Improved understanding of Tasmanian harmful algal blooms and biotoxin events to support seafood risk management

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

Contents

Tables

Figures

<0.8 mg STX eq/kg in (B), where the sensitivity of the Scotia test is shaded (0.2-0.7 mg STX eq/kg) and positive results were expected. The y-axis indicates the numerical value generated by the Scotia Skannex system based on the intensity comparison of the control and test bands of the test. Values ≥0.5 indicate negative samples (●), or positive if <0.5 (●). False positives (●) are those samples with ≤0.20 mg STX eq/kg (LC-FLD analysis) but generated a positive result. See Table S2 (Supplementary material) for complete list of numerical values.

Fig. 3.3.1. IMAS Sarah Ugalde and Juan Dorantes-Aranda, with mussel grower Corrine Ooms from Spring Bay, oyster farmer Hayden Dyke from Little Swanport and scallop fisherman Bob Lister at the IMAS teaching laboratories.

Fig. 3.4.2.1. The probability of detection of PST by Neogen™ rapid test kit at various PST concentrations in experimental series created from four different toxin mixes. From Turnbull et al. (2017)

Fig. 3.4.2.2. The laboratory POD across a range of concentrations for the oyster and mussel experimental series extracted via the standard and optimised protocols. From Dorantes-Aranda et al. (2017)

Fig. 3.4.2.3. The probability of a positive response over a range of PST concentrations across all laboratories. The black dotted line is the average LPOD, and the solid lines are LPOD for 5th and 95th percentile of labs. The numbers above the circles indicate the number of laboratories returning the shown POD. The intersection of the 95% probability of a positive response with the average LPOD results in an average LOD of 0.71 mg STX·2HCl eq/kg.

Fig. 3.4.2.4. The probability of a positive response in oysters over a range of PST concentrations across all laboratories using the standard protocol. The black dotted line is the average LPOD, and the solid lines are LPOD for 5th and 95th percentile of labs. The numbers above the circles indicate the number of laboratories returning the shown POD. The LPOD at the regulatory level was 0.997.

Fig. 4.1. Map of Tasmania, south of the mainland of Australia, showing Sea Surface Temperatures on 27 September 2015 during peak PST, with the East Australian Current (EAC; in red) interacting with the continental shelf. The locations of the main affected shellfish farm areas Moulting Bay, Great Oyster Bay, Little Swanport and Spring Bay are indicated. Source: oceancurrent.imos.org.au.

Fig. 4.2. Shellfish toxicity (mg STX eq./kg) from 2012 to 2016 in Moulting Bay and Little Swanport oysters and Spring Bay mussels. Orange arrows indicate the seasonal 10-15^{o}C temperature window. The 2016 bloom was preceded by a major rainfall event while anomalously cold water in Great Oyster Bay may explain the 2015 bloom.

Fig. 4.3. Light (a,d) and scanning electron micrographs (b,c) of Tasmanian 2015 and 2016 *Alexandrium* field samples. Fig.3a shows ventral pore (arrowed) in the first apical plate; Figs. b,c,d show the extreme fragility of the cells subject to ecdysis within 30-60 min of collection.

Fig. 4.4. Left: *Alexandrium* bloom patch contained on the inner shelf in August 2016, with no cells detected in offshore deeper waters. Middle: Depth profiles of *Alexandrium* cell abundance (top scale), total PST toxins (ng/L) and pg PST eq per cell (bottom scale) in weakly stratified waters of Great Oyster Bay and Spring Bay. Right: *Alexandrium* cyst surveys in August 2016: white dots <0.1; yellow 0.4-0.6; orange 2-3 cysts/g sediment wet weight.

Fig. 5.1. Standard curves for qPCR assays to detect *Alexandrium catenella/fundyense* (primer set US-412-F/US-642-R) and *Alexandrium pacificum* (primer set ACTA-416-F/ACTA-605-R), based on the cultures TRIA-A (green triangles) and CAWD44 (red squares), CS300 (blue diamonds), respectively

Fig.5.2. Standard curves to show the efficiency of the qPCR assay targeting *sxtA4*, against 3 replicate DNA extractions from Tasmanian cultures of *Alexandrium catenella/fundyense* (TRIA-A, triangles and squares) and 2 different strains of *Alexandrium pacificum* (CAWD44, CS300, crosses and stars). All strains amplified efficiently using this primer pair, with efficiency results from 88-96%.

Fig. 5.3. PST cell toxicity of the three *Alexandrium* species present in Tasmania, based on cultures.

Fig. 5.4. Cell PST toxicity of Tasmanian field samples assessed with the Neogen PST kit. Toxicity is highest at low dinoflagellate cell concentrations and in most cases two orders of magnitude higher than toxicity of laboratory cultures.

Fig. 5.5. PST toxin profiles of the three *Alexandrium* species present in Tasmania, based on cultures.

Fig. 5.6. Map of sites for which we took samples in this project to assess the presence and abundance of different toxic species of the *Alexandrium tamarense* complex.

Fig. 5.7. Seasonality of *Alexandrium tamarense* complex (species not discriminated) along eastern Tasmanian coast from 2013-14, based on samples collected by TSQAP and analysed by Analytical Services Tasmania.

Fig. 5.8. Sites at which *Alexandrium* species were detected during Bluefin Cruise, in September 2014.

Fig 5.9. *Alexandrium tamarense* complex detected using qPCR of the *sxtA* gene, compared to PST concentrations in the mussel flesh, at Spring Bay from October 2013 – September 2014

Fig. 5. 10. *Alexandrium tamarense* complex cell concentration detected using qPCR of the *sxtA* gene compared to PST concentrations in the mussel flesh, at Spring Bay from May to November, 2016.

Fig. 5.11. Ratios of *Alexandrium fundyense* and *Alexandrium pacificum* in plankton net samples from sites in Spring Bay and Little Swanport on 15 Sept 2015.

Fig. 5.12. Comparisons of *Alexandrium tamarense* complex detection using cell counts, species-specific qPCR detection, and qPCR of the SxtA4 gene. Samples collected at site 1 in Great Oyster Bay during Southern Cross cruise (22nd-26th August 2016).

Fig. 5.13. Depth profile of *Alexandrium tamarense* species complex, the sxtA4 assay, and a cell count using microscopy, taken on the Southern Cross from 22nd-26th August 2016 at Great Oyster Bay inshore site 2.

Fig. 5.14. Depth profile of *Alexandrium tamarense* species complex, the sxtA4 assay, and a cell count using microscopy, taken on the Southern Cross from 22nd-26th August 2016 at Spring Bay site 3.

Fig. 6.1: Satellite sea surface temperature (SST) from NOAA AVHRR sensors (left) and satellite ocean colour from the NASA MODIS sensor (right) in early spring. Images also show contours of sea surface height (combining data from satellite altimeters and coastal tide gauges) and geostrophic currents estimated from gradients in sea surface height. Further details are available through the IMOS Ocean Current site (http://oceancurrent.imos.org.au/sourcedata/index.php).

Fig. 6.2: Measured median monthly temperature gradient during Spring at the Maria Island National Reference Station. Traffic lights indicate the number of months in each spring that the gradient exceeded a threshold of 0.01°C/m.

Fig. 6.3 : Sources of water arriving at the Maria Island National Reference Station over the period 14-21 November 2016, having dispersed for 7 days at depths of 4 m (left) and 9 m (right). The maps were generated using the dispersal model CONNIE3 (www.csiro.au/connie/) and show the percentage of particles that passed through each cell (approximately 1 km x 1 km) before reaching the reference station cell.

Fig. 6.4: Time series from Orford (Aubin Court) of (a) June rainfall; and (b) August mean minimum temperature (Australian Bureau of Meteorology: http://www.bom.gov.au/jsp/ncc/cdio/weatherData/av?p_nccObsCode=139&p_display_type=dataFile&p_startYear=&p_c=&p_stn_num=092027).

Fig. 6.5: Rainfall during months where *A. tamarense* blooms were present and absent (as detected through analyses of toxin levels in shellfish).

Fig. 6.6: Surface temperature and surface current velocity from the coastal circulation model (Oliver et al. 2016). An east-west vertical temperature section across the mouth of Great Oyster bay is shown at the bottom of each panel. Observed coastal blooms are indicated by red arrows.

Fig. 6.7: Vertical temperature section along the north-south axis of Great Oyster Bay (80 km long with a maximum depth of 70 m) from the coastal circulation model (Oliver 2016). Periods of coastal bloom events at Little Swanport and Spring Bay are framed in red.

Fig. 6.8: Hypotheses for the environmental drivers of HABs off eastern Tasmania: (a) high rainfall potentially accompanied by southerly or onshore winds; and (b) outflow of cold water southward from Great Oyster Bay potentially accompanied by southerly or onshore winds.

Fig. 6.9: Tentative frameworks for assessing the risk of HABs occurring off eastern Tasmania using both existing meteorological data streams and proposed in situ monitoring of coastal waters. Note that quantitative criteria in brackets are based on very few HAB events and require ongoing refinement.

Fig. 6.10: Mock-up of an online HABs portal that combines the environmental conditions used for forcasting with any available biological monitoring and toxicology data.

Fig 7.1. The risk analysis process.

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Executive Summary

Background

During October 2012, a shipment of Blue Mussels (*Mytilus galloprovincialis*) from the poorly monitored east coast of Tasmania, Australia, was tested by Japanese import authorities and found to be contaminated with unacceptable levels of Paralytic Shellfish Toxins (PSTs; 10 mg/kg). Subsequently local oysters, scallops, clams, the viscera of abalone and rock lobsters were also found to be contaminated. This led to a global product recall of all Australian shellfish exported to Japan and loss to the local economy of AUD 23M.

A review of this critical incident (Campbell *et al.* 2013) identified:

1. Failure of plankton monitoring to provide timely results and failure to detect *Alexandrium*;

2. Failure of seafood risk assessment by not recognizing the risk of a new mussel farming venture in a poorly monitored area;

3. Failure of PST monitoring by relying only on plankton monitoring as a first screen rather than including shellfish testing.

The analytical turnaround from Tasmanian biotoxin meat analyses conducted at the National Biotoxin Lab at Advanced Analytical Pty Ltd in Sydney is currently in the order of 7-10 days; but during the peak of the 2012 PST event, this sometimes expanded up to 3 weeks, with sample collection/ transport contributing to over 50% of the turnaround time. Such delays in advice to lease-holders are frustrating and can lead to losses of already harvested stocks when retrospectively found to be tainted with PST toxins.

Need

Improvements in understanding of Tasmanian harmful algal bloom biology, ecology and toxicology are needed to support seafood biotoxin risk management

Aims/objectives

1. Develop, calibrate and adopt screening techniques for rapid detection and evaluation of toxins;

2. Use state-of-the art molecular techniques to elucidate the genetic population structure and biology of toxic *Alexandrium tamarense*- group algae;

3. Oceanographic modelling of time periods and zones at risk, and coordinated data capture to enable prediction of biotoxin event development;

4. Perform a desktop seafood risk assessment as a prelude for a future stage 2 proposal focusing on Paralytic Shellfish Toxin foodweb transfer.

Methodology

1. We assessed the performance of four commercial rapid PST test kits, Abraxis™, Europroxima™, Scotia™ and Neogen™, compared with the official Association of Official Analytical Chemists (AOAC) LC-FLD method for contaminated mussels and oysters.

   We ran training workshops on dinoflagellate toxin extraction and the use of ELISA test kits for staff, sample collectors, and industry willing to do their own sampling.

2. We genetically and toxicologically characterised 40 *Alexandrium* dinoflagellate culture strains as well as Tasmanian field samples collected during the 2012 to 2016 bloom periods.

3. We collated available historical hydrological data from Tasmania's East Coast, and combined this with 2012-2016 Tasmanian Shellfish Quality Assurance Program (TSQAP) biotoxin monitoring data, to define physical conditions, time periods and geographic zones favourable to Tasmanian algal blooms, and as a foundation for establishing a near real-time modelling capability and broader seafood risk assessment in the future.

4. A workshop was conducted with a variety of experts to make a qualitative ranking of the public health risk associated with marine biotoxins in non-bivalve seafood groups that are recreationally and commercially captured in Tasmania. The workshop included representatives from government, commercial and recreational fisheries and experts in risk assessment and marine biotoxins. The scope of the risk ranking exercise was limited to the four toxin groups regulated in the Australian Food Standards Code: Paralytic Shellfish Toxins (PSTs); Diarrhetic Shellfish Toxins (DSTs); Amnesic Shellfish Toxins (ASTs) and Neurotoxic Shellfish Toxins (NSTs). The Tasmanian seafood groups considered were: commercially harvested fisheries products; marine farmed products (with the exception of bivalve molluscs that are already under the control of the Tasmanian Shellfish Quality Assurance Program - oysters, mussels, clams and to some extent scallops); and recreationally harvested seafood.

Results/key findings

1. Abraxis and Europroxima kits underestimated PST in 35-100% of samples when using standard protocols but quantification improved when concentrated extracts were further

diluted (underestimation ≤18%). The Scotia kit (cut off 0.2-0.7 mg STX-diHCl eq/kg) delivered 0% false negatives, but 27% false positives. Neogen produced 5% false negatives and 13% false positives when the cut off was altered to 0.5-0.6 mg STX-diHCl eq/kg, but the introduction of a conversion step eliminated false negatives.

Based on their sensitivity, ease of use and performance, the Neogen kit proved the most suitable kit for use with Tasmanian mussels and oysters. We subsequently conducted a full validation process of this screening test using protocols established by the Association of Official Analytical Chemists (AOAC) in the US, funded through the Department of Agriculture and Water Resources. This work involved a Single Laboratory Validation, whereby a single analyst tested 800 blind samples to examine selectivity, the effect of matrix (oysters vs mussels), probability of detection, repeatability and ruggedness. A further Inter-laboratory Validation was conducted by sending 996 blind samples to 16 laboratories, both domestic and international, ranging from shellfish farmers to fully accredited laboratories.

The Neogen™ test kit performed very well for testing PST in oysters, and on average was satisfactory for mussels. The results were not only faster and cheaper than advanced chemical methods but also much more reliable and highly repeatable between laboratories.

2. While the dinoflagellate *Alexandrium tamarense* had been detected in low concentrations in southeastern Australia since 1987, all previous cultured strains belonged to the mostly non-toxic group 5 (now designated *australiense;* detected since 1987) and weakly toxic group 4 (*pacificum;* detected in 1997). In contrast, the 2012 to 2016 outbreaks were all dominated by highly toxic group 1 (*fundyense*) never detected previously in the Australian region.

Both culture experiments but notably field estimates using both Neogen and LC-MS suggest a very high cellular toxin content up to 100-500 pg STX eq/cell, thus explaining why even very low cell numbers can cause seafood toxicity.

The varying co-occurrence of *fundyense* and *pacificum* in east coast Tasmanian blooms confounds progress in elucidating bloom ecophysiology as well as predict shellfish toxicity. These two species: (1) cannot be discriminated by light microscopy; (2) have different PST toxin profiles; resulting in markedly different cell toxicity; and (3) a may have differing ecophysiology. Total *Alexandrium tamarense* complex cell abundance is therefore not a good predictor of shellfish toxicity. The quantitative PCR assays we developed are capable of distinguishing the different *Alexandrium* species and the SxtA4 gene assay is a useful lead indicator of the subsequent accumulation of PST toxins in shellfish flesh, if routinely measured on a weekly basis.

3. The affected Tasmanian coastal region is classified as a climate change "hotspot" resulting from increasing southward movement of the nutrient-poor East Australian Current. These novel *Alexandrium* blooms are not a simple response to increasing water temperatures however , as they occur in the cold winter-spring months at water temperatures of 10-15^{o}C.

Following low toxicity during 2013 and 2014 and implementation of minimal shellfish farm closures, a more severe bloom event occurred during July-November 2015 and again June-September 2016 (up to 300,000 *Alexandrium* cells/L; 24 mg/kg PST in mussels, 6 mg/kg in *Crassostrea gigas* oysters), also causing 4 human illnesses resulting in hospitalization after consumption of wild shellfish.

In 2016 the peak of the *Alexandrium* bloom coincided with a major high rainfall/ flood event that resulted in salinity stratified coastal waters, while northward flow on the inner shelf was consistent with downwelling favorable conditions along the entire coast. In 2015 the situation was different however with anomalously cold water flowing out of Great Oyster Bay resulting in thermally stratified coastal waters. While both stratified and downwelling conditions are known to favor dinoflagellates over diatoms, further research is in progress on how these processes control *Alexandrium* blooms off eastern Tasmania.

Alexandrium cyst surveys during August 2016 along the entire east coast of Tasmania found consistently low abundances of cysts (0.1-3 cysts per gram of sediment wet weight), but no localized dense cyst beds. Most sediments comprised coarse sands reflective of strong current regimes. Preliminary cyst culture experiments indicated a short dormancy period of 1-2 months, suggestive of rapid cycling between plankton and benthos. The widespread presence of cyst in coastal waters suggest that problems will persist.

4. At the time of the October 2012 PST event there was limited understanding of which seafood species presented a risk to human health from toxin accumulation, and therefore, which species should be tested. Aside from bivalve species (which are known to accumulate high levels of marine biotoxins), PSTs were tested in rock lobsters (*Jasus edwardsii*) giant crabs (*Pseudocarcinus gigas*), abalone (*Haliotis rubra*), sea urchins (*Heliocidaris tuberculata* and *Centrostephanus rodgersii*), calamari (*Sepioteuthis australis*), flathead (unspecified) and banded morwong (*Cheilodactylus spectabilis*). PSTs were found above the bivalve regulatory level in rock lobsters, in low levels in sea urchins and the viscera of giant crabs and abalone, but not found in squid or fish.

   An objective, informed discussion between key stakeholders in the recreational and commercial seafood industries, government officials and scientific experts on the human health risks associated with biotoxins in non-bivalve seafood concluded the toxin:species pairings of PSTs in Southern Rock Lobster (*Jasus edwarsdii*) and abalone (*Haliotis laevigata* and *Haliotis rubra)* to have a risk high enough to warrant inclusion in a management program.

Implications for relevant stakeholders

1. The Neogen PST screening test is recommended for adoption for regulatory purposes. The maximum PST level allowed in the Codex standard is based on the dihydrogen chloride base of saxitoxin, whereas the current Food Standards Code is based on saxitoxin only. This can lead to up to 20% difference in reporting depending on the toxin profile present, and

considerably complicated the interpretation of results between laboratories used in this study.

2. PSTs in Southern Rock Lobster (*Jasus edwarsdii*) and abalone (*Haliotis laevigata* and *H. rubra)* have a risk high enough to warrant inclusion in a management program. A stage 2 FRDC proposal has been prepared to address PST in these two seafood products.

3. To protect tourism and human health, the area has now been sign-posted with 75 permanent public PST warnings, a first for Australia.

4. An integrated on-line bloom prediction portal is being planned through IMOS (Integrated Marine Observing Systems) that continuously feeds in TSQAP shellfish toxicity results, temperature, rainfall and East Australian Current behavior to serve as a green, orange, red traffic light warning for potential impending biotoxin risks.

Recommendations

1. The Neogen PST screening test is recommended for adoption for regulatory purposes, and is already used to provide shellfish growers with a rapid tool for harvesting decisions at the farm gate. Effective rapid screening preventing compliant samples undergoing testing using the more expensive and time consuming LC-FLD method will result in significant savings in analytical costs (estimated $500,000 savings per annum Australia-wide).

   The rapid test kits developed here have the potential to be used to test for PSP contamination of other seafood species such as scallops and rock lobster, but will need to be optimized and validated for each species. While PSP toxins are the most widespread seafood toxin problem in Australia, this product but using different antibodies can readily be applied to other biotoxins.

2. The varying co-occurrence of *Alexandrium fundyense* and *pacificum* in east coast Tasmanian blooms confounds progress in elucidating bloom ecophysiology as well as predict shellfish toxicity. We recommend that every Tasmanian bloom event be subjected to species-specific qPCR detection, and SxtA4 qPCR detection to guarantee the correct interpretation of results. Molecular analyses suggest that *A. fundyense* may have been a cryptic genotype previously present in Tasmania, but newly stimulated by altered water column stratification conditions driven by changing rainfall and temperature patterns. This hypothesis is further being tested during a follow-up ARC Discovery grant funded project (DP170102261) to look at ancient DNA in dated 250-1000 year old sediment depth cores.

3. A submission by SafeFish and the Australian Shellfish Quality Assurance Advisory Committee (ASQAAC) to Food Standards Australia New Zealand is to be made to

amend the need for PST maximum limits to be listed as the di-hydrochloride salt (2HCl) in the Food Standards Australia New Zealand (FSANZ) Food Standards Code.

4. The determination of benchmark PST concentrations against which to judge the performance of the Neogen test kits was complicated by a disturbing lack of consistency in international Toxin Equivalency Factors (TEFs) currently in use by Analytical Laboratories. We recommended the consistent use in Australia of FAO TEFs in line with Codex recommendations.

Keywords

Mytilus galloprovincialis, Crassostrea gigas, Jasus edwarsdii **,** *Haliotis laevigata, Haliotis rubra***,** *Alexandrium tamarense.*

1. Introduction

In 1793 when Captain Vancouver landed on the coast of British Columbia, 5 of his crew became seriously ill after consuming wild mussels collected from what was later named Poison Cove. One crew member died 5 ½ hours later, with medical symptoms described in the Captain's log book as "tingling sensations or numbness around lips; spreading to face and neck, finger tips and toes; ultimately progressing to respiratory paralysis, choking and death". This food poisoning syndrome is now termed "*Paralytic Shellfish Poisoning*" (Fig.1.1). When Vancouver tried to communicate with local Indian tribes about what happened, they made it clear that it was taboo to eat shellfish when the water was bioluminescent. A few years later in 1799 in nearby Southern Alaska further fatalities occurred among workers in the Russian-American sea-otter trade. The causative organism, a microscopic plankton dinoflagellate now called *Alexandrium tamarense,* was not identified until 1936. To prevent further human poisonings, from 1937 onwards American shellfish became regularly tested for toxins by the United States Food and Drug Administration (USFDA) using a crude mouse bioassay. Furthermore the remote Alaskan coastline was permanently closed for shellfish harvesting. The causative compounds, termed saxitoxins, were not chemically characterized until 1975 and briefly used by the CIA in suicide capsules. Today saxitoxin is listed on the Chemical Weapons Convention, and Paralytic Shellfish Poisoning (PSP) is known to be a global seafood poisoning problem, contaminating mussels, oysters, scallops, clams, abalone, and rock lobster. Doses of 1 mg cause moderate symptoms in humans but doses of 10 mg can be lethal. Most seafood growing areas are therefore forced by regulatory authorities to test seafood products on a regular basis, using expensive ($500-$800 per sample) analytical methods such as High-Performance Liquid Chromatography carried out by specialized accredited laboratories.

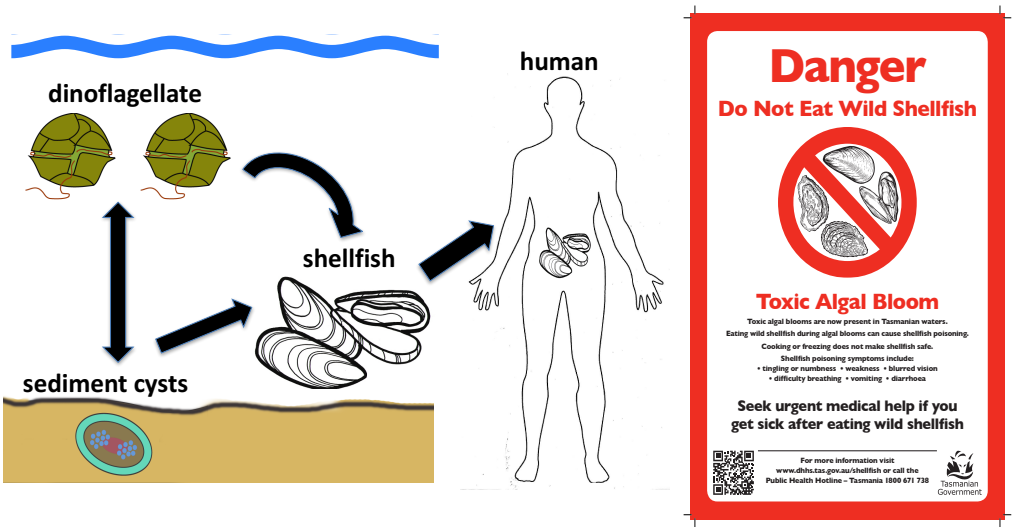

Fig. 1.1. Left: Transfer of dinoflagellate toxins via shellfish to humans. Right: Public warning signs first installed along Tasmania's east coast in 2017.

Shellfish containing more than 0.8 mg PST/kg* are deemed unsuitable for human consumption and prohibited from sale.

Currently Australian regulatory programs conduct over 3800 analyses per year for marine biotoxins, with 90% returning negative results. Slow laboratory turn-around times, combined with transport difficulties from regional areas, often mean that testing results are not available until 3-7 days after samples are taken. This poses a health risk for consumers, and business risk for the seafood industry.

In October 2012, Japanese import authorities detected Paralytic Shellfish Toxins (PSTs) in a consignment of Blue Mussels (*Mytilus galloprovincialis*) harvested from east coast Tasmania. The mussels had bioaccumulated toxins through feeding on a bloom of the dinoflagellate *Alexandrium tamarense* that spread to affect >200km of coastline. Product rejection by Japan resulted in a high-profile recall of product spanning several Australian states and international markets, and Japanese authorities imposed a 2 year 100% border testing regime on all bivalves imported from Australia. This non-compliance event has tarnished the "clean and green" image of Australian seafood and resulted in a $23M loss to the Tasmanian economy. The incident resulted in widespread closures for 3-6 months of commercial and recreational bivalve growing areas, rock lobster, scallop, and crab fisheries. Inability to distinguish toxic and non-toxic dinoflagellate species and strains in early 2013 led to unnecessary harvest closures ($40K loss) in absence of seafood meat toxicity. Following low PST toxicity during 2013 and 2014 and minimal shellfish farm closures, a massive bloom event occurred during July-Nov 2015 (up to 300,000 *Alexandrium* cells/L; 24 mg/kg PST in mussels, 6 mg/kg in *Crassostrea gigas* oysters), also causing four human hospitalisations after consumption of wild shellfish. More severe blooms in 2017 generated 150 mg/kg PST in mussels, 22 mg/kg in oysters, 11 mg/kg in lobster viscera and 1.3 mg/kg in abalone viscera.

These biotoxin events represent a paradigm shift for seafood risk management in Tasmania and Australia as a whole. The causative dinoflagellates are extremely difficult to identify by routine plankton monitoring, and are toxic at very low cell concentrations (50-100 cells/L). Sampling the extensive Tasmanian coast line poses a major logistical challenge. The precise pathway of toxin transfer to other seafood such as abalone or rock lobster is unclear. The presence of dinoflagellate sediment cyst beds in the affected region suggest that problems will persist.

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*The maximum PST level allowed in the Codex standard is based on the dihydrogen chloride base of saxitoxin, whereas the current Food Standards Code is based on saxitoxin only. This can lead to up to 20% difference in reporting depending on the toxin profile present, and considerably complicated the interpretation of results between laboratories used in this study. This discrepancy between Codex and FSANZ has since being resolved.

2. Objectives

1. **Develop, calibrate and adopt screening techniques for rapid detection and evaluation of toxins**

2. **Use state-of-the art molecular techniques to elucidate the genetic population structure and biology of toxic *Alexandrium tamarense*- group algae**

3. **Oceanographic modelling of time periods and zones at risk, and coordinated data capture to enable prediction of biotoxin event development**

4. **Perform a desktop seafood risk assessment as a prelude for a stage 2 proposal focusing on Paralytic Shellfish Toxin foodweb transfer**

3. Develop, assess and adopt screening techniques for rapid detection and evalution of PST toxins

3.1. Preface

The combined transport time and analytical turnaround from Tasmanian biotoxin meat analyses conducted at the National Biotoxin Lab at Advanced Analytical Australia Pty Ltd in Sydney is currently in the order of 7-10 days; but during the peak of the 2012 PST event, this sometimes expanded up to 3 weeks, with sample collection/ transport contributing to over 50% of the turnaround time. Such delays in advice to lease-holders are frustrating and can lead to losses of already harvested stocks when retrospectively found to be tainted with PST toxins. This has led to a global interest in the development of rapid on-site screening tests using mainly immunological platforms previous proven in food diagnostics. A cost effective screening method was therefore required that could be used locally to rapidly sort out harvests with no public health or business risks. Harvests producing negative screen results could be sent direct to market whilst harvests producing positive screen results could then be held on-site, whilst samples are sent for complete chemical analysis.

In the early 1980s Dr Joanna Jellett in Halifax first came up with the idea to develop rapid testing technologies for marine biotoxins (Fig. 3.1.1). A kit based on mouse neuroblastoma cells failed because shipping living cells was too challenging and expensive. A second attempt used a lateral flow immunochromatography platform, essentially similar to that of the home pregnancy kit. A critical component of such kits is the cross-reactivity and sensitivity of the antibodies for PSP toxins. When the first commercial tests came on to the market, they had been fine-tuned for shellfish toxin profiles encountered in North America and Canada. Sadly, it was not well recognized at that time that different geographic strains of even the same dinoflagellate can produce widely different saxitoxin analogues. Our early trials with the Jellett kit in Australia in 2000 were most disappointing and this product received little traction outside the US. The Jellett company faltered and later was renamed Scotia. Triggered by devastating PSP problems facing the Tasmanian shellfish industry in 2012, suffering closures up to 4 months, and an encounter with Dr Katrina Campbell from Queens University of Belfast who just produced much more sensitive antibodies against a wide range of saxitoxin analogues, we decided to revisit the applicability of immunological PST test kits that had almost been given up on. The solution to why this technology did not work previously was to use antibodies with better cross-reactivities, fine-tune mixtures of antibodies to match the toxin analogues to be detected, and to convert (hydrolysis step) difficult to detect to more easy to detect toxin analogues.

Fig. 3.1.1. Juan Dorantes-Aranda (right) and Gustaaf Hallegraeff (left) demonstrating the PST test strip.

We first chemically characterized the full PSP toxin profile of Tasmanian shellfish (Fig. 3.1.2 left). In 2012 GTX2&3 was the major PST analogue (51-100%), followed by STX (14-18%), C1&2 (10-24%) and dcGTX2&3 (5-16%). In contrast, in 2015 GTX1&4 was the major analogue (26-88%), followed by GTX2&3 (8-76%), C1&2 (5-24%) and STX (0-2%).

Subsequently, we assessed the applicability of various commercial test kits that used antibodies with different cross-reactivities. Not surprisingly, Scotia™ (=Jellet), Abraxis™ and Europroxima™ kits that were most sensitive to saxitoxin but failed to pick up neosaxitoxin or gonyautoxins, were not suitable for Tasmanian shellfish. Neogen™ test kits based on Katrina Campbell's antibodies sensitive to neosaxitoxin worked much better, while limited cross-reactivity against gonyautoxins was resolved by us by adding an enzymatic conversion step converting GTX 2,3 to STX, and GTX1,4 to NEO, which both are well detected (arrows below).

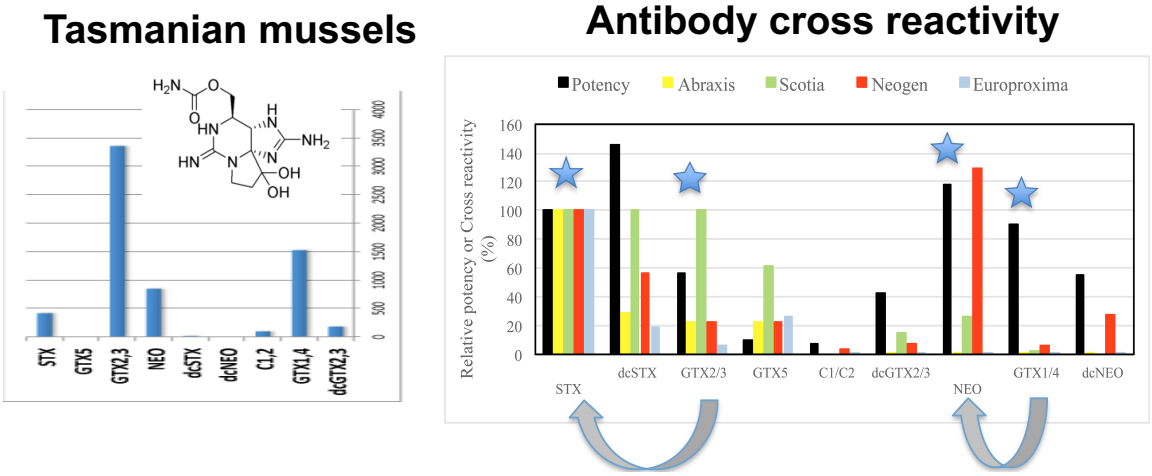

Fig. 3.1.2 Left: PSP analogues in Tasmanian shellfish; Right: Differing abilities by different antibodies (different commercial kits) to detect different analogues. Arrows indicate how enzymatic conversion can be used to turn difficult to detect in more easy to detect analogues. Asterisks mark the key compounds in Australian shellfish and black bars the potency.

Finally, a formal single-lab and international validation process of the Neogen PST kit was funded separately by a separate Package Assisting Small Exporters (PASE) grant awarded to SARDI (Ali Turnbull), IMAS (Gustaaf Hallegraeff) and Katrina Campbell (QUB).

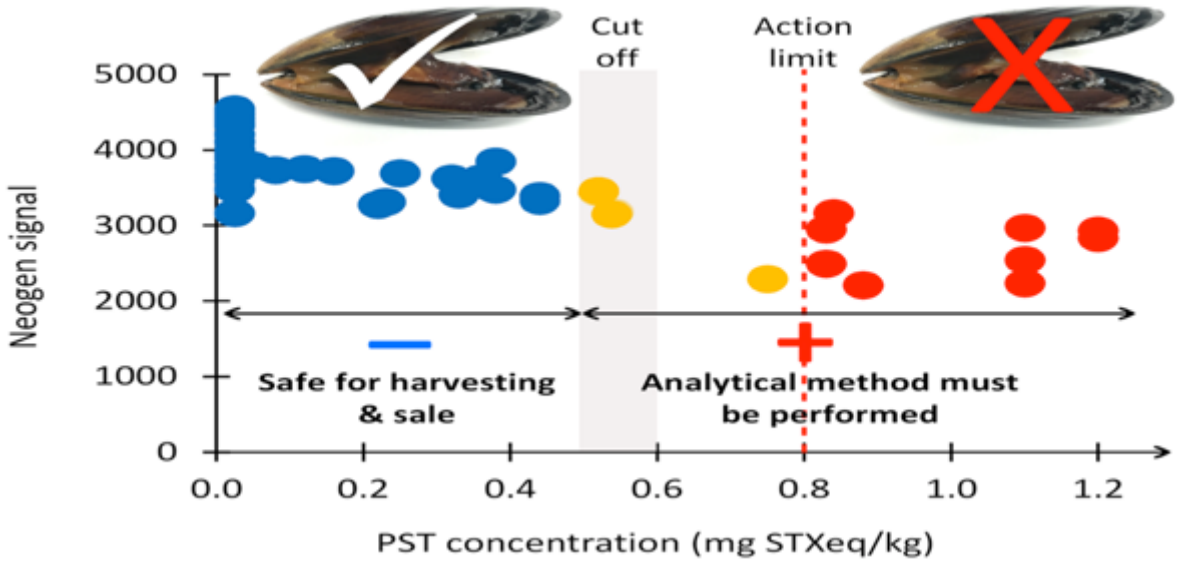

Fig 3.1.3. Proposed use of the Neogen PST screen test once formally implemented into the Tasmanian Shellfish Quality Assurance Program. The sensitivity of the test has been adjusted to a cut off at 0.4-0.5 mg PST/kg, just short of the 0.8 mg/kg action limit at which shellfish farm closures need to be instigated. Negative samples require no further testing, while positive screen test results need to be confirmed by the HPLC method for shellfish closure decisions.

3.2. Comparative performance of four immunological test kits for the detection of Paralytic Shellfish Toxins in Tasmanian shellfish

Juan José Dorantes-Aranda, Katrina Campbell, Andrew Bradbury, Christopher T. Elliott, D. Tim Harwood, Shauna A. Murray, Sarah C. Ugalde, Katrina Wilson, Megan Burgoyne, Gustaaf M. Hallegraeff

Abstract. Blooms of the toxic dinoflagellate *Alexandrium tamarense* (Group 1) seriously impacted the Tasmanian shellfish industry during 2012 and 2015, necessitating product recalls and intensive paralytic shellfish toxin (PST) product testing. The performance of four commercial PST test kits, Abraxis™, Europroxima™, Scotia™ and Neogen™, was compared with the official AOAC LC-FLD method for contaminated mussels and oysters. Abraxis and Europroxima kits underestimated PST in 35-100% of samples when using standard protocols but quantification improved when concentrated extracts were further diluted (underestimation ≤18%). The Scotia kit (cut off 0.2-0.7 mg STX-diHCl eq/kg) delivered 0% false negatives, but 27% false positives. Neogen produced 5% false negatives and 13% false positives when the cut off was altered to 0.5-0.6 mg STX-diHCl eq/kg, the introduction of a conversion step eliminated false negatives. Based on their sensitivity, ease of use and performance, the Neogen kit proved the most suitable kit for use with Tasmanian mussels and oysters. Once formally validated for regulatory purposes, the Neogen kit could provide shellfish growers with a rapid tool for harvesting decisions at the farm gate. Effective rapid screening preventing compliant samples undergoing testing using the more expensive and time consuming LC-FLD method will result in significant savings in analytical costs.

3.2.1 Introduction

In recent years recurrent blooms (up to 300,000 cells/L) by the Paralytic Shellfish Toxin (PST) producing dinoflagellate *Alexandrium tamarense* (Group 1) have seriously impacted the Tasmanian shellfish industry. An initially undetected bloom event in October 2012 led to product recalls with an estimated economic loss of ~US$24 million dollars (Campbell et al., 2013). The regulatory action limit or permissible concentration of PST toxins in shellfish is 0.8 mg STX-diHCl eq/kg shellfish meat (0.8 mg STX eq/kg from now on). During 2015 closures of oyster and mussel farms which lasted for up to 4 months, PST levels were instigated and reached up to 32 mg STX eq/kg, resulting in four documented hospitalizations that occurred after individuals consumed wild mussels (i.e. recreational harvesting) from an affected area with public health warning signs. The current system for shellfish testing by the Tasmanian Shellfish Quality Assurance Program (TSQAP) requires shipping samples to an accredited Sydney laboratory leading to delays (4 to 12 days) for shellfish growers.

The full text of this chapter was published in *Toxicon 125, 110-119 (2017),* and reproduced here with permission.

The AOAC Official Method AOAC.2005.06 (pre-column oxidation or Pre-COX) using liquid chromatography with fluorescence detection (LC-FLD or Lawrence method; Lawrence et al., 2005) is the designated regulatory method for PST in shellfish in Australia. The method is highly specific and sensitive, providing a complete toxin profile and concentration of each PST analogue. However, it has been claimed that the method overestimates gonyautoxin 1&4 (GTX1&4) and neosaxitoxin (NEO), and underestimates gonyautoxin 2&3 (GTX2&3) and sulfocarbamoyl C1&2 compared to AOAC Official method 2011.02 (post-column oxidation or PCOX) (Turner et al., 2014a).

Immunological PST test kits, which were first trialled in the early 2000s (Jellett et al., 2002; Laycock et al., 2000), have the advantage of being sensitive, fast, easy to use and cheaper than HPLC-based analytical methods, and ideally allow farmers to perform tests on site to guide harvesting decisions. However, due to significant variability in PST toxin profiles of different *Alexandrium* species and geographic populations, as well as widely different potency of PST analogues, the applicability of different commercial test kits for local product testing requires careful consideration. Most available kits target saxitoxin (STX), but have low cross-reactivity for GTX1&4 and GTX2&3. The latter are common in Australian shellfish products as well as shellfish in Great Britain (Turner et al., 2014b).

Enzyme-linked immunosorbent assays (ELISA), such as the Abraxis™ and Europroxima™ kits, are quantitative tests that allow the user to calculate the concentration of PST toxins (as mg STX eq/kg) using a STX standard curve. These protocols require laboratory experience to avoid high user errors. By contrast, lateral flow immunoassays (LFIA) are qualitative tests that deliver positive or negative results based on a predetermined cut off limit. Scotia Rapid Test™ (formerly Jellett Rapid Test; Jellett et al., 2002) has a detection limit of ~0.2-0.7 mg STX eq/kg, whereas Neogen™ states that it has a cut off of 0.8 mg STX eq/kg. LFIA kits are more user friendly and simpler to use than ELISA kits, while laboratory experience is not essential. Different commercial immunological tests exhibit highly variable cross-reactivity to different PST analogues (Table 3.2.1). These cross-reactivity profiles do not fully correlate with the toxicity of individual toxins as determined by the mouse bioassay and the toxicity equivalency factors applied in total toxin determination of the LC-FLD method. Therefore, commercial test kits must be shown to be fit for purpose with geographical toxin profiles prior to implementation within testing regimes.

In the present study the performance of four commercially available immunological PST test kits for shellfish testing were evaluated during a major *Alexandrium tamarense* bloom event on Tasmania's East Coast, Australia, between July and November 2015.

Table 3.2.1. Cross-reactivity (mole %) of four immunological test kits as specified by the manufacturers.

	Quantitative		Qualitative	
PST analogue	Abraxis	Europroxima	Neogen[a]	Scotia[b]
STX	100	100	100	100
NEO	1.3	1.4	129	26
GTX2&3	23	5.6	23	100
GTX1&4	<0.2	<0.1	6	1.8[c]
C1&2	nd	0.2	3	nd
GTX5	23	26.2	23	62
dcSTX	29	19.2	56	100
dcNEO	0.6	0.5	28	nd
dcGTX2&3	1.4	0.2	8	15

[a] Jawaid et al. (2015)
[b] Formerly Mist Alert and Jellett (Jellett et al., 2002; Laycock et al., 2000)
[c] If an extra step involving hydrolysis conversion of GTX1&4 to NEO is performed, this cross-reactivity can be increased to 26%
nd = not determined

3.2.2. Materials and Methods

Shellfish samples

Sixty nine shellfish samples, including Blue Mussel *Mytilus galloprovincialis* and Pacific Oyster *Crassostrea gigas*, which originated from 12 farms along the East Coast of Tasmania, Australia were used. Samples (homogenates from whole organisms) were stored at -20°C and analysed within 1 month after harvesting.

Liquid chromatography analysis

Advanced Analytical Australia (AAA), the certified laboratory that TSQAP uses for phycotoxin analysis, determined PST toxin concentration using the AOAC.2005.06, LC-FLD or Lawrence method. Screen and confirmation (when >0.4 mg STX eq/kg were found) analyses of the method were performed.

PST toxins were extracted from 5 g of shellfish homogenate using 3 mL of 1% acetic acid. The mixture was placed in a boiling water bath for 20 min, mixed, allowed to cool and centrifuged at 3600 ×*g* for 10 min. The supernatant was recovered and the pellet resuspended in 3 mL 1% acetic acid, mixed and centrifuged again. Both supernatants were mixed and made up to 10 mL with water. A sample clean-up was performed using a SPE C18 cartridge and screen testing was performed after periodate oxidation of samples. Standards, samples and PST positive certified reference matrices were oxidised with the inclusion of a matrix modifier. The matrix chosen for the matrix modifier reflected the predominant shellfish in the run. Oxidation using the matrix modifier circumvents the need to apply recovery factors for differing shellfish matrices. A further confirmation analysis was performed after peroxide oxidation of C18 cleaned extracts. All results were converted to mg STX-diHCl eq/kg using

EFSA's toxicity equivalency factors (EFSA, 2009) (mentioned as mg STX eq/kg). Subsamples analysed by AAA were returned to IMAS for use in the PST screening with the rapid test kits.

Quantitative Tests

Abraxis™

Abraxis test kits (52255B, lot number 15B5951) were stored at 4°C until analysis. PST toxin quantification was performed according to the manufacturer's protocol.

Extract preparation

A subsample of 10 g of shellfish homogenate was mixed with 10 mL of 0.1 M HCl (modified version of the AOAC.959.08 method, extraction protocol as per the mouse bioassay) and placed in a boiling water bath for 5 min, allowed to cool down and centrifuged at 3500 ×*g* for 10 min. Supernatants were recovered and pH adjusted to 3.0, and diluted in 1x sample diluent (1:1000). Initially, all 69 samples were considered as blind samples and analysed as per the standard test protocol (i.e. 1:1000 dilution). For a second analysis, 15 of these samples were further diluted (i.e. 1:10 or 1:100) based on the known toxin concentration (LC-FLD by AAA) in order to bring them within the working range of the calibration curve.

The Abraxis kit can operate with an alternative extraction method using 80% methanol (MeOH). For this purpose the 15 samples mentioned above (i.e. with extra dilution performed) were tested. Shellfish homogenate (1 g) was mixed with 6 mL of MeOH for 1 min using a Vortex mixer, centrifuged at 3000 ×*g* for 10 min and the supernatant transferred into a clean tube. MeOH (2 mL) was added to the pellet, mixed and centrifuged. Both supernatants were combined and made up to 10 mL with MeOH. Similar to the HCl extracts, the MeOH extracts were analysed using the standard test protocol dilution (1:100) and also with an extra dilution (i.e. 1:10 or 1:100) as required.

Test protocol

A volume of 50 µL of STX standards (provided at 0, 0.02, 0.05, 0.10, 0.20 and 0.40 ng mL^{-1}) and samples (in HCl or MeOH) was transferred into the 96-well coated plate in duplicate, followed by 50 µL of enzyme conjugate and 50 µL of antibody. The microplate was mixed and incubated for 30 min at room temperature. Solutions were decanted and wells rinsed four times with 1x washing buffer solution. Substrate solution was added to all wells (100 µL), mixed and incubated for 30 min in the dark. Stop solution was added to the wells (100 µL) and mixed, with the absorbance read immediately at 450 nm using a microplate reader (FLUOstar OPTIMA, BMG Labtech 413-3350). For data analysis, $\%B/B_0$ values (i.e. average absorbance of STX standards divided by average absorbance of blank standard) were obtained and toxin quantification in samples determined by interpolating response values in the standard curve.

Europroxima™

Europroxima test kits (5191SAXI, lot number QN5327) were stored at 4°C until analysis. Manufacturer's protocol was followed to perform the test. Similar to the Abraxis test kits, all samples were considered as blind samples, with an extra set of 15 samples further diluted (i.e. 1:10, 1:100 or 1:500) based on the known toxin concentration.

Extract preparation

In brief, 1 g of shellfish homogenate was mixed with 5 mL of 0.2 M sodium acetate buffer (freshly prepared), centrifuged at 3000 ×*g* for 10 min and the supernatant recovered for experiments. The extract was diluted in dilution buffer (1:50).

Test protocol

STX standards (0.0125, 0.025, 0.05, 0.1, 0.2 and 0.3 ng mL^{-1}) and samples (50 µL) were transferred into the 96-well plate in duplicate, followed by 25 µL of conjugate and 25 µL of antibody. The plate was shaken for 1 min and incubated for 30 min at room temperature. The solutions were discarded and all wells rinsed three times with rinsing buffer. Substrate was added to all wells (100 µL), the plate was shaken and incubated for 15 min at room temperature in the dark. Stop solution was added (100 µL) and absorbance was read immediately at 450 nm. % maximal OD (optical density) was calculated for all standards and samples using the absorbance readings from the standards with no STX (standard zero, provided by supplier). The concentration of PST in samples was calculated using the calibration curve.

Qualitative Tests

Scotia™

Scotia test kits (PSP Rapid Test, lot number 40000) were stored at 5°C until analysis. These kits use the modified AOAC.959.08 extraction method boiling the shellfish sample in 0.1 M HCl (or mini-AOAC, Jellett et al., 2002), and thus the same extracts prepared for the Abraxis tests were used for the Scotia kits. Manufacturer's protocol was followed for the test. A volume of 400 µL of PSP Scotia rapid buffer was mixed with 100 µL of the shellfish extract, 100 µL of this mix was placed into the test kit sample slot and allowed to develop for 35 min. The test kits were scanned using the Scotia Skannex system, which scans the strip and analyses the bands to give a positive or negative result with a numeric value based on the intensity comparison of both the C (control) and T (test) bands. Values ≥0.5 indicate negative samples, or positive if <0.5 (Turner et al., 2015).

Neogen™

Neogen test kits (NEO9562, lot number 9562-11) were stored at room temperature until analysis. The certificate analysis report accompanying the kits showed that STX-diHCl standards at two concentrations equivalent to 0.16 mg/kg and 0.80 mg/kg returned negative and positive results, respectively, with no further information in performance within that

concentration range. Neogen's standard protocol uses 1 g (±0.05 g) of shellfish homogenate mixed with 30 mL (±0.5 mL) of distilled water in a plastic extraction bag with an inner mesh filter (280 μM pore size, provided with kits) to perform the toxin extraction manually using the metal roller. The filtered liquid (extract) is recovered and 100 μL of this is transferred into a container with buffer saline solution (as provided) and mixed for 30 s. A volume of 100 μL of this mix is then transferred into a microwell and a LFIA test strip inserted and allowed to develop for exactly 5 min, after which it is placed into the AccuScan Pro Reader to obtain the positive or negative result based on the intensity of the test band that appears in the strip (see Jawaid et al., 2015).

Standard curves with the Neogen test kit

The analysis of standard curves of different PST analogues was performed to test the cut off levels for each analogue using the same dilutions as the Neogen standard protocol. Standards of STX, NEO, GTX2&3, GTX1&4 and C1&2 were purchased from the National Research Council Canada (NRC). Saxitoxin standard was tested at concentrations equivalent to 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 mg/kg following the standard Neogen protocol (i.e. 1:31 sample dilution). STX, NEO, GTX2&3, GTX1&4 and C1&2 standards were tested again but now using the adjusted dilution at concentrations of 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 1.2 mg STX eq/kg. Additionally, two mixtures of these standards recreating what has been observed in natural contaminated samples were tested. Mix A: 1, 60, 30 and 9 % of STX, GTX1&4, GTX2&3 and C1&2, respectively, and mix B: 0.5, 90, 4.5 and 5.0 %, respectively. A concentration range of 0.2, 0.4, 0.6, 0.8 and 1.2 mg STX eq/kg were tested. For toxins that were combined, only the epimer with greatest toxicity was used for calculative purposes, using the EFSA toxicity equivalency factors (EFSA, 2009). In addition, because of the low reactivity (6%) of Neogen test towards GTX1&4, two extra batches of GTX1&4 were treated with L-cysteine (Sigma W326305), to explore the possibility of PST analogue conversion (Asakawa et al., 1987) and thus increase the reactivity of the Neogen kit. The second batch of GTX1&4 (at 0.2-1.2 mg STX eq/kg) was incubated with cysteine (2 M) in a water bath at 70°C for 30 min, and the third batch was treated under the same conditions but for 60 min.

Test of shellfish samples

The first dilution step was modified since we observed that STX standard at 0.4 mg/kg returned a positive result when mixing 1 g of shellfish sample (or equivalent standard) with 30 mL of type 1 water, the new dilution was using 45.5 mL of type 1, 18.2 MΩ·cm, water. Sixty nine shellfish samples were tested using 1 g (±0.05 g) of homogenate and extracted with 45.5 mL of type 1 water; the rest of the protocol was as mentioned previously. The Neogen standard protocol (i.e. dilution of 1:31 during extraction) was used only for 11 shellfish samples that included false negatives; a cysteine treatment was also applied to these 11 samples but that were extracted at a dilution of 1:46.5 (extract incubated with 2 M cysteine at 70°C for 30 min). Once all the tests were completed and positive/negative results registered, the data recorded by the Neogen AccuScan Pro Reader was exported to Excel (Microsoft Office™), which includes peak and area values for both the control and test bands, plus the positive or negative result corresponding to the test band.

3.2.3 Results

Using the LC-FLD analytical method, 23 of the 69 samples had PST levels same as or above the action limit of 0.8 mg STX eq/kg (non-compliant), 33 samples had levels ranging between 0.10 and 0.77 mg STX eq/kg, and 13 samples levels of <0.10 mg STX eq/kg (compliant) (Table S1, Supplementary material). Most contaminated samples contained high proportions of the PST analogues GTX1&4 (25-88%) and GTX2&3 (8-70%), followed by C1&2 (4-21%) and STX (0-3%) (Fig. 3.2.1). Due to the varying nature of the shellfish matrices, toxin profiles and concentrations, multiple SPE steps, and pH variances in the oxidation steps, the standard error is fairly high at lower concentrations.

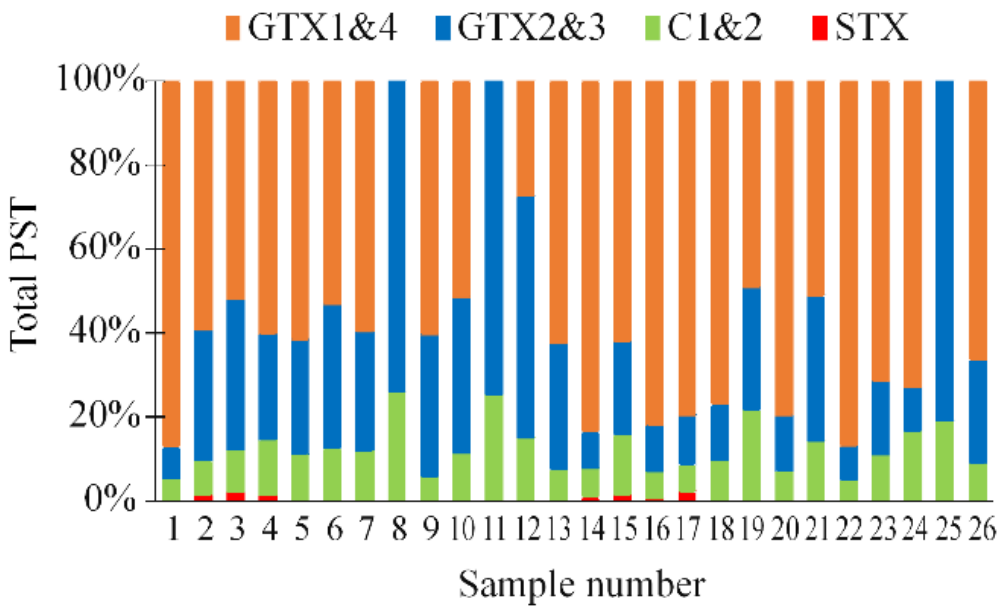

Fig. 3.2.1. Proportion of PST analogues in contaminated shellfish during an *Alexandrium tamarense* (group 1) bloom in Tasmania, Australia, as per Table S1 from Supplementary material. Only samples with ≥0.80 mg STX eq/kg of LC-FLD confirmation analysis are shown.

Quantitative ELISA kits

Abraxis

The Abraxis kit showed poor performance on the 69 shellfish samples when the standard protocol was followed; 8 of the 23 non-compliant samples (34.8%) were underestimated, delivering values below the regulatory action limit (i.e. between 0.24 and 0.77 mg STX eq/kg) in samples containing 0.80-3.29 mg STX eq/kg (as per LC-FLD). Correlation between Abraxis and LC-FLD was poor (r^2=0.33 for linear adjustment) (Fig. 3.2.2A). Performance of the Abraxis kit increased when shellfish extracts were diluted (up to 1:100000) based on the LC-FLD toxin concentrations. Such tests were performed on 15 samples, of which 11 had PST levels >0.8 mg STX eq/kg. Shellfish samples extracted with 80% MeOH showed very similar results as the HCl extracts. Abraxis quantification of HCl and MeOH extracts with extra dilutions improved considerably showing higher correlations with the LC-FLD method,

increasing from r^2=0.38-0.39 to r^2=0.82-0.91 (Fig. 3.2.2B). Despite the improvement in the performance with the extra dilution step (performed in 15 samples, of which 11 were non-compliant), 2 samples (18%) with 0.92-0.97 mg STX eq/kg (LC-FLD) were underestimated by Abraxis, generating values below the action limit (Table 3.2.2).

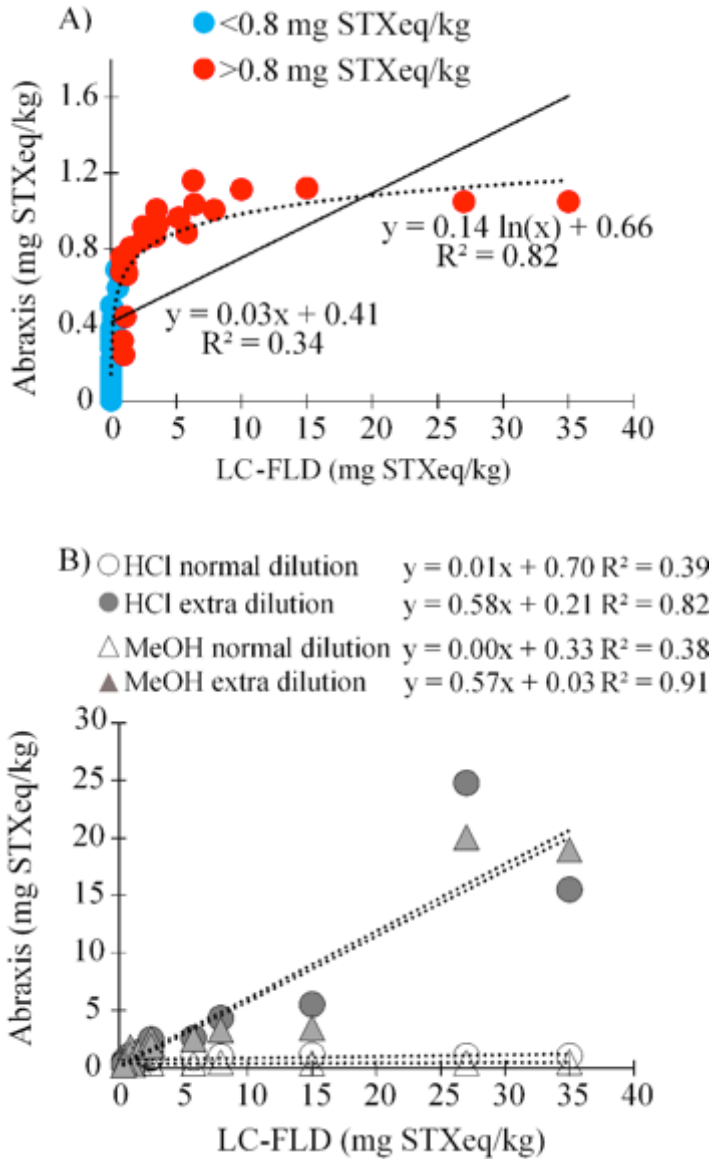

Fig. 3.2.2. Quantification of PST in Tasmanian shellfish samples using the Abraxis™ ELISA kit as compared with the AOAC.2005.06 (LC-FLD) official method. Values are average from duplicate samples (see Table 2). A) All 69 samples with equations from linear and logarithmic regressions. Linear regression is the desired adjustment; however the logarithmic adjustment showed a better correlation, showing the limitation of the Abraxis test for an accurate quantification of concentrated PST extracts. B) Extra analysis of 15 samples using two extraction techniques (HCl and MeOH) with an extra sample dilution step.

Europroxima

The Europroxima kit showed poor performance on the 69 shellfish extracts. All 23 samples with ≥0.8 mg STX eq/kg were underestimated, delivering values of 0.10-0.19 mg STX eq/kg

when manufacturer's protocol was followed (using sample dilution of 1:50 prior). Correlation between Europroxima and LC-FLD quantification was poor (r^2=0.13, linear adjustment) (Fig. 3.2.3A). Quantification was improved by performing an extra dilution to the samples based on their known PST concentrations. Due to the high PST levels in some samples, extra dilutions of up to 1:500 were required (final dilution of 1:150000). The correlation between Europroxima and LC-FLD improved from r^2=0.0004 to 0.91 in the 15 samples that were diluted, which included 11 non-compliant (Fig. 3.2.3B). However, one sample with 0.92 STX eq/kg (LC-FLD) was still underestimated by Europroxima, as having 0.44 mg STX eq/kg (Table 3.2.2).

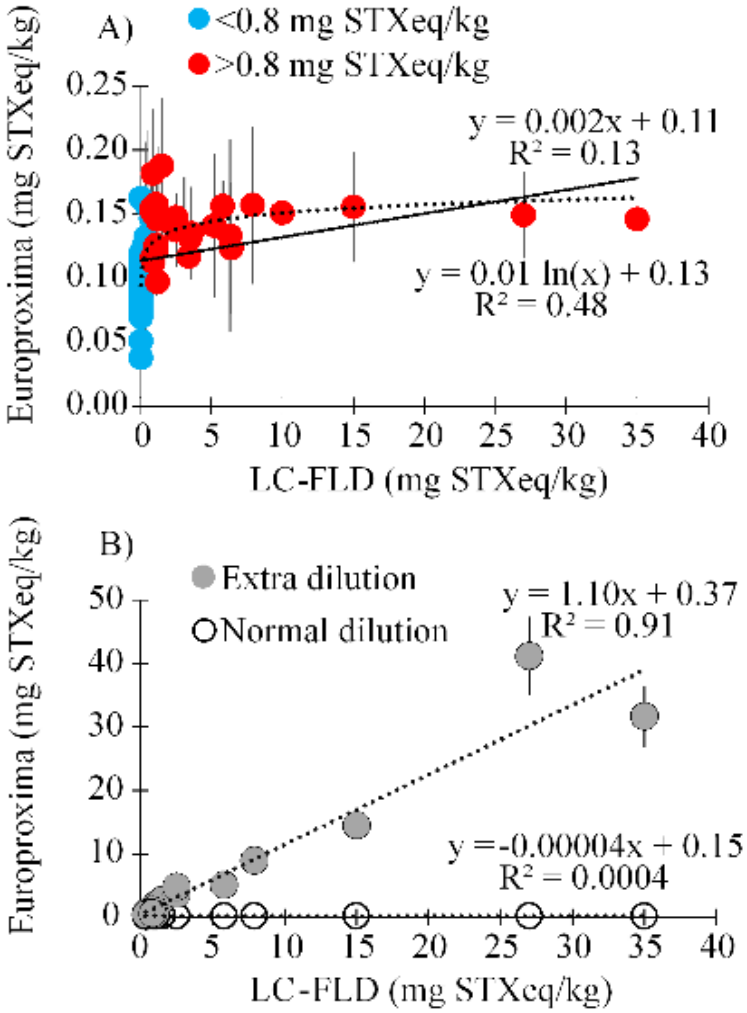

Fig. 3.2.3. Quantification of PST shellfish samples using the Europroxima™ ELISA kit compared to the AOAC.2005.06 (LC-FLD) official method. Values correspond to average of duplicate samples, and bars represent their standard deviations (see Table 2). A) 69 samples with equations from linear and logarithmic regressions are shown for each protocol. Similarly to Abraxis, Europroxima showed limitations in quantification of concentrated samples. B) Extra analysis in 15 samples following the standard protocol and an extra sample dilution.

Qualitative LFIA kits
Scotia

The Scotia kit showed good performance on high PST shellfish. However, considering that the sensitivity is 0.2-0.7 mg STX eq/kg (Scotia Rapid Testing, pers. comm.), the test kits delivered 16 false positives (27%). Among these, 9 contained 0.10-0.19 mg STX eq/kg and the other 7 were reported as <0.10 mg STX eq/kg by the LC-FLD method (Fig. 3.2.4). No false negatives were detected using the Scotia test but only 9 of the 18 shellfish extracts with ≤0.2 mg STX eq/kg were negative (Table 2). The PST concentration in shellfish extracts and numerical values recorded by the Scotia scanner were not well correlated (r^2=0.26-0.45) (Fig. 3.2.4).

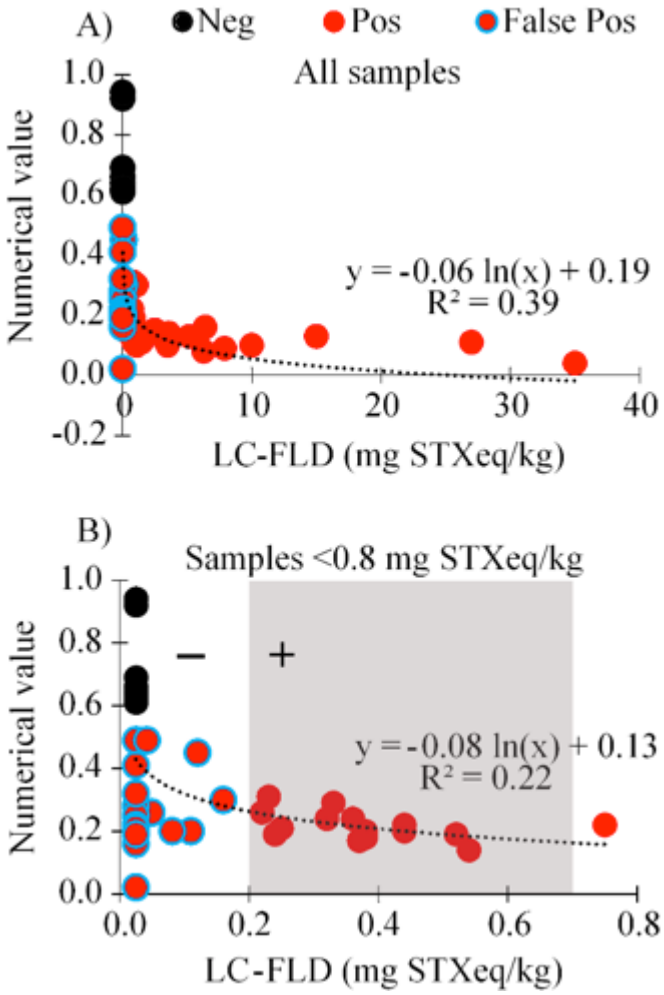

Fig. 3.2.4. PST toxin tests using the Scotia kit in 69 shellfish samples (A) with PST concentration as per the LC-FLD method (mg STX eq/kg) on the x-axis, showing those with <0.8 mg STX eq/kg in (B), where the sensitivity of the Scotia test is shaded (0.2-0.7 mg STX eq/kg) and positive results were expected. The y-axis indicates the numerical value generated by the Scotia Skannex system based on the intensity comparison of the control and test bands of the test. Values ≥0.5 indicate negative samples (●), or positive if <0.5 (●). False positives (●) are those samples with ≤0.20 mg STX eq/kg (LC-FLD analysis) but generated a positive result. See Table S2 (Supplementary material) for complete list of numerical values.

Neogen

Performance on contaminated shellfish samples

Control bands showed an average peak and area of 8192 (±555) and 11755 (±696), respectively. Both values for peak and area for the test bands of the 69 samples were highly correlated. Although peak and area values generally depended on toxin concentration, their quantitative values may not always be indicative due to varying cross-reactivity for different PST analogues.

Neogen performed well on most of the 69 shellfish samples. Considering that the dilution in the extraction step was modified from 1:31 (cut off observed to be 0.4 mg STX eq/kg) to 1:46.5 to set the cut off to 0.5-0.6 mg STX eq/kg, Neogen delivered false positives in only 4 samples (13%), three of which had 0.10 to <0.35 mg STX eq/kg and the other contained 0.03 mg STX eq/kg (as per the LC-FLD method). Two false negatives (5%) were observed in samples with 0.82 and 0.92 mg STX eq/kg (Table 3.2.2). These samples were reanalysed following the Neogen standard protocol (dilution 1:31): the sample with 0.92 mg STX eq/kg turned positive, but the sample with 0.82 mg STX eq/kg was still negative (false negative). To overcome this, cysteine treatment (2 M at 70°C for 30 min) was applied to the two false negative samples (1:46.5 dilution), together with 9 other samples of varying toxin concentrations. Cysteine treatment effectively eliminated the false negatives, although one other false positive was generated (at 0.20 mg STX eq/kg) (Table 3.2.3).

Table 3.2.2. Summary of the performance of the four PST kits on 69 shellfish samples. The confirmation results determined by LC-FLD are included. PST quantification on 15 samples was further investigated with the Abraxis kit by using the two recommended extraction methods (HCl and MeOH), following the standard protocol and by performing an extra dilution step. This was also done for Europroxima. Results shaded in gray indicate false positives (Neogen and Scotia) and in black false negatives (Neogen) based on the sensitivity of each kit (0.2-0.7 mg STX eq/kg for the Scotia test, and altered to 0.5-0.6 mg STX eq/kg for Neogen). NT = not tested.

Sample	LC-FLD (mg STX eq/kg)	Neogen	Scotia	Abraxis HCl				Abraxis MeOH				Europroxima			
				Normal		Extra		Normal		Extra		Normal		Extra	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	9.20	+	+	1.11	0.02	NT		NT		NT		0.15	0.12	NT	
2	5.85	+	+	1.04	0.01	NT		NT		NT		0.12	0.05	NT	
3	4.83	+	+	0.96	0.01	NT		NT		NT		0.14	0.16	NT	
4	5.65	+	+	1.16	0.02	NT		NT		NT		0.13	0.08	NT	
5	3.20	+	+	1.01	0.06	NT		NT		NT		0.13	0.02	NT	
6	3.02	+	+	0.87	0.00	NT		NT		NT		0.12	0.01	NT	
7	3.29	+	+	0.93	0.03	NT		NT		NT		0.13	0.04	NT	
8	1.10	+	+	0.68	0.00	NT		NT		NT		0.10	0.00	NT	
9	0.96	+	+	0.77	0.05	NT		NT		NT		0.13	0.01	NT	
10	0.98	+	+	0.44	0.02	NT		NT		NT		0.12	0.04	NT	
11	1.10	+	+	0.66	0.02	NT		NT		NT		0.15	0.06	NT	
12	0.82	-	+	0.32	0.01	NT		NT		NT		0.11	0.01	NT	
13	31.75	+	+	1.05	0.01	15.51	0.24	0.48	0.02	18.94	3.17	0.15	0.00	31.70	4.81
14	24.44	+	+	1.05	0.01	24.78	0.02	0.41	0.02	20.04	1.58	0.15	0.03	41.22	11.16
15	13.38	+	+	1.12	0.01	5.51	0.15	0.38	0.01	3.48	0.04	0.16	0.04	14.49	0.17
16	7.21	+	+	1.01	0.01	4.32	0.02	0.48	0.00	3.30	0.02	0.16	0.06	8.95	0.75
17	5.23	+	+	0.89	0.00	2.59	0.02	0.37	0.00	2.51	0.00	0.16	0.10	5.07	0.47
18	2.26	+	+	0.85	0.02	2.47	0.04	0.39	0.02	1.90	0.03	0.14	0.03	4.78	0.07
19	0.80	+	+	0.74	0.01	1.09	0.01	0.38	0.01	1.79	0.34	0.18	0.10	1.29	0.41
20	0.97	+	+	0.73	0.02	0.75	0.00	0.31	0.00	0.70	0.01	0.16	0.06	1.93	0.08

21	1.43	+	+	0.81	0.03	1.34	0.03	0.38	0.01	1.23	0.01	0.19	0.05	2.63	0.07
22	2.24	+	+	0.92	0.04	1.94	0.01	0.42	0.01	2.50	0.44	0.15	0.03	3.61	0.37
23	0.92	-	+	0.24	0.00	0.34	0.00	0.31	0.03	0.27	0.00	0.12	0.02	0.44	0.05
24	0.75	+	+	0.69	0.01	0.52	0.00	0.30	0.00	0.61	0.04	0.15	0.08	0.57	0.05
25	0.77	+	+	0.76	0.02	1.08	0.01	0.28	0.00	1.13	0.02	0.15	0.06	1.07	0.35
26	0.42	-	+	0.69	0.01	0.51	0.02	0.28	0.01	0.49	0.01	0.16	0.06	0.65	0.01
27	0.33	-	+	0.43	0.01	0.29	0.00	0.22	0.00	0.23	0.00	0.10	0.03	0.41	0.01
28	0.70	+	+	0.65	0.02	NT		NT		NT		0.15	0.03	NT	
29	0.49	+	+	0.60	0.01	NT		NT		NT		0.12	0.02	NT	
30	0.77	+	+	0.68	0.01	NT		NT		NT		0.12	0.04	NT	
31	0.48	-	+	0.44	0.01	NT		NT		NT		0.11	0.01	NT	
32	0.41	-	+	0.30	0.00	NT		NT		NT		0.10	0.00	NT	
33	0.22	+	+	0.46	0.00	NT		NT		NT		0.12	0.01	NT	
34	0.29	-	+	0.40	0.01	NT		NT		NT		0.12	0.05	NT	
35	0.34	-	+	0.40	0.01	NT		NT		NT		0.13	0.07	NT	
36	0.31	-	+	0.31	0.00	NT		NT		NT		0.12	0.06	NT	
37	0.21	-	+	0.47	0.01	NT		NT		NT		0.12	0.04	NT	
38	0.20	-	+	0.36	0.01	NT		NT		NT		0.11	0.01	NT	
39	0.34	-	+	0.34	0.00	NT		NT		NT		0.12	0.02	NT	
40	0.36	-	+	0.34	0.00	NT		NT		NT		0.12	0.05	NT	
41	0.23	-	+	0.22	0.00	NT		NT		NT		0.10	0.01	NT	
42	0.15	-	+	0.43	0.00	NT		NT		NT		0.09	0.03	NT	
43	0.10	+	+	0.49	0.01	NT		NT		NT		0.11	0.02	NT	
44	0.11	-	+	0.39	0.01	NT		NT		NT		0.11	0.01	NT	
45	0.04	-	+	0.33	0.01	NT		NT		NT		0.12	0.00	NT	
46	0.08	-	+	0.23	0.00	NT		NT		NT		0.10	0.02	NT	
47	0.03	+	+	0.38	0.00	NT		NT		NT		0.11	0.04	NT	
48	<0.35	+	+	0.35	0.01	NT		NT		NT		0.11	0.01	NT	

49	<0.20	-	+	0.38	0.00	NT	NT	NT	0.09	0.02	NT
50	<0.20	-	+	0.33	0.00	NT	NT	NT	0.09	0.01	NT
51	<0.10	-	+	0.13	0.00	NT	NT	NT	0.07	0.01	NT
52	<0.20	-	+	0.28	0.00	NT	NT	NT	0.11	0.02	NT
53	<0.03	-	-	0.05	0.00	NT	NT	NT	0.04	0.00	NT
54	<0.03	-	-	0.07	0.00	NT	NT	NT	0.05	0.00	NT
55	<0.15	-	-	0.20	0.01	NT	NT	NT	0.10	0.00	NT
56	<0.21	-	-	0.21	0.00	NT	NT	NT	0.09	0.01	NT
57	<0.20	-	+	0.16	0.00	NT	NT	NT	0.07	0.01	NT
58	<0.15	-	-	0.05	0.00	NT	NT	NT	0.08	0.01	NT
59	<0.03	-	-	0.06	0.00	NT	NT	NT	0.09	0.00	NT
60	<0.20	-	+	0.14	0.00	NT	NT	NT	0.10	0.03	NT
61	<0.10	-	-	0.04	0.00	NT	NT	NT	0.07	0.01	NT
62	<0.20	-	+	0.28	0.00	NT	NT	NT	0.11	0.03	NT
63	<0.05	-	-	0.10	0.00	NT	NT	NT	0.10	0.01	NT
64	<0.07	-	-	0.00	0.00	NT	NT	NT	0.16	0.04	NT
65	<0.05	-	+	0.04	0.00	NT	NT	NT	0.10	0.01	NT
66	<0.33	-	+	0.50	0.01	NT	NT	NT	0.12	0.02	NT
67	<0.08	-	+	0.10	0.00	NT	NT	NT	0.09	0.01	NT
68	<0.26	-	+	0.04	0.00	NT	NT	NT	0.09	0.01	NT
69	<0.08	-	+	0.06	0.00	NT	NT	NT	0.08	0.01	NT

Table 3.2.3. Comparison of the Neogen standard protocol (dilution 1:31) against two modifications (dilution 1:46.5 with or without cysteine 2 M, 70°C, 30 min). The four main PST analogues in the samples are indicated together with the total concentration (confirmation result). Shaded results indicate false negatives (eliminated when treated with cysteine). Sample numbers are the same as in Table 2. All these samples tested positive with the Scotia kit.

Sample No.	Total PST mg STX eq/kg	STX	GTX1&4	GTX2&3	C1&2	1:31	1:46.5	1:46.5 + cysteine
12	0.82	*	62%	31%	7%	-	-	+
23	0.92	*	61%	24%	7%	+	-	+
30	0.77	*	*	64%	18%	+	+	+
31	0.48	*	*	80%	13%	-	-	+
32	0.41	*	28%	46%	15%	-	-	-
36	0.31	*	47%	46%	7%	-	-	-
38	0.20	*	*	80%	20%	-	-	+
44	0.11	*	41%	*	28%	-	-	-
50	<0.20	NA	NA	NA	NA	-	-	-
67	<0.08	NA	NA	NA	NA	-	-	-
69	<0.08	NA	NA	NA	NA	-	-	-

* Reported as <0.05 mg STX eq/kg
NA = Not available

3.2.4. Discussion

Dinoflagellate blooms of *Alexandrium tamarense* (Group 1) in 2012 and 2015 generated closure of Tasmanian shellfish farms for up to 4 months, causing major economic losses. Mussels and oysters contained gonyautoxins as the major PST analogues. In 2012 GTX2&3 was the major analogue (51-100%), followed by STX (14-18%), C1&2 (10-24%) and dcGTX2&3 (5-16%). In contrast, in 2015 GTX1&4 was the major analogue (25-88%), followed by GTX2&3 (8-70%), C1&2 (4-21%) and STX (0-3%). Due to the low cross-reactivity of commercially available kits for GTX1&4, it was necessary to determine which kit would be the most suitable for shellfish growers to potentially incorporate in their monitoring program. To date few studies have critically compared PST immunological test kit results against AOAC official methods. Most studies used Scotia (formerly Jellett) and Abraxis (Costa et al., 2009; DeGrasse et al., 2014a; Turner et al., 2015; Wong et al., 2010), whereas others used ELISA kits with low or no reactivity to GTX1&4 (Burrell et al., 2016; Sato et al., 2014). The results of the present study are summarised in Table 3.2.4, which compares the main characteristics of the four immunological PST test kits, ease of use and their performance on Tasmanian mussels and oysters.

To date, PST determination in shellfish samples using AOAC official methods is still subject to considerable variability (Burrell et al., 2016; Costa et al., 2009; Turner et al., 2014a). A

Table 3.2.4. Summary of the characteristics and performance of four immunological commercially available test kits on the detection of PST toxins on Tasmanian mussels and oysters, which contained mostly GTX1&4.

	Neogen™	Scotia™	Abraxis™	Europroxima™
Characteristics				
Cut off or detection limit (mg STXeq/kg)	0.4-0.6	0.2-0.7	0.03[a]	0.0038[a]
Cross-reactivity for GTX1&4 (mole %)	6	1.8	<0.2	<0.1
Conversion step for GTX1&4	Yes (newly introduced)	Yes	No	No
Improved cross-reactivity for GTX1&4 (mole %)	129	26	-	-
Cost per test[b] (~US$)	22	22	13[c] to 84[d]	12[c] to 96[d]
Extraction	Distilled water, manually with roller	0.1 M HCl, boil & centrifuge	0.1 M HCl, boil & centrifuge	0.2 M sodium acetate buffer, mix & centrifuge
Dilution for extraction	1:31 (standard) 1:46.5 (present study)	1:2	1:2	1:6
Time for analysis[e]	20-35 min	35-95 min	Up to 3 hrs	Up to 3 hrs
Result	Positive or negative, immediate with reader	Positive or negative, immediate with scanner	mg STXeq/ kg, prior data analysis required	mg STXeq/kg, prior data analysis required
Performance in Tasmanian shellfish (n=69)				
% False positives	13	32	4.6[f]	0[f]
% False negatives	5 →0[g]	0	42.3[h]	100[h]

[a] As minimum concentration detectable in shellfish sample; based on lowest STX standard provided with kits for calibration curve.
[b] Price does not include taxes and reader, scanner or software for data analysis
[c] Considering that the whole 96-well plate is used, 41 or 40 samples can be tested in a single plate with Abraxis or Europroxima, respectively.
[d] Considering only one sample is run at a time (unused wells can be stored and used within the expiry date. Used wells must not be re-used)
[e] It does not include preparation of shellfish homogenate
[f] Reported as non-compliant when in fact they contained <0.8 mg STXeq/kg (as per LC-FLD)
[g] False negatives eliminated with the cysteine conversion step introduced in the present study
[h] Reported as compliant when in fact they contained >0.8 mg STXeq/kg (as per LC-FLD)

recent study did not conclusively select a method of choice but rather concluded that the method to choose should be based on practicality, including access and cost of equipment, and skills of the analyst (Burrell et al., 2016). A continuous input in methodological improvements and innovations is needed to overcome current method limitations, efficiency and sample turnaround time (Boundy et al., 2015; Campbell et al., 2011; Yakes et al., 2012), as well as their application to other matrices, such as human urine and blood serum (DeGrasse et al., 2014b).

Ease of use

The advantage of the Abraxis and Europroxima kits is that they deliver a quantitative PST result. A disadvantage is that both kits require laboratory skills. Abraxis requires extracting the toxin by boiling the shellfish sample in HCl, but this can be avoided by using MeOH and not boiling. The extraction step with Europroxima requires preparing the extraction buffer (0.2 M sodium acetate buffer), while the remaining extraction steps are easy to follow. Both the Abraxis and Europroxima call for pipetting small volumes (10-100 µL and 25-100 µL, respectively) for dilution and perform the assays using 96-well microplates. Stock solutions (i.e. dilution buffer, wash solution, conjugate or antibody) need to be diluted, and different incubation periods are required. Total test times amount to 60 and 45 min for Abraxis and Europroxima, respectively, but including sample extraction and solutions preparation, the protocol can take up to 3 hrs depending on the number of samples to be tested. Data analysis requires a calibration curve to be constructed but no specialised software is required. An adaptation of the 96-well plate Abraxis kit into a compact and easy-to-use shipboard version was successfully used by fishermen during a pilot study in Georges Bank, USA, with good correlations between Abraxis, mouse bioassay and LC-FLD (DeGrasse et al., 2014a). It is noted that the surf clams tested contained mostly STX, for which the Abraxis kit is well suited. However, the Abraxis and the Europroxima test kits had limited applicability to Tasmanian shellfish containing mostly GTX1&4.

The qualitative Scotia and Neogen kits comprise easier steps and laboratory experience is not essential. Scotia, similar to Abraxis, recommends extracting the toxin by boiling shellfish in HCl, but it also has an alternate rapid method using a mix of 2.5 parts of 70% isopropyl with 1 part of 5% acetic acid but this protocol was not performed in the present study. The remainder of the Scotia protocol involves mixing the shellfish extract with Scotia buffer and transfer an aliquot of this mix into the test strip followed by 35-60 min incubation period. The strip then can be scanned using the Scotia Skannex system which delivers an immediate positive or negative result. However, if GTX1&4 is suspected to be the dominant PST analogue, an extra step is recommended which increases the duration of the test by an extra 60 min. The Neogen kit offers the greatest ease of use since the extraction step is achieved using distilled water (or type 1 water, as in this study), and no boiling is required. Extraction is performed using a plastic filter bag which is homogenised mechanically with a roller (both provided). An aliquot of this mix is directly poured into a bottle containing Neogen buffer, which is mixed manually and a subsample used to perform the test. The Neogen strip is

incubated only for 5 min and immediately analysed by the Accuscan Pro reader, which delivers a positive or negative result.

A drawback of Abraxis and Europroxima is that a microplate reader with absorbance detection is necessary. Similarly, a scanner and computer are recommended for Scotia to remove subjectivity of visual interpretation of the bands (Turner et al., 2015). Neogen has also developed their own dedicated reader (Accuscan Pro reader). Both readers have to be purchased from the companies or brand suppliers since they use a specific software or have been calibrated in-house. The advantage of these readers is that they deliver an immediate result. Both Scotia and Neogen kits can be stored at room temperature (Scotia: 4-25°C, Neogen: 18-30°C), whereas the Europroxima and Abraxis kits need to be stored at 2-8°C and 4-8°C, respectively.

Sensitivity and Performance

Both Abraxis and Europroxima tended to underestimate the toxin concentrations in shellfish extracts when manufacturers' recommended protocols were followed. Abraxis underestimated in all 23 samples with ≥0.80 mg STX eq/kg, with 15 of these samples (65%) estimating at 0.8-1.2 mg STX eq/kg (e.g. LC-FLD: 1.43, 9.20, 31.75 mg STX eq/kg = Abraxis: 0.81, 1.11, 1.05 mg STX eq/kg, respectively). Critically, 8 samples (34.8%) were reported below the recommended regulatory action limit, within a concentration range of 0.80-3.29 mg STX eq/kg (LC-FLD), which showed 0.24-0.77 mg STX eq/kg with the Abraxis test. All samples extracted with MeOH were underestimated below the action limit. Europroxima underestimated 100% of samples, including those with up to 31.75 mg STX eq/kg. In some cases, Abraxis and Europroxima overestimated toxin concentration, especially those with ≤0.05 mg STX eq/kg, but none of them above 0.38 mg STX eq/kg.

Shellfish extracts whose PST toxin content were outside the constructed calibration curve were under or overestimated, 30% (HCl extraction) to 60% (MeOH extraction) and 91% of samples were outside the calibration curve for the Abraxis and Europroxima tests, respectively. This could be overcome by performing extra dilutions to make them fit the standard curve; however, re-testing those samples falling outside the calibration curve doubles the cost and time of analysis. Abraxis does recommend a further dilution of 1:10 in highly contaminated samples, however this dilution is not sufficient to obtain a satisfactory quantification since some samples contained up to 31.75 mg STX eq/kg. Extra dilutions of 1:100 or 1:500 were necessary for Abraxis and Europroxima, respectively, which improved toxin quantification significantly, although some samples were still underestimated: 2 with Abraxis and 1 with Europroxima, reporting compliant toxin levels when in fact they contained >0.80 mg STX eq/kg (as per LC-FLD). Costa et al. (2009) also performed extra dilutions to bring mussel and clam extracts within the working range of Abraxis. These authors obtained a correlation (r^2=0.87) comparable to the one we observed (r^2=0.82-0.91) and similarly reported underestimation in some samples above the regulatory action limit, thus recommending that this ELISA kit was not suitable for samples containing multiple PST analogues. DeGrasse et al. (2014a) compared Scotia with a modified Abraxis kit on surf

clams, and reported that Abraxis accurately detected high PST in shellfish contaminated with mostly STX (82%). The difference was claimed due to Scotia having been calibrated against a mixture of PST toxins, whereas Abraxis uses STX for the standard curve. Abraxis proved to be unreliable for samples contaminated with analogues other than STX, especially GTX1&4 for which reactivity is <0.2%.

Although Neogen standard protocol claims to return positive results for samples ≥0.8 mg STX eq/kg, Jawaid et al. (2015) reported a cut off of 0.68 mg STX eq/kg. In our work we observed a cut off of 0.4 mg STX eq/kg, and hence increased the dilution in the extraction step to increase the cut off to 0.5-0.6 mg STX eq/kg as suggested by Tasmanian shellfish growers. Jawaid et al. (2015) observed 38% false positives (samples with 0.265-0.408 mg STX eq/kg), whereas we observed 13% false positives in our samples (samples with 0.03 to <0.35 mg STX eq/kg). While Jawaid et al. (2015) did not observe false negatives, we found 5% false negatives in our Tasmanian studies (two samples containing 0.82 and 0.92 mg STX eq/kg). This difference might be due to the fact that (i) we included more samples in the range 0.5-1.3 mg STX eq/kg, (ii) our samples contained a higher proportion of GTX1&4 (low cross-reactivity by the Neogen and all kits), or (iii) because of the greater dilution we performed. It should be noted that the screen by LC-FLD for one of the false negative samples (with 0.82 mg STX eq/kg, confirmation result) was less than the regulatory limit (0.59 mg STX eq/kg screen result), and homogeneity could play a role in the differentiation of this sample; however, considering the screen result of that particular sample, a positive result was expected. The novel introduction in our work of cysteine treatment effectively overcame the false negatives due to conversion of GTX1&4 to NEO, and GTX2&3 to STX, as it has been shown by this and other thiol compounds (Asakawa et al., 1987; Sakamoto et al., 2000). This conversion step was more convenient with the Neogen kit since it possess the highest reactivity for NEO (129%, Table 3.2.1), and while it could generate false positives, this would not be as serious as for the Scotia kit (Table 3.2.3). Costa et al. (2009) recommended extra sample dilutions for Scotia to avoid false positives, but Turner et al. (2015) concluded that attempting to fine-tune the sensitivity of Scotia through extra dilutions is potentially unsafe. In our work we observed that by adjusting the dilution of the Neogen protocol and introducing a cysteine conversion step (increasing test time by 30 min), it eliminated false negatives. Furthermore, the Neogen kit also proved to perform well for contaminated samples from the 2012 Tasmanian bloom, which were dominated by GTX2&3 (samples used for early work, data not shown).

Turner et al. (2015) reported that Scotia returned negative results in samples up to 0.35 mg STX eq/kg. In their work the use of an extra hydrolysis step increased variability since samples with 0.10-0.70 mg STX eq/kg delivered positive results (improvement in performance), even though some (i.e. 0.10-0.23 mg STX eq/kg) were reported as negative. These authors observed many false positives without the extra step, 50% in samples with <0.1 mg STX eq/kg (including three samples with 0.01-0.08 mg STX eq/kg), and >95% in samples between 0.1 and 0.2 mg STX eq/kg. In the present Tasmanian study we observed 53.8% false positives in samples with <0.1 mg STX eq/kg, and 75% in samples between 0.1 and 0.2 mg STX eq/kg. Costa et al. (2009), DeGrasse et al. (2014a), and Wong et al. (2010)

all previously reported a high percentage of false positives using Scotia (>58%). The kits these authors used were claimed to have a detection limit of 0.4 mg STX eq/kg (Jellett et al., 2002), but DeGrasse et al. (2014a) found that the practical detection limit was 0.1 to 0.2 mg STX eq/kg. The introduction of the Scotia Skannex system improved problems with subjective visual comparison of band intensities (Turner et al., 2015). We did not observe any false negatives with the Scotia kits, but numerical values returned by the Scotia scanner and the toxin concentration determined by LC-FLD were not well correlated (r^2=0.45) which might be due to the higher concentration of GTX1&4 in our samples. The correlation could have been improved by using the extra hydrolysis step, but was not necessary for our samples since we did not obtain any false negatives. The introduction of a conversion step for the Neogen kit presented in this study, by incubating GTX1&4 standards and shellfish samples with cysteine, successfully eliminated false negatives. More work is being performed to fine-tune this extra step.

The Neogen kit was elected as the most suitable tool for our purposes since it offers advantages over other kits: (i) it is more user friendly and laboratory experience is not required, (ii) it returns faster results (~20-35 min), and (iii) is more practical for field use; (iv) it returned a lower number of false positives, and although 5% of false negatives were obtained, the introduction of a conversion step to increase the sensitivity of GTX1&4 successfully eliminated these false negatives. An international validation including Neogen standard protocol and the modification proposed in this study (i.e. altered dilution to change cut off and introduction of cysteine conversion step) is in process to facilitate approval of the Neogen kit for regulatory purposes as a screening tool. Significant savings in analytical costs will result when Neogen negative screen samples will no longer need to be tested using the more expensive and time consuming LC-FLD method.

3.3. Training Workshops

A number of training sessions were organised for Tasmanian shellfish farmers and scallop fishermen in the use of these kits. Sarah Ugalde packed FRDC funded show bags with blenders, scales, boxes of test strips, and scanners for the fishermen to go out and fight the blooming algae on their home fishing grounds. The industry responses have been most encouraging. These workshops will further provide a framework for additional industry training and quality assurance/quality control, as well as the development of a national policy for the use of analytical methods through a new PASE project, which will integrate the kits into industry and government risk management programs.

Fig. 3.3.1. IMAS Sarah Ugalde and Juan Dorantes-Aranda, with mussel grower Corrine Ooms from Spring Bay, oyster farmer Hayden Dyke from Little Swanport and scallop fisherman Bob Lister at the IMAS teaching laboratories.

'..pretty bloody exciting from an Industry perspective!'

'Got to say, (even at this trial stage), we are really loving this test. Especially today as we are sending out a large order to the mainland, so it's great to have that peace of mind and reassurance.'

'We are finding this kit quite easy and straight forward to use, as well as being very practical. Plus not having to stress about possible recalls is a major bonus!'

'..found it very simple to use and took relatively no time at all'

Yeah things are going pretty well. We are doing a few upgrades at work, especially after the hectic summer we had! Yes we've been using and loving the kit. We've actually got some PST

floating around in the bay at the moment at a pretty high level. The kit really helped us out this last fortnight, because TSQAP testing was out of action due to staffing issues. So we used the kit to workout the level. It scored a positive result at 0.5, I used the calculations you gave me a while back to change the detection level to 0.7 where it gave a negative reading. We trusted the kit and sold produce to the mainland, and it paid off. The official PST level in the bay's oyster was 0.61, so chalk up a win for the kit ....

3.4. Validation

3.4.1. Preface

To convince the regulatory bodies of the suitability of this test-kit, we conducted a comprehensive validation exercise using protocols designed by the Association of Official Analytical Chemists in the US. This work involved a Single Laboratory Validation, whereby a single analyst (Sarah Ugalde) tested 800 blind samples to examine selectivity, the effect of matrix (oysters vs mussels), probability of detection, repeatability and ruggedness. The latter refers to degree to of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions such as different laboratories, different analysts, different instruments, different lots of reagents, different elapsed assay times, different assay temperatures, different days, etc. A further Inter-laboratory Validation was conducted by sending 996 blind samples to 16 laboratories, both domestic and international, ranging from shellfish farmers to fully accredited laboratories. The Neogen™ test kit performed very well for testing PST in oysters, on average was satisfactory for mussels, but surprisingly the results were not only faster and cheaper than chemical methods but also highly reliable between laboratories. Two publications were prepared for the *Journal of the Association of Official Analytical Chemists International*, and Alison Turnbull in her capacity as Chair of the Australian Shellfish Quality Assurance Advisory Committee (ASQAAC) is currently pursuing full acceptance of this test for regulatory purposes, not only in Tasmania but throughout Australia. We organized several training courses for shellfish farmers in the operation of the test kits (see 3.3). The responses by industries have been most encouraging. The rapid test kits have the potential to be used with other seafood species such as scallops and rock lobster, but will need to be optimized and validated for each species. While PSP toxins are the most widespread seafood toxin problem in Australia, this product can readily be applied to other biotoxins by using different antibodies.

3.4.2. Detection of Paralytic Shellfish Toxins in Mussels and Oysters using the Neogen™ Qualitative Lateral Flow Immunoassay: Single Laboratory Validation and Collaborative Study

Alison Turnbull, Jessica Tan, Sarah C. Ugalde, Gustaaf M. Hallegraeff, Katrina Campbell, D. Tim Harwood, and Juan José Dorantes-Aranda

Abstract

The Neogen™ rapid test kit, a qualitative lateral flow immunoassay for the detection of paralytic shellfish toxins (PST), with an optimised protocol to improve detection of GTX 1,4 and GTX 2,3, was previously shown to be the best commercially available rapid screening test of PST in bivalves affected by *Alexandrium tamarense* Group 1 in Tasmania, Australia. A validation study was undertaken following AOAC International guidelines for qualitative binary chemistry methods applied to four naturally contaminated mussel and oyster homogenates containing different PST profiles. Appropriate selectivity to known toxins in bivalve shellfish was demonstrated. In the single laboratory validation, the kit used with the optimised protocol consistently detected PST toxins in both matrices with a probability of detection (POD) of 1.0 at the regulatory level (0.8 mg STX·2HCl eg/kg). No significant differences in detection of PST were observed between different production lots of kits. A collaborative study involving 16 laboratories was performed using both the standard kit and optimised protocols. Both methods showed good homogeneity across all laboratories. The mean POD at 0.8 mg STX·2HCl eq/kg in mussels and oysters using the standard protocol was 0.966 and 0.997, and using the optimised protocol, 0.968 and 0.969, respectively. The calculated/estimated limit of detection (LOD) in mussels was 0.308 mg STX·2HCl eg/kg with the standard protocol and 0.682 mg STX·2HCl eg/kg with the optimised protocol, while for oysters, it was 0.701 and 0.724 mg STX·2HCl eg/kg for standard and optimised protocols respectively. The results show the Neogen™ rapid test kit is acceptable for regulatory screening purposes for oysters in accordance with European Commission directives, with the standard protocol providing a probability of a negative response at the regulatory level of < 0.034 on 95% of occasions.

3.4.2.1 Introduction

A large bloom of *Alexandrium tamarense* Group 1, previously not seen in Tasmania, occurred on the east coast of Tasmania, Australia in 2012. The bloom caused extensive closures of the bivalve fisheries, but also impacted rocklobster and abalone fisheries, which had not been impacted by closures previously (Campbell et al. 2013, McLeod et al. 2014).

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It has since been discovered that this organism is extremely toxic, with record cell potencies of up to 500 pg STX eq per cell (Hallegraeff et al, 2017, in press). As with blooms from many dinoflagellate species, *A. tamarense* Group 1 has since recurred annually, usually in winter at low levels, however, significant cell numbers were recorded in 2015. The main paralytic shellfish toxin (PST) analogues accumulated in seafood have been gonyautoxin 1,4 (GTX 1,4) and gonyautoxin 2,3 (GTX 2,3) (Dorantes-Aranda et al. 2016).

Due to the high intracellular toxicity of this alga, PST levels in bivalve shellfish from affected areas rise rapidly, even at low algal cell numbers. Algal monitoring therefore has limited efficacy, and local authorities and businesses have been relying on weekly biotoxin analysis to manage the risk. Delays in transport from the regional areas to the national analytical facility in Sydney, combined with analysis times and the rapid increase in toxin levels have resulted in a number of exceedances of the regulatory level occurring in marketed shellfish. Since the 2012 bloom, there have been 21 domestic and 3 international recalls of Tasmanian shellfish due to biotoxins, all related to *A. tamarense* blooms (Food Standards Australia New Zealand and Department of Agriculture and Water Resources data, August 2016). In addition, there have been 5 cases of mild PST poisoning affecting recreational fishers, despite numerous public health warnings in the media and signs in local areas (Department of Health and Human Services data, August 2016).

Tasmanian businesses have experienced reputational damage and expenses associated with the recalls, and are concerned about the resultant risk of illness. There has been negative media nationally and internationally, and export restrictions to some countries. Broader ramifications have occurred also in other shellfish growing states in Australia; the situation in Tasmania has resulted in all Australian states increasing their bivalve biotoxin monitoring frequency, and hence, costs of risk management have substantially increased. However, the rate of detection of toxins is low. Nationally (including Tasmania), 90% of the analyses conducted for biotoxins are negative (Advanced Analytical Australia data for 2015/2016). Businesses and regulators alike are looking for cost effective, rapid tests for PST that can be used locally and at higher frequency to screen samples for toxins, avoid high risk product going to market and reduce monitoring costs.

A recent review of rapid biotoxin test kits for the Scottish Food Safety Authority found that of the 21 kits on the market for rapid shellfish toxin analysis, only two had completed an AOAC or Eurachem validation process (McLeod et al. 2015). There is likely to be a number of reasons for this, not the least that validation studies are a significant and costly volume of work.

Dorantes-Aranda et al. (2017) compared the four commercially available PST kits, for use with the profiles currently in Tasmania. Two kits were quantitative enzyme-linked immunosorbent assay (ELISA) and two were qualitative lateral flow immunoassay (LFIA). All used different extraction methods. All the kits had good reactivity to saxitoxin (STX), for which they were designed, but poor cross reactivity with GTX 1,4, which is a major

component of PST from the *A. tamarense* blooms in Tasmania, and only the Scotia kit had good reactivity to GTX 2,3, also a major component of PST from these blooms. Dorantes-Aranda et al. found that the Neogen™ rapid test kit was the best fit for purpose. The extraction procedure for this kit is simply to mix the sample with water and filter before adding the LFIA strips. In order to increase the cross-reactivity of the Neogen™ rapid test kits to gonyautoxins, Dorantes-Aranda et al. optimised the extraction procedure, using cysteine to convert GTX 1,4 to neosaxitoxin (NEO), and GTX 2,3 to STX, both of which have good cross reactivity, thus increasing the performance of this test kit for toxin profiles high in GTX 1,4 and GTX 2,3.

The aim of this study was to validate the preferred kit, the Neogen™ rapid test kit, so it could be used with confidence by growers to reduce business risk, and for consideration by regulators for potential inclusion as a screening tool in bivalve risk management programs.

3.4.2.2. Validation protocol

The validation followed the recently released AOAC guidelines for validation of qualitative binary chemistry methods (AOAC International, 2014). Two matrixes, *Crassostrea gigas* (Pacific oysters) and *Mytilus galloprovincialis* (Mediterranean mussels) were studied, with over 1600 samples analyzed blind, and compared against the pre-column oxidation (Pre-COX) HPLC method (AOAC 2005.06). The AOAC guideline breaks the validation into three components: a selectivity study, a matrix probability of detection (POD) study that determines the probability of detecting toxins at different concentrations in the matrixes of concern and a collaborative study that looks at the repeatability, reproducibility and POD of the method across a range of testing sites. The selectivity and matrix POD studies were conducted on the optimised extraction protocol only, as a single laboratory study had already occurred on the standard protocol, and the collaborative study was conducted on both the standard and optimised protocols. Due to the proprietary nature of the test kits, which are produced in batches, a ruggedness study was also undertaken to look for any differences between batches, or between results within batches on different days. No significant differences between batches were found (data not shown, see Turnbull et al. 2017 for details).

3.4.2.3. Selectivity Study

The selectivity study looked at the ability of the rapid test kit to appropriately identify the toxins of interest (i.e. PST), and checked that the kit does not react with other toxins that might be expected to be present (e.g. amnesic or diarrhetic shellfish toxins, or tetrodotoxin). Certified standards were spiked into blank shellfish matrix at a concentration of 0.57 mg STX·2HCl eq/kg for target toxins, and at the regulatory level for non-target toxins. The rapid test kits used with the optimised extraction protocol were able to identify STX, GTX 1,4, GTX 2,3, N-sulfocarbamoyl-gonyautoxin-2&3 (C1,2) and NEO, but did not identify the non-target toxins tetrodotoxin, okadaic acid and domoic acid.

3.4.2.4. Matrix POD Study

The matrix POD study determined the ability of the rapid test kits to detect PST at various concentrations in the matrix of interest, in this case, mussels and oysters. Two dilution series of oyster homogenates and two of mussels homogenates were tested, each with different toxin profiles. The homogenates used all originated from natural blooms of *A. tamarense* Group 1 and *Gymnodinium catenatum* in Tasmania. One oyster homegenate was spiked with STX and one with GTX 1,4 to better represent profiles found in other areas of the world such as the Gulf of Maine (USA) and the United Kingdom (Turner, et al. (2014), Harrison et al. (2016), DeGrasse et al. (2014)). The origin and composition of the homogenates are summarised in Table 3.4.1.

Table 3.4.2.1. Origin and toxin profile of contaminated shellfish homogenates in the single laboratory validation.

Stock mix	Matrix	Origin	Toxin profile (% of Total PST mg STX-2HCl eq/kg)
1	Oyster	*A. tamarense* bloom, East Coast of Tasmania, 2015, + STX[+]	37% GTX2&3, 26% GTX1&4, 25% STX, 11% C1&2
2	Oyster	*A. tamarense* bloom, East Coast of Tasmania, 2015 & 2016, + GTX 1,4[+]	46% GTX1&4, 40% GTX2&3, 14% C1&2
3	Mussel	*A. tamarense* bloom, East Coast of Tasmania, 2015	45% GTX2&3, 44% GTX1&4, 10% C1&2
4	Mussel	*Gymnodinium catenatum* bloom, Sullivans Cove, Hobart, Tasmania, 2015	56% dcSTX, 18% dcGTX2&3, 14% C1&2, 11% C3&4, 0.4% GTX6

[†] Stock mixes 1&2 were fortified with STX and GTX 1,4 respectively.

For each experimental series, stock homogenates were diluted to produce samples of approximately 0, 0.25, 0.4, 0.6, 0.8 and 1.2 mg STX·2HCl eq/kg. The number of replicates prepared per series was 110. More replicates were included between 0.6 and 0.8 mg STX·2HCl eq/kg, as this is where the POD was expected to change rapidly. An additional ten samples of approximately 0.2 mg STX·2HCl eq/kg were analysed for mussels following the determination that the POD was higher at lower PST concentrations for this matrix. All samples were analysed for PST using the same batch of Neogen™ test kits.

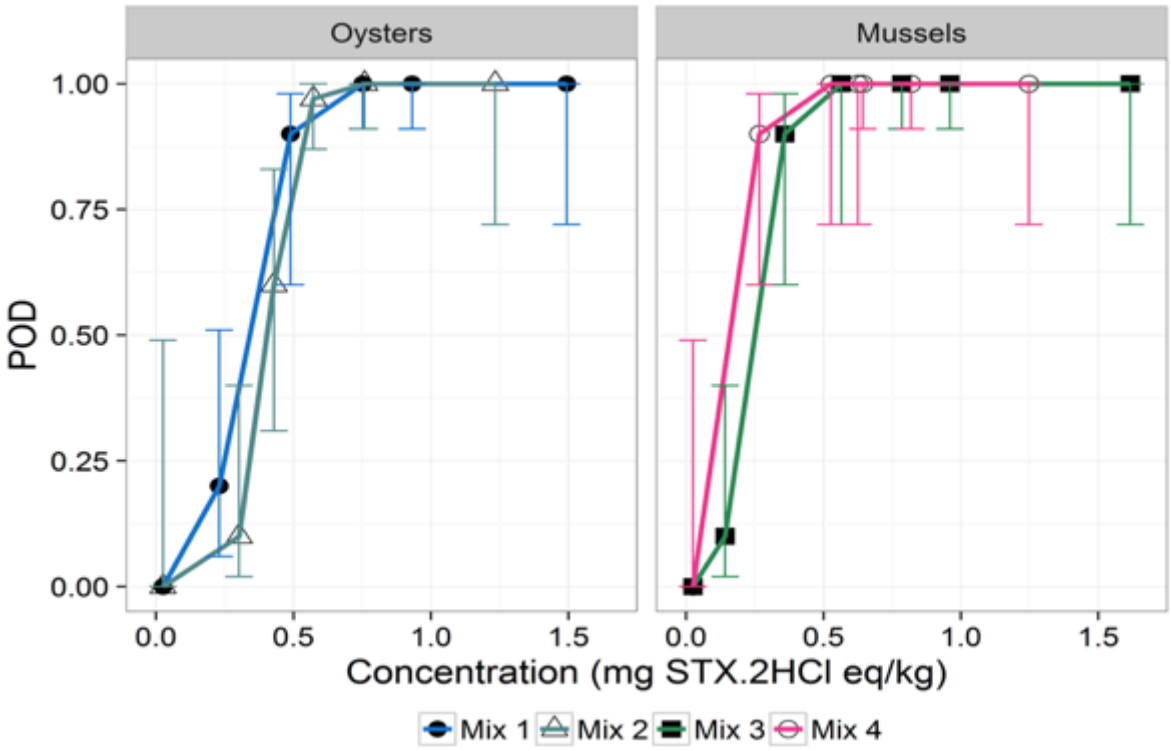

Figure 3.4.2.1. The probability of detection of PST by Neogen™ rapid test kit at various PST concentrations in experimental series created from four different toxin mixes. From Turnbull et al. (2017). *J AOAC International 101(2)*.https://doi.org/10.5740/jaoacint.17-0135; reproduced with permission.

The results of the matrix POD study are shown in Fig. 3.4.2.1. The POD of toxins in all the samples with <0.025 mg STX·2HCl eq/kg was zero as negative results were returned for all replicates in all experimental series. As the PST concentration increased, a higher proportion of replicates returned positive results, resulting in an increased POD. For both oyster series, a rapid rise in POD occurred between 0.2 and 0.5 mg STX·2HCl eq/kg. For both the mussel series, the POD rose rapidly between 0 and 0.4 mg STX·2HCl eq/kg. All replicates returned positive results for all concentrations above 0.7 STX·2HCl eq/kg in oysters, and above 0.4 mg STX·2HCl eq/kg in mussels.

The POD at selected PST concentrations was obtained by a binominal logistic regression, using the statistical software R. The model predicted a POD of 1.0 at 0.8 mg STX·2HCl eq/kg (the bivalve regulatory level) for all homogenates.

Thus, the single laboratory study showed that the rapid test kits used with the optimised extraction protocol showed appropriate selectivity and appropriate certainty of detection at the regulatory level in both oyster and mussel matrixes, giving sufficient confidence to proceed with a collaborative study.

3.4.5. Collaborative Study

The collaborative study is a comparison of repeatability, reproducibility and POD across a range of testing sites similar to those where the test will be used. Sixteen laboratories from Australia, New Zealand, USA, Europe and the United Kingdom participated in the study. The laboratories ranged from accredited facilities to oyster hatcheries. Both the standard and optimised extraction protocols were tested on eight naturally contaminated homogenates: four from oysters, two of which were spiked to increase STX and GTX 1,4 content; and four from mussels. There were 6 replicates of each homogenate per laboratory, resulting in 96 replicates per homogenate across all of the laboratories.

As with the matrix POD study, each of the shellfish homogenates used in this study had a different toxin profile. Shellfish were used from Tasmanian blooms, some of which were spiked with STX and GTX 1,4 to mimic profiles observed in other parts of the world. The major analogues were GTX 2,3, GTX 1,4, C toxins and STX. The source and composition of the homogenates used in the collaborative study are given in Table 3.4.2.

There was a strong homogeneity of results from the different laboratories in each matrix at low and high PST levels. All laboratories returned negative results for all the samples in both matrices when PST levels were <0.025 mg STX·2HCl eq/kg. Only one of 96 replicates above 0.8 mg STX·2HCl eq/kg was negative for oysters using both protocols, whilst only one replicate above 0.8 mg STX·2HCl eq/kg was negative for mussels, using the standard protocol. Data were analysed in accordance with the AOAC protocol, following the statistical methods outlined in Wehling et al. (2011), LaBudde & Harnly (2012) and Macarthur & von Holst (2012). Low repeatability, laboratory and reproducibility variations were found at both low and high PST levels, and the homogeneity test at these levels showed no significant difference between laboratories ($p > 0.05$), indicating the method is reproducible.

The data are presented in Figure 3.4.2.2. As with the single laboratory study, the laboratory POD (i.e. the probability of detection across all laboratories, LPOD) in oysters using both protocols was relatively low at the 0.25 mg STX·2HCl eq/kg concentration, and increased as PST level increased. Also, as with the single laboratory study, the test was shown to be highly sensitive for mussels with high LPOD at relatively low PST levels. The LPOD was ≥0.95 for all PST concentrations above 0.20 mg STX·2HCl eq/kg in this matrix.

Table 3.4.2.2. Total PST and average toxin profile of naturally contaminated mixes used in the collaborative study. From Dorantes-Aranda et al. 2017

Matrix	Bloom source (all from Tasmania, Australia)	mg STX·2HCl eq/kg[a]	Average toxin profile (% of Total mg STX·2HCl eq/kg)
Oyster	Nil	<0.02	-
	Alexandrium tamarense 2015	0.25	67% GTX 2,3, 29% GTX 1,4, 13% C 1,2
	A. tamarense 2015/16 + STX	0.74	33% GTX 2,3, 32% GTX 1,4, 26% STX, 9% C 1,2
	A. tamarense 2015 +GTX1&4	0.97	58% GTX 1,4, 31% GTX 2,3, 10% C 1,2
Mussel	Nil	<0.02	-
	Gymnodinium catenatum 2015	0.22	50% dcSTX, 24% dcGTX2,3, 13% C 1,2
	A. tamarense 2015	0.70	50% GTX 2&3, 41% GTX 1&4, 8% C 1,2
	A. tamarense 2012	1.13	48% GTX 2,3, 37% STX, 10% C 1,2, 2% GTX 5, 2% dcGTX 2,3

[a] determined by the AOAC.2005.06 Official method performed by three laboratories, calculated using FAO/WHO TEFs (FOA/WHO 2016).

The results were plotted as shown in Fig. 3.4.2.3, and used to estimate prediction intervals within which 95% of laboratories are expected to give a positive result. The limit of detection (LOD) was estimated from linear interpolation of the LPOD curves. For example, in Figure 3, the average LOD of the rapid test kits for PST in oysters extracted via the standard protocol was found by taking 95% LPOD on the y axis horizontally across to the average LPOD, and dropping to the x axis, where it crosses at 0.71 mg STX·2HCl eq/kg. The intersection of the same 95% LPOD horizontal line with the 95th percentile prediction interval showed that 95% of laboratories were predicted to have an estimated LOD of less than 0.731 mg STX·2HCl eq/kg.

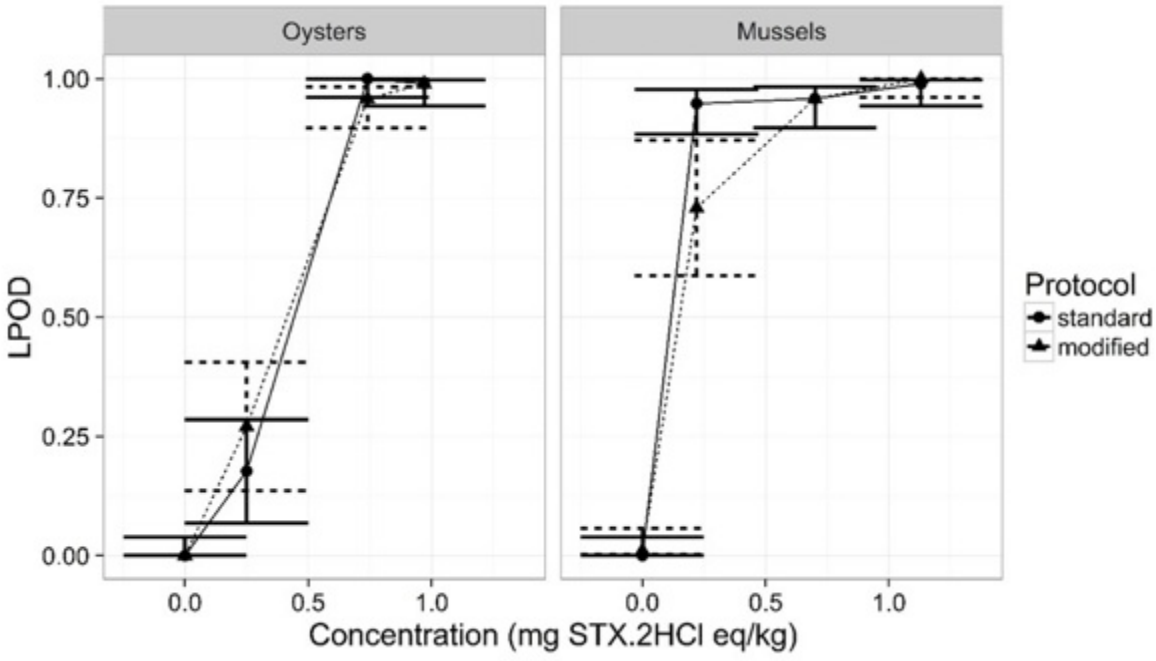

Fig. 3.4.2.2. The laboratory POD across a range of concentrations for the oyster and mussel experimental series extracted via the standard and optimised protocols. From Dorantes-Aranda et al. (2017). *J AOAC International 101(2)*https://doi.org/10.5740/jaoacint.17-022; reproduced with permission.

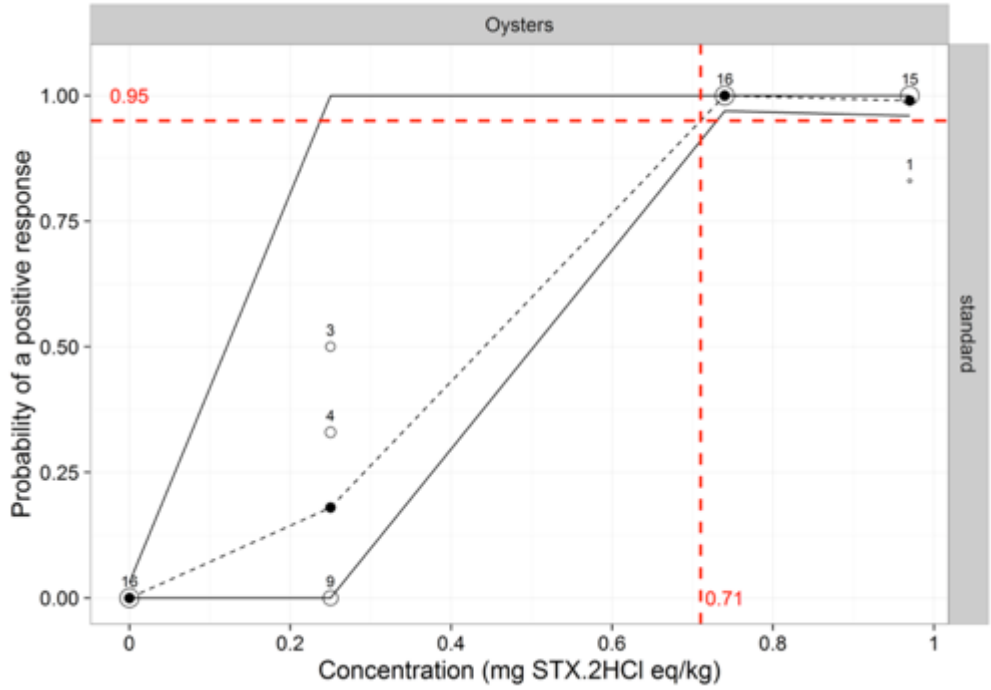

Fig. 3.4.2.3. The probability of a positive response over a range of PST concentrations across all laboratories. The black dotted line is the average LPOD, and the solid lines are LPOD for 5th and 95th percentile of labs. The numbers above the circles indicate the number of laboratories returning the shown POD. The intersection of the 95% probability of a positive response with the average LPOD results in an average LOD of 0.71 mg STX·2HCl eq/kg. From Dorantes-Aranda et al. (2017). *J AOAC International 101(2)*https://doi.org/10.5740/jaoacint.17-022; reproduced with permission.

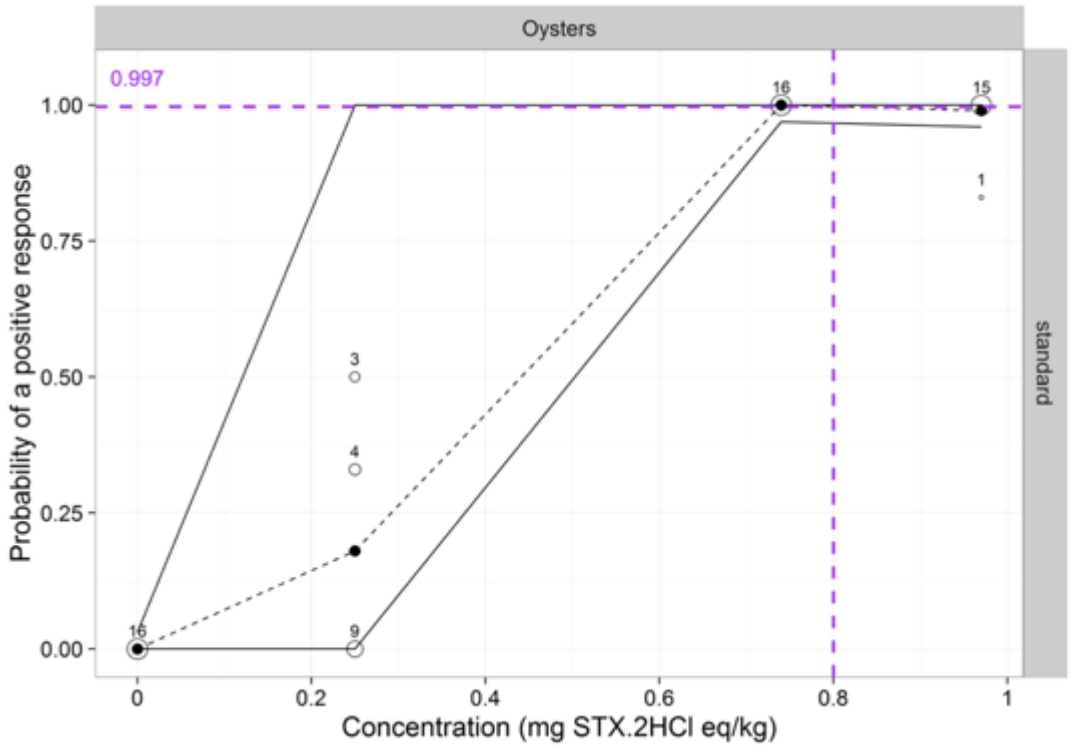

Fig. 3.4.2.4. The probability of a positive response in oysters over a range of PST concentrations across all laboratories using the standard protocol. The black dotted line is the average LPOD, and the solid lines are LPOD for 5th and 95th percentile of labs. The numbers above the circles indicate the number of laboratories returning the shown POD. The LPOD at the regulatory level was 0.997. From Dorantes-Aranda et al. (2017). *J AOAC International 101(2)*https://doi.org/10.5740/jaoacint.17-022; reproduced with permission.

The LPOD at the regulatory level was also found through linear interpolation by projecting a line vertically from the x axis at 0.8 mg STX·2HCL eq/kg to intersect with the average LPOD, and then crossing horizontally to the y axis. For the example shown in Figure 3.4.2.4 (oysters extracted with the standard protocol), the LPOD at the regulatory level was 0.997.

The β-error of qualitative tests is defined as the laboratory probability of a negative response, and can be obtained by subtracting the LPOD from 1. The β-error at the regulatory limit is used in the European Union to determine if an analytical method is suitable for use as a screening method.

European Commission decision 2002/657/EC, section 2.2 states that the β-error at the regulatory limit must be <0.05 and that screening methods must be validated in a documented manner, if they are to be used in conformity with Directive 96/23/EC (37). The Neogen™

rapid test kit used for both oysters and mussels complied with this requirement when both extraction protocols were used (Table 3.4.2.4).

For the oyster matrixes, logistic regression models were also used to obtain estimates of the β-error. This method was not able to be used on the mussel experimental series as the model fit was poor. The models estimated a β-error of 0.00 using both the standard protocol and optimised extraction protocol.

Table 3.4.2.3. Summary of the results for LOD and LPOD at the regulatory level in mussels and oysters obtained from the collaborative study.

	Oysters		**Mussels**	
Protocol	**Estimated LPOD at regulatory level (95% prediction interval)**	**Estimated LOD (mg STX·2HCL eq/kg)**	**Estimated LPOD at regulatory level (95% prediction interval)**	**Estimated LOD (mg STX·2HCL eq/kg)**
Standard	0.997 (0.967, 1.00)	0.710 (0.731)	0.966 (0.763,1.00)	0.316 (1.113)
Optimised	0.966 (0.925, 1.00)	0.734 (0.921)	0.968 (0.771,1.00)	0.682 (1.098)

The results of both the standard and the optimised extraction protocols from the collaborative study are shown in Table 3.4.2.4. For oysters, both extraction methods had a LPOD at the regulatory level that met the requirement of the EC, and average LOD below the regulatory level, however, the standard protocol performed better overall, as it had a higher 95% prediction interval for the LPOD at the regulatory level and the LOD that 95% of labs could reach was lower (0.731 mg STX·2HCL eq/kg cf 0.921 mg STX·2HCL eq/kg for the optimised protocol).

With the mussels, the average LPOD at the regulatory level was high, although the 95% prediction interval covered a greater range, and was below 0.8 at the lower levels. Whilst the average LOD was low at 0.316 and 0.682 mg STX·2HCL eq/kg for the standard and optimised protocols respectively, the LOD achievable by 95% of laboratories was high at over 1.0 mg STX·2HCL eq/kg.

3.4.2.6. Conclusion

The Neogen™ rapid test kit for PST analysis was demonstrated to be appropriate for screening of PST in Pacific oysters, and we would recommend the use of the standard extraction protocol, which has an easier and faster extraction and performs better. The use of the rapid test kits is slightly more complicated for Mediterranean mussels, and whilst on average it met the EU requirements, we recommend that the performance of the technique is checked carefully in the laboratory in which it will be used. The use of the Neogen™ rapid test kit will reduce toxin monitoring costs, allow a greater frequency of monitoring, and the rapid return of results, particularly for oysters and for mussels grown in areas that are not prone to toxic blooms. The high POD of PST in mussels at low PST concentrations complicates the use of this test in areas where PST is present. In general, the result will be an overly cautious response to mussel harvest closures, particularly during bloom development and depuration phases.

The results from these studies have accepted for publication by the Journal of the Association of Official Analytical Chemists as two separate manuscripts, one on the single laboratory study of selectivity, matrix POD and ruggedness, and one on the collaborative study. There is considerable interest from Australian shellfish businesses to use this screening technique to reduce the cost of recalls and the risk of causing illness. There is also interest in exploring how the screening techniques might be incorporated into official control programs under certain circumstances, in line with the American National Shellfish Sanitation Program (NSSP) use of screening techniques that have submitted acceptable validation studies (e.g. Abraxis Shipboard ELISA and the Scotia Rapid Test LFIA (NSSP, 2016)) and the recent acceptance of rapid test kits for mycotoxins that have undergone validation according to AOAC guidelines in the EU (European Commission Regulation No 519/2014).

Australia will be developing guidelines for the use of screening tools for incorporation into the Australian Shellfish Quality Assurance Program, along with industry training and accreditation programs, and investigating the use of this rapid test kit in other seafood matrixes such as lobster viscera.

4. Unprecedented *Alexandrium* blooms in a previously low biotoxin risk area of Tasmania, Australia

Gustaaf Hallegraeff , Christopher Bolch , Scott Condie , Juan José Dorantes-Aranda, Shauna Murray, Rae Quinlan , Rendy Ruvindy , Alison Turnbull , Sarah Ugalde, Kate Wilson

Abstract

During October 2012, a shipment of blue mussels (*Mytilus galloprovincialis*) from the poorly monitored east coast of Tasmania, Australia, was tested by Japanese import authorities and found to be contaminated with unacceptable levels of Paralytic Shellfish Toxins (PSTs; 10 mg/kg). Subsequently local oysters, scallops, clams, the viscera of abalone and rock lobsters were also found to be contaminated. This led to a global product recall and loss to the local economy of AUD 23M. Following low toxicity during 2013 and 2014 and implementation of minimal shellfish farm closures, a more severe bloom event occurred during July-November 2015 and again June-September 2016 (up to 300,000 *Alexandrium* cells/L; 24 mg/kg PST in mussels, 6 mg/kg in *Crassostrea gigas* oysters), also causing 4 human illnesses resulting in hospitalization after consumption of wild shellfish. While *Alexandrium tamarense* had been detected in low concentrations in southeastern Australia since 1987, all cultured strains belonged to the mostly non-toxic group 5 (now designated *A. australiense;* detected since 1987) and weakly toxic group 4 (*A. pacificum;* detected in 1997). In contrast, the 2012 to 2016 outbreaks were dominated by highly toxic group 1 (*A.fundyense*) never detected previously in the Australian region. Molecular analyses suggest that *A. fundyense* may have been a cryptic genotype previously present in Tasmania, but newly stimulated by altered water column stratification conditions driven by changing rainfall and temperature patterns. Increased seafood and plankton monitoring of the area now include the implementation of *Alexandrium* qPCR, routine Neogen™ immunological and HPLC PST tests, but ultimately may also drive change in harvesting strategies and aquaculture species selection by the local seafood industry.

4.1. Introduction

Starting in 1985, the Tasmanian shellfish industry has become used to annually recurrent closures and public warnings of paralytic shellfish poisoning (PSP) risk inflicted by *Gymnodinium catenatum* blooms (reviewed by Hallegraeff *et al.* 2012). This large chain-forming dinoflagellate can be readily recognised by light microscopy, and, in the past, the affected area was primarily confined to the Huon River and d'Entrecasteaux Channel, near the capital city of Hobart.

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Over time, mussel farms in the most severely affected Huon River all closed business and an economic decision was made to declare the area unsuitable for shellfish farming with no new leases allowed. Early HAB surveys of other Tasmanian locations since 1987, including the east coast, had detected low concentrations of *Alexandrium tamarense* (Hallegraeff *et al.* 1991; Bolch & Hallegraeff 1990; Bolch & de Salas 2007). However, all cultured strains proved to be non-toxic and belonged to what was initially termed the "Tasmanian ribotype" (now designated group 5 or *Alexandrium australiense*; Scholin *et al.* 1995, John *et al.* 2014). A single small bloom event in Spring Bay in 1997 was caused by toxigenic group 4 (or *Alexandrium pacificum*), also widespread along the New South Wales and Victorian coasts of Australia (Hallegraeff *et al.* 1991; Farrell *et al.* 2013). Despite this event, the Tasmanian east coast continued to be classified as a low biotoxin risk and hence was subject to very limited plankton and biotoxin monitoring. Unexpectedly, in October 2012, a shipment of blue mussels (*Mytilus galloprovincialis*) from the east coast of Tasmania tested by Japanese import authorities was found to be contaminated with unacceptable levels of Paralytic Shellfish Toxins (PSTs; 10 mg/kg). This incident triggered a recall of all Australian shellfish exported to Japan. Subsequent monitoring of the area confirmed PST in mussels, oysters, scallops, clams and rock lobster.

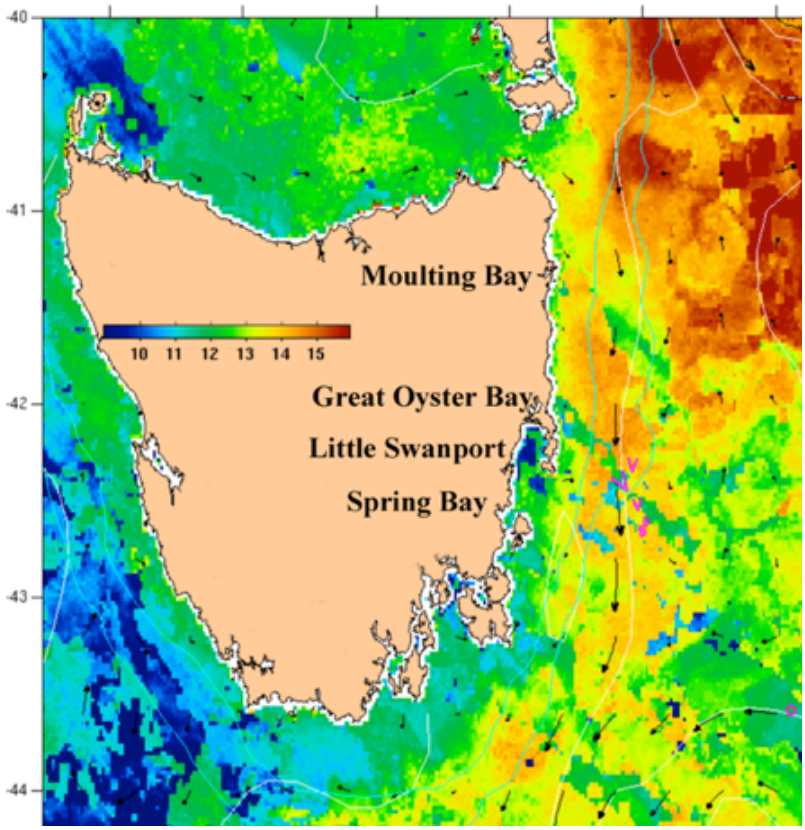

Fig. 4.1. Map of Tasmania, south of the mainland of Australia, showing Sea Surface Temperatures on 27 September 2015 during peak PST, with the East Australian Current (EAC; in red) interacting with the continental shelf. The locations of the main affected shellfish farm areas Moulting Bay, Great Oyster Bay, Little Swanport and Spring Bay are indicated. Source: oceancurrent.imos.org.au.

A review of this critical incident (Campbell *et al.* 2013) identified: 1. Failure of plankton monitoring to provide timely results and failure to detect *Alexandrium*; 2. Failure of seafood risk assessment by not recognizing the risk of a new mussel farming venture in a poorly monitored area; 3. Failure of PST monitoring by relying only on plankton monitoring as a first screen rather than including shellfish testing. Here we review the results of increased *Alexandrium* plankton and seafood PST monitoring since the 2012 incident with the aim to identify key regions and seafood species at risk as well as environmental variables driving the blooms.

4.2. Material and Methods

Shellfish toxins were monitored at weekly intervals at >20 Tasmanian east coast sites by the Tasmanian Shellfish Quality Assurance Program (TSQAP) using the AOAC approved Liquid Chromatography with fluorescence detection (LC-FLD) method (Lawrence *et al.* 2005). Satellite oceanography of the area was monitored as part of the Integrated Marine Observing System (IMOS; Fig.4.1). At the height of the August 2016 bloom, additional plankton, toxin and hydrological data were collected along inshore-offshore transects aboard the RV *Southern Cross.* A Seabird SBE 19PlusV2 CTD was used to collect temperature and salinity depth profile*s.* Plankton counts were obtained by settling 1L Lugol's iodine preserved samples. PST estimates were conducted on 3L of 8µm filtered water using the Neogen^{TM} Reveal 2.0 immunological test kit (modified after Dorantes-Aranda *et al.* 2017). Cyst sediment samples were collected using a Craib corer and processed using primulin staining (Yamaguchi *et al.* 1995).

4.3. Results and Discussion

4.3.1. Shellfish toxins

Following low PST detection in 2013 and 2014 (both low rainfall years) with implementation of minimal shellfish farm closures, a more severe bloom event occurred during July-November 2015 (up to 300,000 *Alexandrium* cells/L; 15 mg/kg STX eq. in mussels, 6 mg/kg in *Crassostrea gigas* oysters), also causing 4 human hospitalizations after consumption of wild shellfish. More severe blooms recurred in 2016, following a major flood event in May and blooms lasting until September when up to 24 mg PST/kg was recorded in mussels (Fig. 4.2). In 2015, the highest PST concentration was measured in the south in Spring Bay, but in 2016 highest PST occurred further north in Little Swanport and Great Oyster Bay. Most shellfish contained high proportions of GTX1&4 (26-88%) and GTX2&3 (8-76%), followed by C1&2 (5-24%) and STX (0-2%) (Dorantes-Aranda *et al.* 2017).

4.3.2. Increased PST flesh testing

The current protocol for sample processing by the Tasmanian Shellfish Quality Assurance Program involves shipping samples to an accredited laboratory in Sydney, leading to frustrating delays (4-12 days) for shellfish growers. The performance of four commercial

PST test kits, Abraxis™, Europroxima™, Scotia™ and Neogen™, was compared with the LC-FLD method for contaminated mussels and oysters. Based on their sensitivity, ease of use and performance, the Neogen kit proved the most suitable kit for use with Tasmanian mussels and oysters. Neogen produced 5% false negatives and 13% false positives when the cut off was altered to 0.5-0.6 mg STX-diHCl eq/kg, whereas the introduction of a hydrolysis conversion step eliminated false negatives. A full single lab and international validation process were conducted and once formally approved for regulatory purposes, the Neogen kit will provide shellfish growers with a rapid tool for on-farm harvesting decisions. Rapid screen tests to prevent compliant samples undergoing testing using the expensive LC-FLD method will also result in significant savings (estimated $200k/yr) in analytical costs.

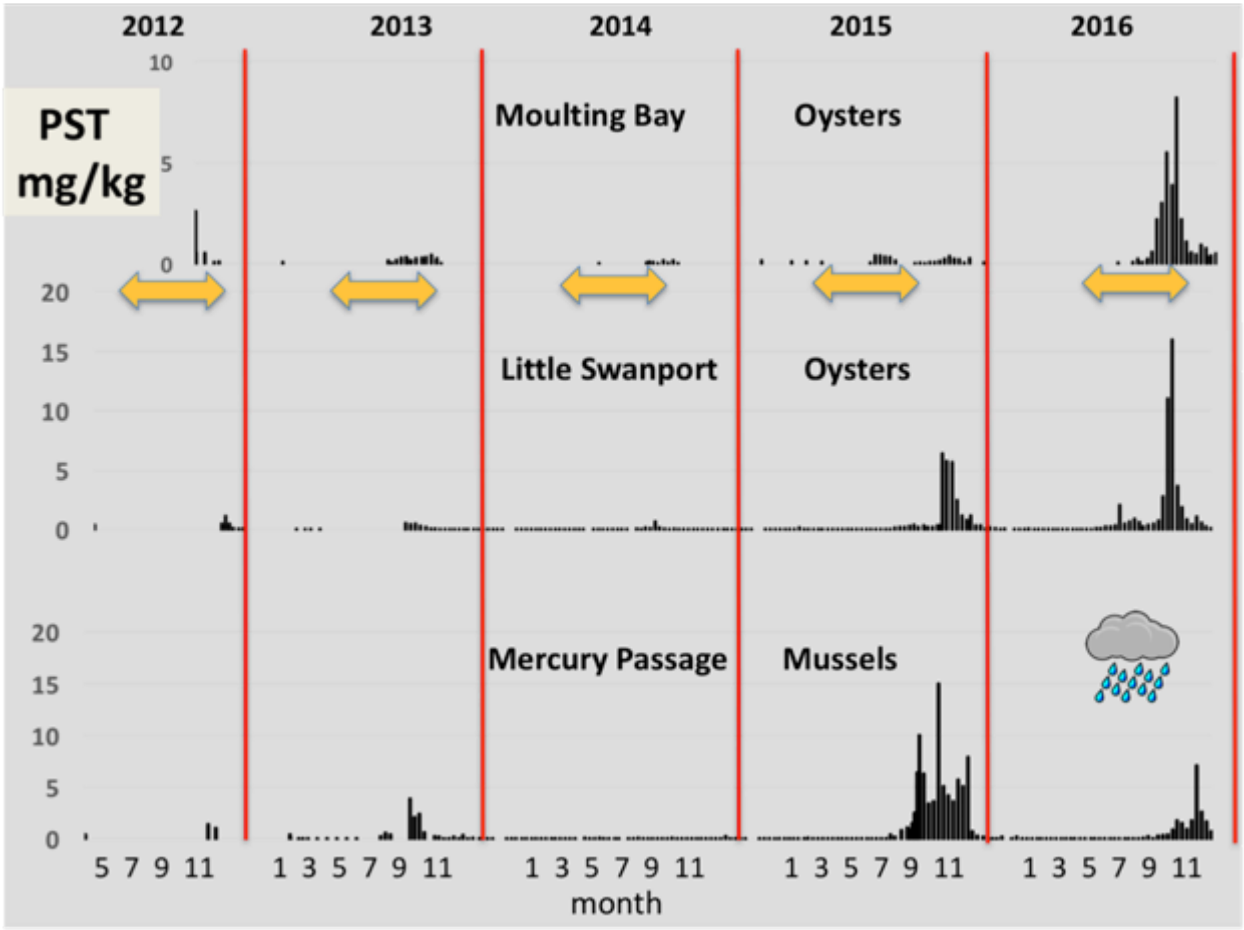

Fig. 4.2. Shellfish toxicity (mg STX eq./kg) from 2012 to 2016 in Moulting Bay and Little Swanport oysters and Spring Bay mussels. Orange arrows indicate the seasonal 10-15ºC temperature window. The 2016 bloom was preceded by a major rainfall event while anomalously cold water in Great Oyster Bay may explain the 2015 bloom.

4.3.3. Causative dinoflagellates

The causative dinoflagellates morphologically agreed with *Alexandrium fundyense*, possessing a ventral pore in the 1st apical plate (Fig.4.3a, arrow), and occurring as single cells or division pairs. An unusual feature of field samples was the extreme fragility of cells, ecdysing within 30-60 min after collection (Figs 4.3 b,c,d). Unexpectedly all cultured strains established during 2012 and 2015 belonged to group 1 never before detected in Australian waters in over 30 years of observations. Unique microsatellite signatures of these cultures (U. John, pers. comm.) suggest an endemic cryptic population being newly stimulated by changing environmental conditions. Paleogenomic research is in progress using dated sediment depth cores from the area to document historic shifts in abundance of *Alexandrium tamarense* genotypes 1, 4 and 5.

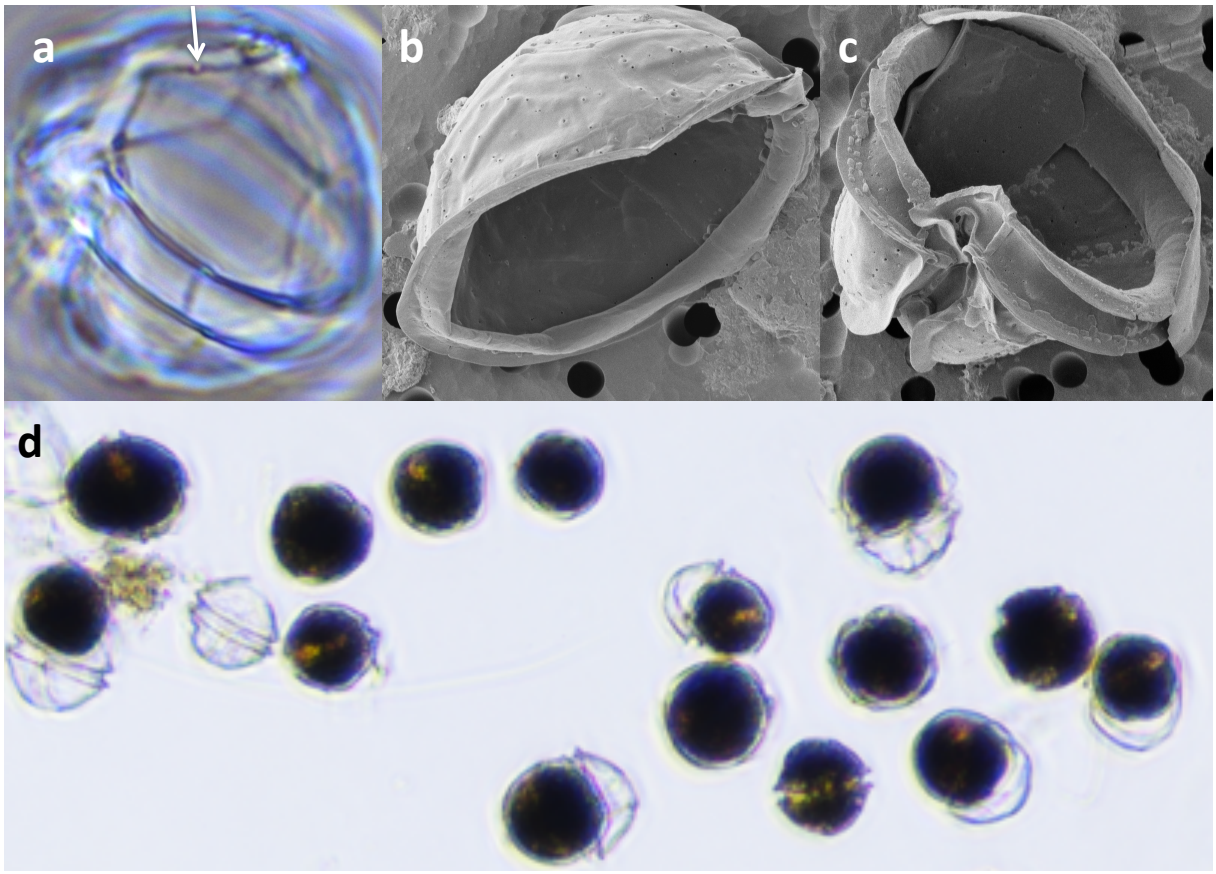

Fig. 4.3. Light (a,d) and scanning electron micrographs (b,c) of Tasmanian 2015 and 2016 *Alexandrium* field samples. Fig.3a shows ventral pore (arrowed) in the first apical plate; Figs. b,c,d show the extreme fragility of the cells subject to ecdysis within 30-60 min of collection.

4.3.4. Preliminary views on bloom conditions

The affected Tasmanian coastal region is classified as a climate change "hotspot" resulting from increasing southward movement of the nutrient-poor East Australian Current (Fig.4.1). These novel *Alexandrium* blooms are not a simple response to increasing water temperatures however (2.3ºC increase since the 1940s), as they occur in the cold winter-spring months at water temperatures of 10-15ºC. An observed trend of decreased silica concentrations in these waters would favor dinoflagellates and select against competing diatom blooms (Thompson *et al.* 2009). Preliminary culture growth experiments showed *Alexandrium* growth rates as high as 0.5-0.8 divisions/day, and a preference for low phosphorus and stimulation by humics (R.Quinlan, unpublished). Both culture experiments but notably field estimates using the Neogen test suggest a high cellular toxin content up to 100-500 pg STX eq/cell (Fig. 4.4, middle). In August 2016 *Alexandrium* populations were abundant in inner shelf waters (35-50m deep) (Fig. 4.4, left) and just inside the sand bars of the main shellfish growing estuaries of Little Swanport, Great Oyster Bay and Moulting Bay. However *Alexandrium* were virtually absent from the shallow (1-2m) turbid waters of those estuaries, and also were absent from deeper (100m) offshore waters dominated instead by spring bloom diatoms.

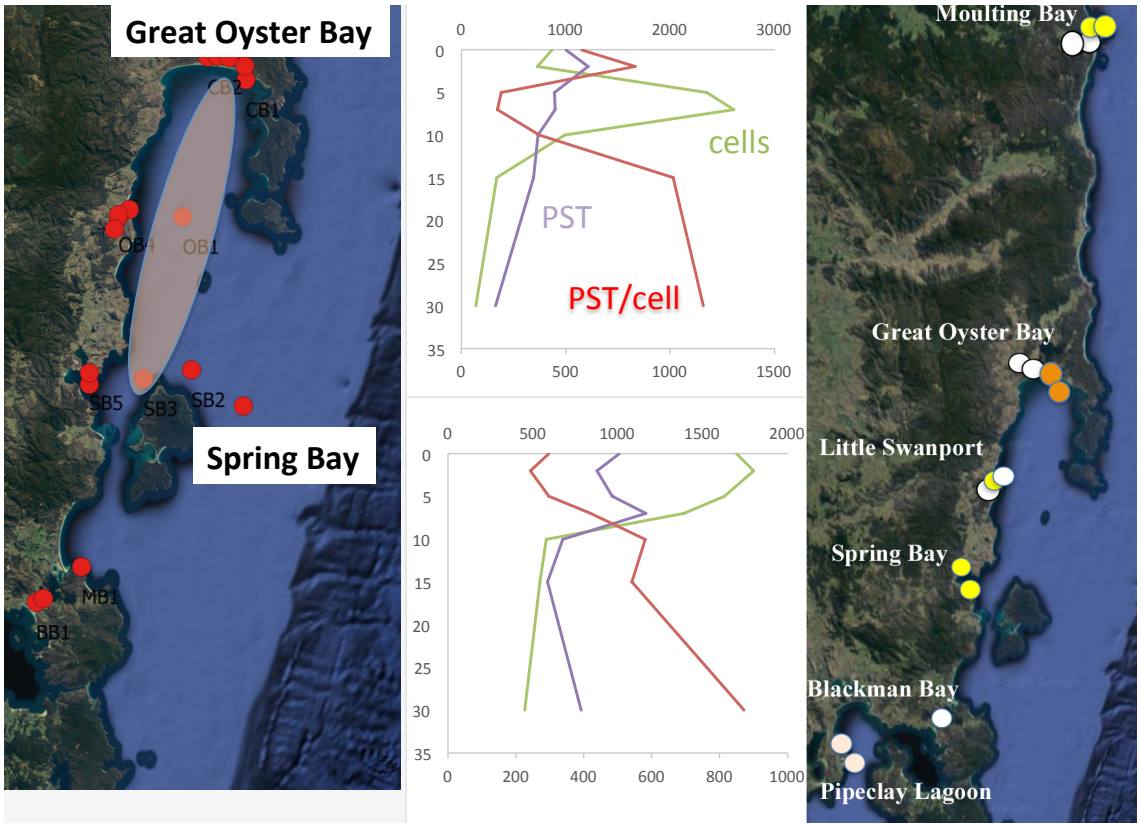

Fig. 4.4. Left: *Alexandrium* bloom patch contained on the inner shelf in August 2016, with no cells detected in offshore deeper waters. Middle: Depth profiles of *Alexandrium* cell abundance (top scale), total PST toxins (ng/L) and pg PST eq per cell (bottom scale) in weakly stratified waters of Great Oyster Bay and Spring Bay. Right: *Alexandrium* cyst surveys in August 2016: white dots <0.1; yellow 0.4-0.6; orange 2-3 cysts/g sediment wet weight.

In 2016 the peak of the *Alexandrium* bloom coincided with a major high rainfall/ flood event that resulted in salinity stratified coastal waters (Fig. 4.2), while northward flow on the inner shelf was consistent with downwelling favorable conditions along the entire coast. In 2015 the situation was different however with anomalously cold water flowing out of Great Oyster Bay resulting in thermally stratified coastal waters. While both stratified and downwelling conditions are known to favor dinoflagellates over diatoms (Condie & Bormans 1997, Condie & Sherwood 2006), further research is in progress on how these processes control *Alexandrium* blooms off eastern Tasmania.

Alexandrium cyst surveys during August 2016 along the entire east coast of Tasmania found consistently low abundances of cysts (0.1-3 cysts per gram of sediment wet weight), but no dense cyst beds. Most sediments comprised coarse sands reflective of strong current regimes. Preliminary cyst culture experiments indicated a short dormancy period of 1-2 months (compare Hallegraeff *et al.* 1998 for New South Wales *Alexandrium* cysts) suggestive of rapid cycling between plankton and benthos. To protect tourism and human health, the area has now been sign-posted with permanent public PST warnings, which is a first for Australia.

5. Use state-of-the art molecular techniques to resolve the population structure and biology of toxic *Alexandrium tamarense*- species complex algae

Shauna Murray, Christopher Bolch, Gustaaf Hallegraeff, Rendy Ruvindy

The single species that was formerly known as "*Alexandrium tamarense*" is now known to consist of a group of five closely related species, a 'species complex' (*A. catenella/fundyense*, A. tamarense, A. mediterraneum*, *A. pacificum, A. australiense*, or also designated as Groups 1 to 5). These species are not distinguishable using morphological information alone, but are genetically distinct (John et al 2014). Species also differ from one another in toxin production and ecological preferences (John et al 2014, Anderson et al 2012). These species are known to co-occur at several sites in the world (Brosnahan et al 2010; Toebe et al 2013). This would suggest that, if we are to predict the causes and consequences of PST blooms by *Alexandrium tamarense* species in Tasmanian waters, more detailed information is required on their precise identity.

Prior to 2012, the species *A. australiense* (group 5) and *A. pacificum* (group 4) had been found along the east coast of Tasmania, and cultures isolated (Bolch & de Salas, 2007), which confirmed PST production (Murray et al 2011, 2012). However, their identity, distribution and toxicity were little studied as they were not generally abundant. The 2012 bloom may have been dominated by the species, *A. catenella/fundyense* (Group 1), as all the *Alexandrium* strains cultured from the 2012 event belonged to Group 1 (Bolch et al. 2014). However, it is not known to what extent different species of this '*Alexandrium tamarense*' complex may contribute to PST toxins in the Tasmanian region, and whether this may differ between locations and years.

Therefore, we developed and tested methods for distinguishing the five species of *Alexandrium tamarense* complex in the region. We isolated cultures, identified them to species and tested them for toxicity. Finally, we examined the distribution and relative abundance of the PST-producing species of *Alexandrium* for the period 2013-2016, using the molecular tools we had developed, to assess the risk of bloom formation in this region.

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*The Nomenclature Committee for Algae in February 2017 decided that *A. fundyense* and *A. catenella* are conspecific, with the name *Alexandrium catenella* having nomenclatural priority over *Alexandrium fundyense* (Prud'homme van Reine, 2017). In the present report we refer to *Alexandrium catenella/fundyense* or *Alexandrium tamarense* group 1.

5.1. Development of molecular genetic detection assays for species of the '*Alexandrium tamarense'* species complex

5.1.1. Molecular detection assays

In order to distinguish the five morphologically similar species present, the use of molecular probes for their quantification and identification is critical in advancing our understanding of bloom dynamics and the environmental factors that drive them. We therefore developed assays to quantify these species in environmental samples.

5.1.2 rRNA Quantitative PCR Assays

qPCR assays for the three PST producing *Alexandrium* species that occur in the region (*A. catenella* /*A. pacificum*/*A. australiense* , Groups 1/4/5, Table 5.1) were adapted from existing literature (Erdner et al 2010, Hosoi Tanabe and Sako 2005, de Salas, unpublished). Primers were designed to target known LSU rDNA sequence differences among the three *Alexandrium* target groups. We compared 2-3 primer sets to determine the most specific and efficient primers to quantify these species from Tasmanian waters, using Sybr green methods.

Standard curves were generated for strains of *A. catenella/fundyense* (TRIA-A, TRIA-E) and *A. pacificum* (CAWD44, CS300) using the assays in Table 5.1 (Fig 5.5). The efficiency of the assays for the strains of *A. pacificum* CAWD44 and CS300 with catF/catR primers were 98.9% and 95.8% respectively, and 102.2% and 97.79% respectively with ACTA-416-F/ACTA-605-R primers. The efficiency of the assays of *A. catenella/fundyense* TRIA-A with ACT-US-408-F/ACT-US-645-R and US-412-F/US-642-R primers were 96.45% and 101.27% respectively, and 96% for the Erdner et al (2010) primers. This suggests that all primers were highly efficient at reliably quantifying *A. catenella/fundyense* and *A. pacificum*.

In testing the specificity of the assays targeting *A. pacificum,* the primer set ACTA-416-F/ACTA-605-R was more species-specific than the catF/catR primer set, as the catF/catR primer set also amplified a strain of the species *A. australiense* (Table 5.1). Therefore the primer set ACTA-416-F/ACTA-605-R is the preferred primer set for the quantification of *A. pacificum* cells in environmental samples. For *A. catenella/fundyense,* all the three primer sets/assays were strictly species-specific, and, as their efficiency was equally high, any of these primer sets could be chosen to quantify the presence of *A. catenella/fundyense.*

Table 5.1: Specificity tests of rRNA gene targeted qPCR assays. + refers to a positive result, - to a negative result, N/D means this was not studied.

Assays	Reference	Strains tested							
		A. pacificum		*A.australiense*	*A. catenella*			*A. minutum*	
		CAWD44	CS300	ATCJ33	TRIA-E	ATSTHM	TRIAA	CAWD12	CS324
A.catenella									
ACT-US-408-F/ACT-US-645-R	de Salas (unpublished)	-	-	-	+	+	N/D	-	-
US-412-F/US-642-R	de Salas (unpublished)	-	-	-	+	+	N/D	-	-
AlexLSUf2/ Alexgp1 RevAF1	Erdner et al (2010)	-	-	-	+	+	N/D	-	-
A.pacificum									
catF/catR	Hosoi Tanabe & Sako (2005)	+	+	+	-	-	N/D	-	-
ACTA-416-F/ACTA-605-R	de Salas (unpublished)	+	+	-	-	-	N/D	-	-
A.australiense									
AusTv2-F/AusTv2-R	de Salas (unpublished)	-	-	+	-	-	N/D	-	-

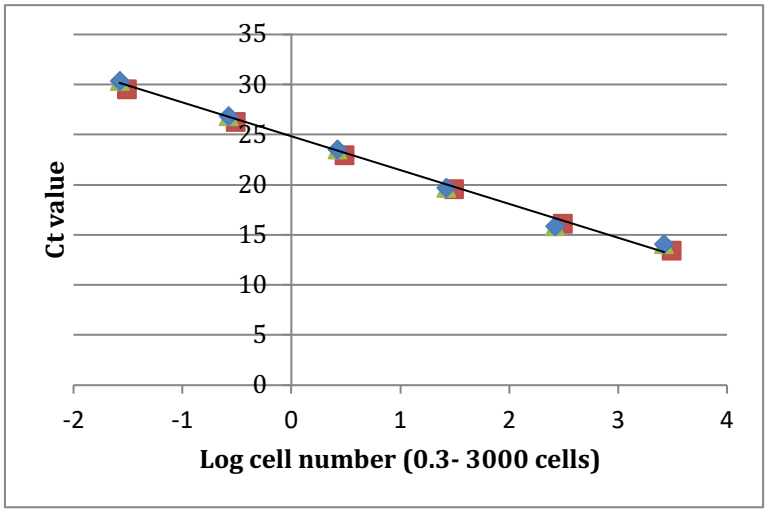


Fig. 5.1. Standard curves for qPCR assays to detect *Alexandrium catenella/fundyense* (primer set US-412-F/US-642-R) and *Alexandrium pacificum* (primer set ACTA-416-F/ACTA-605-R), based on the cultures TRIA-A (green triangles) and CAWD44 (red squares), CS300 (blue diamonds), respectively.

5.1.3. *sxtA4* saxitoxin gene qPCR assay

A further method that can be used to identify and quantify the possibility of a bloom of PST producing *Alexandrium* is an assay based on the detection of genes involved in PST toxin biosynthesis. This assay is based on the quantification of a gene, *sxtA4,* gene which is specific for PST biosynthesis in dinoflagellates (Murray et al. 2011).

We tested the specificity of the *sxtA4* assay against a series of toxic and non-toxic *Alexandrium* species that are commonly found in Australian waters (Table 5.2). This was important, as all of these species are morphologically relatively similar, and can only be distinguished by skilled expert taxonomists, however, some are never toxic, while others potentially can product PST toxins. All six non-toxic *Alexandrium* species assayed (*A. affine, A. concavum, A. leei, A. margalefi, A. fraterculus, A. pseudogonyaulax*) resulted in negative *sxtA4* assay outcomes. Toxic strains of *A. catenella/fundyense* (TRIA-A, TRIA-E, AT-STH-M) and *A. pacificum* (CAWD44, CS300) and *A. australiense* (ATNWB01) from Tasmanian waters all tested positive with the *sxtA4* assay.

Table 5.2: Specificity tests of amplification with the *sxtA4* assay, showing it was specific to *Alexandrium* strains producing PST toxins.

Strain Code	Species Name	*sxtA* Assay
CCMP112	*Alexandrium affine*	-
CAWD52	*Alexandrium concavum*	-
CCMP2933	*Alexandrium leei*	-
CS322	*Alexandrium margalefi*	-
CAWD97	*Alexandrium fraterculus*	-
CAWD54	*Alexandrium pseudogonyaulax*	-
CB1214	*Alexandrium* sp. (Calabash Bay)	-
CS300	*Alexandrium pacificum*	+
TRIA-A	*Alexandrium catenella*	+
ATCJ33	*Alexandrium australiense*	+
CS324	*Alexandrium minutum*	+
CAWD135	*Alexandrium ostenfeldii*	+

Standard curves required for quantification were established for Tasmanian *A. catenella/fundyense* (TRIA-A, TRIA-E, AT-STH-M) and *A. pacificum* (CAWD44, CS300), and the assay validated on bloom and/or environmental samples spiked with cultured cells (Fig. 5. 6).

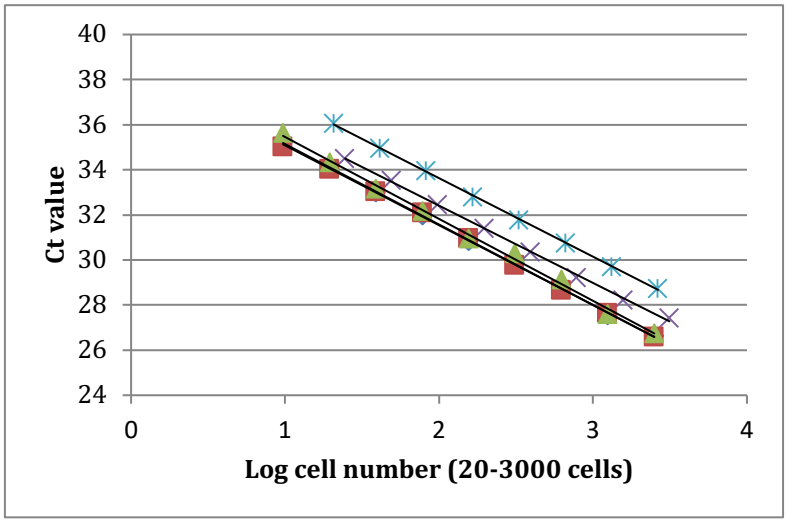


Fig 5.2. Standard curves to show the efficiency of the qPCR assay targeting *sxtA4*, against 3 replicate DNA extractions from Tasmanian cultures of *Alexandrium catenella/fundyense* (TRIA-A, triangles and squares) and 2 different strains of *Alexandrium pacificum* (CAWD44, CS300, crosses and stars). All strains amplified efficiently using this primer pair, with efficiency results from 88-96%.

Table 5.3: The addition of known cell numbers of *A. catenella/fundyense* and *A. pacificum* to natural seawater samples to assess the amplification efficiency using the sxtA assay and species specific assays.

Species	Cell Numbers	Assays							
		SxtA4		ACT-US-408-F/ACT-US-645-R		US-412-F/US-642-R		catF/catR	
		Expected Ct	Average Actual Ct	Expected Ct	Average Actual Ct	Expected Ct	Average Actual Ct	Expected Ct	Average Actual Ct
Alexandrium pacificum (CS300)	1065	30.0	33.1	N/D	N/D	N/D	N/D	14.2	18.1
	5680	27.5	31.4	N/D	N/D	N/D	N/D	11.7	16.3
Alexandrium fundyense (AT-STH-M)	1496	29.5	29.4	16.8	12.3	16.5	11.0	N/D	N/D
	4488	27.8	28.0	15.3	10.8	14.8	9.9	N/D	N/D

We evaluated whether or not samples with a normal seawater phytoplankton community background would amplify as efficiently as cultures, by spiking a known cell number into a natural seawater sample, and performing a DNA extraction followed by qPCR using *sxtA* and

species-specific primer pairs. We found that some differences were apparent, however these were less in the *sxtA4* assay than the species-specific qPCR assays. The use of standard curves generated using spiked data would allow us to take any minor differences into account.

5.1.3. Evaluation of DNA extraction methods for qPCR of *Alexandrium*

As it is known that the DNA extraction methods can influence the amount of DNA recovered, and also, the purity of the DNA, which impacts the qPCR assay, it was necessary to test different DNA extraction methods on *Alexandrium* samples to assess their relative performance. Five different DNA extraction methods were tested to determine the most efficient, reliable and simplest DNA purification method for the subsequent quantitative PCR (qPCR) analysis. Additionally, cells were lysed using a simple cell lysis (BioGX lysis tubes), and qPCR was performed to determine whether DNA purification is required for reliable qPCR results. The isolated DNA/cell extracts were tested in qPCR assays.

- PureLyse DNA extraction kit, automated DNA purification (ClaremontBio, USA)
- Fast DNA plant kit (MPBio, USA)
- MagPurix Nucleic Acid Bacterial DNA kit: automated for up to 12 samples in parallel
- Viogene plant genomic DNA kit (Viogene BioTek, Taiwan)
- CTAB DNA extraction using acid washed glass beads for cell fragmentation

An initial lysis step is necessary for all applied DNA extraction methods. The highest yield of DNA was obtained by using the CTAB method (Table 5.4). The other methods were similar in the overall obtained DNA yield, after some adjustments (i.e. lysis of cells using glass beads and bead beater). The MagPurix automated DNA extraction is very convenient, and useful to generate a standardized DNA extraction method without any differences in the purification procedure.

Table 5.4. DNA yield using different extraction methods using triplicate samples of ~1000 cells of cultured strains.

DNA extraction methods *A. minutum* CAWD 12, *A. pacificum* CAWD44, *A. ostenfeldii* CAWD 136, *A. pacificum* CS300/01	DNA ng μl^{-1}
PureLyse DNA	~19
Fast DNA plant kit	~ 20-25
MagPurix	~ 6-15
Viogene plant genomic kit	~ 10-20
CTAB	~ 40-50

The amplification efficiency using crude cell lysate (BioGX lysis tubes), DNA isolated with the Fast DNA plant kit and DNA isolated by CTAB were tested using the *sxtA4* assay. The lowest Cycle Threshold (Ct) value in the qPCR assay (data not shown) was achieved using the FAST DNA plant kit (28 Ct). The Cts generated by using crude cell lysate or DNA isolated with the CTAB method were nearly equal (31-32 Ct). This suggests that using crude cell lysate preferred using the BioGX lysis tubes may be a preferred method, as it is fastest extraction method (~10 min, as opposed to 1-4 hours) and generates reliable results. This method has the further advantage that it can be conducted with minimal equipment, and can be carried out on site in aquaculture operations

5.2 Strain isolation and toxin analysis of species of the '*Alexandrium tamarense* group' occurring in east coast Tasmanian waters

In this study, we wanted to determine whether cultured strains of the different '*Alexandrium tamarense'* species complex showed differences in PST toxin production, as found in other studies internationally (Anderson et al 2012). Therefore, we isolated strains at several sites on the east coast of Tasmania, and tested them for PST toxins. During 2013-2014, a total of 36 "*Alexandrium tamarense*-complex" unialgal dinoflagellate strains were cultured, genotyped and analysed for PST using LCMS/MS (Fig 5.3, 5.4, 5.5). The cultures were isolated from a range of sites along the northern, eastern and south-eastern Tasmanian coasts: St Helens (16 strains), Triabunna (8), Norfolk Bay (2), Tamar Estuary (7), Devonport (1), Emu Bay (1) and North West Bay (1). Figs 5.1 and 5.3 show that the average per cell toxicity was highest in cultures of *Alexandrium catenella/fundyense* (Group 1), followed by *Alexandrium pacificum* (Group 4). Strikingly, some Tasmanian cultured strains (ATTRIA-F; 527 pg STX eq/cell) as well as Triabunna field samples (Sept 2015; 538 pg STX equivalent/cell) rank among the most potent on a global scale (Fig 5.3, 5.4, 5.5, see Anderson et al 2012 for global comparison).

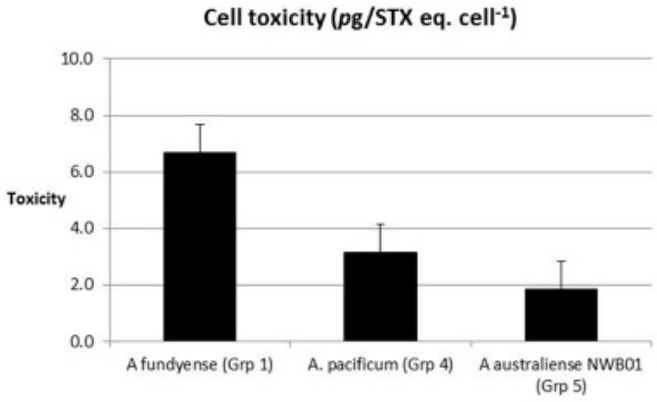


Fig. 5.3. PST cell toxicity of the three *Alexandrium* species present in Tasmania, based on cultures.

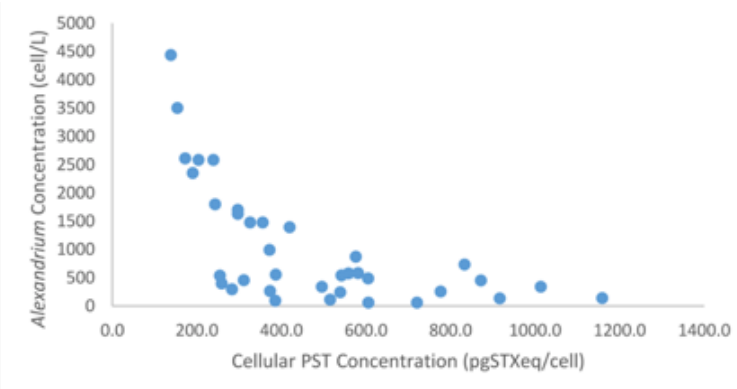


Fig. 5 4. PST cell toxicity of Tasmanian field samples assessed with the Neogen PST kit. Toxicity was highest at low dinoflagellate cell concentrations and in most cases two orders of magnitude higher than toxicity of laboratory cultures

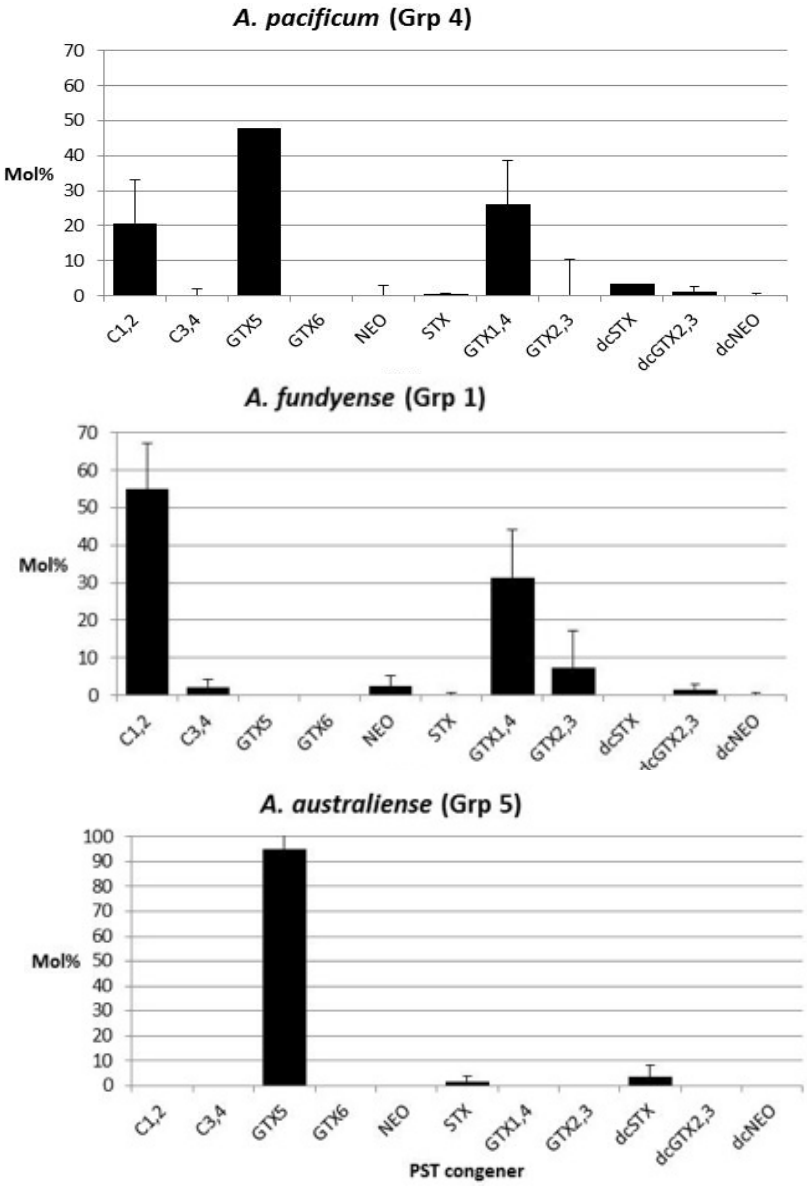


Fig. 5.5. PST toxin profiles of the three *Alexandrium* species present in Tasmania, based on cultures.

These results of the examination of toxin profiles using LCMS/MS showed that cultures of the three species exhibited differences in toxin profiles. *Alexandrium australiense* (Group 5), which had the lowest toxicity of the three species (Fig 5.3), showed an unusual profile dominated by GTX5 analogs. In contrast, *A. catenella/fundyense* (Group 1) showed a profile high in C1,2 analogs, and *A. pacificum* (Group 4) showed a profile high in GTX1,4 analogs, as well as GTX5.

The high toxicity per cell of field samples (Fig 5.4) shows that low *Alexandrium* cell concentrations can be associated with significant PST toxicity. This poses serious challenges for the reliable detection of *Alexandrium* spp. using field samples and microscopy, as the identification and detection of *Alexandrium* spp. at low cell abundances is particularly challenging. Therefore, sensitive detection methods such as those using molecular genetic approaches are necessary to detect *Alexandrium* cells at such low abundances.

5.3. Distribution and abundance of *Alexandrium tamarense* species complex species in Tasmanian east coast sites, 2013-2016

5.3.1 Sample collection

In order to determine the distribution of each *Alexandrium tamarense* species and their associated PST toxicity risk, we collected samples along the eastern Tasmanian coast during 2013-2016, using a combination of shore collections and research vessels (Table 5.5, Fig. 5.6). Samples were also collected at two fixed sites, Triabunna, on the lease of Spring Bay Seafoods (samples were collected by Spring Bay Seafoods), and at Storm Bay, as part of a project conducted by IMAS (Table 5.5, Fig. 5.6).

We analysed data from: 1) the species-specific qPCR assays that we developed (see Section 5.1); 2) qPCR analyses conducted on farm (Spring Bay Seafoods) using the *sxtA4* gene probe and cell lysis tubes method; 3) PST analyses conducted by TSQAP and; 4) light microscopy-based cell counts, where available.

We synthesised this information to analyse the distribution and abundance of *Alexandrium* species and their associated PST toxins in the region. We also examined a dataset collected by TSQAP and AST from 2013-2014, based on light microscopy and showing the abundance of *Alexandrium* species (all Groups) in the region.

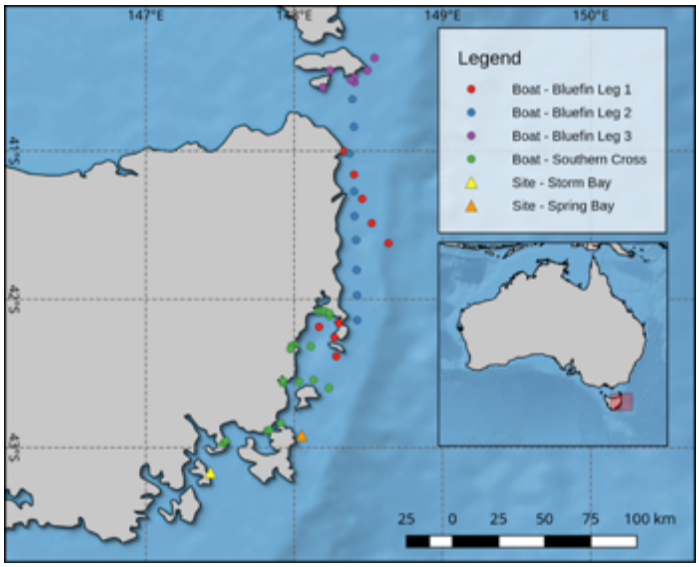


Fig. 5.6. Map of sites for which we took samples to assess the presence and abundance of different toxic species of the *Alexandrium tamarense* complex.

Table 5.5. Sites and dates for samples collected 2013 - 2016 to determine *Alexandrium* species presence and distribution in Tasmanian shellfish growing areas.

Sample set	Location	Boat collection or shore collection	Dates	Type of sample	Analysis methods
1	Little Swanport – Spring Bay	Shore collection	15th September 2015	Net samples	qPCR
2	Eddystone Point - Freycinet	Boat - Bluefin Leg 1	September 2014	1 L seawater	qPCR and microscopy
3	Flinders Island – North East Coast	Boat Bluefin Leg 2	September 2014	1 L seawater	qPCR
4	Storm Bay	Shore collection	December 2014-April 2015	1 L seawater	qPCR
5	Spring Bay	Boat collection	2013-2014, 2016	500 ml seawater	qPCR, microscopy
6	Freycinet – Pipe Clay Lagoon	Boat- Southern Cross	22-26 August 2016	1 litre seawater	qPCR, microscopy

5.3.2 Seasonality and annual variation of *Alexandrium tamarense* species complex

The *Alexandrium* abundance data collected by TSQAP during 2013- 2014 showed that misidentifications of other species as *Alexandrium tamarense* complex species can occur using light microscopy (Fig 5.7). Based on these data from 2013/2014, it appears that species of the *Alexandrium tamarense* species complex occur most frequently during winter/autumn months on the east and south-east coasts (Fig. 5.7). In general, species were first detected during June-August in 2013/2014, and continued until September-October.

During winter-autumn 2013 and 2014, abundances of *Alexandrium tamarense* complex species were generally low along the eastern coast of Tasmania (Figs 5.7, 5.8), based on data from TSQAP, as well as samples that we collected for this study (Figs 5.8, 5.9).

Offshore and shelf/edge samples were collected in September 2014 during a RV Bluefin cruise (Fig 5.8). Cells were not detected by microscopy-based cell counting at any sites except Ansons Bay (site 1). This is likely due to the fact that *Alexandrium tamarense* species complex cells were either absent or below the limit of detection of the method (< 10 cells/L) (Figs 5.8). Using qPCR, we detected the presence of the *sxtA4* gene in several samples, in low abundances. As cell abundances were very low (< 20 cells/L), no PST toxins were found in shellfish samples in this region (Bluefin Legs 1-2, see Table 5.5) at this time.

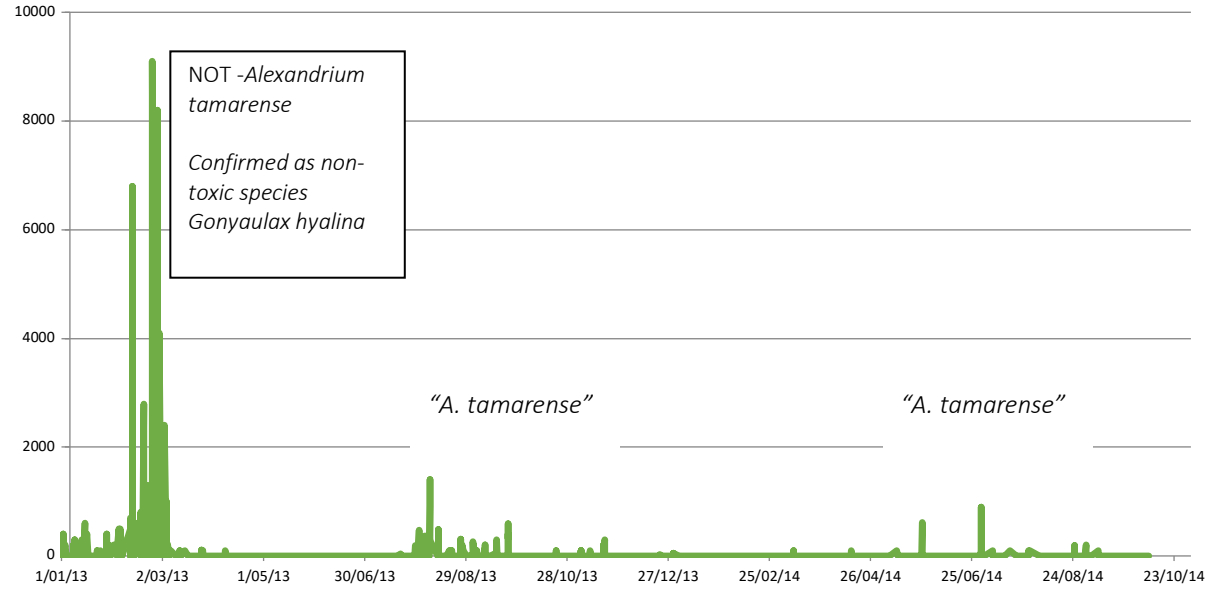


Fig. 5.7. Seasonality of *Alexandrium tamarense* complex species (not discriminated) along eastern Tasmanian coast from 2013-14, based on samples collected by TSQAP and analysed by Analytical Services Tasmania.

During 2013-2014, samples collected at Spring Bay (Table 5.5) showed very low but detectable PST levels in shellfish, consistent with low equivalent *Alexandrium tamarense* species complex cell concentrations, which were detected using the *sxtA4* gene qPCR (Fig 5.9). Considerably higher shellfish toxicity and *Alexandrium tamarense* species complex cell concentrations were detected at Spring Bay during July – October 2016 (exceeding 8 mg/kg) (Fig. 5.10). We found a strong temporal correlation with increasing and decreasing copy numbers of the *sxtA4* gene detected by qPCR, with the peak in *sxtA4* gene abundance preceding the peak toxicity in shellfish flesh by several weeks (Fig 5.10). We therefore found that *sxtA4* abundance is a potentially useful lead indicator for subsequent shellfish toxin accumulation. The abundance of *sxtA4* was used by Spring Bay Seafoods as an indicator of the impending PST bloom, and based on the qPCR results, as well as the initial

low PST samples, Spring Bay Seafoods undertook a voluntary closure during mid 2016. Spring Bay Seafoods has continued to undertake weekly *sxtA4* qPCR assays during 2017.

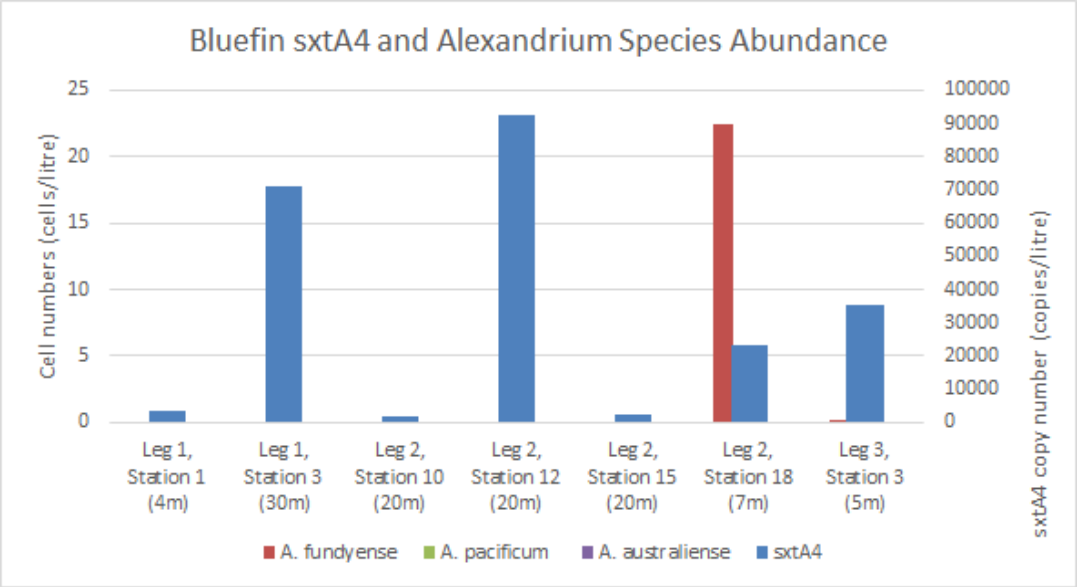


Fig. 5.8. Sites at which *Alexandrium* species were detected during the Bluefin Cruise, in September 2014.

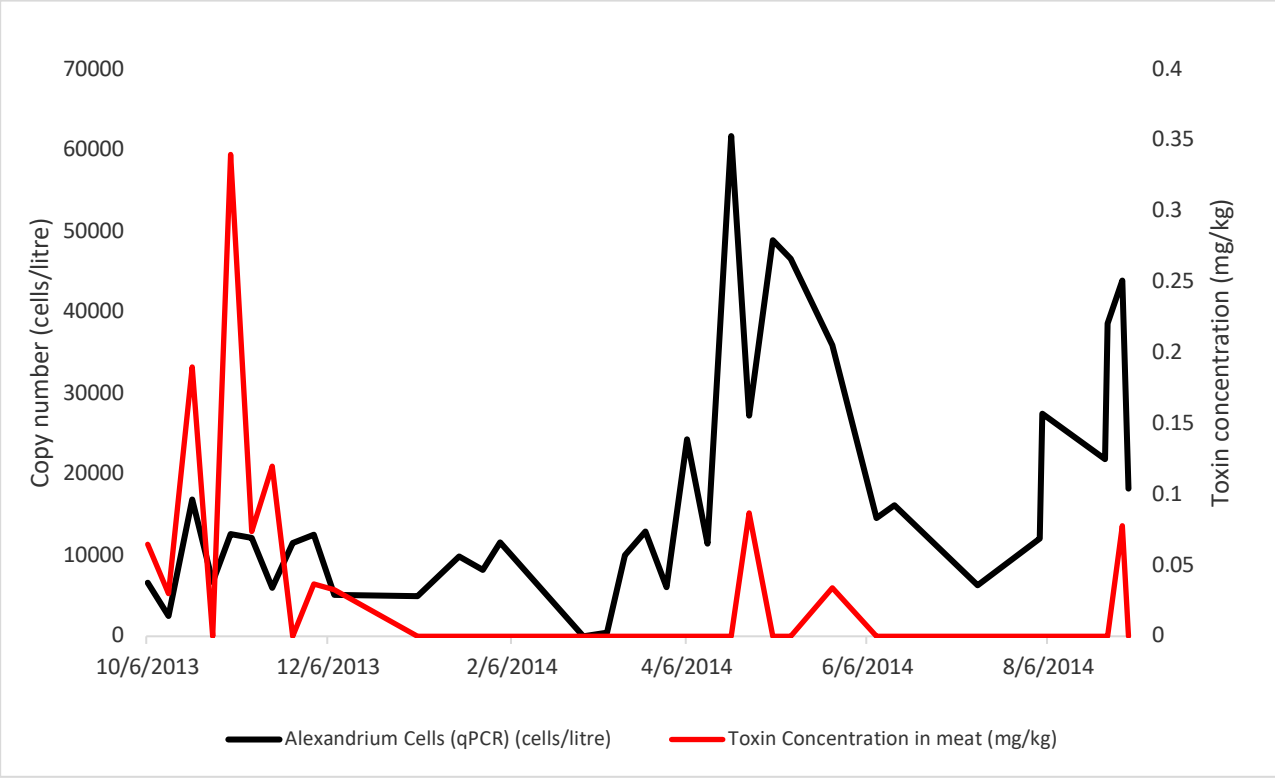


Fig 5.9. *Alexandrium tamarense* complex detected using qPCR of the *sxtA* gene, compared to PST concentrations in the mussel flesh, at Spring Bay from October 2013 – September 2014.

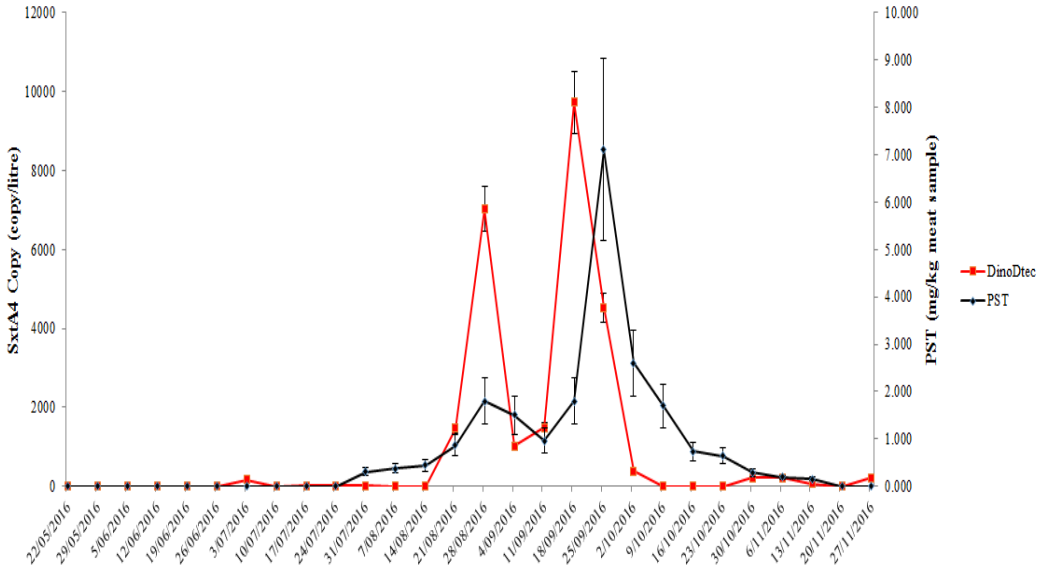


Fig. 5. 10. *Alexandrium tamarense* complex cell concentration detected using qPCR of the *sxtA* gene compared to PST concentrations in the mussel flesh, at Spring Bay from May to November, 2016.

5.3.3 Relative abundances of different species of the *Alexandrium tamarense* species complex, and contribution to PST toxicity

In order to gain an understanding of the dynamics of blooms of *Alexandrium tamarense* complex species, we used species specific qPCR assays, *sxtA* assays, and light microscopy to distinguish species in samples taken at several sites on the 15th September 2015, as well as during the Southern Cross research cruise (Table 5.5), conducted during August 2016, which was a time of high *Alexandrium tamarense* species complex abundance and PST detection (Fig. 5.10).

From the samples collected using net tows at six locations on a single day in September 2015 at Spring Bay, Blackmans Bay and Little Swanport) qPCR ananalysis showed that the population consisted of mixtures of *Alexandrium catenella* (Group 1) and *A. pacificum* (Group 4) (Fig. 5.5). At Spring Bay, Mercury passage sites were dominated by *A. catenella*, whereas near-shore sites contained *A. fundyense* with significant proportions (circa 50%) *A. pacificum*, as did samples on the shelf near Little Swanport, and near shores samples at Blackman Bay Jetty (Fig. 5.5).

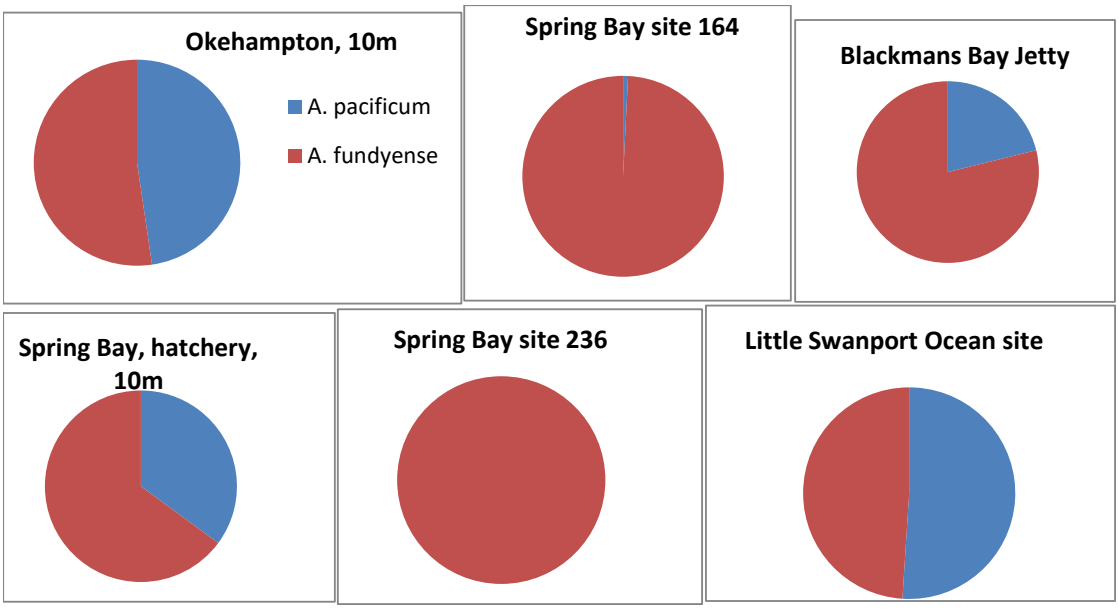


Fig. 5.11. Ratios of *Alexandrium catenella/fundyense* (Group 1) and *Alexandrium pacificum* (Group 4) in plankton net samples from sites in Spring Bay and Little Swanport on 15 Sept 2015. Abundances were generally low at this time.

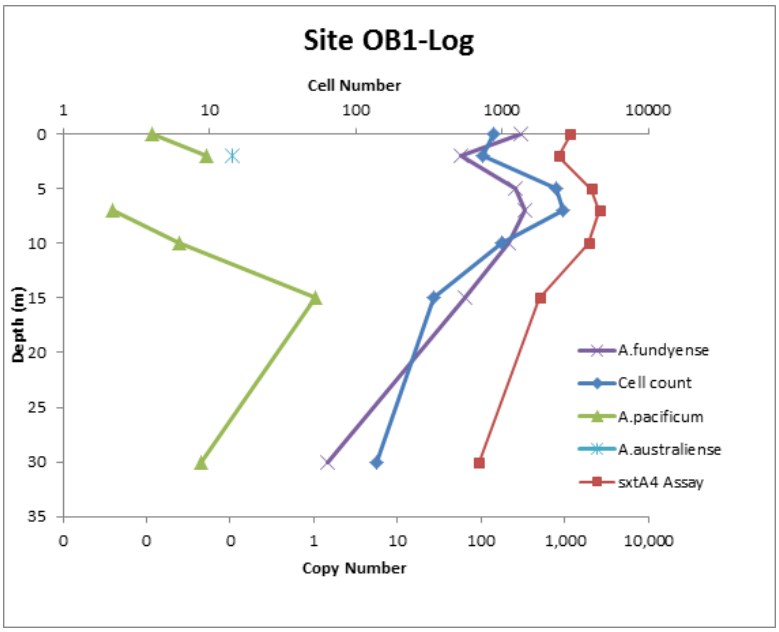


Fig. 5. 12. Comparisons of *Alexandrium tamarense* complex detection using cell counts, species-specific qPCR detection, and qPCR of the *sxtA4* gene. Samples collected at site 1 in Great Oyster Bay during Southern Cross cruise (22nd-26th August 2016).

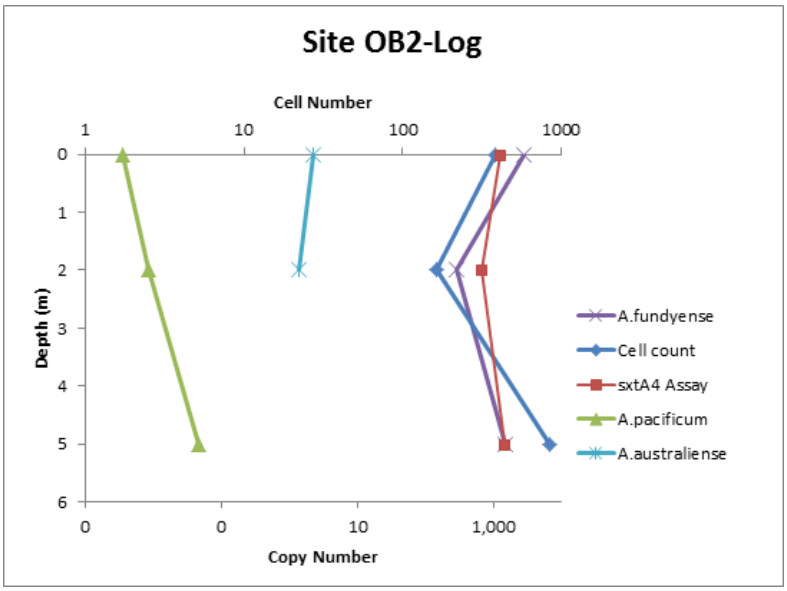


Fig. 5.13. Depth profile of *Alexandrium tamarense* species complex, the sxtA4 assay, and a cell count using microscopy, taken on the Southern Cross from 22nd-26th August 2016 at Great Oyster Bay inshore site 2.

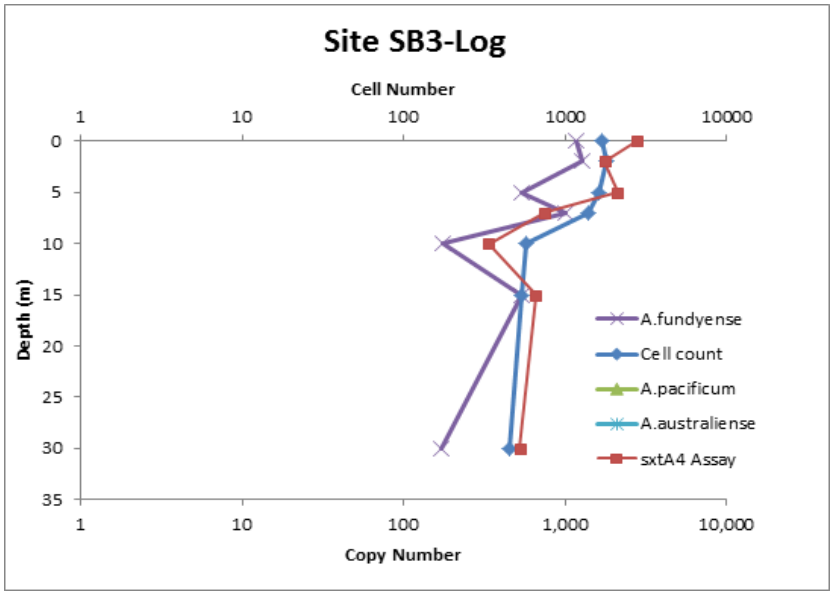


Fig. 5. 14. Comparisons of *Alexandrium tamarense* complex detection using cell counts, species-specific qPCR detection, and qPCR of the *sxtA4* gene. Samples collected Spring Bay site 3 during the Southern Cross cruise (22nd-26th August 2016).

Samples taken from Great Oyster Bay and Spring Bay during August 2016 (Figs 5.12, 5.13, 5.14) had high concentrations of *Alexandrium tamarense* species (generally >1000 cells/L). The samples showed a strong depth stratification of *Alexandrium tamarense* complex species, with several fold changes in cell abundances at the lowest depths (30 m, Fig 5.12). Each of the three samples analysed were dominated by *A. catenella/fundyense* Group 1, with comparatively low concentrations of *Alexandrium pacificum* and negligible concentrations of *A. australiense* detected at this time. The relative abundance of *sxtA4* gene copy number was strongly correlated with the cell counts based on light microscopy.

5.4. Conclusions

1. Three species of the *Alexandrium tamarense* species complex can co-occur in Tasmania, with *A. australiense* (Group 5) to date appearing to be of negligible toxicity in most areas and not contributing significantly to shellfish toxicity on the eastern coast.
2. *A. catenella/fundyense* (Group 1) is to date confined predominantly to the eastern coast and as far south as the western side of the Tasman Peninsula. However, qPCR evidence indicates that it occurs in low concentrations as far north as the southern coast of Flinders Island. *Alexandrium catenella/fundyense* is capable of producing very high cellular toxicity, particularly at low cell concentrations, thus explaining the accumulation significant PST in shellfish
3. *Alexandrium catenella/fundyense* (Group 1) is the dominant species during blooms on the eastern coast, but blooms likely contain highly variable proportions of *Alexandrium pacificum.*
4. The quantitative PCR assays developed are capable of distinguishing the different species, and the *sxtA4* gene assay is a useful lead indicator of the subsequent accumulation of PST toxins in shellfish flesh, if routinely measured on a weekly basis.

The varying co-occurrence of *Alexandrium catenella/fundyense* and *Alexandrium pacificum* in east coast Tasmanian blooms confounds progress in elucidating bloom ecophysiology as well as predicting of shellfish toxicity. These two species (1) cannot be discriminated by light microscopy; (2) have different PST toxin profiles; resulting in markedly different cell toxicity; (3) and may have differing ecophysiology. Total *Alexandrium tamarense* complex cell abundance is therefore not a good predictor of shellfish toxicity. We recommend that every Tasmanian *Alexandrium* bloom event be subjected to species-specific qPCR detection, and that larger shellfish farms consider the use of routine on site *sxtA4* qPCR detection, to guarantee the correct interpretation of results and to act as an early warning system.

6. Use available east coast Tasmania oceanographic data, current patterns and models (including satellite imagery) to define time periods and geographic zones most at risk of physical conditions favourable to algal blooms

Scott Condie, Brian Hatfield and Gustaaf Hallegraeff

Experiences across a diverse range of marine and freshwater systems indicate that environmental conditions can have a major influence on the development of harmful algal blooms or HABs (Hallegraeff *et al.*, 1995; Bormans & Condie, 1998; Weise *et al.*, 2002; Anderson *et al.*, 2012; Yamamoto *et al.*, 2013). We investigated the influences of meteorological and oceanographic conditions on the development of blooms of the toxic dinoflagellate species, *Alexandrium tamarense*, off eastern Tasmania. The results represent a significant first step towards forecasting the development of HABs in this region.

There are major challenges associated with forecasting HABs, particularly in relation to the complexity of the marine environment across a broad range of temporal and spatial scales. However, based on a very limited number of observed HAB events off eastern Tasmania (during 2012, 2015, 2016 and a more localized event in 2013) our analyses suggest that environmental factors, such as water column stratification and coastal circulation, are important drivers that can provide a foundation for forecasting future blooms.

6.1. Methods

We have used a combination of weather information, in situ ocean measurements, remote sensing and hydrodynamic model outputs to explore potential links between the occurrence of *A. tamarense* blooms and environmental conditions off eastern Tasmania. Key data sets are summarised in Table 6.1. The location and timing of blooms were inferred from toxin levels detected in shellfish. Weather information was accessed through the Bureau of Meteorology website and remote imaging of sea surface temperature and surface chlorophyll was accessed through the Integrated Marine Observing System (IMOS) website. In situ profiles of temperature and salinity from the Maria Island Reference Station were also accessed through IMOS.

Presented by Scott Condie at the second Australian Forum for Operational Oceanography conference held in Fremantle on 25-27 July 2017.

Table 6.1. Meteorological and oceanographic data used in the analysis of HABS off eastern Tasmania.

Region	Data description	Data source
Tasman Sea	Satellite SST (2012-2016) Satellite ocean colour (2012-2016) Altimeter sealevel (2012-2016) Geostrophic current velocity (2012-2016)	http://oceancurrent.imos.org.au
Tasmanian continental shelf	Ocean temperatures from Maria Island monthly sampling (1956-2015) Modelled shelf circulation (2012-2015)	http://imos.org.au/nrsvesselsampling.html (Oliver *et al.*, 2016)
Tasmanian east coast	Rainfall from coastal weather stations (1951-2016) Air temperature from coastal weather stations (1968-2016)	http://www.bom.gov.au/climate/data/

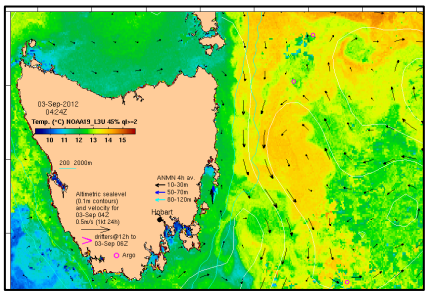
The hydrodynamic model included realistic forcing by river discharges, winds and offshore conditions, but excluded tides (Oliver et al., 2016). Model results were used to explore coastal stratification and circulation patterns, as well as forming the basis for dispersal modelling using the CONNIE3 analysis and visualisation tool (www.csiro.au/connie/). Statistical analyses (Mann-Whitney U test and z test) were also undertaken to identify significant relationships between monthly rainfall conditions and the presence of HABs.

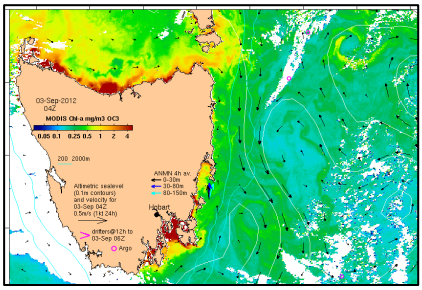
6.2. The offshore oceanographic environment

While there is no evidence that *A. tamarense* blooms originate offshore in the Tasman Sea, the offshore environment may play a role in moderating coastal water conditions. The East Australia Current (EAC) flows southward, although off eastern Tasmania current direction is strongly dependent on the location of individual ocean eddies. Flow on the adjacent continental shelf has been predominantly northward in spring, with relatively uniform SST and chlorophyll decreasing offshore (Figs 6.1 a, b, c, e). 2015 was an exceptional year with warm EAC water intruding over the shelf in the north, then separating from the coast at Freycinet Peninsular. South of Freycinet, shelf waters were anomalously cold (Figure 6.1d) consistent with high rates of heat loss to the atmosphere.

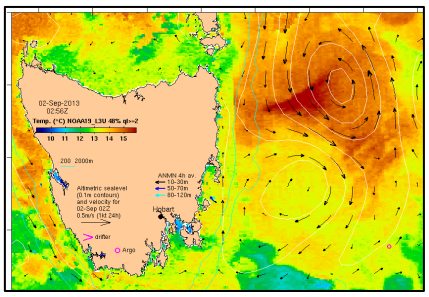
Thermal stratification over the continental shelf usually developed over spring (Fig. 6.2). Since 2009 there has been a tendency towards earlier development of stratified conditions. Interestingly, the strongest and most persistent stratification over the 60-year record (1956-2015) occurred in 1963, 2009, 2012 and 2015, suggesting a potential correlation with HAB events, although there is no evidence that *A. tamarense* blooms extend this far offshore. While water samples collected from the Maria Island National Reference Station (MINRS) in November 2016 contained anomalously high concentrations of other dinoflagellate species, *A. tamarense* cell counts remained low (Ruth Eriksen, pers comm.). This is consistent with dispersal modelling for this period, which does not reveal any exchange from known coastal HAB sites to the MINRS (Fig. 6.3).

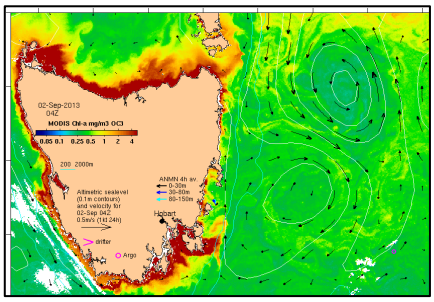
(a) 2012



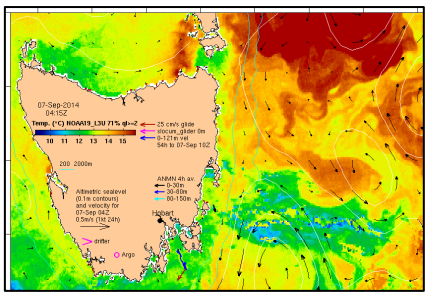


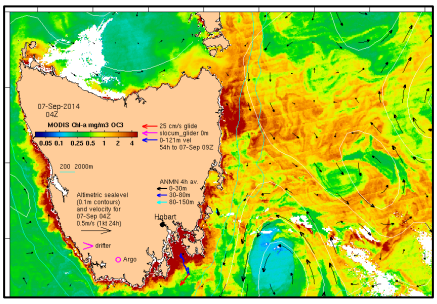
(b) 2013



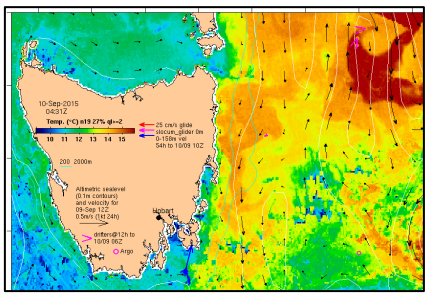


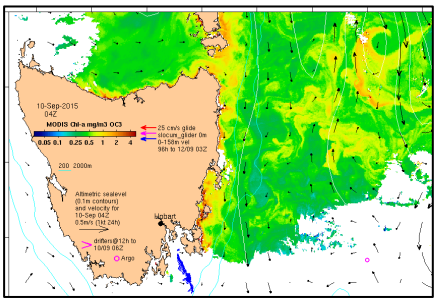
(c) 2014



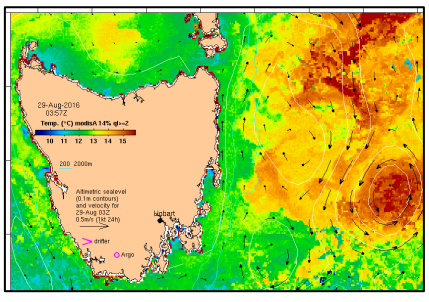


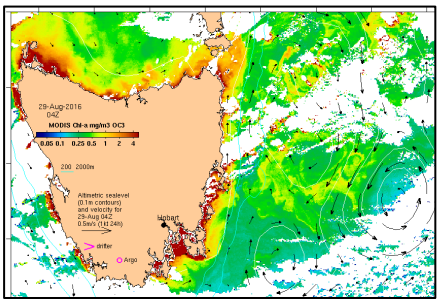
(d) 2015





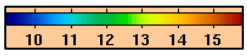
(e) 2016





→0.5 m/s

SST (°C)



Chl-*a* (mg/m3)

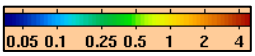


Fig. 6.1: Satellite sea surface temperature (SST) from NOAA AVHRR sensors (left) and satellite ocean colour from the NASA MODIS sensor (right) in early spring. Images also show contours of sea surface height (combining data from satellite altimeters and coastal tide gauges) and geostrophic currents estimated from gradients in sea surface height. Further details are available through the IMOS Ocean Current site (http://oceancurrent.imos.org.au/sourcedata/index.php).

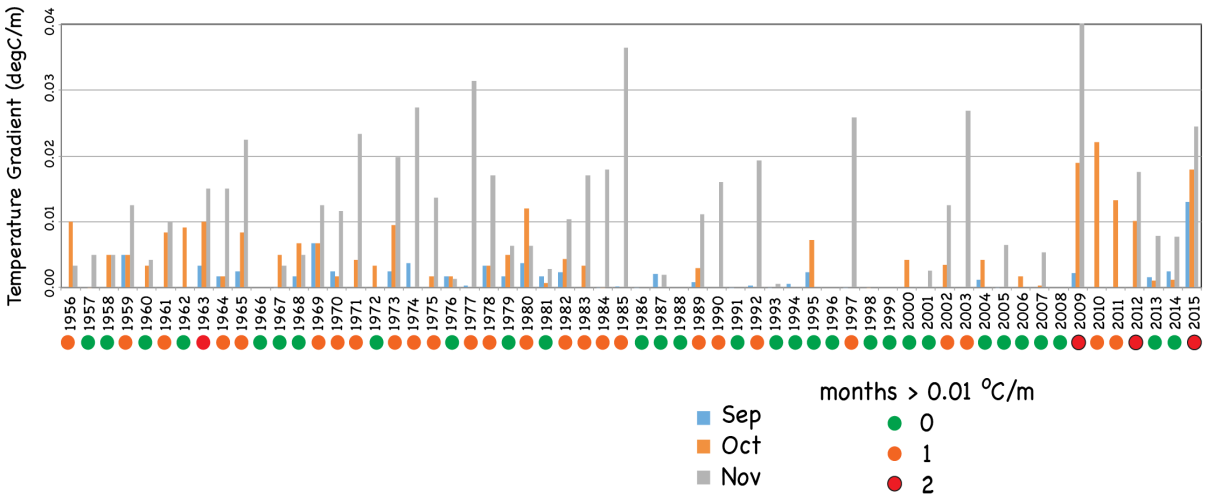


Fig. 6.2 . Measured median monthly temperature gradient during Spring at the Maria Island National Reference Station. Traffic lights indicate the number of months in each spring that the gradient exceeded a threshold of 0.01°C/m.

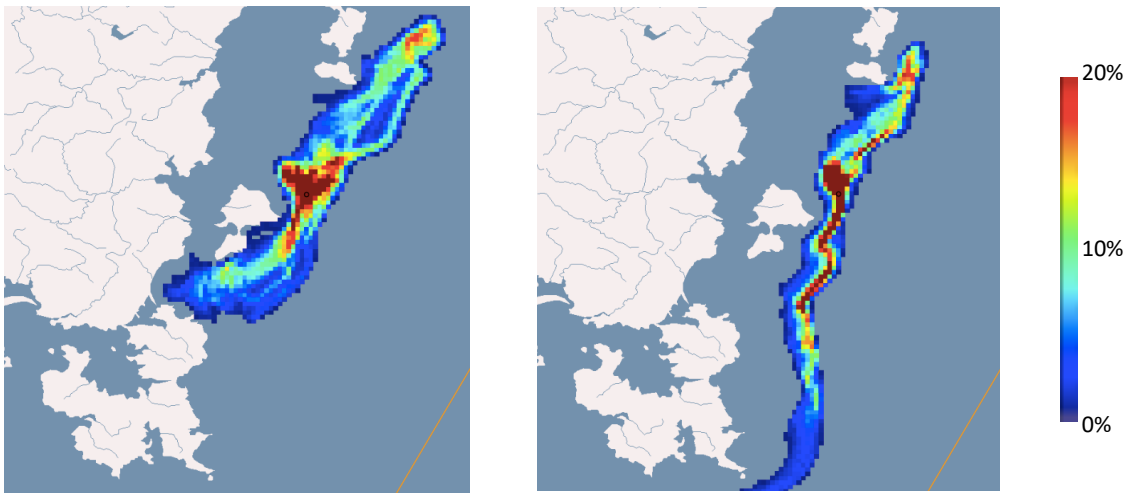


Fig. 6.3 . Sources of water arriving at the Maria Island National Reference Station over the period 14-21 November 2016, having dispersed for 7 days at depths of 4 m (left) and 9 m (right). The maps were generated using the dispersal model CONNIE3 (www.csiro.au/connie/) and show the percentage of particles that passed through each cell (approximately 1 km x 1 km) before reaching the reference station cell.

6.3. Meteorological conditions

Rainfall off eastern Tasmania is highly variable (Fig. 6.4a) and has the potential to significantly influence coastal conditions and associated marine ecological processes. In particular, high monthly rainfall appears to be a consistent precursor to HABs. In months when water temperatures were within a favourable range (10-15°C), high rainfall (> 80 mm per month) was followed by blooms events in 2012, 2013 and 2016 (Table 6.2, Fig. 6.5). Considering all months with favourable water temperatures within the period 2012-2016, the median monthly rainfall was 40 mm (inter-quartile range = 25 mm) for months when blooms were not detected and 47 mm (inter-quartile range of 107 mm) for months when blooms were detected. However, the relationship was not significant ($U = 643$, $N_{HAB} = 21$, $N_{clear} = 78$, $z = 1.50$, $p < 0.05$; Mann-Whitney U test and z test). It was only when the 2015 data was removed that a significant relationship emerged ($U = 268$, $N_{HAB} = 16$, $N_{clear} = 60$, $z = 2.70$, $p < 0.05$; Mann-Whitney U test and z test). The 2015 bloom event observed south of Great Oyster Bay was exceptional in that it occurred during a low rainfall period. However, 2015 was also an exceptionally cold winter along the east coast of Tasmania. Monthly mean minimum water temperature dropped to record levels in the southeast (Fig. 6.4b) and there was associated cooling of coastal waters (Fig. 6.1d). The implications of this cooling for water column stratification will be explored below.

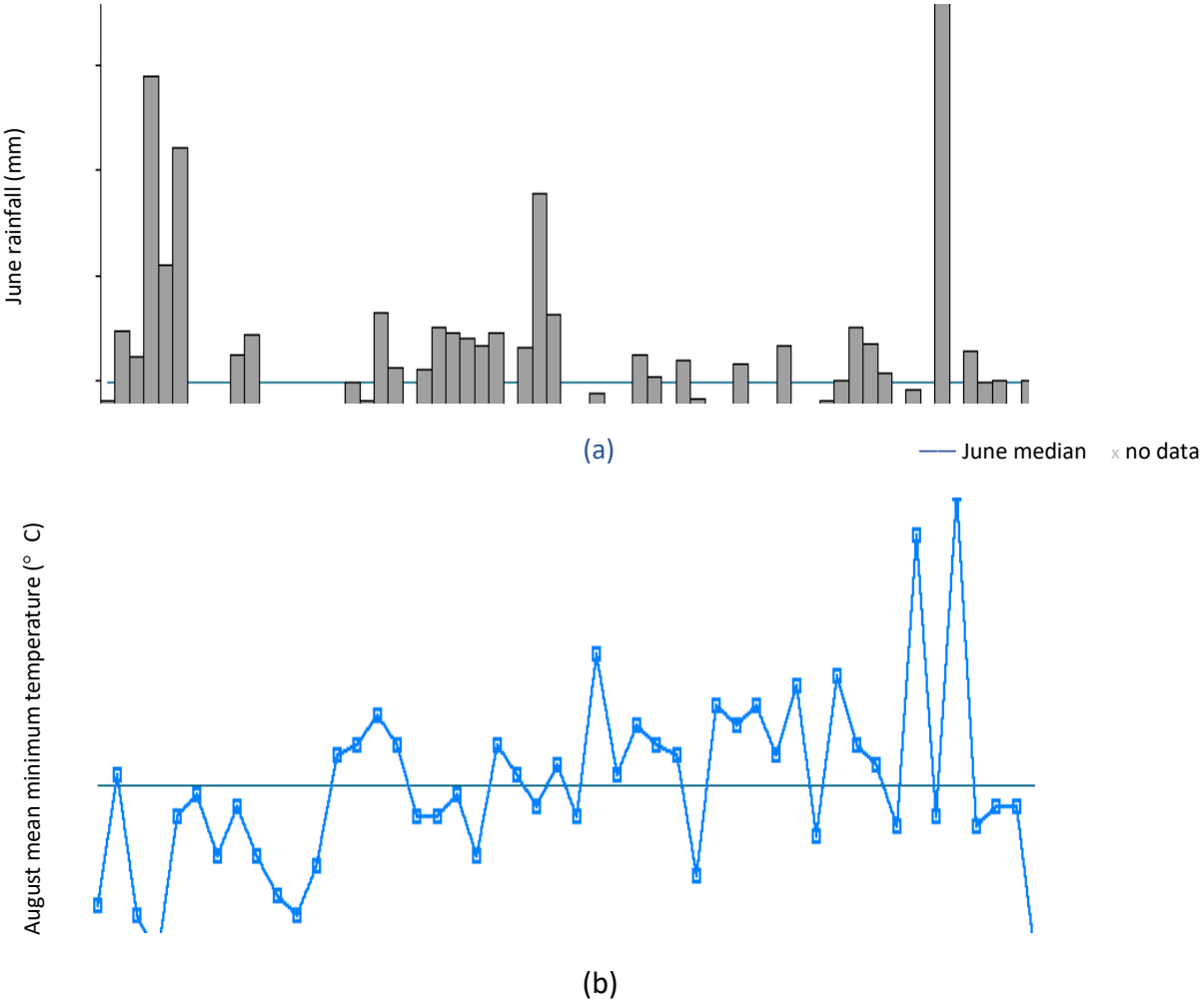


Fig. 6.4: Time series from Orford (Aubin Court) of (a) June rainfall; and (b) August mean minimum temperature (Australian Bureau of Meteorology: http://www.bom.gov.au/jsp/ncc/cdio/weatherData/av?p_nccObsCode=139&p_display_type=dataFile&p_startYear=&p_c=&p_stn_num=092027).

Table 6.2: Rainfall in east coast regions. Highlighted are months with rainfall > 80 mm (underlined), water temperatures in the range conducive to blooms (pale orange) and those during which local blooms were present (as detected through analyses of toxin levels in shellfish, red).

REGION	YEAR	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
MOULTING BAY (ST HELENS AERODROME)	2012	19.6	61	79.6	35.6	179	78.4	15.6	25.8	86.4	22.8	44.6	33.8
	2013	19.8	60.6	65.6	19.6	35.6	88	45.2		62	70	291	60.6
	2014	29.8	13.2	65.4	71.8	11.8	45.2	65.6	20.6	34.2	63	13.2	65.4
	2015	66.6	61.6	40.6	44.2	37.8	38.8	44	34	52.6	9.4	112	28
	2016	193	16.2	107	26.2	104	180	70	25	96	49.8		
GREAT OYSTER BAY (SWANSEA – FRANCIS STREET)	2012	21.4	28	32.4	42.4	115	50	23.5	26.8	40.5	36.2	28.4	31.4
	2013	16.2	51.4	26.9	39.4	27.8	62.9	42.8	20.8	37.8	44.2	230	19
	2014	8.6	19.3	26.8	26.6	20.8	45.2	55.3	14.6	11.2	40.2	16.6	54.4
	2015	92.8	66.1	56.7	30.5	14.6	38.7	14	22.6	39.2	12.2	44	25
	2016	242	29.5	50.2	9.4	67	153	34.6	18	106	62.4		
GREAT OYSTER BAY (COLES BAY – FREYCINET)	2012	25.1	21.2	56	60.5	140	57.1	22	42.6	62.2	34.6	46.2	27.9
	2013	16.8	77	51	36.9	31.9			24	53.8	30	235	29.6
	2014	13.1	33.8	31.8	33.2	38.2	19	78.2	51.8	6.5	36.2	77.8	59.2
	2015	137	53.2	75.6	41.6	16.4	42	32.5	38.6	71.8	10.1	85.6	24.2
	2016	182	21.2	38.5	13.7	99	241	29.5	20.4	145			
LITTLE SWANPORT (LISDILLON FARM)	2012							17.2	26.6	45.8	30.6	47	19.6
	2013	24.2	57.6	31.8	17.2	19.4	58.2	53	39.2	43.6	53.4	210	19.4
	2014	5.2	23.2	29	24.2	27.4	30.6	69	14.8	8.8	48.8	16.8	55.6
	2015	98.6	54.4	49.2	20.2	21.2	49.4	7.6	22.8	30.4	19.8	46.8	22
	2016	283	41.6	37.6	7	75.6	184	50.6	16	130	56		
SPRING BAY (AUBIN COURT)	2012	30.2	26	34.8	50.6	160	48.8	21.4	37.4	51.3	50.2	43.2	33
	2013	30	46.2	46.6	24.6	22.8	50.1	77	46.8	63.4	42.6	184	22.9
	2014	9.9	20	24.6	22	34	38.5	67.1	58.8	13.7	44.4	29.8	79.8
	2015	116	43.4	50.2	26	26.6	49.8	19.9	20.2	23.6	14	43.8	30.2
	2016	236	29.4	18.4	10.6	74.8	206	48.6	12.2	132	45		
ORFORD SOUTH	2012	31.2	27.2	31.8	46.2	166	36.2	21.2	31	36	50.6	44	36.8
	2013	30.6	56.4	31.2	22.8	21.2	58.8	83	54.2	37.4	42.4	171	22
	2014	11.4	21.4	19.2	20.2	35.8	35.8		40.6	11.4	37.4	33	69
	2015	112	33	64	20.2	14.2	50.6	19	44	22.2	8.6		26
	2016	286	30.6	22.6	6	84.8	143	39.2	20.8	104	45		

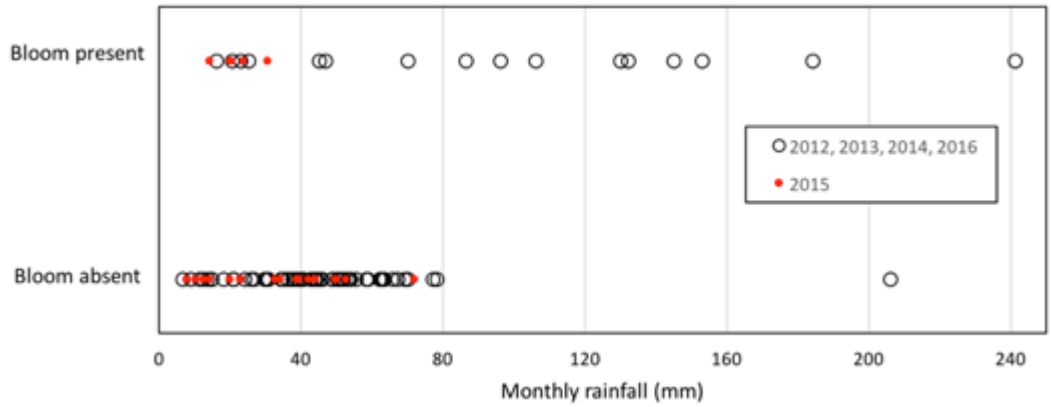


Fig. 6.5: Rainfall during months where *A. tamarense* blooms were present and absent (as detected through analyses of toxin levels in shellfish).

6.4. Coastal circulation

Results from the coastal circulation model indicate that bloom events were generally accompanied by persistent (subtidal) surface currents directed onshore (Fig 6.6). This implies downwelling flow along the coast, which in this system could be driven by winds from the south, southeast or east (historically around 30% of spring winds: http://www.bom.gov.au/climate/averages/wind/selection_map.shtml). These flows would be expected to retain positively buoyant algae near the coast, and this was confirmed by the dispersal modelling. For example, 7-day dispersal plumes around the 2016 HAB sites extended only 2-4 km (not shown) compared to 50 km around the offshore MINRS site (Fig. 6.3).

Another notable feature of the model results is the production of anomalously cold water (< 10°C) inside Great Oyster Bay in July and August 2015 (Figs 6.6 and 6.7). As this cold water sank and flowed out of the mouth of the bay beneath warmer shelf water, it created a large region of enhanced thermal stratification encompassing Little Swanport (Fig 6.7d). Similarly, cooling in the shallows of Mercury Passage (west of Maria Island in Fig 6.6d) enhanced the stratification around Spring Bay.

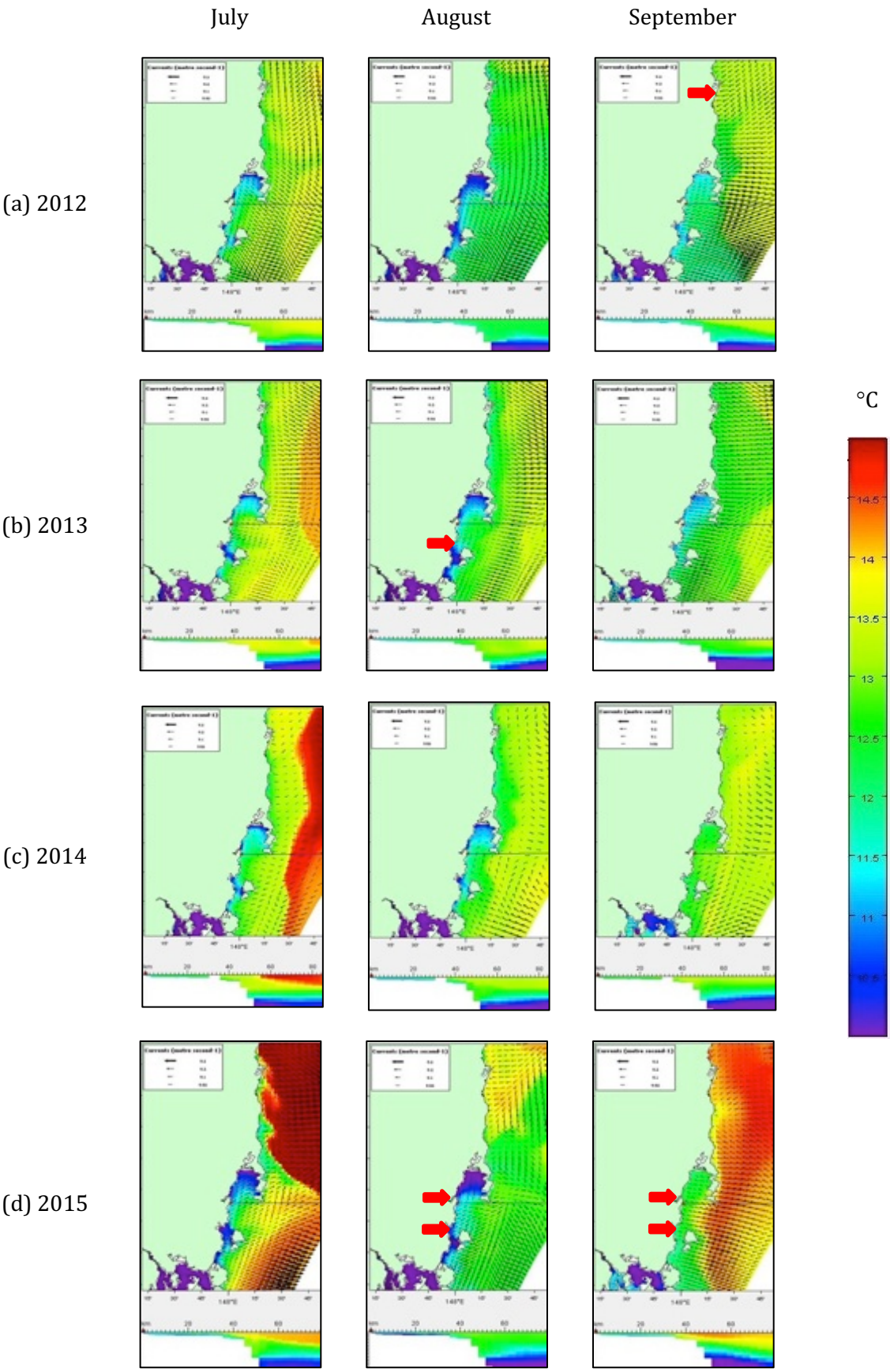
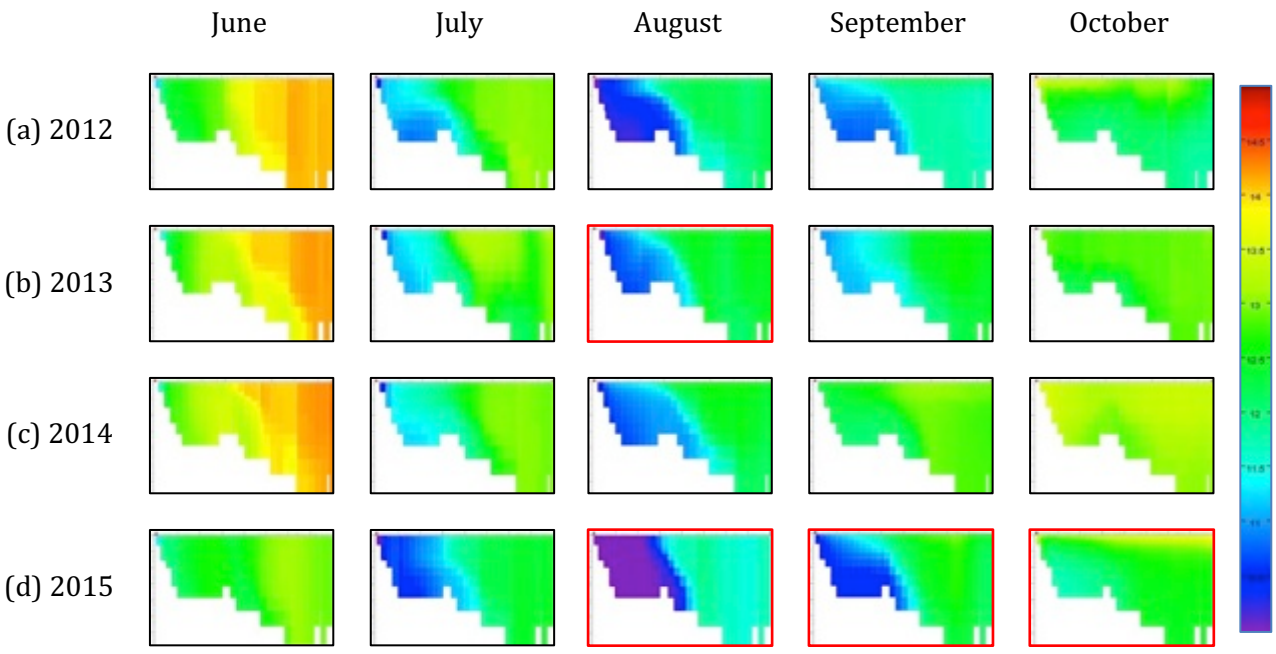
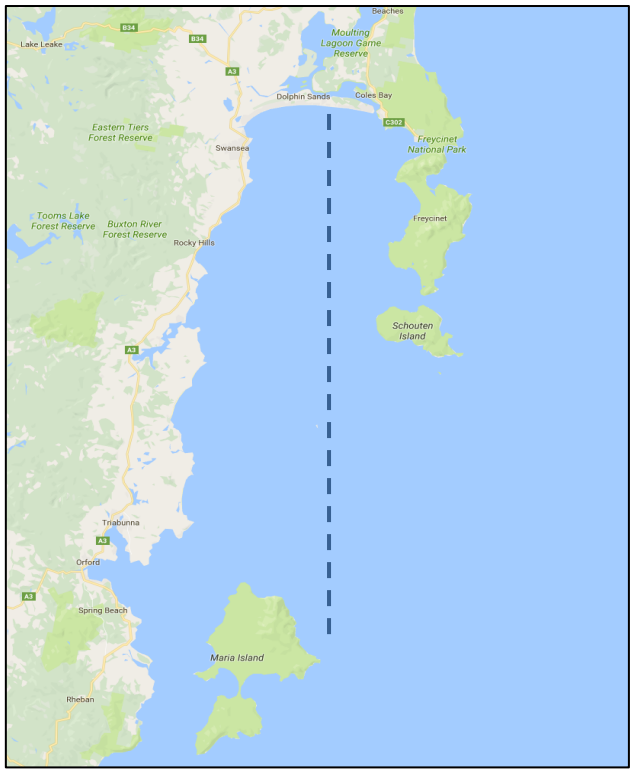

July
August
September
(a) 2012
(b) 2013
(c) 2014
(d) 2015
°C
14.5
14
13.5
13
12.5
12
11.5
11

Fig. 6.6: Surface temperature and surface current velocity vectors from the coastal circulation model (Oliver et al. 2016). An east-west vertical temperature section across the mouth of Great Oyster bay is also shown at the bottom of each panel. Observed coastal bloom event are indicated by red arrows.





(e) North-south section through Great Oyster Bay

Fig. 6.7: Vertical temperature section along the north-south axis of Great Oyster Bay (80 km long with a maximum depth of 70 m) from the coastal circulation model (Oliver 2016). Periods of coastal bloom events at Little Swanport and Spring Bay are framed in red.

6.5. Environmental drivers

The observations and model results describe above suggest that bloom events in 2012, 2013 and 2016 each followed periods of high local rainfall. The associated freshwater river plume enhanced coastal stratification, thereby suppressing vertical mixing of nutrients and plankton (Fig. 6.8a). Coastal stratification was also enhanced during the 2015 bloom period, albeit through a different mechanism involving the outflow of anomalous cold water in Great Oyster Bay and Mercury Passage (Fig. 6.8b).

While a range of environmental processes can potentially interact with *A. tamarense* ecology, stratification appears to be a strong underlying driver for bloom formation. This hypothesis is consistent with observations elsewhere (Anderson *et al.*, 2012; Yamamoto *et al.*, 2013) and theoretical ideas suggesting that the stability associated with a stratified water column can provide dinoflagellate species with a significant competitive advantage over diatom species (Condie & Bormans, 1997). In stably stratified systems diatoms tend to sink out of the photic zone, whereas positively buoyant dinoflagellates accumulate in the photic zone. Additionally, the absence of vertical mixing can lead to rapid depletion of nutrients (nitrogen and silicate) in the photic zone, which is again much more limiting for diatoms than for dinoflagellates.

Results from the coastal circulation model also reveal that blooms were associated with onshore surface flow (Fig. 6.6), which implies downwelling flow near the coast and depletion of coastal nutrients. These conditions are also advantageous to positively buoyant dinoflagellates and limiting to diatoms. Specifically, diatoms tend to follow downwelling flow offshore and descend below the euphotic zone, whereas dinoflagellates rising toward the surface tend to be carried onshore where they accumulate (Condie & Sherwood, 2006).

6.6. Implications of longer term environmental change

The ocean environment east of Tasmania is well documented as a climate change hotspot, characterized by a stronger EAC and rapidly increasing ocean temperatures (Suthers *et al.*, 2011). Higher water temperatures are likely to favour earlier winter blooms (as observed in 2016). However, 2015 conditions could also become more common if these warm waters spread over colder inner shelf waters so as to enhance the thermal stratification across the shelf. Decreased nutrient concentrations (including silicate) associated with a strengthening EAC (Condie & Dunn, 2006) are also likely to favour dinoflagellates and *A. tamarense* over diatoms.

Climate driven changes in seasonal rainfall are also potentially important. Downscaled climate projections from the IPCC Fourth Assessment Report predict relatively little change in east coast winter rainfall (Grose *et al.*, 2013). However, rainfall projections for this region are highly uncertain due to the dependence on episodic high-intensity cutoff lows in the atmosphere, that tend to be poorly resolved by most climate models (Grose *et al.*, 2012).

6.7. Implications for forecasting HABs off eastern Tasmania

There have been only a small number of *A. tamarense* blooms recorded off eastern Tasmania and the available environmental data is limited. However, there are patterns emerging that are broadly consistent with theoretical expectations and empirical research conducted in other regions where *A. tamarense* has been problematic over a number of decades. Early identification of the biophysical relationships that might underpin a forecasting system should help to guide ongoing research by shifting the focus towards risk assessment and mitigation.

We propose a tentative framework as a first step towards developing a HABs forecasting system for the region (Fig. 6.9). The approach includes use of existing (low cost) meteorological information such as rainfall, air temperature and wind direction, as well as proposing new in situ monitoring (and modelling) of coastal waters. Hence, it should be feasible to implement a limited forecasting system quite rapidly, with a view to expanding its capability over the next few years. Given the short history of *A. tamarense* bloom observations off eastern Tasmania, implementation of any forecasting system will require ongoing assessment, refinement and validation.

It is also proposed that any forcasting system should be incorporated into an online HABs portal that would provide management agencies and industry with comprehensive and timely information on the status of HABs off eastern Tasmania (and potentially other regions into the future). For example, the mock-up shown in Fig. 6.10 combines the environmental conditions used for forcasting, with data streams from in situ biological monitoring (e.g. cell counts, molecular probes) and any toxicology data available through seafood quality assurance programs. This combination would provide stakeholders with an integrated view of the current and near-future risk of HABs in their region.

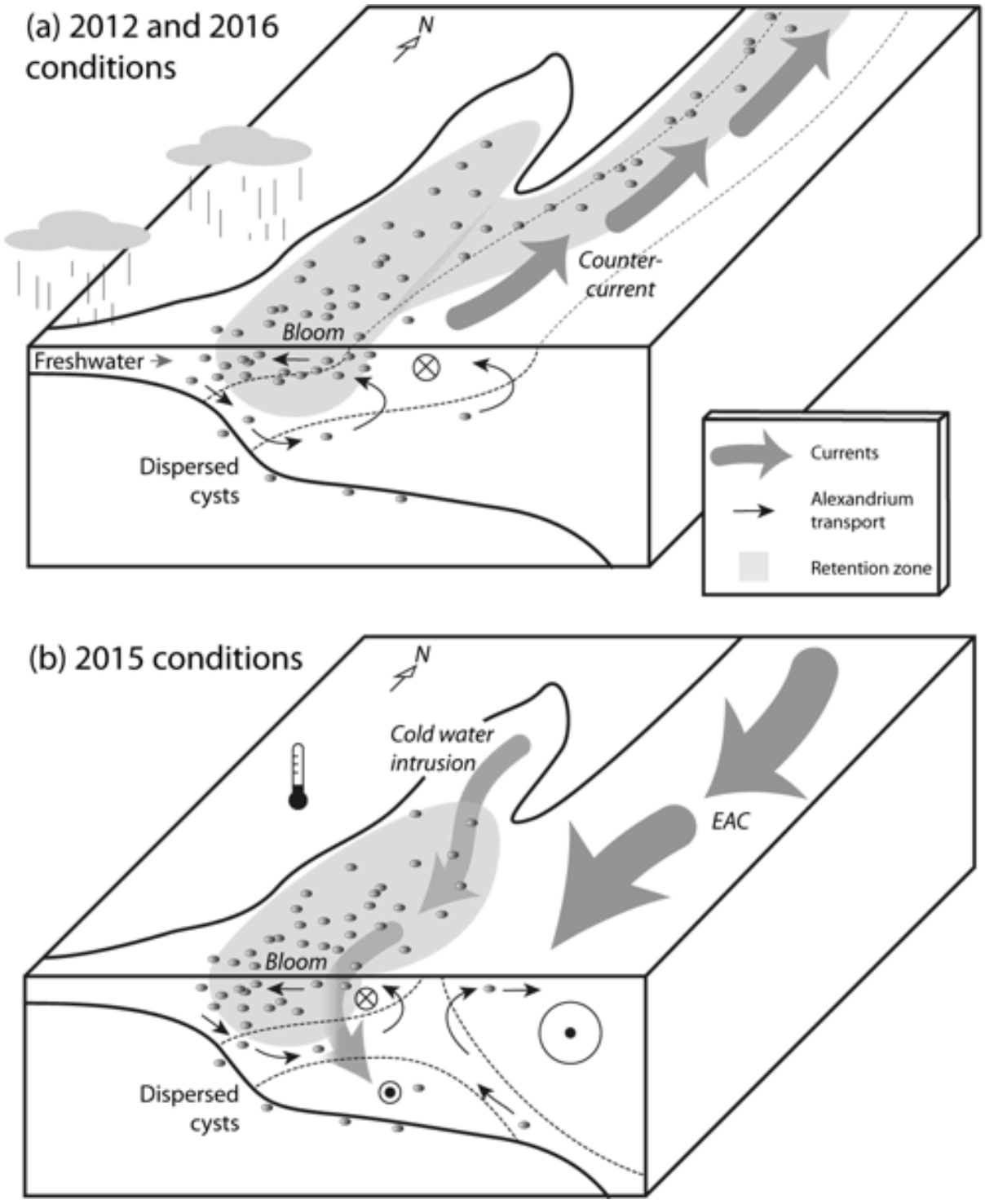


Fig. 6.8: Hypotheses for the environmental drivers of HABs off eastern Tasmania: (a) high rainfall potentially accompanied by southerly or onshore winds; and (b) outflow of cold water southward from Great Oyster Bay potentially accompanied by southerly or onshore winds.

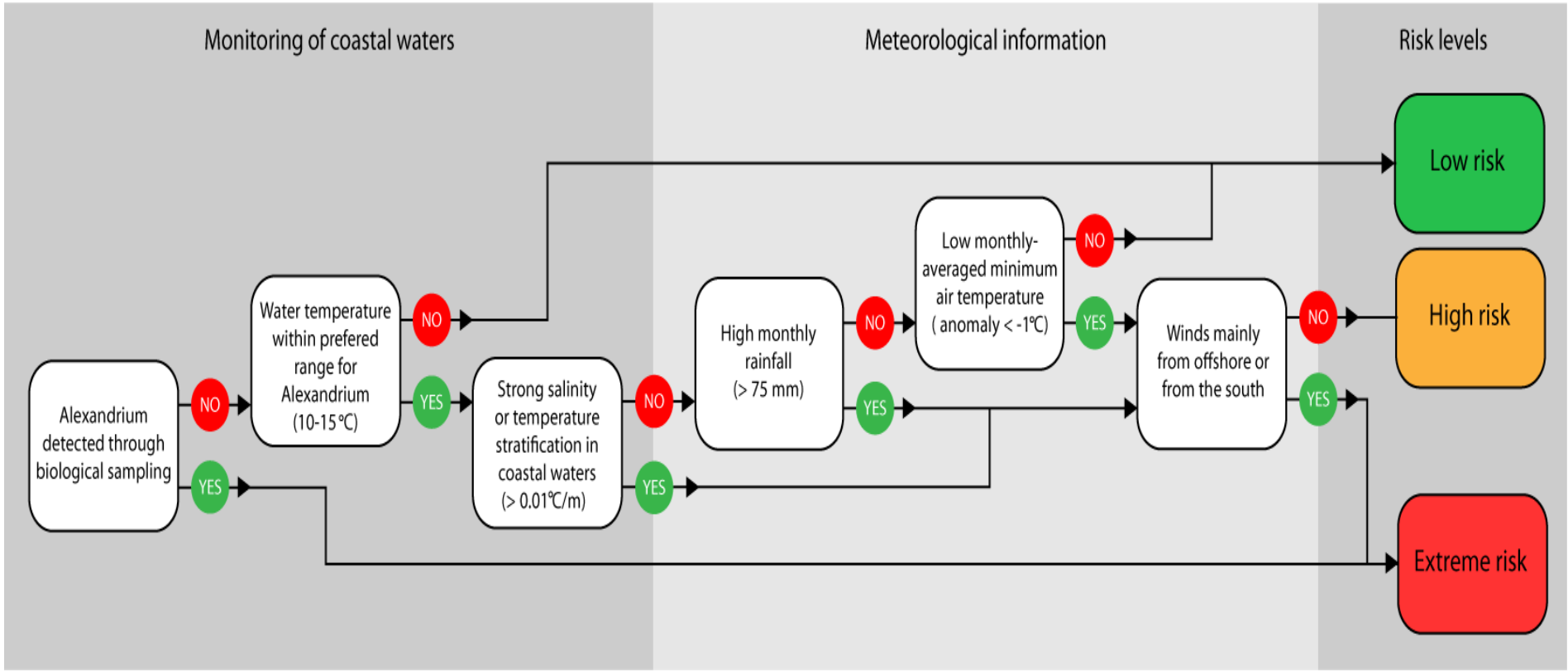


Fig. 6.9: Tentative frameworks for assessing the risk of HABs occurring off eastern Tasmania using both existing meteorological data streams and proposed in situ monitoring of coastal waters. Note that quantitative criteria in brackets are based on very few HAB events and require ongoing refinement.

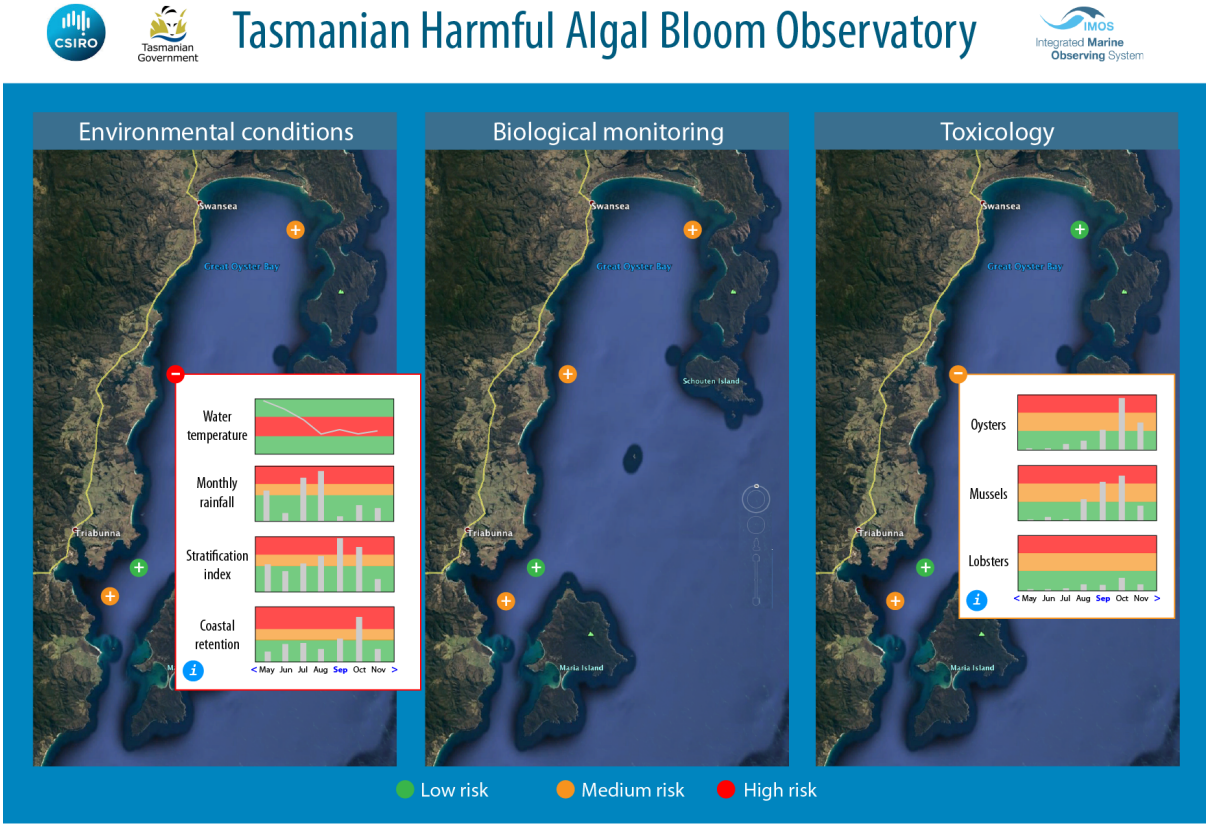


Fig. 6.10: Mock-up of an online HABs portal that combines the environmental conditions used for forcasting with any available biological monitoring and toxicology data.

7. Risk ranking of marine biotoxins in Tasmanian non-bivalve seafoods

Alison Turnbull, Navreet Malhi and Stephen Pahl

A significant bloom of *Alexandrium tamarense,* a paralytic shellfish toxin (PST) producing alga, occurred off the east coast of Tasmania in October 2012. Toxins were present from Eddystone Point on the north-east corner to Marion Bay on the lower east coast. At the time there was limited understanding of which seafood species presented a risk to human health from toxin accumulation, and therefore, which species should be tested. Aside from bivalve species (which are known to accumulate high levels of marine biotoxins), PSTs were tested in rock lobsters (*Jasus edwardsii*), giant crabs (*Pseudocarcinus gigas*), abalone (*Haliotis rubra*), sea urchins (*Heliocidaris tuberculata* and *Centrostephanus rodgersii*), calamari (*Sepioteuthis australis*), flathead (unspecificed) and banded morwong (*Cheilodactylus spectabilis*). PSTs were found above the bivalve regulatory level in rock lobsters (3.9 mg/kg, in low levels in sea urchins and the viscera of giant crabs (0.5 mg/kg) and abalone (0.3 mg/kg), but not found in squid or fish.

An independent review of the incident highlighted the need for an objective basis for risk management of non-bivalve seafood species. The review recommended that *"A risk ranking exercise for fisheries species to ascertain which ones should be included in the management plan and a relevant associated framework for regulation"* was a short-term priority for fisheries management (Campbell *et al.* 2013). The review also recommended that a long-term priority should be to use the results of the risk ranking exercise to underpin development of a state-wide biotoxin management plan.

A workshop was conducted with a variety of experts to make a qualitative ranking of the public health risk associated with marine biotoxins in non-bivalve seafood groups that are recreationally and commercially captured in Tasmania. The workshop included representatives from government, commercial and recreational fisheries and experts in risk assessment and marine biotoxins.

The scope of the risk ranking exercise was limited to the four toxin groups regulated in the Australian Food Standards Code: Paralytic Shellfish Toxins (PSTs); Diarrhetic Shellfish Toxins (DSTs); Amnesic Shellfish Toxins (ASTs) and Neurotoxic Shellfish Toxins (NSTs). The Tasmanian seafood groups considered were: commercially harvested fisheries products; marine farmed products (with the exception of bivalve molluscs that are already under the control of the Tasmanian Shellfish Quality Assurance Program - oysters, mussels, clams and to some extent scallops); and recreationally harvested seafood.

The Tasmanian seafood groups considered important were based on DPIPWE commercial and recreational catch data. These species included:

- Giant crab (*Pseudocarcinus gigas)*
- Southern Rock Lobster (*Jasus edwarsdii*)
- Southern calamari (*Sepioteuthis australis)*
- Octopus (*Octopus maorum)*
- Gould's squid (*Nototodarus gouldi)*
- Abalone (*Haliotis laevigata* & *Haliotis rubra)*
- Native sea urchin (*Heliocidaris erythrogramma* and *Heliocidaris tuberculate*)
- Long spined sea urchin (*Centrostephanus rodgersii*)
- Garfish (unspecified)
- Southern rock cod (*Lotella* & *Pseudophycis* spp.)
- Australian salmon (*Arripis trutta* & *Arripis trutta)*
- Jack mackerel (*Trachurus declivis* & *Trachurus murphyi)*
- Silver trevally (*Pseudocaranx dentex)*
- Warehou (unspecified) (*Serollella* spp.)
- Banded morwong (*Cheilodactylus spectabilis*)
- Pike (*Dinolestes lewini, Sphyraena novaehollandiae)*
- Barracouta (*Thyrsites atun)*
- Wrasse (unspecified)
- Bastard trumpeter (*Latridopsis forsteri) and* Striped trumpeter (*Latris lineata)*
- Mullet (*Aldrichetta forsteri* & *Mugil cephalus)*
- Blue mackerel (*Scomber australasicus)*
- Tuna (unspecified)
- Whiting (unspecified)
- Black bream (*Acanthopagrus butcheri)*
- Flounder (unspecified)
- Flathead (unspecified)
- Gurnards (unspecified)
- Leatherjacket (unspecified)
- Shark and rays (unspecified)
- Atlantic salmon (*Salmo salar)*
- Trout (*Salmo trutta, Oncorhynchus mykiss, Salvelinus fontinalis).*

The overarching objective of the risk ranking exercise was to make recommendations on which seafood species should be included in any future biotoxin risk management program developed for Tasmania. The toxin groups included: Paralytic Shellfish Toxins (PSTs) known as the saxitoxin group; Diarrhetic Shellfish Toxins (DSTs) known as OA group toxins; Amnesic Shellfish Toxins (ASTs) - domoic acid and isomer; and Neurotoxic Shellfish Toxins (NSTs), known as the brevetoxin group (BTX).On the basis on the information provided and the expertise of workshop participants, the consensus reached was:

- The risk associated with ASTs in non-bivalve seafood from Tasmania is negligible. This primarily relates to the low levels of AST found in Tasmania and the limited number of international illnesses associated with AST.
- The risk associated with NSTs in non-bivalve seafood from Tasmania is negligible. This primarily relates to restricted distribution of NSTs internationally.
- The level of risk associated with DSTs in most Tasmanian seafood is negligible. This primarily relates to the low severity of the disease and the low level of DSTs generally found in Tasmania. However, it was acknowledged that DSTs associated with crabs

and gastropods (particularly carnivorous gastropods) did cause a significant level of illness internationally due to the ability of these organisms to accumulate DSTs to very high levels. Given the unknown ability of Tasmanian crabs and gastropods to accumulate DSTs, these pairings were ranked a slightly higher risk than DSTs in the other non-bivalve seafood groups, resulting in a "very low" ranking.

- PSTs are a potential risk that require closer scrutiny. The consensus was that PSTs in all non-bivalve seafood groups represented a considerably lower risk for producing illness than PSTs in bivalve shellfish. PST: seafood pairings were assessed as negligible, low or medium risk. Where a medium risk was deemed (whole abalone and whole Southern Rock Lobster), the Group considered the risk of illness could be controlled through either a monitoring program or through processing steps to remove contaminated tissues.

Table 7.1. Marine biotoxin groups known to accumulate in seafood and their regulatory maximum levels (MLs)

Toxin Group	Symptoms	ML (mg/kg)
Paralytic Shellfish Toxins (PST) = saxitoxin group (STX)	Variety of neurological symptoms, ranging from mild (e.g. tingling sensations in extremities, headaches, dizziness, nausea) through serious (e.g. muscle/limb paralysis) to severe (e.g. respiratory distress and in extreme cases, death).	0.8
Diarrhetic Shellfish Toxins (DST) = OA group toxins: okadaic Acid (OA), dinophysistoxins (DTX) and derivatives	Predominant symptoms are diarrhoea, nausea, vomiting and abdominal pain. Symptoms are dose dependent, but are not considered lethal, and hospitalisation is not normally required.	0.2 (0.16 EU, NZ)
Amnesic Shellfish Toxins (AST) = domoic acid and isomers (DA)	Characterised by a number of clinical symptoms and signs involving multiple organ systems, including the gastrointestinal tract, the central nervous system and the cardiovascular system In severe cases death may ensue or neuronal damage can persist for years after exposure to the toxin.	20
Neurotoxic Shellfish Toxins (NST) = brevetoxin group (BTX)	Acute neurological and gastrointestinal effects (e.g. nausea, diarrhoea, numbness, temperature reversal, slurred speech, respiratory distress). Multiple symptoms often occur at the same time, with neurological symptoms lasting longer Neurological symptoms can be serious (seizures).	200 MU (0.8 Pb-TX2)

The Risk Ranking Group identified several knowledge gaps constraining the current management of biotoxins in Tasmania. The working group made the following recommendations to better understand potential risk levels associated with specific toxin:seafood pairings:

1. Further sampling of PSTs in giant crab should be undertaken during bloom events.
2. Sampling of DSTs in giant crab and abalone should be undertaken during bloom events.
3. Sampling of PSTs and DSTs in the developing fisheries of periwinkles and whelks should be undertaken during bloom events.

Furthermore, the working group acknowledged the lack of understanding on the formation of toxin blooms in certain geographical areas, including the west coast of Tasmania and recommended more information should be obtained regarding these zones.
The Group recommended stakeholders should unite to produce a state-wide approach to biotoxin management for Tasmanian seafood on the basis of the risk ranking conducted. By combining resources, a cost-effective plan/s covering all relevant geographical areas could be designed that takes into consideration the economic, social, political and legal landscape. The plan/s should cover bivalves, Southern Rock Lobster and abalone. Wherever possible, additional information/partners that are monitoring marine algae for other reasons should be included in the plan/s, e.g. salmon aquaculture and environmental researchers.

Outcomes Achieved

An objective, informed discussion between key stakeholders in the recreational and commercial seafood industries, government officials and scientific experts on the human health risks associated with biotoxins in non-bivalve seafood made the following determinations:

1. Toxin:species pairings considered to have a risk high enough to warrant inclusion in a management program:

- PSTs in Southern Rock Lobster (*Jasus edwarsdii*) and abalone (*Haliotis laevigata & Haliotis rubra)*.

2. Toxin:species pairings which were considered to be of very low risk and not necessary to include in any future management procedures:

- NSTs and ASTs in giant crab, Southern Rock Lobster, Southern calamari, octopus, Gould's squid, abalone, native sea urchins, long spined sea urchin, garfish, Southern rock cod, Australian salmon, jack mackerel, silver trevally, warehou, banded morwong, pike, barracouta, wrasse, bastard trumpeter, striped trumpeter, mullet, blue mackerel, tuna,

whiting, black bream, flounder, flathead, gurnards, leatherjacket, shark and rays, Atlantic salmon and trout.

- DSTs in Southern Rock Lobster, Southern calamari, octopus, Gould's squid, native sea urchin, long spined sea urchin, garfish, Southern rock cod, Australian salmon, jack mackerel, silver trevally, warehou, banded morwong, pike, barracouta, wrasse, bastard trumpeter, striped trumpeter, mullet, blue mackerel, flathead, gurnards, leatherjacket, shark and rays, Atlantic salmon and trout.

- PSTs in Southern calamari, octopus, Gould's squid, native sea urchin, long spined sea urchin, garfish, Southern rock cod, Australian salmon, jack mackerel, silver trevally, warehou, banded morwong, pike, barracouta, wrasse, bastard trumpeter, striped trumpeter, mullet, blue mackerel, tuna, whiting, black bream, flounder, flathead, gurnards, leatherjacket, shark and rays, Atlantic salmon and Trout.

3. Toxin : species pairings for which little or no data exists, and which therefore could not be ranked with confidence at present:

- DSTs in giant crab and abalone and the low volume, developing fisheries of periwinkles and whelks. Potential risk for DST accumulation was identified for crabs and gastropods, in particular carnivorous gastropods.

- PSTs in giant crab, periwinkles and whitebait. Whitebait is a recreational fishery including whitebait (*Lovettia sealii*), jollytail (*Galaxias maculatus*), climbing galaxias (*Galaxias brevipinnis*), spotted galaxias (*Galaxias truttaceus*), Tasmanian mudfish (*Neochanna cleaveri*) and Tasmanian smelt (*Retropinna tasmanica*).

Outputs

- A risk ranking report for public health risk associated with the regulated marine biotoxins in Tasmanian non-bivalve seafood.
- A comprehensive report describing background information for future risk assessment activities, including: hazard identification and characterisation; information on catch volumes and distributions for recreational and commercial fisheries; export volumes; a review of the evidence for the accumulation of toxins in associated seafood groups internationally, and a review of illnesses associated with toxins in seafood.
- A single database containing all historical biotoxin analysis results for seafood species in Tasmania, including methods of analysis.

7.1. Introduction

A significant bloom of toxin-producing *Alexandrium tamarense* occurred off the east coast of Tasmania in October 2012. Toxins were first detected in exported mussels *Mytilus galloprovincialis* from Spring Bay on the central east coast. Subsequent testing in bivalve

aquaculture zones along the east coast showed the toxins were present from Eddystone Point on the north-east corner to Marion Bay on the lower east coast [Campbell et al. 2013].

The public health response to avoid illness and restore consumer confidence resulted in testing of a number of seafood species for paralytic shellfish toxins (PSTs). However, at the time there was limited information to determine which species could accumulate PSTs and therefore which species should be sampled. Following testing, significant closures of recreational and commercial fisheries occurred. PSTs were found above the bivalve regulatory level in scallops (*Pecten fumatus*) and rock lobsters (*Jasus edwardsii*) in addition to the bivalve shellfish managed under the Tasmanian Shellfish Quality Assurance Program: oysters (*Crassostrea gigas*), mussels (*Mytilus galloprovincialis)* and clams/cockles (*Venerupis largillierti* and *Katelysia scalarina*). Low levels of PSTs were detected in the viscera of giant crabs (*Pseudocarcinus gigas*) and abalone (*Haliotis rubra*), and in sea urchins (*Heliocidaris tuberculata, Centrostephanus rodgersii*) (0.5, 0.24 and 0.12 mg STX eq kg^{-1} respectively). While lower levels of PSTs were detected in giant crabs and sea urchins, it is possible that sampling of these species was not undertaken at the highest time and place of risk during the bloom. Seafood that were tested and found not to contain PSTs were calamari (*Sepioteuthis australis*), flathead (predominantly *Platycephalus bassensis)* and banded morwong (*Cheilodactylus spectabilis*).

The Campbell et al. 2013 review of the incident highlighted the need for an objective basis for risk management of non-bivalve seafood species. The review recommended as one of two short term priorities for fisheries management: *"A risk ranking exercise for fisheries species to ascertain which ones should be included in the management plan and a relevant associated framework for regulation"*.

The review also recommended several longer term priorities, among them the risk ranking exercise was noted to be an important factor underpinning development of a state-wide biotoxin management plan: *"Integrate risk management protocols for higher-risk fisheries species (e.g. potentially scallops, rocklobster, abalone, crabs) into the Plan, which currently covers bivalves. The fisheries species to be included in the plan should be based on recommended risk ranking exercise (to be undertaken in the short-term)"*.

7.2. Scope

It was agreed that the scope of this risk assessment was to be limited to the four toxin groups regulated in the Australian Food Standards Code (see Table 7.1), and covered by the current bivalve shellfish monitoring program in Tasmania. The toxin groups included: Paralytic Shellfish Toxins (PSTs) known as the saxitoxin group; Diarrhetic Shellfish Toxins (DSTs) known as OA group toxins; Amnesic Shellfish Toxins (ASTs) - domoic acid and isomer; and Neurotoxic Shellfish Toxins (NSTs), known as the brevetoxin group (BTX).

The risk ranking exercise considered these marine biotoxins in the following Tasmanian seafood:

- Commercially harvested fisheries products;
- Marine farmed products, with the exception of bivalve molluscs that are already under the control of the Tasmanian Shellfish Quality Assurance Program (oysters, mussels, clams and to some extent scallops); and
- Recreationally harvested seafood.

Objectives

The specific objectives were to:

1. Identify seafood species harvested in Tasmania that are known to accumulate significant levels of marine biotoxins and any associated human health impacts, both in Tasmania and internationally.
2. Identify intensity of fishing/farming effort for each seafood species in Tasmanian waters.
3. Undertake a risk ranking exercise to establish the relative risk of the various Tasmanian seafood species causing illness in consumers through consumption of the regulated toxins.

The overarching objective was to make recommendations on which seafood species should be included in any future biotoxin risk management plan/s developed for Tasmania.

7.3. Method

The risk ranking broadly follows the approach suggested by the EFSA scientific opinion on the development of a risk ranking framework on biological hazards [Anonymous 2004]. The process followed was:

1. Define the risk ranking question and scope
2. Collate all the information available for risk analysis (toxin-producing algae and toxin levels found in seafood in Tasmania, records of toxins in seafood internationally, production volumes, serving sizes, food web data)
3. Summarise all data according to each toxin group in each seafood
4. Bring relevant stakeholders together with independent experts
5. Determine risk metrics[1]
6. Determine risk ranking method/s

[1] A risk metric is the output from the model that is used to compare the risk rating of each hazard-food pairing. It may be qualitative, such as high, medium, low or negligible, or a quantitative number such as the number of adverse outcomes, cumulative exposure, or summary health measures (e.g. DALY – disability adjusted life years or QALY – quality adjusted life years)

7. Determine inputs for risk ranking method/s
8. Rank risks.

The risk ranking scope was determined through discussion with stakeholders at the initiation of the project.

The process for risk analysis was as follows:

- Identify the seafood of concern and the associated fishing effort;
- Collate data on serving size and calculate the number of serves produced per species;
- Summarise the potential food web relationships between species;
- Identify and analyse toxic algae and marine biotoxins known in seafood in Tasmania;
- Identify levels of toxins associated with these types of seafood internationally; and
- Identify any illnesses caused by the hazard/seafood pairing locally or internationally.

As scant information was available on many of the species, seafoods were grouped into similar trophic levels based on their modes of feeding and key food sources. One page summaries were made of all relevant factors for easy referencing during the risk ranking process. These summaries included:

- Species group title
- Species included
- Trophic position
- Commercial and recreational production levels in tonnage and number of serves per annum
- Toxin levels in Tasmania for each toxin group, noting the level of information available, whether regulatory maximum levels (MLs) have been exceeded, and whether toxicity has been measured during bloom events
- Toxin levels from literature, noting the volume and relevance of information available.

On the day additional information regarding export figures and catch distribution were presented. Further information on phytoplankton and cyst distribution and consumption patterns was elicited from experts in the room on the day of the workshop.

Stakeholder groups in Tasmania that are impacted by marine biotoxins in non-bivalve seafood, or would be involved in potential risk management activities, were identified. These broadly fitted into four categories: public health risk managers, fisheries managers, commercial fishers and recreational fishers.

The participants in the risk ranking process were chosen as representative of the groups most impacted. Participants are listed in Table 7.2. Two risk assessment experts were included in the process: Tom Ross from the University of Tasmania and John Sumner (M & S

Consultants). Gustaaf Hallegraeff, who has expertise in toxic algae and marine biotoxins, was also included.

Table 7.2. Risk ranking participants and affiliations

#	Name	Organisation	Position/Expertise
1	Hilary Revill	Department of Primary Industries, Parks, Water and Environment (DPIPWE)/ Fisheries	Principal Fisheries Management Officer (Crustacean and Scallop Fisheries)
2	Kate Wilson	DPIPWE/ Biosecurity Tasmanian Shellfish Quality Assurance Program (TSQAP)	TSQAP Manager
3	Professor Gustaaf Hallegraeff	University of Tasmania (UTAS)	International expert in harmful algal blooms
4	Sarah Ugalde	University of Tasmania (UTAS)	Research Assistant
5	Julian Harrington	Tasmanian Seafood Industry Council	Chief executive
6	Dean Lisson[1]	Abalone Council of Australia Ltd.	Chair
7	John Sansom	Tasmanian Rock Lobster Fisherman's Association	Executive Officer
8	Bob Lister	Tasmanian Scallop Fishermen's Association	Executive Officer
9	Mark Nicolai	Tasmanian Association for Recreational Fishing	Executive Officer
10	Rosalind Harrison	Department of Health and Human Services	Senior Scientific Officer (Toxicology)
11	Stewart Quinn	Department of Health and Human Services	Senior Program Officer, Food Safety
12	Associate Professor Tom Ross	University of Tasmania (UTAS)	International expert in risk assessment
13	John Sumner	M&S Consultants	International expert in risk assessment
14	Alison Turnbull	South Australian Research and Development Institute (SARDI)	Seafood Program Manager
15	Navreet Mahli	South Australian Research and Development Institute (SARDI)	Scientific Officer

[1] Unable to be present on the day

Participants were supplied with the relevant information one week prior to the risk ranking workshop. The workshop took place in Hobart on the 9th September 2015 from 10:30am to 4pm.

The choice of risk metrics is to a large extent dependent on the risk ranking approach taken. For the relatively low resources invested in this approach, qualitative or semi-quantitative metrics were discussed.

The risk ranking workshop followed the agenda presented in Appendix 2. The aims of the day, process to follow and summary information were presented in the first session. The

second session focused on risk assessment processes and potential models for risk ranking. The third session brought these together and produced qualitative relative rankings for the marine biotoxin:seafood pairings. The fourth session discussed the rankings, and how these related to current risk management processes and to trade risks. Stakeholders were asked to identify significant knowledge gaps and considerations for a statewide management plan.

7.4. Results

The process of risk analysis is divided into risk assessment, risk management and risk communication in accordance with Codex principles (see Fig. 7.1) The importance of maintaining a separation between risk assessment and risk management was presented, as well as having a transparent process that adequately states knowledge gaps, uncertainties and constraints. The results should be reassessed over time as more data becomes available.

The knowledge gaps of particular note identified during the initial phase of the risk ranking were:

- Presence and distribution of toxin-producing algae on the west coast of Tasmania and in offshore waters.
- Information on accumulation of PSTs during toxin-producing blooms in giant crab, Gould's squid, Southern calamari, octopus, periwinkles, urchins and fish.
- Information on the accumulation of DSTs, ASTs and NSTs during toxin-producing blooms in all Tasmanian non-bivalve seafood.
- Reliable information on serving size and consumption patterns for some species, especially with respect to recreational fishers.

Participants in the risk ranking workshop considered that trade was an important consideration for any future risk management approach due to the significant value of seafood exported from Tasmania and the potential social and economic impacts of any disruption to trade. As resources were not sufficient to address both trade and human health risk, trade considerations were noted rather than assessed.

For the risk ranking exercise participants were divided into risk assessors and risk managers. Risk assessors included Gustaaf Hallegraeff, Julian Harrington, John Sansom, Bob Lister, Ros Harrison, Tom Ross, John Sumner and Sarah Ugalde. The risk managers were Hilary Revill, Kate Wilson, Mark Nicolai, Alison Turnbull, Megan Burgoyne and Stewart Quinn.

The overall aim of the exercise was to provide an assessment of which toxin:seafood pairings needed to be included in a biotoxin management plan, and which species should be considered for testing in future bloom situations.

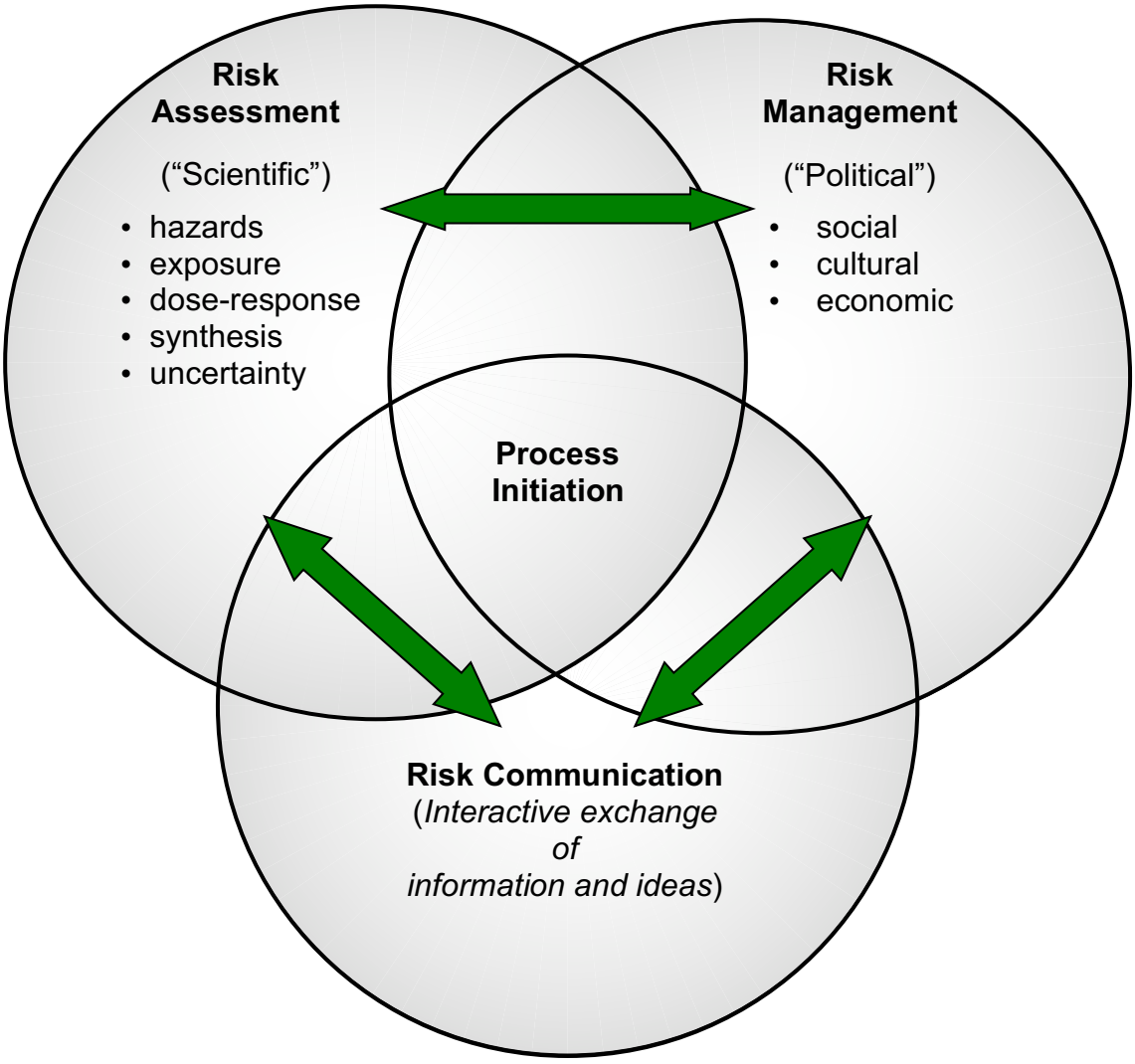


Fig 7.1. The risk analysis process

The risk managers defined the risk assessment question as follows:

- To make a qualitative determination of the public health risk associated with the hazard food pairing of the four regulated marine biotoxins in Australia (PSTs, DSTs, ASTs and NSTs) and the non-bivalve seafood caught in significant quantities in the commercial and recreational sector. These seafood groups are: giant crab, Southern Rock Lobster, abalone, Gould's Squid, Southern calamari, octopus, periwinkles, urchins and fish .

- If time permits, to make a semi-quantitative determination of the public health risk of those deemed to have significant level of risk.

The tools considered for qualitative assessment were those of Huss et al. [2000], Ross and Sumner [2000] and Anonymous (2000) [CSIRO]. The CSIRO tool was chosen as appropriate and examples of the risk of illness through marine biotoxins in oysters under managed and unmanaged regimes presented (Tables 7. 3 and 7.4). These were reflective of commercial and recreational scenarios and provided a benchmark for the exercise.

Table 7.3. Qualitative risk assessment of marine biotoxins in oysters under a managed regime using the CSIRO tool

Product	Oysters
Hazard name	PSP
Hazard severity	High
Likelihood of hazard occurring	Low
Growth required to reach infective dose	No
Effect of processing	No Reduction
Is there a consumer cooking step	No
Are there epidemiological links?	Yes
Risk	Very Low

Table 7.4. Qualitative risk assessment of marine biotoxins in oysters under an unmanaged regime using the CSIRO tool

Product	Oysters
Hazard name	PSP
Hazard severity	High
Likelihood of hazard occurring	Moderate
Growth required to reach infective dose	No
Effect of processing	No Reduction
Is there a consumer cooking step	No
Are there epidemiological links?	Yes
Risk	High

To provide context, it was pointed out that Australians consume about 800 million serves of fish/annum. During the period 1998-2014 there have been no recorded outbreaks of food poisoning related to the regulated marine biotoxins in non-bivalve seafood.[2] There have

[2] An outbreak consists of more than one case.

however been eight probable or suspected cases of PST in bivalve shellfish from Tasmania, one of which is documented in the scientific literature [Turnbull et al. 2013].

The CSIRO risk assessment tool was designed for microbiological hazards that can have a growth step and be impacted by processing and/or cooking. Marine biotoxins do not have a growth step and are heat and acid stable, so are not significantly impacted by cooking. Thus these factors were not considered during the exercise. The risks may however be impacted by processing (e.g. to remove viscera), so processing was considered for seafood where the whole product may not be consumed (crab, abalone, squid, calamari and fish).

The risk ranking questions were thus defined as:

1. What is the severity of illness?
2. What is the likelihood of a toxic dose accumulating in the seafood in the portion consumed?
3. Are there epidemiological links to the toxin:seafood pairing?
4. What is the level of consumption (including both number of serves per annum and volume consumed)?

7.5. The Four Hazards

The risk ranking group first considered the hazard severity and likelihood for each toxin group. Paralytic shellfish poisoning (PSP) was considered a high severity and PSTs were thought likely to occur in Tasmania, at various levels in the different seafood groups. It was the Group's decision that this hazard needed to be considered across all the seafood groups.

Diarrhetic shellfish poisoning was considered a low severity. The likelihood of DSTs occurring at high levels in Tasmania was judged as low[3], and their ability to accumulate in the Tasmanian seafood groups was acknowledged as unknown (noting the ability to accumulate in some seafood internationally). Whilst this hazard was not judged a high priority it was noted that if significant blooms did occur, seafood known to cause illness internationally should be tested, namely crabs and gastropods (primarily giant crab and abalone, but also the low volume developing fisheries of periwinkles and whelks if they are present in the area of concern). Southern Rock Lobster was not included in this list due to the lack of evidence internationally of illness caused by DSTs in lobsters.

Amnesic shellfish poisoning (ASP) was considered of medium-high severity. ASTs were acknowledged as rarely an issue in Tasmania[3], with no samples from the bivalve industry exceeding the maximum regulatory levels thus far. There has only been one outbreak of ASP internationally. This hazard was judged a negligible risk and not taken any further in the risk ranking exercise.

[3] All likelihood estimates acknowledged the lack of information in offshore and West Coast waters.

Neurotoxic shellfish poisoning (NSP) was considered of medium severity. NSTs were acknowledged as unlikely to be an issue in Tasmania given that the international distribution is confined to the Gulf of Mexico and to a lesser extent, New Zealand. This hazard was judged a negligible risk and not taken any further in the risk ranking exercise.

7.6. Risk Ranking of PST: Seafood Pairings

The risk of illness from PST:seafood pairings was assessed against the remaining risk assessment questions (i.e. are there epidemiological links to the hazard:seafood pairing and what is the level of consumption, including both number of serves per annum and volume consumed per sitting) for all the identified seafood groups. The inputs determined are shown in Table 7.5.

Giant crab, Southern Rock Lobster and abalone were considered as whole product and meat only product. A recent risk assessment for PSTs in abalone found that abalone viscera may be consumed, particularly in Asian countries [McLeod et al. 2014]. On occasion, products comprising only viscera may be consumed, e.g. viscera sashimi. A survey of recreational fishers in Tasmania found that 15% of people on the east coast of Tasmania consumed the hepatopancreas of rock lobsters, whilst 21% of fishers on the east coast consumed the hepatopancreas of rock lobster. The Group did not have any knowledge on the consumption of tissues from giant crab in either a commercial or recreational setting.

No epidemiological evidence of illness from marine biotoxins ingested by consumption of Tasmanian non-bivalve seafood groups was identified. Epidemiological evidence was deemed as negligible or low for each toxin:seafood pairing based on the following criteria:

- *Negligible level of evidence*: no evidence of illness from consumption of PSTs in any seafood from a similar group internationally (e.g. any crab species for giant crabs, any squid species for calamari and Gould's squid,).
- *Low level of evidence*: reports available of PSTs in other seafood species from related groups causing illness.

Generally fish do not accumulate PSTs in their flesh as fish tissues are sensitive to PSTs, resulting in death before toxins can accumulate. However there are international reports of whole planktivorous fish, including mullet, causing illness. The Group considered that this would not normally be an issue for most fish in Australia, as only the flesh is consumed. The exception is whitebait, which are consumed whole and often in large quantities.

Consumption was considered from the point of view of the number of serves per annum and the volume consumed per sitting. The Group considered the estimates for serving size for giant crab, Southern Rock Lobster, scallops and abalone to be questionable, and raised the issue that recreational fishers are likely to eat significantly larger volumes of these species

than consumers obtaining their seafood from commercial sources. Dr John Sumner pointed to the Australian Seafood Catering Manual to provide better estimates for serving sizes [Kane 1994]. Despite this, the significant differences in catch volumes allowed a relative ranking from low to high for this category (see Table 7.5).

The final risk ranking estimate for each PST:seafood pairing considered all the inputs. It also took into account the ability of active management to reduce the risk estimate. This follows the example of oysters above, where unmanaged oysters represent a high risk of illness through consumption of PSTs, whereas managed oysters represent a very low risk. Final risk metrics selected for PSTs in seafood groups were negligible, low, and medium. High was not considered a necessary category, as all seafood groups were considered a significantly lower risk than bivalves. The risk ranking result for each PST:seafood pairing is shown in Table 7.5.

Following the risk ranking exercise, the Group discussed current risk management practices and whether these were appropriate in light of the information supplied and the risk estimates reached. There is currently a formal biotoxin management plan for Southern Rock Lobster on the east coast of Tasmania. This program is administered by DPIPWE with support from the rock lobster industry. There is also informal biotoxin management of abalone in the south-east of Tasmania, and on the east coast of Tasmania when large blooms are present. This program is managed between the Department of Agriculture, DPIPWE, and the abalone industry. Both the Southern Rock Lobster and abalone risk management approaches rely heavily on information provided through the TSQAP.

There was agreement that the current inclusion of rock lobster and abalone in risk management activities during blooms of PST producing algae was appropriate. Crabs, periwinkles, whelks and whitebait were identified as worthy of sampling to obtain further information on the potential accumulation of PSTs in these species which were recognised as relatively low catch species.

Table 7.5. Risk ranking inputs and summaries for PSTs in Tasmanian non-bivalve seafood

	Severity (of illness)	Likelihood (of a toxic dose in the tissue consumed)	Epidemiological evidence in the target population	Consumption	Risk estimate
Crab whole	High	Medium (uncertain)	Low	Low (uncertain eating patterns)	Low
Crab meat	High	Low (uncertain)	Low	Low (uncertain eating patterns)	Negligible
SRL whole	High	Medium	Low	Medium	Low if managed. Medium if unmanaged
SRL meat	High	Nil	Negligible	Medium	Negligible
Southern Calamari	High	Low	Low	Low	Negligible
Goulds Squid	High	Low	Low	Medium	Negligible
Octopus	High	Low	Low	Low	Negligible
Abalone - whole	High	Medium	Negligible	High-medium	Low if managed. Medium if unmanaged
Abalone foot	High	Low	Negligible	High-medium	Negligible
Periwinkles	High	Unknown	Low	Low	Low by virtue of low consumption

Sea urchins	High	Low (uncertain)	Negligible	Very low	Low by virtue of low consumption
Omnivorous & carnivorous fish	High	Nil	Negligible	High	Negligible
Planktivorous Fish (eg small pelagics)	High	Uncertain	Low	Low	Low

7.7. Discussion

The purpose of this risk ranking exercise was to make a qualitative determination of the public health risk associated with the hazard:food pairings from the four regulated marine biotoxins in Australia (PST, DST, AST and NST) and the non-bivalve seafood caught in significant quantities in the commercial and recreational sector. The estimates produced are relative to each other and are not intended to be compared with other hazard:food pairings in a quantitative sense. The aim was to provide guidance to risk managers on which species should be included in any future state-wide biotoxin management plan/s.

The consensus of the Risk Ranking Group was:

- The risk associated with ASTs and NSTs in non-bivalve seafood from Tasmania is negligible. This primarily relates to the low levels of AST found in Tasmania, the limited number of international illnesses associated with AST and the internationally restricted distribution of NSTs.

- The level of risk associated with DSTs in most Tasmanian seafood is negligible. This primarily relates to the low severity of the disease and the low level of DSTs generally found in Tasmania. However, the Group acknowledged that DSTs associated with crabs and gastropods (particularly carnivorous gastropods) did cause a significant level of illness internationally due to the ability of these organisms to accumulate DSTs to very high levels. Southern Rock Lobster was not included in this group due to the lack of any international evidence of illness through consumption of DSTs in lobsters. Given the unknown ability of Tasmanian crabs and gastropods to accumulate DSTs, these pairings were ranked a slightly higher risk than DSTs in the other seafood groups, resulting in a "very low" ranking.

- PSTs are a potential risk that require closer scrutiny. The consensus was that PSTs in all non-bivalve seafood groups represent a considerably lower risk for producing illness than PSTs in bivalve shellfish. PST:seafood pairings were assessed as negligible, low or medium risk. Where a medium risk was deemed, the Group considered the risk of illness was able to be controlled through either a monitoring program or through processing steps to remove the contaminated tissues.

The risk ranking of each toxin:seafood group pairing is given in Table 7.6. The only pairings for which the risk was judged as medium if unmanaged were PSTs in Southern Rock Lobster and whole abalone. All other pairings had a negligible to low risk estimate.

The Group acknowledged that trade risk was an important consideration and often formed the economic driver for biotoxin management, as trade requirements are generally conservative and set at levels below that at which human health risks will occur. The resources available for this project did not allow an analysis of trade risk, however this was discussed. Abalone and Southern Rock Lobster represent the greatest trade volume and value, adding to the reasons to undertake some level of biotoxin risk management for PSTs in these species. Of

potential concern for trade and public health is the lack of information regarding PSTs on the west coast of Tasmania where large volumes of abalone and Southern Rock Lobster are sourced. At present, there is no monitoring of toxic algae or toxins in seafood on this coast.

Table 7.6. Risk ranking of toxin:seafood pairings

Toxin group	Seafood group	Risk estimate
AST	All	Negligible
NST	All	Negligible
DST	All except crabs and gastropods	Negligible
DST	Crabs and gastropods	Very Low[1]
PST	Crab meat, Rock Lobster meat, Southern calamari, Gould's squid, octopus, abalone foot, omnivorous and carnivorous fish	Negligible
PST	Periwinkles and sea urchins	Low (by virtue of low consumption)
PST	Whole crab and planktivorous fish	Low
PST	Whole Southern Rock Lobster and whole abalone	Low if managed. Medium if unmanaged

1. Potentially negligible, but no/little information on accumulation in Tasmanian species

The only toxin:seafood pairings resulting in a medium risk to consumers if unmanaged were whole abalone and whole Southern Rock Lobster. A recent provisional risk assessment for wild caught Australian abalone rated PSTs in whole abalone as a low risk of causing illness, and PSTs in abalone viscera products as a low to medium risk. Currently risk management activities for abalone are instigated when bivalve species indicate PSTs are a potential issue. Zones in areas impacted by significant toxic blooms are closed for harvest until such time as abalone tissue samples indicate it is safe to re-open. Sampling and analysing for biotoxins in abalone may often be delayed for economic reasons in low production zones where there is no active fishing planned. This level of management was considered to match the outputs of the current risk ranking exercise, noting the lack of monitoring on the west coast of Tasmania.

A biotoxin management plan is currently in place for Southern Rock Lobster on the east coast of Tasmania. This plan relies heavily on information from the bivalve monitoring program to raise alerts when PSTs are found above the regulatory level. When this occurs, the Rock Lobster fishery is closed and sampled for toxins. Further actions are defined in the management plan based on PST levels found. This level of management was considered consistent with the outputs of the current risk ranking exercise, noting the lack of monitoring

on the west coast of Tasmania. Potential changes to the details of the current risk management plan following completion of the current research work into PSTs in Southern Rock Lobster were flagged.

The risk management of recreational fisheries was discussed. There is a desire to see a continuation of the current policy where the opening and closing of recreational fisheries is managed in accordance with that of commercial fisheries.

The issue of reduced distribution of bivalve shellfish farms in the high-risk south-east corner of Tasmania was discussed. Recently bivalve aquaculture farms in the lower Huon Estuary (Deep Bay in Port Cygnet) and Port Esperance have become inactive due to the financial difficulties of operating in areas that are often closed due to PSTs. These were key monitoring sites for the south-east region, offering early warning of blooms to adjacent areas, and supporting public health management of the region. The risks to recreational harvesting caused by these closures were highlighted. A state-wide, collaborative approach to monitoring program/s that included monitoring of shellfish from salmon aquaculture sites in these zones is one potential means of filling this gap. The Group recommended that salmon aquaculture be included in any discussions on biotoxin management in Tasmania.

7.8. Implications

By including a wide range of stakeholders from the government, commercial and recreational fishing sectors, supported by risk assessment and marine biotoxin experts from SARDI and the University of Tasmania, the Group was able to reach consensus on a scientifically sound risk ranking of biotoxin:seafood pairings in Tasmania.

The results of this risk ranking exercise can be used to underpin a state-wide approach to biotoxin monitoring in Tasmania. Key species to monitor during major bloom events were identified. Discussions were initiated on including this ranking into a cost-effective state-wide approach for biotoxin risk management.

At present, monitoring and management of toxins in seafood is highly dependent on the monitoring conducted by the Tasmanian Shellfish Quality Assurance Program. Significant knowledge gaps on the potential for toxic blooms on the west coast of Tasmania where there are no bivalve aquaculture sites exist. If industry decides PSTs in Southern Rock Lobster are a trade risk then this issue should be addressed. In addition, the reduction of bivalve aquaculture farms in key sites in the high risk D'Entrecasteaux Channel region has implications for on-going management of recreational and commercial fisheries in these popular and high catch regions.

7.9. Recommendations

1. Stakeholders should unite to produce a state-wide approach to biotoxin management for Tasmanian seafood on the basis of the risk ranking conducted. By combining resources, a cost-effective plan/s covering all relevant geographical areas could be designed that takes into consideration the economic, social, political and legal landscape. The plan/s should cover bivalves, Southern Rock Lobster and abalone. Wherever possible, additional information/partners that are monitoring marine algae for other reasons should be included in the plan/s, e.g. salmon aquaculture and environmental researchers.

2. Several key knowledge gaps were identified that would reduce uncertainty of the risk estimates. The most significant gap that needs to be addressed is information on the potential for toxin-producing blooms to form in offshore waters and on the west coast of Tasmania. Other knowledge gaps that need to be filled before a more comprehensive risk assessment could be conducted include: consumption patterns of giant crab; the ability of giant crab to accumulate PSTs and DSTs during toxin-producing blooms; the ability of abalone to accumulate DSTs during toxin-producing blooms; the ability of periwinkles and whelks to accumulate PSTs and DSTs; and the ability of whitebait to accumulate PSTs during toxin-producing blooms.

7.10. Further Developments

A state-wide biotoxin management program involving all stakeholders and considering economic, social, political and legal factors should be developed using the risk ranking platform developed in this report. This management program should consider the information arising in the current FRDC research project led by Professor Gustaaf Hallegraeff to better manage PST producing blooms in Tasmania. It should also include other entities that are currently monitoring for toxic marine algae for other reasons, e.g. fish health or ecosystem health. The program may consist of a single biotoxin management plan covering all species, or multiple plans covering each of the seafood species of concern.

7.11. Extension and Adoption

The completed report was sent to all stakeholder groups present on the day and to Oysters Tasmania for wider dissemination and discussion. It was also be made available on the SafeFish website. The report should prompt further discussion within Tasmania on the development of a state-wide biotoxin management program. Professor Gustaaf Hallegraeff and Alison Turnbull will support these discussions.

The risk ranking project was discussed at the recent Australian Shellfish Quality Assurance Advisory Committee in Queenscliff on the 18th September 2015. Several states requested copies of the supporting information document. This will occur following finalisation.

8. Conclusions

1. The seafood industry along the east coast of Tasmania in the period 2012-2016 increasingly has been impacted by toxic dinoflagellate blooms of *Alexandrium tamarense,* causative organism of paralytic shellfish poisoning in humans. This has led to seasonal industry closures, loss of market access, damage to reputation and product recalls.

2. A multidisciplinary research team led by the University of Tasmania helped introduce, calibrate and validate a rapid screening tests for paralytic shellfish toxins (30 min @ $30 compared to 7-10 days @$500 per sample).

3. We integrated new knowledge on the biology, genetics and toxicology of these dinoflagellate blooms into a biotoxin risk decision-tree model which is driven by temperature, rainfall and East Australian Current behaviour.

4. Apart from PST biotoxin risks for bivalves (mussels, oysters, scallops) we identified Southern Rock Lobster (*Jasus edwarsdii*) and abalone (*Haliotis laevigata* and *Haliotis rubra)* to have a risk high enough to warrant inclusion in a management program.

9. Implications

5. The Neogen PST screening test is recommended for adoption for regulatory purposes. Effective rapid screening preventing compliant samples undergoing testing using the more expensive and time consuming LC-FLD method will result in significant savings in analytical costs (estimated $500,000 savings per annum Australia-wide).

6. The maximum PST level allowed in the Codex standard is based on the dihydrogen chloride base of saxitoxin, whereas the current Food Standards Code is based on saxitoxin only. This can lead to up to 20% difference in reporting depending on the toxin profile present, and considerably complicated the interpretation of results between laboratories used in this study.

7. *Alexandrium* cyst surveys along the entire east coast of Tasmania found consistently low abundances of cysts (0.1-3 cysts per gram of sediment wet weight), but no localized dense cyst beds.. The presence of cysts suggest that problems will persist.

8. Both culture experiments but notably field estimates using both Neogen and LC-MS suggest a very high cellular toxin content up to 100-500 pg STX eq/cell, thus explaining why even very low cell numbers can cause seafood toxicity.

9. To protect tourism and human health, the east coast of Tasmania has now been sign-posted with 75 permanent public PST warnings, which is a first for Australia.

10. Recommendations

1. The Neogen PST screening test is recommended for adoption for regulatory purposes, and is already used to provide shellfish growers with a rapid tool for harvesting decisions at the farm gate.

2. The varying co-occurrence of *Alexandrium fundyense* and *Alexandrium pacificum* in east coast Tasmanian blooms confounds progress in elucidating bloom ecophysiology as well as predicting of shellfish toxicity. We recommend that every Tasmanian bloom event be subjected to species-specific qPCR detection, and SxtA4 qPCR detection to guarantee the correct interpretation of results.

3. A submission by SafeFish and the Australian Shellfish Quality Assurance Advisory Committee (ASQAAC) to Food Standards Australia New Zealand is to be made to amend the need for PST maximum limits to be listed as the di-hydrochloride salt (2HCl) in the Food Standards Australia New Zealand (FSANZ) Food Standards Code.

4. The determination of benchmark PST concentrations against which to judge the performance of the Neogen test kits was complicated by a disturbing lack of consistency in international Toxin Equivalency Factors (TEFs) currently in use by Analytical Laboratories. We recommended the consistent use in Australia of FAO TEFs in line with Codex recommendations

11. Further developments

1. A stage 2 FRDC proposal has been prepared to address PST in rock lobster and abalone. The rapid test kits have the potential to be used with other seafood species such as scallops and rock lobster, but will need to be optimized and validated for each species. While PSP toxins are the most widespread seafood toxin problem in Australia, this product but using different antibodies can readily be applied to other biotoxins.

2. Molecular analyses suggest that *A. fundyense* may have been a cryptic genotype previously present in Tasmania, but newly stimulated by altered water column stratification conditions driven by changing rainfall and temperature patterns. This hypothesis is further being tested during a follow-up ARC Discovery grant funded project DP170102261 to look at ancient DNA in dated 1000 year old sediment depth cores.

3. An integrated on-line bloom prediction portal is being developed through IMOS (Integrated Marine Observing Systems) that continuously feeds in TSQAP shellfish toxicity results, temperature, rainfall and East Australian Current behavior to serve as a green, orange, red traffic light warning for impending biotoxin risks.

12. Extension and Adoption

We played a key role (Chair Ali Turnbull) in running two Australian Shellfish Quality Assurance Committee (ASQAAC) science days, one in Hobart 2014 and one in Sydney 2016. We also presented our work at three Shellfish Futures Conferences in St Helens 2015, Sorell 2016 and Hobart 2017.

We ran dedicated PST Training sessions during ASQAAC Hobart and St Helens Shellfish Futures , as well as full hands-on workshop in the IMAS laboratories in Hobart .

We hosted a broader Biotoxin Risk assessment Workshop and an FRDC project industry information workshop , both in Hobart.

13. Project coverage

This work attracted Australia- wide media attention

- http://www.utas.edu.au/latest-news/utashomepage-news/bloom-diagnostic-test-to-aid-shellfish-industry
- http:// www.abc.net. Au/news/ 2014-10-17/ a- new-fast-test-for-screening-shellfish-toxins-has/ 5823414? Section=tas].

Parts of this work were presented at national (Perth, Sept. 2017) and international conferences (Brasil, Oct. 2016; Galway, Ireland, May 2017).

14. Project materials developed

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FRDC FINAL REPORT CHECKLIST

Project Title:	Improved understanding of Tasmanian harmful algal blooms and biotoxin events to support seafood risk management		
Principal Investigators:	Gustaaf Hallegraeff, Chris Bolch, Katrina Campbell, Scott Condie, Juan Dorantes, Shauna Murray, Alison Turnbull, Sarah Ugalde		
Project Number:	2014/032		
Description:	Development, calibration and validation of improved monitoring and management tools to limit the impact of biotoxin outbreaks on the Tasmanian seafood industry		
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Key Words:	*Mytilus galloprovincialis, Crassostrea gigas, Jasus edwarsdii* **,** *Haliotis laevigata, Haliotis rubra***,** *Alexandrium tamarense.*		

Please use this checklist to self-assess your report before submitting to FRDC. Checklist should accompany the report.

	Is it included (Y/N)	**Comments**
Foreword (optional)	N	
Acknowledgments	Y	
Abbreviations	N	
Executive Summary	Y	
- **What the report is about**	Y	
- **Background – why project was undertaken**	Y	
- **Aims/objectives – what you wanted to achieve at the beginning**	Y	
- **Methodology – outline how you did the project**	Y	
- **Results/key findings – this should outline what you found or key results**	Y	
- **Implications for relevant stakeholders**	Y	
- **Recommendations**	Y	
Introduction	Y	
Objectives	Y	
Methodology	Y	
Results	Y	

Discussion	Y	
Conclusion	Y	
Implications	Y	
Recommendations	Y	
Further development	Y	
Extension and Adoption	Y	
Project coverage	Y	
Glossary	N	
Project materials developed	Y	
Appendices	N	