and the Total- and Free- forms of the DSTs).

Scenario 2: Implement Neogen DST rapid kit testing in laboratory in Tasmania, replacing confirmatory DST testing in low risk areas in 3 out of 4 weeks

Incremental LC-MS testing costs for the laboratory:	Weekly testing for DSTs continues for each of the 24 growing areas (as in BAU), but in laboratory Neogen rapid test kit replaces the current LC-MS laboratory test in 3 out of every 4 weeks. Incremental LC-MS testing cost savings for the laboratory is therefore current average testing cost of $339.50 multiplied by 39 weeks (i.e. ¾ x 52 weeks) multiplied by 24 growing areas.
Incremental capital cost to laboratory:	Neogen rapid test capital equipment costs applied in Year 0, and again at intervals according to the assumed replacement schedule under frequent use (section 0). It is assumed in that 5 sets of testing equipment are purchased every 5 years by the laboratory and used on-rotation before being renewed at the beginning of the next 5-year period.
Incremental transport costs for growers:	No change is assumed in grower transport requirements for sample to the laboratory, because a sample of 12 oysters is still required weekly by the laboratory for other shellfish toxins testing regardless of the frequency of DST testing.
Incremental tech staff costs:	As in the case of '*incremental LC-MS testing costs for the laboratory*', this calculation assumes that one DST Neogen rapid test result is needed for each growing area (24 areas) for 3 out of every 4 weeks of the year (39 weeks). This implies a total of 24 x 39 = 936 rapid test results required per year, for an average of 936/365.25 = 2.56 results per week. The number of samples required to get a test result is then used to estimate the total amount of tech staff time required per week (on the average). The *Cost of tech staff time* assumption is applied to estimate the incremental tech staff costs for the laboratory in implementing the rapid test in 3 out of every 4 weeks for each growing area in place of the LC-MS test for those weeks.
Incremental consumables costs to laboratory:	As in the case of '*incremental LC-MS testing costs for the laboratory*', this calculation assumes that one DST rapid test result is needed for each growing area (24 areas) for 3 out of every 4 weeks of the year (39 weeks). This implies a total of 24 x 39 = 936 rapid test results required per year. The number of samples required to get a test result for the Neogen test is then used to calculate the total cost of consumables for a year of operating the Neogen rapid test assay at the current laboratory.
Validation and implementation costs:	Validation and implementation costs have been based on those costs incurred in the case of the PST rapid test technology (now implemented on farms in Tasmania). (*PASE project: Implementing PST screening test kits to the Bivalve Industry*).

Scenario 3: Implement qPCR testing on-farm in Tasmania, replacing confirmatory DST testing in low risk areas in 3 out of 4 weeks

Incremental LC-MS testing costs for the laboratory:	Weekly testing for DST continues for each of the 24 growing areas (as in BAU), but on-farm qPCR testing replaces the current LC-MS laboratory test in 3 out of every 4 weeks. Incremental LC-MS testing cost savings for the laboratory is therefore current average testing cost of $339.50 multiplied by 39 weeks (i.e. ¾ x 52 weeks) multiplied by 24 growing areas.
Incremental capital cost for on-farm testing to growers:	qPCR test capital equipment costs are applied in Year 0, and then again at intervals according to the assumed service life the equipment. All testing equipment assumed to be renewed at the same time *(NB: some equipment may have longer or shorter replacement times, but complete renewal at regular intervals is considered good practice).* It is assumed in this analysis that one set of testing equipment will be required for each growing area in the State.
Incremental transport costs for growers:	No change is assumed in grower transport requirements for sample to the laboratory, because a sample of 12 oysters is still required weekly by the laboratory for other shellfish toxins testing regardless of the frequency of DST testing.
On-going costs for on-farm testing to growers:	As in the case of '*incremental LC-MS testing costs for the laboratory*', this calculation assumes that one on-farm qPCR test result is needed in each growing area (24 areas) for 3 out of every 4 weeks of the year (39 weeks). This gives the total requirement of 24 x 39 = 936 independent on-farm test results per year. The number of samples required to get a test result is used to estimate the consumables cost per result, and the amount of grower time per result. The cost of grower time per result is then estimated using *Opportunity cost of grower time* assumption. The annual on-going costs to growers for implementing the on-farm qPCR testing technology is the cost of the consumables plus cost of grower time per test result, multiplied by the total number of independent on-farm test results that are needed each year.
Validation and implementation costs:	Validation and implementation costs have been based on those costs incurred in the case of the PST rapid test technology (now implemented on farms in Tasmania). (*PASE project: Implementing PST screening test kits to the Bivalve Industry*).

6.3 Results

Results from our analysis are shown below in Table 1. Detailed calculations for Scenarios 1, 2, and 3 are presented in Appendices A, B and C, respectively. As shown in Table 1, all scenarios considered in this report would represent a net cost saving over 10 years when compared with the current practice of weekly LC-MS tests conducted by the laboratory service provider (i.e., the BAU scenario).

Table 1: Result for the Net Present Value of Savings accrued over the 10-year time horizon under Scenario1, Scenario 2 and Scenario 3 when compared to the BAU scenario of weekly LC-MS testing undertaken by a laboratory service provider. The analysis in this report compares each Scenario on the common basis of one (1) sample being required to obtain a reliable result from either the Neogen or the qPCR tests.

Scenario	Net Present Value of Savings
Scenario 1: Implement Neogen DST rapid kit testing on-farm in Tasmania, replacing confirmatory DST testing in low risk areas in 3 out of 4 weeks.	$1,610,113.86
Scenario 2: Implement Neogen DST rapid kit testing in laboratory in Tasmania, replacing confirmatory DST testing in low risk areas in 3 out of 4 weeks.	$1,984,644.08[#]
Scenario 3: Implement qPCR testing on-farm in Tasmania, replacing confirmatory DST testing in low risk areas in 3 out of 4 weeks.	$1,554,974.18

[#] Please note: these savings do not account for Quality Assurance (QA) costs and potential National Association of Testing Authorities (NATA) accreditation for the new testing methodologies.

The highest savings in our analysis occurred under Scenario 2, in which the Neogen technology is implemented centrally by the laboratory service provider. While this scenario represented the greatest Net Present Value of Savings over the 10-year time horizon of $1,984,644.08, it is important to note that our analysis has not accounted for the Quality Assurance (QA) costs, and potential National Association of Testing Authorities (NATA) accreditation, of Neogen testing method in-lab. These costs are difficult to determine without fully understanding the implementation standards for each test), however would need to be costed and accounted for following a full validation of this testing method.

The next greatest savings occurred under Scenario 1, in which the Neogen rapid test kit is implemented on-farm in each of the 24 growing areas monitored. Scenario 1 resulted in a Net Present Value of Savings of $1,610,113.86 over the 10-year time horizon. This was followed by Scenario 3, in which the qPCR test (water/shellfish) is implemented on-farm in each of the 24 growing areas. This resulted in a Net Present Value of Savings of $1,554,974.18 over the 10-year time horizon.

In both Scenario 1 and Scenario 3 our analysis assumes that only one (1) sample is needed to generate a test result using the respective on-farm technologies (i.e., the Neogen rapid test, and the qPCR test). However, this may not be the case and the cost efficiency of either technology increases when fewer samples are required to generate the result. Therefore, understanding the exact number of samples required by the Neogen and qPCR technologies to ascertain a reliable result is an important area of further research. A sensitivity analysis of our results with respect to the number of samples required to obtain a result is shown in Appendix D.

Reduction in commercial loss and economic impact from potentially harmful DST blooms in Tasmania following the introduction of rapid diagnostic testing

In this section we apply economic benefit versus cost analysis to investigate the reduction in commercial loss and economic impact from potentially harmful DST blooms in Tasmania following the introduction of the rapid diagnostic testing. We measure the reduction in commercial loss and economic impact as being the change in the *expected cost* of a product recall event associated with each of the scenarios (Scenario 1, Scenario 2, Scenario 3) considered by this report. We consider this 'avoided cost' for the case of the Pacific Oyster industry in Tasmania, however our results extend more generally to other shellfish sectors in the State.

The analysis in this section is based on:

- The likelihood of a DST outbreak, and the likelihood of an infected oyster leaving a farm before the outbreak is detected (given that the outbreak has occurred under the testing regime)
- The potential loss of sales due to the impact of a recall event (which could be valued at the 2018/19 average price per dozen oysters, and an assumed recovery profile in sales)

Method

A biotoxin event introduces significant costs for industry and government. Following the official notification of a contamination incident there will generally be a period during which the affected products are banned from sale in the market until the contamination source is identified, all the affected products are withdrawn from the market channel, and the source of contamination is brought under control. Even after the products are allowed back into the market channel, consumption levels may not rebound immediately due to continued perceptions of risk by consumers.

The total cost of product recall event (TC^{recall}) therefore includes:

- The administrative cost of the recall programme,
- The cost of the lost sales over the duration of the recall programme, and
- The cost of lost sales across a group of similar seafood products due to reputation damage following the product recall.

Given the total cost of a product recall event, the expected cost (EC^{recall}) of the recall event is given in equation (1) below.

$$EC^{recall} = P(leaves\ farm \mid DST\ outbreak) \times P(DST\ outbreak) \times TC^{recall} \quad (1)$$

Where *P(leaves farm | DST outbreak)* is the probability that an infected oyster leaves the farm (and enters the market channel) *given that* there has been a DST outbreak in the food sources consumed by shellfish in that growing area; and *P(DST outbreak)* is the naturally occurring probability of DST appearing in the water column within that growing area. *P(DST outbreak)* may not be identically and independently distributed in all 24 growing areas, for example correlations might be expected between adjacent areas subject to the same ocean currents or tidal systems, and occurrences in an area might be influenced by idiosyncratic factors (like differences in sea surface temperature or the relative strength of upwelling). *P(leaves farm | DST outbreak)* is determined by the efficacy of the testing regime at identifying affected shellfish.

Results

Tasmania has adopted an internationally accepted program for the reduction of food safety risks of shellfish consumption. The ShellMAP programme in Tasmania provides Regulatory Services that are consistent with the Australian Shellfish Quality Assurance Program (ASQAP). The basis of this program is to improve the safety of bivalve shellfish, by monitoring harvest waters for the presence of biological or chemical hazards and using comprehensive risk management systems to reduce the risk of food-borne illness. ASQAP requires each growing area to have:

- A comprehensive sanitary survey which includes the approval classification (i.e., if there are any conditions attached to the harvest of shellfish from a growing area) and the development of a management plan (specifies trigger points for the closure and reopening of a growing area, *inter alia*),
- An ongoing bacteriological monitoring program,
- A continuous environmental monitoring program to ensure that harvesting only takes place within management plan criteria,
- A biotoxin monitoring program and management plan,
- A chemical residue testing program, and
- An annual review of both the recent data collected and the current management plan.

The program has been successfully carried out since the mid-1980s, and it is generally now accepted that when a weekly regime of biotoxin testing is applied within Tasmanian growing areas, the probability *P(leaves farm | DST outbreak)* effectively meets the government standard of being zero (0) for all practical purposes. Therefore, under the BAU case the expected cost of a product recall event is effectively:

$$EC^{recall} = \quad 0 \quad \times P(DST\ outbreak) \times TC^{recall} \ = \ 0 \qquad (1^*)$$

To be endorsed and implemented as part of the ShellMAP programme, we assume that any new testing regime would necessarily have to maintain this standard as a minimum condition (i.e., any new test would need to be implemented at a frequency and scale that ensured the maximum risk of an infected product leaving a Tasmanian growing area continues to be negligible). We would therefore not anticipate any change in EC^{recall} due to a fully validated and approved testing regime; and by extension, we would not estimate a change in the *expected cost* of a product recall event in each scenario (Scenario 1, Scenario 2, Scenario 3) considered in this report, assuming that they would be fully validated and approved for use prior to their implementation.

6. 4 Summary/Conclusion

The analysis presented in this report is intended to satisfy the requirements of Objective 5 of FRDC 2017-203:

- Conduct an economic impact analysis to estimate the reduction/annual savings in monitoring costs for regulatory authorities in Tasmania by implementing rapid diagnostic testing for DSTs.
- Calculate the reduction in commercial loss and economic impact from potentially harmful DST blooms in Tasmania following the introduction of the rapid diagnostic testing.

A summary of our findings for each component of Objective 5 is presented below.

Reduction/annual savings in monitoring costs for regulatory authorities in Tasmania by implementing rapid diagnostic testing for DSTs.

While we can say that the qPCR or Neogen technology both offer cost advantages when compared with the laboratory service provider (BA) practice, we are unable to quantify the exact extent of this without further work to validate the two alternative testing technologies. All scenarios considered in this report would represent a net cost saving over 10 years when compared with the current practice of weekly LC-MS tests for DST conducted by a laboratory service provider (BAU). The highest savings in our analysis occur under Scenario 2, in which the Neogen technology is centralised in the laboratory and spread across all 24 growing areas.

However, we were not able to account for QA costs and potential NATA accreditation as these were difficult to ascertain without understanding the implementation standards for the test. The exact number of samples needed to obtain a result (Neogen rapid test, qPCR test) was also important for the projected savings under Scenarios 1 and 3. While our analysis assumed that one sample would be required in each of these scenarios, this may not be the case and the cost efficiency of both technologies increases substantially when fewer samples are needed to generate a result. A sensitivity analysis of our results with respect to the number of samples required to obtain a result is shown in Appendix D.

Therefore, a full validation study covering each of the major testing methods examined in this project (Neogen rapid test, qPCR test) is recommended an important area of further research. The need for on-going internal QA of kit batches should also be assessed, as the efficacy of the rapid test kits can be impacted by processes outside of the testing laboratory's control that occur in the production and/or transport of the kits.

Reduction in commercial loss and economic impact from potentially harmful DST blooms in Tasmania following the introduction of the most appropriate diagnostic testing.

To be implemented under the ShellMAP programme, we expect that any new testing regime would need to be implemented at a frequency and scale that ensures the risk of an infected product leaving a Tasmanian growing area continues to be negligible. Therefore, we would not estimate any change in the *expected* commercial loss and economic impact from potentially harmful DST blooms in Tasmania following the introduction of the most appropriate diagnostic testing considered under this project. This risk would continue to be negligible for Tasmanian growers.

The scenarios considered in this study are for domestic supply, in compliance with potential use under the ASQAP programme. Further considerations would be needed for use in any export programme.

Potential increases in the per sample LC-MS cost (due to lower sample volume) have not been estimated. Prior to implementation of a change to the monitoring regime, it is also recommended that industry and the regulator determine an agreed harvest area management protocol for dealing with Neogen RTK positive result and/or elevated qPCR results.

6.5 References

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Appendix A: detailed benefit-cost tables for implementation Neogen rapid testing on farms in Tasmania

Scenario 1: Implement Neogen DST rapid kit testing on-farm in Tasmania, replacing confirmatory DST testing in low risk areas in 3 out of 4 weeks:

Description of scenario

DST testing in lab decrease to once per month but other shellfish toxin testing (at lab) remains the same schedule (i.e., weekly).

Neogen rapid test kit to be used weekly for the remainder of the month (e.g. a 3 week on-farm to 1 week in-lab schedule)

Year	0	1	2	3	4	5	6	7	8	9	10
Incremental LC-MS testing costs for the laboratory											
Number of growing areas monitored in Tas.	24	24	24	24	24	24	24	24	24	24	24
Current LC-MS lab tests done per year *(i.e. weekly testing)*	1248	1248	1248	1248	1248	1248	1248	1248	1248	1248	1248
Revised LC-MS in lab per year *(@ 1 in 4 weeks)*	312	312	312	312	312	312	312	312	312	312	312
Cost to service provider per test *(NB: cost recovery in pricing)*	$340	$340	$340	$340	$340	$340	$340	$340	$340	$340	$340
Service provider costs ($)	-$317,772	-$317,772	-$317,772	-$317,772	-$317,772	-$317,772	-$317,772	-$317,772	-$317,772	-$317,772	-$317,772
Incremental capital cost for on-farm testing to growers											
Number of growing areas monitored in Tas.	24	24	24	24	24	24	24	24	24	24	24
Raptor Solo Diagnostic Reader	$4,000	$0.00	$0.00	$0.00	$0.00	$4,000	$0.00	$0.00	$0.00	$0.00	$0.00
Heater Block	$500	$0.00	$0.00	$0.00	$0.00	$500	$0.00	$0.00	$0.00	$0.00	$0.00
Transfer Pipette 100-1000uL	$500	$0.00	$0.00	$0.00	$0.00	$500	$0.00	$0.00	$0.00	$0.00	$0.00
Vortex	$400	$0.00	$0.00	$0.00	$0.00	$400	$0.00	$0.00	$0.00	$0.00	$0.00
Capital costs ($)	$129,600.00	$0.00	$0.00	$0.00	$0.00	$129,600.00	$0.00	$0.00	$0.00	$0.00	$0.00
Incremental transport costs for growers											
	No change in transport requirements to the lab in the case of Tasmania, because a sample of 12 Pacific Oysters is still required weekly by the lab for other testing, regardless of the frequency of DST testing.										
Transport Cost ($)	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
On-going costs for on-farm testing to growers											
Cost of consumables per sample											
8mL x analytical grade methanol (4L @ $75)	$0.1500	$0.1500	$0.1500	$0.1500	$0.1500	$0.1500	$0.1500	$0.1500	$0.1500	$0.1500	$0.1500
0.1mL x NaOH (2.5M) (1kg salt @ $159)	$0.0019	$0.0019	$0.0019	$0.0019	$0.0019	$0.0019	$0.0019	$0.0019	$0.0019	$0.0019	$0.0019
0.1mL x HCl (2.5 M) (2.5L @ $200)	$0.0080	$0.0080	$0.0080	$0.0080	$0.0080	$0.0080	$0.0080	$0.0080	$0.0080	$0.0080	$0.0080
10mL x deionized water (10L @ $30)	$0.0300	$0.0300	$0.0300	$0.0300	$0.0300	$0.0300	$0.0300	$0.0300	$0.0300	$0.0300	$0.0300
6 x pipette tips (1000 @ $336)	$2.0160	$2.0160	$2.0160	$2.0160	$2.0160	$2.0160	$2.0160	$2.0160	$2.0160	$2.0160	$2.0160
1 x glass vial (100 @ $50)	$0.5000	$0.5000	$0.5000	$0.5000	$0.5000	$0.5000	$0.5000	$0.5000	$0.5000	$0.5000	$0.5000
2 x 10ml falcon tube (500 @ $500)	$2.0000	$2.0000	$2.0000	$2.0000	$2.0000	$2.0000	$2.0000	$2.0000	$2.0000	$2.0000	$2.0000
1 x Neogen test (24 samples @ $974.50)	$40.6042	$40.6042	$40.6042	$40.6042	$40.6042	$40.6042	$40.6042	$40.6042	$40.6042	$40.6042	$40.6042
Consumables per sample ($)	$45.31	$45.31	$45.31	$45.31	$45.31	$45.31	$45.31	$45.31	$45.31	$45.31	$45.31
Number of samples required to get an on-farm DST test result	1	1	1	1	1	1	1	1	1	1	1
Number of on-farm DST test results required per year	936	936	936	936	936	936	936	936	936	936	936
Cost of consumables per year ($)	*$42,410.21*	*$42,410.21*	*$42,410.21*	*$42,410.21*	*$42,410.21*	*$42,410.21*	*$42,410.21*	*$42,410.21*	*$42,410.21*	*$42,410.21*	*$42,410.21*
Cost of Grower Time											
Hourly rate for grower time	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74
Number of samples required to get an on-farm DST test result	1	1	1	1	1	1	1	1	1	1	1

Number of hours needed per DST test result	1	1	1	1	1	1	1	1	1	1	1
Cost of time per result ($)	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74
Number of growing areas monitored in Tas.	24	24	24	24	24	24	24	24	24	24	24
Number of results needed per growing area per year	39	39	39	39	39	39	39	39	39	39	39
Total number of results needed per year	936	936	936	936	936	936	936	936	936	936	936
Cost of time per result ($)	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74
Cost of grower time per year ($)	*$38,133.89*	*$38,133.89*	*$38,133.89*	*$38,133.89*	*$38,133.89*	*$38,133.89*	*$38,133.89*	*$38,133.89*	*$38,133.89*	*$38,133.89*	*$38,133.89*
Total for on-going testing ($)	$80,544.10	$80,544.10	$80,544.10	$80,544.10	$80,544.10	$80,544.10	$80,544.10	$80,544.10	$80,544.10	$80,544.10	$80,544.10
Validation and implementation costs											
Validation	$95,000.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
Training modules	$35,000.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
Salary	$25,500.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
Project management and travel	$52,280.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
Readers and test kits ($75k)	$20,000.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
Validation and implementation ($)	$227,780.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
Net present cost											
										-	-
Incremental LC-MS testing costs for the laboratory	-$317,772.00	-$317,772.00	-$317,772.00	-$317,772.00	-$317,772.00	-$317,772.00	-$317,772.00	-$317,772.00	-$317,772.00	$317,772.00	$317,772.00
Incremental capital cost for on-farm testing to growers	$129,600.00	$0.00	$0.00	$0.00	$0.00	$129,600.00	$0.00	$0.00	$0.00	$0.00	$0.00
Incremental transport costs for growers	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
On-going costs for on-farm testing to growers	$80,544.10	$80,544.10	$80,544.10	$80,544.10	$80,544.10	$80,544.10	$80,544.10	$80,544.10	$80,544.10	$80,544.10	$80,544.10
Validation and implementation costs	$227,780.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
										-	-
Net cost	*$120,152.10*	*-$237,227.90*	*-$237,227.90*	*-$237,227.90*	*-$237,227.90*	*-$107,627.90*	*-$237,227.90*	*-$237,227.90*	*-$237,227.90*	*$237,227.90*	*$237,227.90*
										-	-
Present value of Net cost	*$120,152.10*	*-$225,931.33*	*-$215,172.70*	*-$204,926.38*	*-$195,167.98*	*-$84,329.28*	*-$177,023.11*	*-$168,593.44*	*-$160,565.18*	*$152,919.22*	*$145,637.35*
Net present cost	*-$1,610,113.86*										

⟹Net Present Value of Savings Over 10 Years $1,610,113.86

Appendix B: detailed benefit-cost tables for implementation Neogen rapid testing by laboratory service provider in Tasmania

Scenario 2: Implement Neogen DST rapid kit testing in laboratory in Tasmania, replacing confirmatory DST testing in low risk areas in 3 out of 4 weeks.

Description of scenario

Current DST testing in lab continues at once per month (rather than weekly). Oher shellfish toxin testing (at lab) remains the same schedule (weekly).

Neogen rapid test kit at the lab replaces current DST testing suit for three in four weeks. Assume the current confirmatory test used for one out of four weeks in each growing area.

No change in grower transport costs (as with other scenarios), as PST testing continues to be done weekly under BAU.

Year	0	1	2	3	4	5	6	7	8	9	10
Incremental LC-MS testing costs for the laboratory											
Number of growing areas monitored in Tas.	24	24	24	24	24	24	24	24	24	24	24
Current LC-MS lab tests done per year *(i.e. weekly testing)*	1248	1248	1248	1248	1248	1248	1248	1248	1248	1248	1248
Revised LC-MS in lab per year *(@ 1 in 4 weeks)*	312	312	312	312	312	312	312	312	312	312	312
Cost to service provider per test *(NB: cost recovery in pricing)*	$340	$340	$340	$340	$340	$340	$340	$340	$340	$340	$340
Service provider costs ($)	-$317,772	-$317,772	-$317,772	-$317,772	-$317,772	-$317,772	-$317,772	-$317,772	-$317,772	-$317,772	-$317,772
Incremental capital cost to laboratory											
Number of testing stations implemented @ lab	5	5	5	5	5	5	5	5	5	5	5
Raptor Solo Diagnostic Reader	$4,000	$0.00	$0.00	$0.00	$0.00	$4,000	$0.00	$0.00	$0.00	$0.00	$0.00
Heater Block	$500	$0.00	$0.00	$0.00	$0.00	$500	$0.00	$0.00	$0.00	$0.00	$0.00
Transfer Pipette 100-1000uL	$500	$0.00	$0.00	$0.00	$0.00	$500	$0.00	$0.00	$0.00	$0.00	$0.00
Vortex	$400	$0.00	$0.00	$0.00	$0.00	$400	$0.00	$0.00	$0.00	$0.00	$0.00
Capital costs ($)	$27,000.00	$0.00	$0.00	$0.00	$0.00	$27,000.00	$0.00	$0.00	$0.00	$0.00	$0.00
Incremental tech staff costs											
based on: 1.5 hours per batch of 10 samples, with a minimum of 1 hour per batch											
Cost of Tech Staff Time											
Hourly rate for tech staff time	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74
Number of low-risk growing areas monitored in Tas.	24	24	24	24	24	24	24	24	24	24	24
Number rapid test results needed per growing area per year	39	39	39	39	39	39	39	39	39	39	39
Number of samples required for a rapid test result	1	1	1	1	1	1	1	1	1	1	1
Total number of rapid test samples conducted at lab per year	936	936	936	936	936	936	936	936	936	936	936
Average number of rapid test samples conducted per day	2.56	2.56	2.56	2.56	2.56	2.56	2.56	2.56	2.56	2.56	2.56
Average tech staff hours required per day	1.09	1.09	1.09	1.09	1.09	1.09	1.09	1.09	1.09	1.09	1.09
Cost of tech staff time per day ($)	$44.28	$44.28	$44.28	$44.28	$44.28	$44.28	$44.28	$44.28	$44.28	$44.28	$44.28
Cost of tech staff time per year ($)	*$16,172.61*	*$16,172.61*	*$16,172.61*	*$16,172.61*	*$16,172.61*	*$16,172.61*	*$16,172.61*	*$16,172.61*	*$16,172.61*	*$16,172.61*	*$16,172.61*
Tech Staff ($)	$16,172.61	$16,172.61	$16,172.61	$16,172.61	$16,172.61	$16,172.61	$16,172.61	$16,172.61	$16,172.61	$16,172.61	$16,172.61
Incremental consumables costs to laboratory											
Cost of consumables per sample											
8mL x analytical grade methanol (4L @ $75)	$0.1500	$0.1500	$0.1500	$0.1500	$0.1500	$0.1500	$0.1500	$0.1500	$0.1500	$0.1500	$0.1500
0.1mL x NaOH (2.5M) (1kg salt @ $159)	$0.0019	$0.0019	$0.0019	$0.0019	$0.0019	$0.0019	$0.0019	$0.0019	$0.0019	$0.0019	$0.0019
0.1mL x HCl (2.5 M) (2.5L @ $200)	$0.0080	$0.0080	$0.0080	$0.0080	$0.0080	$0.0080	$0.0080	$0.0080	$0.0080	$0.0080	$0.0080
10mL x deionized water (10L @ $30)	$0.0300	$0.0300	$0.0300	$0.0300	$0.0300	$0.0300	$0.0300	$0.0300	$0.0300	$0.0300	$0.0300
6 x pipette tips (1000 @ $336)	$2.0160	$2.0160	$2.0160	$2.0160	$2.0160	$2.0160	$2.0160	$2.0160	$2.0160	$2.0160	$2.0160
1 x glass vial (100 @ $50)	$0.5000	$0.5000	$0.5000	$0.5000	$0.5000	$0.5000	$0.5000	$0.5000	$0.5000	$0.5000	$0.5000
2 x 10ml falcon tube (500 @ $500)	$2.0000	$2.0000	$2.0000	$2.0000	$2.0000	$2.0000	$2.0000	$2.0000	$2.0000	$2.0000	$2.0000

1 x Neogen test (24 samples @ $974.50)	$40.6042	$40.6042	$40.6042	$40.6042	$40.6042	$40.6042	$40.6042	$40.6042	$40.6042	$40.6042	$40.6042
Consumables per sample ($)	$45.31	$45.31	$45.31	$45.31	$45.31	$45.31	$45.31	$45.31	$45.31	$45.31	$45.31
Total number of rapid test samples conducted at lab per year	936	936	936	936	936	936	936	936	936	936	936
Cost of consumables per year ($)	*$42,410.21*	*$42,410.21*	*$42,410.21*	*$42,410.21*	*$42,410.21*	*$42,410.21*	*$42,410.21*	*$42,410.21*	*$42,410.21*	*$42,410.21*	*$42,410.21*
Consumables ($)	$42,410.21	$42,410.21	$42,410.21	$42,410.21	$42,410.21	$42,410.21	$42,410.21	$42,410.21	$42,410.21	$42,410.21	$42,410.21
Validation and implementation costs											
Validation	$95,000.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
Training modules	$35,000.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
Salary	$25,500.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
Project management and travel	$52,280.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
Readers and test kits ($75k)	$20,000.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
Validation and implementation ($)	$227,780.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
Net present cost											
										-	-
Incremental LC-MS testing costs for the laboratory	-$317,772.00	-$317,772.00	-$317,772.00	-$317,772.00	-$317,772.00	-$317,772.00	-$317,772.00	-$317,772.00	-$317,772.00	$317,772.00	$317,772.00
Incremental capital cost to laboratory	$27,000.00	$0.00	$0.00	$0.00	$0.00	$27,000.00	$0.00	$0.00	$0.00	$0.00	$0.00
Incremental tech staff costs	$16,172.61	$16,172.61	$16,172.61	$16,172.61	$16,172.61	$16,172.61	$16,172.61	$16,172.61	$16,172.61	$16,172.61	$16,172.61
Incremental consumables costs to laboratory	$42,410.21	$42,410.21	$42,410.21	$42,410.21	$42,410.21	$42,410.21	$42,410.21	$42,410.21	$42,410.21	$42,410.21	$42,410.21
Validation and implementation costs	$227,780.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
										-	-
Net cost	*-$4,409.18*	*-$259,189.18*	*-$259,189.18*	*-$259,189.18*	*-$259,189.18*	*-$232,189.18*	*-$259,189.18*	*-$259,189.18*	*-$259,189.18*	*$259,189.18*	*$259,189.18*
										-	-
Present value of Net cost	*-$4,409.18*	*-$246,846.83*	*-$235,092.22*	*-$223,897.35*	*-$213,235.58*	*-$181,926.29*	*-$193,410.95*	*-$184,200.91*	*-$175,429.44*	*$167,075.65*	*$159,119.67*
Net present cost	*-$1,984,644.08*										

⟹Net Present Value of Savings Over 10 Years $1,984,644.08

Appendix C: detailed benefit-cost tables for implementation of qPCR testing on-farms in Tasmania

Scenario 3: Implement qPCR for *Dinophysis* spp. testing on-farm in Tasmania, replacing confirmatory DST testing in low risk areas in 3 out of 4 weeks.

Description of scenario

DST testing in lab decrease to once per month but other shellfish toxin testing (at lab) remains the same schedule (i.e., weekly).
qPCR rapid test kit to be used for weekly testing for the remainder of the month (e.g. a 3 week on-farm to 1 week in-lab schedule)

Year	0	1	2	3	4	5	6	7	8	9	10
Incremental LC-MS testing costs for the laboratory											
Number of growing areas monitored in Tas.	24	24	24	24	24	24	24	24	24	24	24
Current LC-MS lab tests done per year *(i.e. weekly testing)*	1248	1248	1248	1248	1248	1248	1248	1248	1248	1248	1248
Revised LC-MS in lab per year *(@ 1 in 4 weeks)*	312	312	312	312	312	312	312	312	312	312	312
Cost to service provider per test *(NB: cost recovery in pricing)*	$340	$340	$340	$340	$340	$340	$340	$340	$340	$340	$340
Service provider costs ($)	-$317,772	-$317,772	-$317,772	-$317,772	-$317,772	-$317,772	-$317,772	-$317,772	-$317,772	-$317,772	-$317,772
Incremental capital cost for on-farm testing to growers											
Number of growing areas monitored in Tas.	24	24	24	24	24	24	24	24	24	24	24
qPCR machine	$10,000	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
Sampler	$500	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
Transfer Pipette 100-1000uL	$500	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
Mini Centrifuge	$600	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
Vortex	$400	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
Capital costs ($)	$288,000.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
Incremental transport costs for growers											
	No change in transport requirements to the lab in the case of Tasmania, because a sample of 12 Pacific Oysters is still required weekly by the lab for other testing, regardless of the frequency of DST testing.										
Transport Cost ($)	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
On-going costs for on-farm testing to growers											
Cost of consumables per sample											
1 x PCR tubes (120 @ $360)	$3.0000	$3.0000	$3.0000	$3.0000	$3.0000	$3.0000	$3.0000	$3.0000	$3.0000	$3.0000	$3.0000
1 x Cell lysis tubes (50 @ $250)	$5.0000	$5.0000	$5.0000	$5.0000	$5.0000	$5.0000	$5.0000	$5.0000	$5.0000	$5.0000	$5.0000
1 x kit assay ($25 per sample)	$25.0000	$25.0000	$25.0000	$25.0000	$25.0000	$25.0000	$25.0000	$25.0000	$25.0000	$25.0000	$25.0000
3 x pipette tips (1000 @ $442)	$1.3260	$1.3260	$1.3260	$1.3260	$1.3260	$1.3260	$1.3260	$1.3260	$1.3260	$1.3260	$1.3260
1 x 8 micrometre filter paper (100 @ $72)	$0.7200	$0.7200	$0.7200	$0.7200	$0.7200	$0.7200	$0.7200	$0.7200	$0.7200	$0.7200	$0.7200
1 x Syringe (40 @ $26)	$0.6500	$0.6500	$0.6500	$0.6500	$0.6500	$0.6500	$0.6500	$0.6500	$0.6500	$0.6500	$0.6500
1 x Syringe filter holder (12 @ $320) *[note: can be used mult. times]*	$2.6667	$2.6667	$2.6667	$2.6667	$2.6667	$2.6667	$2.6667	$2.6667	$2.6667	$2.6667	$2.6667
Consumables per sample ($)	$38.36	$38.36	$38.36	$38.36	$38.36	$38.36	$38.36	$38.36	$38.36	$38.36	$38.36
Number of samples required to get an on-farm DST test result	1	1	1	1	1	1	1	1	1	1	1
Number of on-farm DST test results required per year	936	936	936	936	936	936	936	936	936	936	936
Cost of consumables per year ($)	*$35,907.46*	*$35,907.46*	*$35,907.46*	*$35,907.46*	*$35,907.46*	*$35,907.46*	*$35,907.46*	*$35,907.46*	*$35,907.46*	*$35,907.46*	*$35,907.46*
Cost of Grower Time											
Hourly rate for grower time	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74

Number of samples required to get an on-farm DST test result	1	1	1	1	1	1	1	1	1	1	1
Number of hours needed per DST test result	1	1	1	1	1	1	1	1	1	1	1
Cost of time per result ($)	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74
Number of growing areas monitored in Tas.	24	24	24	24	24	24	24	24	24	24	24
Number of results needed per growing area per year	39	39	39	39	39	39	39	39	39	39	39
Total number of results needed per year	936	936	936	936	936	936	936	936	936	936	936
Cost of time per result ($)	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74	$40.74
Cost of grower time per year ($)	*$38,133.89*	*$38,133.89*	*$38,133.89*	*$38,133.89*	*$38,133.89*	*$38,133.89*	*$38,133.89*	*$38,133.89*	*$38,133.89*	*$38,133.89*	*$38,133.89*
Total for on-going testing ($)	$74,041.34	$74,041.34	$74,041.34	$74,041.34	$74,041.34	$74,041.34	$74,041.34	$74,041.34	$74,041.34	$74,041.34	$74,041.34
Validation and implementation costs											
Validation	$95,000.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
Training modules	$35,000.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
Salary	$25,500.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
Project management and travel	$52,280.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
qPCR machine and kits and consumables ($75k)	$75,000.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
Validation and implementation ($)	$282,780.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
Net present cost											
				-	-	-	-	-	-	-	-
Incremental LC-MS testing costs for the laboratory	-$317,772.00	-$317,772.00	-$317,772.00	$317,772.00	$317,772.00	$317,772.00	$317,772.00	$317,772.00	$317,772.00	$317,772.00	$317,772.00
Incremental capital cost for on-farm testing to growers	$288,000.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
Incremental transport costs for growers	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
On-going costs for on-farm testing to growers	$74,041.34	$74,041.34	$74,041.34	$74,041.34	$74,041.34	$74,041.34	$74,041.34	$74,041.34	$74,041.34	$74,041.34	$74,041.34
Validation and implementation costs	$282,780.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
				-	-	-	-	-	-	-	-
Net cost	$327,049.34	-$243,730.66	-$243,730.66	$243,730.66	$243,730.66	$243,730.66	$243,730.66	$243,730.66	$243,730.66	$243,730.66	$243,730.66
				-	-	-	-	-	-	-	-
Present value of Net cost	$327,049.34	-$232,124.43	-$221,070.89	$210,543.70	$200,517.81	$190,969.35	$181,875.57	$173,214.83	$164,966.50	$157,110.95	$149,629.48
Net present cost	-$1,554,974.18										

⟹Net Present Value of Savings Over 10 Years $1,554,974.18

Appendix D: sensitivity analysis of the Net Present Value of Savings over 10 Years with respect to the number of samples required to obtain a result

Table D.1: Sensitivity analysis of the Net Present Value of Saving over 10 years for each of Scenario 1, Scenario 2, and Scenario 3 as the number of samples required to get a result from the new testing tech in each scenario (i.e., the Neogen or qPCR test) increases from 1 sample needed for a (reliable) result, up to 10 samples needed for a (reliable) result.

Net Present Value of Savings over 10 Years vs. the number of samples required to obtain a result:	*Number of samples required to obtain a reliable test result (Neogen or qPCR, depending on the Scenario in the far-left column)*									
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Scenario 1: Implement Neogen DST rapid kit testing on-farm in Tasmania, replacing confirmatory DST testing in low risk areas in 3 out of 4 weeks.	$1,610,113.86	$1,221,745.80	$833,377.74	$445,009.67	$56,641.61	-$331,726.45	-$720,094.51	-$1,108,462.57	-$1,496,830.64	-$1,885,198.70
Scenario 2: Implement Neogen DST rapid kit testing in laboratory in Tasmania, replacing confirmatory DST testing in low risk areas in 3 out of 4 weeks1.	$1,984,644.08	$1,596,276.01	$1,207,907.95	$696,964.08	$308,596.02	-$79,772.04	-$468,140.10	-$979,083.97	-$1,367,452.04	-$1,755,820.10
Scenario 3: Implement qPCR testing on-farm in Tasmania, replacing confirmatory DST testing in low risk areas in 3 out of 4 weeks1.	$1,554,974.18	$1,204,844.01	$854,713.85	$504,583.68	$154,453.52	-$195,676.65	-$545,806.81	-$895,936.98	-$1,246,067.14	-$1,596,197.31

7. Conclusions

This study had five main aims with respect to generating new knowledge about DSTs and their detection methods in Australian shellfish. In the process of the work undertaken to meet these aims, a summary of conclusions is as follows:

- *Conducting an initial assessment of DST profiles present in Australian shellfish and assessing laboratory capabilities to detect these toxins;*

A review of DST profiles in Australian shellfish found that okadaic acid was the prominent DST analogue reported. Dinophysis and reports of DSTs are common in relation to Australian shellfish. The four laboratories that participated in an interlaboratory comparison for LC-MS/MS or LC-MS were able to detect DSTs when present and correctly assess when not present in all matrices. We found all toxins in all species could be recovered by all laboratories using LC-MS/MS and LC-MS, however, DST recovery at low and mid-level concentrations (<0.1 mg/kg) was variable (0-150%), while recovery at high-level concentrations (>0.86 mg/kg) was higher (60-262%).

- *Generating knowledge about commercially available DST test kits and rapid molecular techniques for toxin and species detection;*

A review of available DST test kits led to the assessment of five kits, and investigations into the use of qPCR to detect DST producing species.

- *Comparing the efficacy of DST toxin detecting kits across oysters, mussels and pipis;*

A comparison of five DST test kits (three quantitative ELISA kits by Beacon™, Eurofins/Abraxis™ and EuroProxima™; a quantitative PP2A kit by Eurofins/Abraxis™, and a qualitative LFA kit by Neogen™) did not support the use of any of the five kits tested as a stand-alone quality assurance method at this time. While no clear differences were observed between shellfish, all kits delivered an unacceptably high level (25-100%) of falsely compliant results for spiked samples.

The LFA (Neogen) and the PP2A (Abraxis) kits performed satisfactorily for naturally contaminated pipis (0% and 5% falsely compliant results, respectively). Due to other factors such as such as method cost, preparation time, test complexity, and extra equipment required, the PP2A kit has potential, but continued collaboration with the manufacturer to refine its test procedure is necessary. The LFA kit, on the other hand is relatively simple to use, returns a faster result than other kits, and shows promising results for naturally contaminated shellfish. Further validation work on this kit is recommended.

- *Developing a DST qPCR assay for species detection for onsite farm use;*

A qPCR assay to detect DST producing species was developed, and strongly matched field observations of microscopic cell counts during a pilot study. Further validation work is recommended.

- *Providing cost versus benefit analysis of improved testing of DSTs in Tasmanian shellfish:*

The cost benefit analysis (CBA) assessed the use of qPCR and the Neogen test kit in comparison to current weekly testing protocols in Tasmania. The CBA reported that a net cost saving was possible by using these technologies. The need for further validation of the methods has limited the utility of the CBA, but it shows that these methods offer promising cost savings, if they could be sufficiently improved. Each state and shellfish sector would need to conduct their own DST risk analysis, as Tasmania is a comparatively low DST risk state, and the economic benefit analysis may be very different for other states and fisheries.

8. Implications

We conducted a replicated, quality-controlled laboratory-based study to compare the performance of a range of commercially available rapid test kits on DSTs standards spiked in oyster, mussel and pipi samples. Overall, considering the highly varied, and sometimes erroneous results, along with other factors such as method cost, preparation time, test complexity, and extra equipment required, our results do not support the use of any DST rapid test kit as a stand-alone quality assurance measure at this time.

Quantitatively, the Abraxis PP2A kit outperformed all other rapid test kits (notably in naturally contaminated pipis) and may be suitable for screening purposes. Using this kit however, one sample took ~ 3 hours to complete. This kit also requires more rigorous validation to determine the statistics around its false compliant results. Continued collaboration with the manufacturer to refine this test procedure should be undertaken to improve its potential. Qualitatively, the Neogen test kit performed well for naturally contaminated Pipis (0% falsely compliant results at the regulatory level) but appeared much less reliable (63% false negative results at regulatory level) for spiked pipis, oysters, and mussels. These results suggest possible differences in kit performance dependent on the shellfish matrix analysed, or whether the shellfish is naturally contaminated or artificially spiked. The reason(s) for differing results between naturally contaminated shellfish and spiked samples however, remains unclear, particularly when toxin determination using LC-MS did not result in any significant difference between these two matrices in the present study. The Neogen kit is, however, relatively simple to use, returns a faster result than other kits, and as discussed above, shows promising results for naturally contaminated shellfish. A single laboratory validation study such as carried out by for paralytic shellfish toxins in mussels and oysters ((Turnbull et al., 2018), followed by an international validation study (Dorantes et al. 2017), is recommended prior to approval of any rapid test kit for regulatory purposes.

We then developed a rapid, sensitive and efficient quantitative real-time qPCR assay to detect species belonging to the genus Dinophysis spp. In this study, we demonstrated a remarkable similarity between the qPCR and microscopy quantification methods, suggesting that this assay is a valuable early warning tool for HAB monitoring. Future work would need to include the development and validation of a simplified and commercialised qPCR pipeline for the detection of Dinophysis spp. for on farm usage.

We also provided a cost versus benefit analysis of improved testing of DSTs in Tasmanian shellfish. While the qPCR or Neogen technology both offer cost advantages when compared with the laboratory service provider practice, we were unable to quantify the exact extent of this without further work to validate the two alternative testing technologies. Furthermore, to be implemented under the ShellMAP programme, we would expect that any new testing regime would need to be implemented at a frequency and scale that ensures the risk of a contaminated product leaving a Tasmanian growing area continues to be negligible.

Finally, we demonstrated the use of the Neogen rapid test kit and the qPCR assay to the farmers in the Manning River and Wallis Lake oyster harvest area. The farmers were both interested and engaged in the technology.

9. Recommendations

I. All four tested laboratories offering marine biotoxin analysis to the Australian seafood industry can detect all analogues in all shellfish matrices with a reasonable error level. The seafood industry can have confidence in the results of the laboratories that are available to provide LC-MS/MS and LC-MS services for marine biotoxin analysis to the Australian shellfish industry. Regulators should be aware that all LC-MS/MS and LC-MS standard methods are associated with a level of standard error, which was typically around +/- 20%. Regulation of the shellfish aquaculture industry based on LC-MS/MS and LC-MS detection of DST toxins needs to be conservative to account for the standard level of variability of the LC-MS DST analysis method.

II. The use of any of the currently commercially available rapid DST test kits as a standalone method for DST analysis in Australia is currently not recommended due to unacceptably high levels of incorrect results at the regulatory level. However, the shellfish aquaculture industry in each state should review the information gained in this study to determine whether the potential benefits in cost savings and reduction in turn-around time of using rapid test kits warrants further examination or development of rapid methods in their state context. Considerable savings could be achieved using these kits and/or the qPCR assay for Dinophysis species detection developed in this study, if they could be sufficiently improved.

III. If any state decided that potential savings warranted further validation of rapid methods of DST or Dinophysis detection, then we would suggest that validation of the Abraxis PP2A and/or Neogen LFA and qPCR assay for Dinophysis species detection could be costed and carried out in accordance with Association of Official Agricultural Chemists (AOAC) procedures for the validation of such tests. We suggest that individual manufacturers of the appropriate methods be approached to contribute to such studies, were they to go ahead.

10. Extension, Adoption and Project Materials Developed

Outreach and project materials developed during this project include:

1. Five milestone reports submitted to FRDC

2. Presentation to stakeholders at the Australian Shellfish Quality Assurance Program's Science Day, 18-19 Sept 2019, Perth, Western Australia. This day was organised by the Australian Shellfish Quality Assurance Advisory Committee, chaired by DPI's NSW Food Authority, and included industry representatives of from all states (~50 attendees in total).

3. Workshops to train farmers in rapid diagnostic testing (Aim 6):

Seafood Industry Field Day, Wednesday 16th June 2021, Graham Barclay Oysters, Little Street, Forster Wallis Lake, NSW and Thursday 17th June 2021, Coastal Oysters, 41 Ferry Road, Croki (Manning River) NSW. Approximately 30 and 20 attendees respectively gathered to discuss the latest scientific research including a demonstration on the use of the Neogen rapid test kit for the detection of DSTs in shellfish and qPCR for the detection of *Dinophysis* in water samples (species which produce DSTs).

4. A draft manuscript, *A comparative analysis of methods (LC-MS/MS and Rapid Test Kits) for the determination of diarrhetic shellfish toxins in oysters, mussels and pipis*, Penelope A. Ajani, Chowdhury Sarowar, Alison Turnbull, Hazel Farrell, Anthony Zammit, Stuart Helleren, Gustaaf Hallegraeff and Shauna A. Murray, was submitted to the NSW Shellfish Committee and FRDC for endorsement. The manuscript was submitted to *Toxins* special issue "Marine Toxins from Harmful Algae and Seafood Safety" on 8 July 2021 and accepted for publication on 9 August 2021. The methodology and findings of this research are included in this report.

11. Appendices

Appendix 1. Methods, detection limits, limit of quantification/reporting and measurement uncertainty as reported by each laboratory for LC-MS/MS and LM-MS determination of DSTs in shellfish

	Method	Limit of Detection	Limit of Quantification (LOQ)/Limit of reporting (LOR)	Measurement Uncertainty
Lab 1 LC-MS/MS	LC-MS/MS Method similar to McNabb (2005) and Villar-Gonzalez et al. (2011) and the EU-Harmonised method from the EU Reference Lab. That is, an 80% MeOH extraction, with two portions of the extract analysed after 1) hexane-cleanup, 2) alkaline hydrolysis (to convert esters to acids).	0.004 mg/kg OA, DTX-1, DTX-2	0.01 mg/kg OA, DTX-1, DTX-2	25% OA 26% DTX-1 24% DTX-2 (at a confidence level of 95%)
Lab 2 LC-MS/MS	Multitoxin LC-MS/MS method for lipophilic toxins based on McNabb 2005 with IANZ (ISO 17025) accreditation	0.001-0.002 mg/kg OA, DTX-1, DTX-2	0.01 mg/kg OA, DTX-1, DTX-2	21% at 0.01 mg/kg
Lab 3 LC-MS	Sample extraction was performed using the method as described by McNabb et al. (2005). OA analysis was conducted using a Thermo Scientific™ Q EXACTIVE™ high resolution mass-spectrometer equipped with an electrospray ionization. Chromatographic separation was performed on a Thermo Scientific™ ACCELA™ UPLC system.	0.006 mg/kg OA 0.007 mg/kg DTX-1 0.007 mg/kg DTX-2	0.021 mg/kg OA 0.023 mg/kg DTX-1 0.024 mg/kg DTX-2	19% OA 21% DTX-1 12 % DTX-2
Lab 4 LC-MS/MS	LC-MS/MS using the instrument AB ScieX Triple Quad 6500.	~5-10 x lower than the LOQ/LOR	0.025 mg/kg OA, DTX-1 0.015 mg/kg DTX-2	20% Total OA 20% Total DTX-1 20% Total DTX-2 15% Free OA 15% Free DTX-1 10% Free DTX-2

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