Improved risk management of Paralytic Shellfish Toxins in Southern Rock Lobster

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

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Table of abbreviations

AMSA	Australian Marine Science Association
ASQAP	Australian Shellfish Quality Assurance Program
AST	Analytical Services Tasmania
BT	Biosecurity Tasmania, Natural Resources and Environment Tasmania (NRE; previously Department of Primary Industries, Parks, Water and Environment, DPIPWE)
DAFF	Department of Agriculture, Fisheries and Forestry, previously Department of Agriculture, Water and Environment (DAWE)
DAWE	Department of Agriculture, Water and the Environment
dcNEO	Decarbamoylneosaxitoxin
dcSTX	Decarbamoylsaxitoxin
DH	Department of Health, previously Department of Health and Human Services (DHHS)
doSTX	Deoxycarbamoylsaxitoxin
DPIPWE	Department of Primary Industries, Parks, Water and Environment, Tasmania (now NRE)
FRDC	Fisheries Research & Development Corporation
GTX	Gonyautoxin
HP	Hepatopancreas
IMAS	Institute for Marine and Antarctic Studies
LC-FLD	Liquid chromatography fluorescence detector
LC-MS	Liquid chromatography – mass spectrometry
ML	Maximum level (regulatory level[1])
Neo	Neosaxitoxin
NRE	Department of Natural Resources and Environment Tasmania
NZ RLIC	New Zealand Rock Lobster Industry Council
POD	Probability of detection
PST	Paralytic shellfish toxin
SARDI	South Australian Research and Development Institute
ShellMAP	Shellfish Market Access Program
SRL	Southern Rock Lobster
STX	Saxitoxin
TEF	Toxic equivalency factor
TRLFA	Tasmanian Rock Lobster Fishermen's Association
TSIC	Tasmanian Seafood Industry Council
WFM	Wild Fisheries Management Branch, NRE

[1] The CODEX bivalve regulatory level for PST is 0.8 mg STX.2HCl equiv. kg^{-1} (CODEX Standard 292-2008) and the Australian bivalve regulatory level for PST is 0.8 mg STX equiv. kg^{-1} (FSANZ Standard 1.4.1).

Executive Summary

During 2012 an extensive bloom of the toxic dinoflagellate *Alexandrium catenella* occurred on the east coast of Tasmania causing paralytic shellfish toxins (PST) to accumulate in bivalve shellfish at 12 times the regulatory maximum level (ML; 0.8 mg STX equiv. kg^{-1}). Southern Rock Lobster, accumulated PST to 3.9 mg STX.2HCl equiv. kg^{-1}, resulting in the first closure of an Australian lobster fishery due to marine biotoxins. Recurrent blooms since 2012 have had an on-going impact on both the commercial fishery in Tasmania, valued at AUD 97 M, and the significant recreational fishing sector.

The present body of work aimed to address the following knowledge gaps exposed by this novel risk: (1) the toxicokinetics associated with PST uptake and depuration in Southern Rock Lobster from *A. catenella* blooms; (2) the impact of PST on lobster health; (3) the supply chain risk of PST accumulation in Southern Rock Lobster; and (4) to determine and validate (where appropriate) cost-effective methods to adequately monitor and manage PST accumulation in lobster in the field. In elucidating these questions, information was sought that could inform management of public health and market access risks and determine whether PST accumulation could adversely affect lobster performance, health, and catchability.

To examine toxicokinetics of PST in Southern Rock Lobster, an experimental study was undertaken in a biosecure aquaculture facility in South Australia. Adult male lobsters were fed highly toxic mussels (6 mg STX.2HCl equiv. kg^{-1}) sourced from the Tasmanian east coast for 4 weeks, then allowed to depurate for a further 5 weeks. Control (fed non-toxic mussels) and exposed lobster were harvested at regular intervals, tissues dissected and analysed for PST. The lobsters rapidly accumulated PST in the hepatopancreas (exponential rate of 6% per day), exceeding the bivalve ML within one week, and reaching a maximum of 9.0 mg STX.2HCl equiv. kg^{-1}. Once toxic feed was removed, the lobster depurated at a rate of 7% per day. Toxins were found in lobster antennal glands at concentrations two orders of magnitude lower than found in the hepatopancreas. This is the first report of PST in lobster antennal glands which, along with the gills, represent possible excretion routes for PST. However, PST were not detected at significant levels in the lobster haemolymph, which rules out the possibility of non-destructive sampling of lobsters for biotoxin analyses.

During the experiment, lobster health was assessed to determine the impact of PST accumulation in the hepatopancreas. A comprehensive range of behavioural (vitality score, righting ability and reflex impairment score), health (haemocyte count, bacteriology, gill necrosis and parasite load), nutritional (hepatopancreas index and haemolymph refractive index) and haemolymph biochemical (21 parameters including electrolytes, metabolites, and enzymes) parameters were examined. Accumulation of PST increased the apparent feed intake but did not result in mortality nor significant changes in the behavioural, health, or nutritional measures suggesting limited gross impact on lobster performance. Furthermore, most haemolymph biochemical parameters measured exhibited no significant difference between control and exposed animals. However, in the lobsters fed toxic mussels, the concentration of potassium in the haemolymph increased with PST, whilst the concentration

of lactate and the sodium:potassium ratio decreased with PST. In addition, lobsters with elevated levels of PST in the hepatopancreas showed a hyperglycaemic response, indicative of stress. These findings suggest that PST accumulation results in some measurable indicators of stress for lobsters, but these changes appear to be within the adaptive range for Southern Rock Lobster and do not result in a significant impairment of gross performance.

To determine whether Southern Rock Lobster could accumulate PST in the supply chain, a controlled experiment was conducted where adult male lobsters were exposed to highly toxic cultures of *A. catenella* at field relevant concentrations (2×10^5 cells L^{-1}) over a period of 21 days. In contrast to the feeding experiment, no PST accumulated in lobster from exposure to toxic algal cells. The same lobster health parameters were assessed, with no change seen in any of the behavioural, health, nutritional or haemolymph biochemical parameters examined.

To assess appropriate management strategies, regulatory monitoring results since September 2012 were combined with field studies to examine uptake and depuration of toxins in Southern Rock Lobster on the east coast of Tasmania during *A. catenella* blooms. Results from 499 lobster hepatopancreas PST samples were analysed. A high degree of variability was seen across years, months, sites and between individuals. The highest risk sites are on the central east coast, with exceedances of the bivalve ML occurring between July and January. Mussel sentinel lines were installed in each lobster management zone on the east coast of Tasmania and monitored fortnightly during high-risk periods. These lines proved effective in indicating a risk of elevated PST in lobster hepatopancreas. Field uptake and depuration rates of PST in lobster were similar to those seen during the experimental studies (maximum of 2% and 3% per day respectively), but always less than rates measured simultaneously in mussels. Statistical analysis of hepatopancreas PST levels during bloom development and decline occurred to determine the level of confidence in the regulatory sampling regime (5 individual lobster hepatopancreas samples are analysed from each site during an event). When PST in the hepatopancreas of all lobsters sampled is < 0.42 mg STX equiv. kg^{-1} there is 97.5% confidence that any lobster from that site would be below the bivalve ML.

The Neogen™ rapid test kit, a qualitative lateral flow immunoassay for the detection of PST, was previously shown to be the best commercially available rapid screening test of PST in bivalves affected by *Alexandrium catenella* in Tasmania. In the present study we confirmed its applicability to rock lobster hepatopancreas matrices. Based on the analysed toxin profiles, the test kit provides high confidence in detecting PST at levels above 0.4 mg STX.2HCl equiv. kg^{-1} in lobster hepatopancreas tissues. That means that during years with dense widespread blooms the Neogen rapid test could facilitate monitoring programs by making faster decisions when PST levels are less than half the regulatory limit. Negative Neogen results would not need to be followed up with more expensive LC-MS analysis. Based on our field validation, this could present an average annual cost savings of $5,138 - $6,154 ($41,100 - $49,228 across the eight biotoxin seasons monitored) and provide increased sample turn-around (1-1.5 days vs. 2-3 days for confirmatory LC-MS analysis), which in turn translates to shorter fishery closures (should no PST be detected).

Further work testing samples contaminated with *Gymnodinium/A. minutum* derived PST is required to ascertain how applicable the Neogen assay is to these blooms (positive Neogen results at lower PST concentrations may limit the cost-effectiveness of the assay during blooms of these species).

The combined field and experimental work has informed improvements to the biotoxin risk management program for Southern Rock Lobster in Tasmania. Implications for biotoxin risk monitoring are: (1) lobsters continue to feed during bloom periods and high concentrations of PST can occur; (2) animal toxin monitoring should be frequent at the start of a bloom in case of a rapid accumulation of PST; (3) mussel sentinel lines are a cost-effective early warning system for toxin accumulation; (4) it is adequate to sample 5 individuals per site as long as a reduced trigger level of closure of harvest is employed; (5) depuration is relatively quick so that sampling to confirm re-opening should occur soon after bloom collapse (as indicated by mussel PST levels); (6) non-lethal sampling is not possible as haemolymph PST levels do not reflect levels in the hepatopancreas. Importantly, (7) lobsters exposed to toxic algae during wet storage in long supply chains (on vessel, in sea-cages or at processing facilities) do not take up PST. Furthermore, (8) survival, quality, and safety of this high-value product are not impacted by accumulation of PST or by exposure to toxic cells in the water.

In conducting this research, it became apparent that international regulations for maximum levels of PST in seafood and research into PST accumulation vary in the units used for PST concentration. Some standards/research reports use mg STX.2HCl equiv. kg^{-1}, some mg STX equiv. kg^{-1} (effectively producing total PST results that are 24% lower), and some only state mg kg^{-1}. Similarly, the toxic equivalency factors (TEF) used to convert analogue concentrations to total PST in toxicity equivalents are varied, and often not stated. A call for uniform reporting units was published to highlight this issue, recommending countries and researchers adopt the Codex Alimentarius Commission protocols of reporting in mg STX.2HCl equiv. kg^{-1}, using FAO/WHO TEFs. To align with international standards, PST concentrations in this report are expressed as STX.2HCl equiv. kg^{-1}, except for Chapter 6. This chapter directly relates to current Tasmanian PST regulations which are aligned with the Food Standards Australia New Zealand (FSANZ) biotoxin standard and the Australian bivalve regulatory PST level. An application to harmonise the Australian biotoxin standard (PST expressed as STX equiv. kg^{-1}) with the international CODEX standard (PST expressed as STX.2HCl equiv. kg^{-1}) has been submitted to FSANZ by SafeFish and is currently being processed.

Keywords. Southern Rock Lobster, *Alexandrium catenella,* Paralytic Shellfish Toxins, marine biotoxin, PST analogues, spiny lobster, hepatopancreas, toxicokinetics, non-traditional vector, uptake, depuration, risk management.

1. Introduction

Starting in October 2012 the east coast of Tasmania has suffered from significant outbreaks of seafood contamination by paralytic shellfish toxins (PST) from dinoflagellate blooms of *Alexandrium tamarense* genotype 1 (now designated *Alexandrium catenella*). A previous FRDC project 2014/032 focused on the impact on oyster and mussel industries which experienced seasonal closures up to 4 months, most severely in 2012, 2015, 2016 and 2017. A key achievement of this project includes the introduction into Tasmania, calibration, fine-tuning and full validation of a simple immunological test kit for PST toxins that presents considerable analytical cost savings and increased sample turn-around (20 min to result) by testing shellfish directly at the farm gate.

The $97 M lobster industry has also been impacted by seasonal Tasmanian closures (up to 5+ months) due to PST contamination of hepatopancreas, notably in the St Helens and Maria Island regions), and up to Flinders Island, with an estimated lost revenue of $780,000 (Campbell et al 2013). While only trace levels of PSTs have been detected to date in lobster tail meat (Madigan et al., 2018a), hepatopancreas contamination poses significant trade barriers for key markets such as China and Hong Kong. Cooking of lobsters does not release biotoxins from the hepatopancreas into the cooking water or tail meat (Turnbull et al. 2018). Hepatopancreas is a sought after foodstuff (mainly used as a dipping sauce for tail meat), consumed by 16% of fishers on the Tasmanian east coast and 22% on the west coast (Madigan et al. 2018b). Limited evidence suggests that PST contamination of lobsters may also occasionally occur in South Australia and Victoria (Madigan et al. 2018a, Turnbull et al. 2020).

Since the 2012 *Alexandrium* bloom, the monitoring, management, and mitigation approaches for biotoxin risks in crustaceans in Tasmania have been continually refined and are spelled out in the DPIPWE Rock Lobster Biotoxin Monitoring and Decision Protocols (2020). The plan uses bivalve PST monitoring as a trigger for lobster testing, with a limited number (n=5) animals tested per management zone, at a cost of ~$2,340 for each zone and each sampling event. The fishery in the affected zone is closed during sampling and a decision to remain closed or open made based on the results of confirmatory PST analysis. Any result >0.5 mg STX equiv. kg^{-1} in the hepatopancreas leads to an assessment of risk, potentially resulting in the zone remaining closed, while any individual returning a result of >0.8 mg STX equiv. kg^{-1} always results in the zone remaining closed. Once closed, reopening of fishing zones is dependent upon the results of continued hepatopancreas sampling and bloom activity in the zone.

Previous improvements in Southern Rock Lobster (SRL) biotoxin risk management options have occurred following SARDI project 2013-713. From this project (CI Madigan), we have experience with Southern Rock Lobster in-tank feeding experiments providing them PST contaminated Spring Bay (Tas) mussels. This work reported biotoxin accumulation of 3.7 mg STX.2HCl equiv kg^{-1} hepatopancreas after 28 days of feeding. Using the new $350,000 SARDI biotoxin contamination facility grant (FRDC 2017-051, CI Turnbull), the present

project builds upon these initial observations to further refine existing monitoring and management of PST in Tasmanian Southern Rock Lobster through a series of laboratory and field studies, including:

- Aquaculture tank trials exposing lobster to naturally PST contaminated mussels to determine PST uptake and depuration rates, as well as exploring the feasibility of non-destructive sampling by testing for PST in the haemolymph (Chapter 3).
- Assessments of the potential impacts of PST accumulation on lobster behaviour and health during these tank trials (Chapter 4).
- Exposure of lobsters to live *Alexandrium* microalgal cultures to determine if lobster are at risk of PST accumulation/health impacts during wet storage (Chapter 5).
- A field validation of the current Tasmanian Southern Rock Lobster PST monitoring program, including assessment of PST variability between individual animals, sampling sites, sampling months and years, as well as determination of PST uptake rates in the field (Chapter 6).
- A single laboratory validation of the qualitative Neogen PST test kit to determine if this assay can reliably detect PST in lobster hepatopancreas tissues (Chapter 7).
- An evaluation of the Neogen test kit using naturally contaminated lobster hepatopancreas samples to assess the cost-effectiveness of incorporating the assay into existing biotoxin monitoring protocols (Chapter 8).

The project findings are summarised (Chapter 9) to provide detailed recommendations for improved biotoxin management and additional research to further refine the cost-effectiveness of biotoxin monitoring operations.

2. Objectives

1. Assess the applicability of Neogen PST test kits for lobster viscera.

2. Exploration of lobster haemolymph as a proxy for lobster viscera PST contamination, and of the Neogen PST test kit for use with haemolymph if appropriate.

3. Determination of the impact of PST accumulation on lobster vigour.

4. Improved definition of East Coast Tasmanian lobster management zones, based on improved understanding of dietary origin of PST (from tank studies), and improved understanding of variability (from field studies).

3. Paralytic shellfish toxin uptake, tissue distribution, and depuration in the Southern Rock Lobster

Alison Turnbull, Navreet Malhi, Andreas Seger, Tim Harwood, Jessica Jolley, Quinn Fitzgibbon & Gustaaf Hallegraeff

Abstract. Up to 13.6 mg STX.2HCl equiv. kg^{-1} of paralytic shellfish toxins (PST) have been found in the hepatopancreas of Southern Rock Lobster on the east coast of Tasmania. Blooms of the toxic dinoflagellate *Alexandrium catenella* have been reported in this region since 2012. Experimental work was undertaken to improve the understanding of the uptake and depuration mechanisms involved. Adult male lobsters were fed highly toxic mussels (6 mg STX.2HCl equiv. kg^{-1}) sourced from the impacted area. The apparent feed intake of the lobster was positively correlated to increasing PST levels in the hepatopancreas. Toxins accumulated rapidly in the hepatopancreas reaching a maximum of 9.0 mg STX.2HCl equiv. kg^{-1}, then depurated at a rate of 7% per day once toxic fed was removed. However, PST were not detected at significant levels in the haemolymph of these animals. Notable increases occurred in the relative amount of several PST analogues, including GTX2&3, C1&2 and decarbomoyl toxins in the hepatopancreas in comparison to the profile observed in contaminated mussel feed. The concentration of PST in lobster antennal glands was two orders of magnitude lower than concentrations found in the hepatopancreas. This is the first report of PST in lobster antennal glands which, along with the gills, represent possible excretion routes for PST. Implications for the risk monitoring program are: monitoring should be frequent at bloom start to cover the potential for a rapid accumulation of PST; non-lethal sampling is not possible as toxicity in the haemolymph does not represent toxicity in the hepatopancreas; lobsters will continue to feed during bloom periods and thus high concentrations of PST can occur.

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3.1 Introduction

Blooms of the paralytic shellfish toxin (PST) producing dinoflagellate *Alexandrium catenella* (Whedon and Kofoid) Balech, previously referred to as *A. tamarense* Group 1 (Litaker et al. 2018), have been occurring annually on the east coast of Tasmania, Australia, since 2012 (Condie et al. 2019). These blooms result in accumulation of PST in filter feeding bivalve shellfish and in the hepatopancreas of Southern Rock Lobster. Toxin accumulation creates a public health risk for recreationally and commercially harvested product (McLeod et al., 2018). It also creates a market access risk for the Tasmanian lobster fishery (Campbell et al., 2013), which is largely export focused and worth AUD $93 M per annum (Mobsby, 2018).

Current biotoxin risk management practices require destructive sampling of many animals to address the high variability found in PST accumulation (Madigan et al., 2018). Lobster haemolymph can be readily extracted and therefore offered an opportunity for non-lethal sampling. PST have been identified in the blood of humans (Gessner et al., 1997; Llewellyn et al., 2002), sea turtles (Dorantes and Hallegraeff 2018, pers. comm), and PST-like substances were also previously detected in the haemolymph of xanthid crabs (Llewellyn, 1997). Other marine biotoxins have been commonly detected in the blood of marine animals, for instance: domoic acid in dolphins, seals and sea lions (Gulland, 2000; Lefebvre et al., 2016; Schwacke et al., 2010); brevetoxins in fish, dolphins, sea turtles and sea birds (Fauquier et al., 2013a; Fauquier et al., 2013b; Fire et al., 2007; Woofter et al., 2005); and ciguatoxins in fish (O'Toole et al., 2012). If PST concentrations in Southern Rock Lobster haemolymph were correlated to those in the hepatopancreas, this would offer an alternative to lethal sampling of this high value product.

Uptake of PST in lobsters occurs upon the consumption of contaminated prey, with PST concentrated in the hepatopancreas but not in the meat (Haya et al., 1992; Madigan et al., 2018). The mechanisms for uptake and depuration of PST in Southern Rock Lobster are poorly understood, and toxin distribution across other tissues is unknown. Previous studies have shown slow depuration of PST from American and Southern Rock Lobster hepatopancreas (Desbiens and Cembella, 1995; Haya et al., 1994; Madigan et al., 2018), with slightly higher rates in fed compared to starved animals (Madigan et al., 2018). The main excretion route for PST in mammals is via urine (Andrinolo et al., 1999; Gessner et al., 1997). The antennal glands act as the equivalent renal system in lobster (Mantel and Farmer, 1983), although nitrogenous compounds are also lost through the gills. Toxic compounds such as heavy metals can be taken in through the gills.

In this study, we examined the uptake and depuration of PST in Southern Rock Lobster following consumption of highly toxic mussels collected during an *A. catenella* bloom on the Tasmanian east coast. Hepatopancreas, hindgut, antennal gland and gill tissues were analysed for PST to determine uptake and depuration rates, and potential routes of excretion. The levels of PST in the haemolymph were studied to determine whether the haemolymph could provide an alternative, non-lethal method of sampling for risk management purposes.

3.2 Methodology

Experimental system

Adult male Southern Rock Lobster, (n=80), weighing 600 – 800 g, were sourced from fishing vessels landing at Port Lincoln and Kangaroo Island, South Australia in March 2018. The lobsters were held in individual 30 L tanks in mesh cages in a flow through system as described by Madigan et al. (2018). Briefly, water temperature in the lobster tanks was maintained between 13.2 and 14.8 ^{o}C, similar to temperatures found in Tasmania during winter (BOM, 2018), and salinity was maintained between 35.5 and 37.0. Water quality was maintained by biological filtration using pre-conditioned sponge biofilters, and by adjusting flow rate in response to nutrient measurements. Flow rates in each tank ranged from 2 – 10 L h^{-1}. Water quality parameters of pH and conductivity (HACH KTO sensION), temperature and dissolved oxygen (DO, Oxyguard, Polaris 2) were analysed daily in each tank prior to cleaning; whilst ammonia (API® test strips) and nitrite (HACH NitriVer®3 pillow reagents and DR900 Colorimeter) were analysed on a rotating basis every third day; and nitrate (HACH NitraVer5® pillow reagents) was analysed every 10 days. Bicarbonate soda was added to the sea water supply as required, maintaining pH between 7.8 - 8.2. Dissolved oxygen mean and standard deviation were 94.7 ± 3.9% saturation. Mean and standard deviation of ammonia, nitrite and nitrate levels were 0.67 ± 0.68, 0.26 ± 0.24 and 1.7 ± 1.0 mg L^{-1}, respectively. Faeces and surplus food were removed daily by siphoning and immediately refilling the tank with seawater. Low level lighting (10 lumens in the lobster cages) was provided on a 12:12h light:dark cycle.

Lobster treatments

Lobsters were held in the experimental system for 13 days to acclimatise prior to the experiment. During acclimation, all animals were fed with in-shell blue mussels (*Mytilus galloprovincialis* Lamarck), sourced from Port Lincoln, South Australia, during a period with no known algal blooms. Two pooled mussel samples, each containing 12 randomly selected mussels, were analysed to confirm there was no detectable PST in the mussels (see analytical method below). For the first week, each lobster received one mussel initially, and then an additional mussel each day, if all mussels presented had been consumed. For the following 7 days, lobsters were fed one additional mussel to what they had eaten the previous day, to a maximum of 3 mussels per day.

Lobsters (n = 77) were randomly divided into control and exposed groups. Lobsters were then further divided into harvest groups of 7 replicates, such that a control and exposed group could be harvested on days 7, 17, 27, 41 and 63 with an additional control group harvested on day 0. Seven replicates were used to allow statistical rigour whilst minimising the number of experimental animals for ethical reasons. Lobsters were randomly allocated to each group using R software (R Core Development Team, version 3.4.3, November 2017).

During the experiment, lobsters were fed up to a maximum of 3 mussels per day until day 7, then 2 mussels a day following this to reduce nutrient build up in the tanks. Control animals were fed non-toxic mussels, whilst exposed animals were fed toxic in-shell blue mussels during the uptake phase (days 0-26), and non-toxic mussels during the depuration phase (days 27-62). Toxic mussels were sourced from Great Oyster Bay, Tasmania, during an *A. catenella* bloom in 2017. The toxin content of the mussels, determined by averaging two pooled samples of 12 randomly selected mussels, was 6.10 mg saxitoxin (STX.2HCl) equiv. kg^{-1}. On day 2, toxic mussels were mistakenly fed to control animals. The toxic mussels were quickly removed from the tanks, but not before some lobsters had partially consumed them.

Apparent Feed Intake

The apparent feed intake (AFI) of each harvest group was measured in the week prior to harvest following Fitzgibbon et al. (2017). Feed control tanks (one for non-toxic and one for toxic mussels) were included in the random allocation, with no lobsters placed in these tanks. The feed control tanks received the same number of mussels as the exposed and control lobsters. At the beginning of each day, uneaten mussel meat from each tank (control and exposed animals, and feed control tanks) was collected, the meat was removed from the shell, and frozen cumulatively in ziplock plastic bags. Subsequently, the uneaten food was dried at 105 ^{o}C for 24 h and the dry weight of each bag was measured. The AFI of each lobster was calculated by subtracting the dry weight of the uneaten food from that of the respective control tank, and dividing by the number of days over which the mussels were collected.

Harvest protocol, haemolymph sampling and dissection

For experimental sampling, lobsters were removed from the water, euthanised in an ice slurry, and haemolymph samples (5-15 mL) immediately taken from the sinus under the right 5th leg joint. Haemolymph was then distributed into chilled vials and frozen. The animals were kept on ice over-night and dissected the following day to collect the hepatopancreas, antennal glands, gills, and hind-gut, all of which were weighed. The hepatopancreas was homogenised (VELP OV5) and all tissue samples frozen at -80 ^{o}C.

Analytical methods

All PST samples were analysed at Cawthron Institute, New Zealand, by LC-MS/MS (Waters Acquity UPLC i-Class system coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer with electrospray ionization) following the method described by Boundy et al. (2015) and Turner et al. (2015). Results were reported in mg STX.2HCl equiv. kg^{-1} calculated using FAO toxicity equivalency factors (FAO/WHO, 2016). Sample preparation used a different solvent-to-sample ratio than detailed in the methods described above and involved weighing 2.0 ± 0.1 g of homogenised lobster hepatopancreas (or alternative matrix; hindgut, antennal gland or gill) into a 50 mL centrifuge tube followed by the addition of 18 mL of 1% acetic acid. The mixture was vortex mixed before being placed into a boiling water bath for 5 min. Samples were then cooled for 5 min in an ice bath before further vortex mixing. Insoluble debris was pelleted by centrifugation at 3,200 x g for 10 min before a 1 mL aliquot was

transferred into a 1.5 mL polypropylene tube, followed by the addition of 5 µL of ammonium hydroxide (NH_4OH; 25% ammonia) before clean-up. The solid-phase extraction clean-up procedure was performed manually with amorphous graphitized polymer carbon Supelco ENVI-Carb 250 mg/3 mL cartridges. The cartridges were conditioned with 3 mL of acetonitrile–water–acetic acid (20:80:1, v/v/v), followed by 3 mL of water–25% NH_4OH (1,000:1, v/v). Sample extracts (400 µL) were loaded onto the conditioned cartridges and then washed with 700 µL of MilliQ water. PST were eluted with 2 mL of acetonitrile–water–acetic acid (20:80:1, v/v/v) into a labelled 15 mL polypropylene tube. The eluent was mixed and diluted by transferring 100 µL to a polypropylene autosampler vial and adding 300 µL of acetonitrile. Sample analysis used hydrophilic interaction LC-MS/MS, as described by Boundy et al. and Turner et al. Method performance was assessed by fortifying PST-free blank matrix generated from control animals and determining recovery. Results reported as part of this study were corrected based on spike recoveries observed for the different sample matrices analysed. The limit of reporting for each PST analogue differed for each matrix tested.

For the haemolymph, sample preparation differed in that 2 g of coagulated material was weighed into a 15 mL polypropylene tube and just 2 mL of 1% acetic acid added. The mixture was vortex mixed before being placed into a boiling water bath for 5 min. Samples were then cooled for 5 min in an ice bath before further vortex mixing. Insoluble debris was pelleted by centrifugation at 3,200 x g for 10 min before a 1 mL aliquot was transferred into a 1.5 mL polypropylene tube. The remaining sample preparation steps were then the same as described above. To increase the sensitivity of the haemolymph analysis, the eluant from the solid phase extraction step was reduced in volume under nitrogen to give an up to 10-fold concentration.

Data analysis

All statistical analyses were performed using R Software (R Core Development Team version 3.6, April 2019). Data were checked for homoscedasticity using Levene's test and normality using the Shapiro-Wilk test, and appropriate transformations made if required prior to testing for significant differences using analysis of variance.

The retention of PST in the hepatopancreas of each lobster after the first week of exposure was calculated from a comparison between the amount of PST consumed (the product of the concentration of PST and weight of mussels consumed) against the amount of PST increase in the hepatopancreas (the product of the concentration of PST and weight of the hepatopancreas at day 7 for each exposed animal minus the mean amount of PST in the day 0 control lobsters). PST uptake rate was calculated from exposed animals harvested on days 0, 7, 17 and 27, using non-linear least squares modelling. PST depuration rates were calculated from exposed animals harvested on days 27, 41 and 63. Only lobsters with total PST > 0.02 mg STX.2HCl equiv. kg^{-1} were used for rate calculations. Rates were compared using least square means and the Tukey method for comparison of means. Differences were considered statistically significant when $p < 0.05$.

3.3 Results

Stocking animals

The lobsters were an approximately equal mix of red and brindle (pale red), indicating they had been sourced from a mix of shallow and deep habitats (Chandrapavan et al. 2010); 76 lobsters were male and 1 was female. At harvest, the lobsters weighed 598 – 905 g, with carapace lengths of 110-131 mm. One male lobster died (control, day 7 harvest group), and two lobsters successfully moulted (exposed, day 41 and 63 harvest groups). One exposed and one control animal were noted during dissection to have thicker inner skins with soft shells, indicating they were in preparation for a moult.

Apparent Feed Intake (AFI)

There was no significant difference in AFI across days (p >0.05), but AFI was significantly greater in the exposed animals fed toxic mussels compared to control lobsters (p=0.04, Figure 3.1). The difference between the mean of the control and exposed animals was 0.3 g day^{-1}.

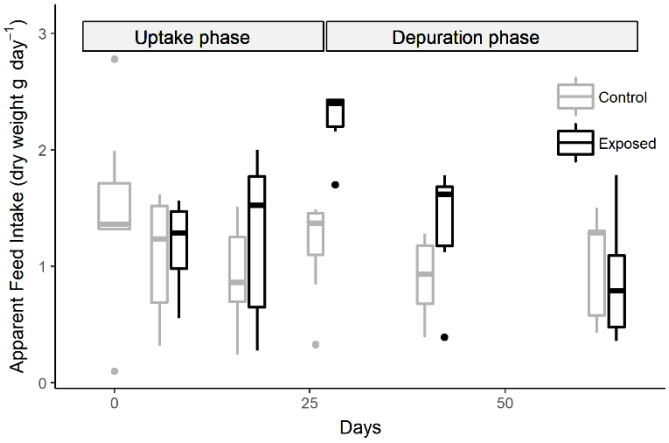

Figure 3.1 Apparent feed intake of lobster, Southern Rock Lobster, fed non-toxic (control group) and toxic mussels (exposed group) during, uptake and depuration phases of the experiment.

Total PST accumulation and depuration in the hepatopancreas of Southern Rock Lobster

Surprisingly, low levels of PST were detected in the hepatopancreas of all control lobsters. Control lobsters on day 0 ranged from 0.01 to 0.08 mg STX.2HCl equiv. kg^{-1} Control lobsters harvested on day 7 (after the feeding error) showed statistically significantly higher levels than day 0, 41 and 63 (p = 0.02, 0.04 and 0.003 respectively). There was a significant difference between the PST in the control and exposed animals and across days (p < 0.001). After one week of feeding on toxic mussels, each exposed lobster had consumed 52.6 ± 17.9 g of mussels. Given the average PST concentration of the mussels was 6.10 mg STX.2HCl equiv.

kg^{-1}, this equates to a total intake of 321 ± 109 µg STX.2HCl equiv. At the end of the first week, only 14 ± 5% of the total toxin intake was retained in the hepatopancreas (Table 3.1).

Over the 27 days of uptake, PST accumulated to high levels in the hepatopancreas of the exposed lobsters, mean of 6.66 ± 1.66 mg STX.2HCl equiv. kg^{-1}, maximum concentration of 9.0 mg STX.2HCl equiv. kg^{-1} on day 27). The estimated exponential uptake rate over this period was 0.06 mg STX.2HCl equiv. kg^{-1} day^{-1} .

Following 36 days of depuration, the PST concentration in the hepatopancreas decreased to 0.26 ± 0.17 mg STX.2HCl equiv. kg^{-1}. The estimated exponential depuration rate of total PST over this period was -0.07 mg STX.2HCl equiv. kg^{-1} day^{-1}.

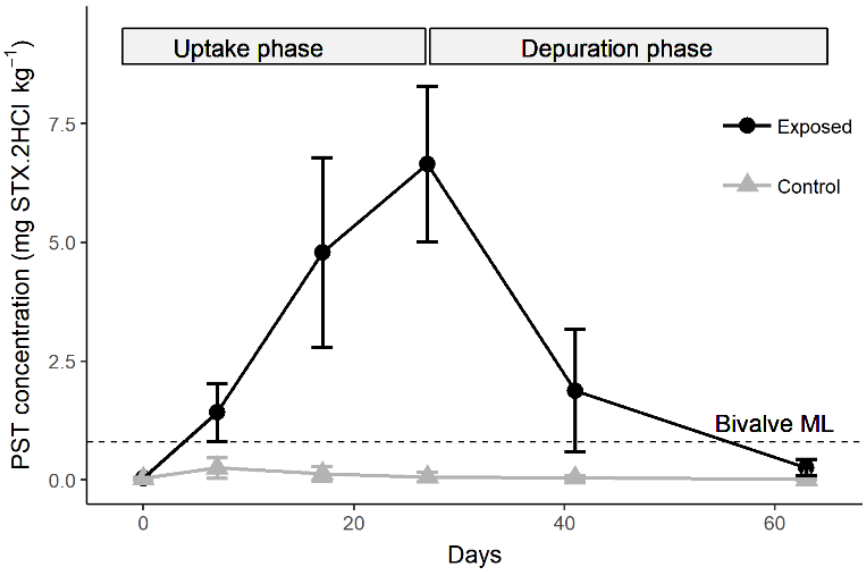

Figure 3.2 PST concentrations (mean ± standard deviation) in the hepatopancreas of lobster, Southern Rock Lobster. Control lobsters (grey triangles) fed non-toxic mussels; exposed lobsters (black circles) fed toxic mussels until day 27 (uptake phase), then non-toxic mussels until day 63 (depuration phase). The bivalve regulatory maximum level (ML) is shown as the dotted line.

Table 3.1 Retention of PST in *J. edwardsii* hepatopancreas after one week of feeding on toxic mussels.

Replicate	Wet weight mussels consumed (g)	PST consumed (μg STX.2HCl equiv.) *	Hepato-pancreas weight (g)	PST in hepato-pancreas (mg STX.2HCl equiv. kg^{-1})	PST increase in hepato-pancreas# (μg STX.2HCl equiv.)	Retention (%)
1	40.2	245.1	31.5	1.1	35.2	14.4
2	48.6	296.7	21.9	0.9	19.1	6.4
3	61.7	376.6	30.1	2.1	63.2	16.8
4	69.9	426.4	28.7	1.5	42.9	10.1
5	17.1	104.1	36.0	0.7	24.1	23.2
6	58.5	356.8	31.0	1.2	35.8	10.0
7	72.5	442.2	32.7	2.3	74.7	16.9
Mean ± SD	52 ± 18	321 ± 109	30.3 ± 4.0	1.4 ± 0.6	43.1 ± 18.7	14.0 ± 5.2

* Mean PST concentration of mussels was 6.1 mg STX.2HCl equiv. kg^{-1}

Amount PST in hepatopancreas at Day 7 minus mean amount PST present in hepatopancreas in control animals on day 0 (0.9 μg STX.2HCl equiv.)

Table 3.2 Exponential uptake and depuration rates of PST analogues in the hepatopancreas of Southern Rock Lobster hepatopancreas fed contaminated mussels for 27 days followed by depuration for 36 days. Rates sharing a letter are not significantly different.

PST Analogue	Uptake rate *mg STX.2HCl equiv. kg^{-1} day^{-1}*	Depuration rate mg STX.2HCl equiv. kg^{-1} day^{-1}
STX	0.087^{a}	-0.066^{a}
GTX1&4	0.040^{b}	-0.169^{b}
GTX2&3	$0.068^{a,b}$	-0.062^{a}
C1&2	$0.059^{a,b}$	$-0.079^{a,b}$
dcSTX	0.103^{a}	-0.065*
dcGTX2&3	$0.061^{a,b}$	-0.059*
Total PST	0.06	-0.070

* Depuration rate not compared due to low and/or infrequent detection values

Impact of PST on feed intake

Higher concentrations of PST in the hepatopancreas significantly correlated with higher feed intake in the exposed animals (AFI = 0.18*PST + 0.85, correlation coefficient r= 0.72, Figure 3.3).

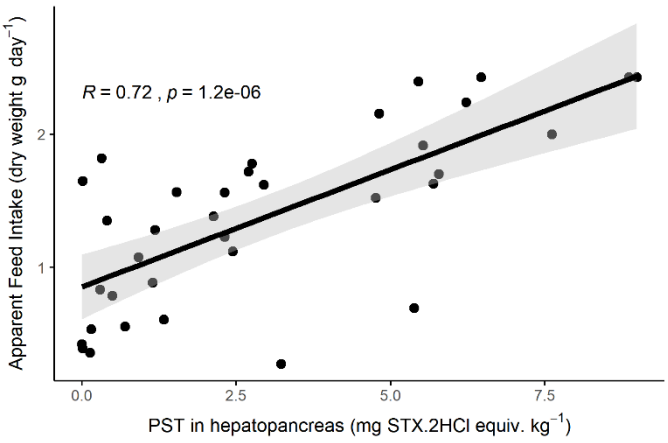

Figure 3.3 Apparent feed intake of exposed Southern Rock Lobster in relation to concentrations of PST in the hepatopancreas, including regression line and 95% confidence interval.

Total PST in other Southern Rock Lobster tissues

PST were analysed in the hindgut, haemolymph, antennal glands, and gills of lobsters at the peak of the uptake phase (day 27, harvest 4), and compared to that found in the hepatopancreas (Table 3.2). Clearly quantifiable PST levels were detected in all the hindgut samples (0.06 – 1.86 mg STX.2HCl equiv. kg^{-1}), which contained variable amounts of faecal material. PST were also consistently detected in the antennal glands (0.06 – 0.18 mg STX.2HCl equiv. kg^{-1}), although approximately two orders of magnitude lower than the concentrations in the hepatopancreas. Very low levels of PST were detected in 6 of the 7 lobster gill samples (maximum 0.033 mg STX.2HCl equiv. kg^{-1}). However, PST were only detected above the reporting limit in two haemolymph samples, both at extremely low levels of 0.32 and 0.86 ng STX.2HCl equiv. mL^{-1} (replicates 3 and 7 respectively).

Table 3.3 PST concentrations in the Southern Rock Lobster tissues after lobsters had been feeding on PST contaminated mussels for 27 days. Concentrations expressed as mg STX.2HCl equiv. kg^{-1}.

Replicate	Hepatopancreas	Hindgut	Antennal Glands	Gills
1	4.82	0.81	0.06	0.031
2	5.45	1.86	0.09	0.010
3	5.78	0.64	0.08	0.033
4	6.22	1.24	0.07	0.016
5	6.46	0.44	0.07	<LOR
6	8.86	0.79	0.18	0.027
7	8.99	0.06	0.13	0.004
Mean ± SD	6.65 ± 1.64	0.83 ± 0.58	0.09 ± 0.04	0.02± 0.01

PST were analysed in the hindgut, haemolymph, antennal glands, and gills of lobsters at the peak of the uptake phase (day 27, harvest 4), and compared to that found in the hepatopancreas (Table 3.2). Clearly quantifiable PST levels were detected in all the hindgut samples (0.06 – 1.86 mg STX.2HCl equiv. kg^{-1}), which contained variable amounts of faecal material. PST were also consistently detected in the antennal glands (0.06 – 0.18 mg STX.2HCl equiv. kg^{-1}), although approximately two orders of magnitude lower than the concentrations in the hepatopancreas. Very low levels of PST were detected in 6 of the 7 lobster gill samples (maximum 0.033 mg STX.2HCl equiv. kg^{-1}). However, PST were only detected above the reporting limit in two haemolymph samples, both at extremely low levels of 0.32 and 0.86 ng STX.2HCl equiv. mL^{-1} (replicates 3 and 7 respectively).

PST analogues in contaminated mussels and lobster tissues

Most of the PST analogues observed in contaminated mussels on a molar percentage basis were gonyautoxins (GTX), with GTX1&4 and GTX2&3 accounting for 56% and 32% of total PST observed respectively (Figure 3.4). Other analogues present in lower concentrations were STX, C1&2, and M2.

In comparison, GTX2&3 was the dominant analogue in the lobster hepatopancreas, consistently constituting approximately 50% of the toxin profile observed during the uptake phase (Figure 4.4). The relative amount of GTX1&4 found in the hepatopancreas was lower than that in the mussels, with 12% found in the hepatopancreas initially, decreasing further during the uptake phase. After GTX2&3, C1&2 was the major analogue observed in lobster hepatopancreas (approximately 20%), although C1&2 was only observed to be a minor proportion of PST in the contaminated mussels. Higher proportions of STX and decarbomoyl saxitoxin (dcSTX) were also observed in the lobster hepatopancreas during uptake than was observed in the mussels (Figure 3.4). This was reflected in relatively high uptake rates for STX

and dcSTX, although these were only statistically significantly different to that of GTX1&4 (Table 3.2).

During depuration, GTX2&3 remained the dominant analogue, whilst the relative amount of GTX1&4 decreased. This was reflected in the depuration rate for GTX1&4, which was significantly higher than for all other analogues except C1&2 (Table 3.2). The relative amount of C1&2 also decreased during depuration whilst STX, dcSTX and dcGTX2&3 increased.

The hindgut consisted of faecal matter and the gut lining. The PST profile in the hindgut was similar to that in the hepatopancreas, particularly at the end of the depuration phase. As with the hepatopancreas and the hindgut, GTX2&3 dominated the toxin profile of the antennal glands and gills. The antennal glands also contained a similar relative amount of GTX1&4 as the hepatopancreas, and low levels of C1&2, but neither of these analogues were detected in the gills (noting that the concentration of PST in the gill tissue was low; Figure 3.4). The proportion of dcSTX and dcGTX2&3 was higher in the antennal glands and gills (approximately 50% of the toxin profile) than in the hepatopancreas.

Of the nine haemolymph samples analysed from animals with elevated hepatopancreas PST levels, there was only a detection of a single analogue (GTX1&4) in two samples above the limit of detection. Trace detection of other analogues was observed, but at levels well below the limit of detection of the assay (approx. 15 ng STX.2HCl equiv. mL^{-1}).

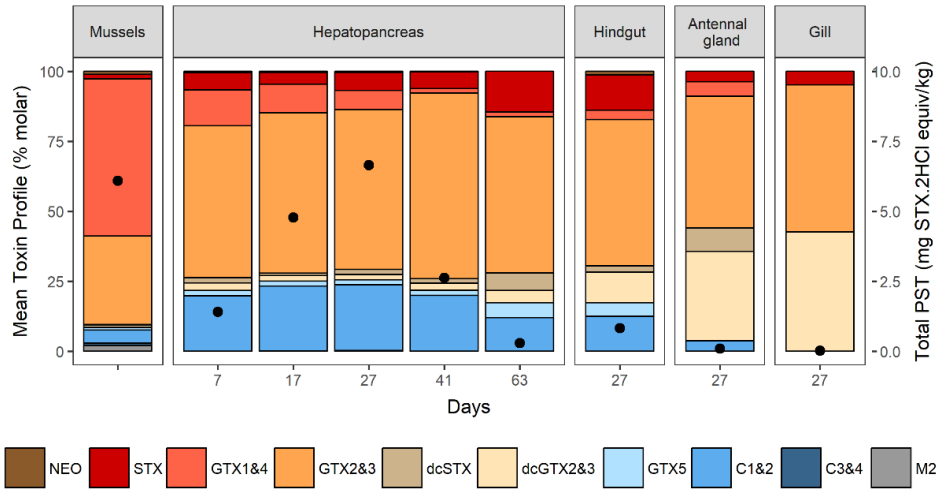

Figure 3.4 PST analogue mean profiles (percent molar) in contaminated blue mussels; hepatopancreas of exposed Southern Rock Lobster during uptake (days 7-27) and depuration (days 28-63) phases; and lobster hindgut, antennal glands and gills at the end of the uptake phase (day 27). Total PST (mg STX.2HCl equiv. kg^{-1}) shown as black circles. Only samples with total PST >0.02 mg STX.2HCl equiv. kg^{-1} are included in the calculation of the mean percent molar profiles.

3.4 Discussion

Feeding toxic mussels to Southern Rock Lobster resulted in a rapid and large accumulation of PST in the hepatopancreas: extrapolation of the hepatopancreas PST data shows concentrations would have exceeded the bivalve maximum allowable level of 0.8 mg STX.2HCl equiv. kg^{-1} (Codex Alimentarius Commission, 2008) within four days. The potential for lobster to rapidly accumulate PST if highly toxic prey species are available was also demonstrated by Hay et al. (1992 & 1994) (Table 3.4), and is a concern for lobster biotoxin risk management.

Southern Rock Lobster hepatopancreas PST concentrations reached approximately four times that established by Madigan et al. (2018) in a similar experimental tank study, and close to the maximum of 13.6 mg STX.2HCl equiv. kg^{-1} found in field monitoring of this species from the east coast of Tasmania in 2017 (unpublished data). This is well below the PST concentrations reached during experimental studies with American Lobster, *Homarus americanus* (maximum 32 mg STX equiv. kg^{-1}, summarised in Table 3.4). However, the large PST loads in this study did not affect the survival of Southern Rock Lobster.

After feeding on highly toxic mussels for one week, only 14% of the total toxin consumed was retained in the hepatopancreas of Southern Rock Lobster. This is reflected in similarity in uptake and depuration rates. When reviewing detoxification kinetics in bivalve molluscs, Bricelj and Shumway (1998) divided them into two groups: rapid to moderate detoxifers (e.g. *Mytilus edulis* and *Crassostrea gigas*) and slow detoxifers (e.g. *Saxidomus giganteus* and

Spisula solidissima), with elimination rates of above or below 4% per day respectively. The depuration rate for total PST determined in this work translates to a toxin elimination of 7% per day. Previous work by Madigan et al. (2018) found a lower rate of 2% per day for fed Southern Rock Lobster (depuration rate of 0.02 mg STX.2HCl equiv. kg^{-1} day^{-1}). These two depuration rates place this species in the low end of the moderately fast detoxifers, or the high end of the slow detoxifers respectively. This finding is positive for the Southern Rock Lobster fishery as it means that lobster will not retain PST for long periods after the bloom has passed. In contrast, depuration rates reported for American Lobster vary, with one study finding 3% per day and another finding no significant depuration over 53 days (Table 3.4).

Low concentrations of PST were detected in all control lobsters, including lobsters harvested on day 0. This indicates that the animals in the field had been exposed to PST, and is the second report of low level PST in lobsters from South Australia (Madigan et al., 2018). Animals were sourced from this area as there are no known problems with PST blooms.

Current practises for monitoring PST in Southern Rock Lobster require destructive sampling of multiple high-value animals at each sampling occasion, due to the high variability between individuals. Such sampling is both costly and wasteful (Madigan et al., 2018). Dorantes-Aranda and Hallegraeff have detected PST in sea turtle blood (unpublished 2018), and studies on brevetoxin (BTX) in fish, sea birds and sea turtles, have shown that blood samples can be an useful indicator of intoxication for this toxin (Fauquier et al., 2013a; Fauquier et al., 2013b; Woofter et al., 2005). However, despite the high concentrations of PST in the hepatopancreas achieved during this study, PST concentrations in the haemolymph were below the level of detection for most of the samples analysed. As lobsters were harvested a minimum of 18 hours after the mussels were presented to them, one explanation could be that PST have a short half-life in lobster haemolymph, as found for PST in the blood of humans (Gessner et al., 1997), cats (Andrinolo et al., 1999) and rats (Hines et al., 1993). In a symposium abstract, Cembella and Desbiens (1994) similarly reported no PST in the haemolymph of American Lobsters with high levels of PST in the hepatopancreas. This important finding unfortunately demonstrates that haemolymph sampling would not be an effective non-lethal method for risk management.

Observations during a previous PST contamination experiment with Southern Rock Lobster raised the question of potential reduction of feed intake when Southern Rock Lobster are exposed to PST, but AFI was not measured in that study (Madigan et al., 2018). In the present experiment, exposed

Table 3.4 Summary of experimental studies on PST uptake and depuration in rock lobster hepatopancreas.

Lobster species/Geographic region	Method of Analysis	Feed details & water temperature	Uptake data	Depuration data	Reference
Homarus americanus Bay of Fundy	AOAC Mouse bioassay + Mouse neuroblastoma	*Placopecten magellanicus,* 40.0 mg STX equiv. kg^{-1} Fed for twice a week for 16 weeks, 12-14° C	PST increased to week 2, no significant increase thereafter. Range 2.7 – 32 mg STX equiv. kg^{-1}	NA	Haya et al. (1992) (symposium abstract only)
Homarus americanus Bay of Fundy	AOAC Mouse bioassay + Chemical method (not referenced)	Scallop (spp and PST concentration not stated) Fed toxic feed every 2nd day for 8 weeks, then non-toxic feed, 10°C	21.5 mg STX equiv. kg^{-1} after 4 weeks, 15.5 mg STX equiv. kg^{-1} after 8 weeks. C1&2 did not transfer from scallop, GTX1,2 & NEO proportions decreased cf scallop feed	Half-life of 23 days (equates to exponential depuration rate of 0.03 mg STX equiv. kg^{-1} day^{-1}). Below regulatory level in 70 d. Proportion of STX, NEO and dcSTX increased and GTX1,4 decreased during depuration	Haya et al. (1994) (symposium abstract only)
Homarus americanus Quebec	AOAC Mouse bioassay + HPLC-FD (in-house method)	Lobster collected in bloom, some starved and depurated, others fed toxic *Mya arenaria* (PST concentration not stated) 12-14 °C	Not stated. No significant PST in haemolymph	Harvest animals 0.45-15.5 mg STX equiv. kg^{-1} (MBA), 0.45-12.0 (HPLC). No significant depuration after 53 d	Cembella & Desbiens (1994) (symposium abstract only) Desbiens & Cembella (1995 & 1997)
Homarus americanus Bay of Fundy	AOAC Mouse bioassay	*Spisula solidissima* 0.03-22.4 mg kg^{-1}, one feeding, 18 °C	Max 1.1 mg kg^{-1}	NA	Yentsch & Balch (1975)
Panulirus stimpsoni China	AOAC 959.08 Mouse bioassay & HPLC (Oshima, 1995). Large differences between two method results.	*Chlamys nobili* 0.85 mg STX equiv. kg^{-1} Fed for 6 days, then fed non-toxic *C. nobili* or squid for 6 days, paired or single replicates only, 21-24 °C	0.08-0.12 mg STX equiv. kg^{-1}, 0.9 -2.6% retention of toxin. Toxin proportions similar to toxic feed.	Depuration in 6 d when fed non-toxic squid; Proportions of GTX2&3 increased, C1&2 and dcSTX decreased during depuration	Jiang et al. (2006)
Jasus edwardsii Australia	AOAC 2005.06 HPLC (Lawrence et al., 2005)	*Mytilus galloprovincialis* 1.51 mg STX.2HCl kg^{-1}	Exceeded regulatory level on day 16, max 3.7 mg STX. 2HCl kg^{-1}.	Below regulatory level in 42 d, exponential depuration rate fed = 0.019 mg STX. 2HCl kg^{-1} day^{-1},	Madigan et al. (2018)

Lobster species/Geographic region	Method of Analysis	Feed details & water temperature	Uptake data	Depuration data	Reference
		Fed 2 toxic mussels per day for 28 days, then either starved or fed 2 non-toxic mussels a day for 70 days, 13-16ºC	Proportions of STX and GTX2&3 increased and C1&2 decreased cf toxic feed.	starved = 0.013 mg STX. 2HCl kg^{-1} day^{-1}. Similar toxin proportions during depuration.	
Jasus edwardsii Australia	LC-MS/MS (Boundy et al., 2015, Turner et al., 2015)	*Mytilus galloprovincialis* 6.10 mg STX.2HCl kg^{-1} Fed 2-3 toxic mussels per day for 26 days, then 2 non-toxic mussels a day for 36 days, 13.2 - 14.8ºC	Exceeded regulatory level within 4 d, max level 9.0 mg STX.2HCl kg^{-1}, 14 % retention of toxin. Proportion GTX1&4 decreased and GTX2&3, C1&2, STX and dcSTX increased cf toxic feed	Exponential depuration rate 0.07 mg STX. 2HCl kg^{-1} day^{-1} Proportion GTX1&4 and C1&2 decreased, STX, dcSTX and dcGTX2&3 increased	Present work

lobsters increased their feeding in correlation with increasing concentrations of PST in the hepatopancreas. Thus the accumulation of PST is unlikely to reduce the growth and nutritional status of lobsters during a bloom. This finding is positive for the Australian and New Zealand Southern Rock Lobster fisheries, which previously expressed concern around the productivity impacts of recurrent toxic dinoflagellate blooms. It also suggests that lobster catchability when using baited traps, as occurs in the recreational and commercial fisheries in Australia and New Zealand, will not be negatively impacted by toxin accumulation. This highlights the need for risk management activities to protect public health and market access during bloom periods.

Depuration of PST from marine invertebrates is achieved through a combination of elimination and biotransformation, with several studies demonstrating that PST analogues are metabolised in different ways by different species (see reviews by Bricelj & Shumway, 1998, and Wiese, 2010). The different profiles seen here between the mussel and lobster tissues could be indicative of either differing rates of retention and elimination or biotransformation. It should also be noted that not all PST consumed by the lobster was absorbed: substantial concentrations were found in the hindgut of exposed lobsters, most likely in the faecal material. Interestingly, the profile of toxins in hindgut varied from that in the mussels, but was very similar to that in the hepatopancreas throughout uptake and depuration. The similar profiles in these two tissues indicate that the processes resulting in the changed profile were occurring in both the digestive tract and hepatopancreas.

During the uptake phase we observed a substantial decrease in the relative amount of GTX1&4 (associated with a higher depuration rate relative to other analogues) and a concomitant increase in GTX2&3 and C1&2 in Southern Rock Lobster hepatopancreas and hindgut when compared to the PST-contaminated mussel feed. Also, changes in the relative amount of the decarbomoyl toxins (dcSTX and dcGTX2&3) was observed between lobster tissues, with a greater amount of these analogues in the gills and antennal glands than in the hepatopancreas (the former possibly skewed by the low level of PST detected in these tissues). This demonstrates a shift from high potency analogues in the toxic feed to lower potency analogues in the lobster tissue. On a molar percentage basis, 59% of the toxin analogues present in mussels had TEFs $\geq$ 0.7, whereas this proportion was always less than 19% in the lobster tissue. This change in potency is advantageous to the lobster, as it results in a reduced total toxicity relative to molar concentration of toxin present. The impact of PST on lobster health was also examined in this study, and will be reported in a following manuscript.

The significant difference in depuration rates between GTX1&4 and GTX2&3 is particularly interesting as the only structural difference between these two compounds is a hydroxyl group. This slight structural difference is therefore somehow causing either selective retention or faster elimination of this analogue in the lobster tissue. Previous studies have described a reduction of GTX1&4 relative to GTX2&3 in *H. americanus* (Jiang et al., 2006) and scallop homogenates (Oshima, 1995; Shimizu and Yoshioka, 1981), and decarbamoylation in surf clams (Bricelj and Cembella, 1995; Bricelj et al., 1996; Cembella et al., 1993; Sullivan et al., 1983; Turner et al., 2013). Interestingly, Lin et al. (2015) postulated that the hydroxyl group on GTX1&4 and NeoSTX was increasing the affinity of these analogues to STX-binding

proteins in crab haemolymph relative to others, effectively decreasing their contribution to total toxicity. However, our study was not designed to distinguish between biotransformations and selective retention and elimination, nor to examine STX-binding proteins in the lobster haemolymph. Thus we cannot confirm what is driving the change of toxin proportions.

In mammals, the primary elimination route for PST is via the kidneys (Andrinolo et al., 1999; Garcia et al., 2004; Gessner et al., 1997; Hines et al., 1993; Naseem, 1996). The equivalent organs maintaining concentrations of ions, nutrients and other solutes in lobster are the antennal glands (Binns and Peterson, 1969; Mantel and Farmer, 1983), with crustacean gills also contributing to osmotic/ion regulation and ammonia excretion (reviewed by Henry et al., 2012). PST have been detected in crustacean gills previously (Jester et al., 2009; Sephton et al., 2007). However, this is the first report of PST in crustacean antennal glands and suggests that both the antennal glands and gills represent potential routes of PST excretion from crustaceans.

3.5 Conclusions

Southern Rock Lobster can rapidly accumulate PST in the hepatopancreas if feeding on highly toxic prey, surpassing accepted maximum regulatory levels for bivalve molluscs within a matter of days. Despite high levels of PST accumulated in the hepatopancreas, these toxins were not consistently found in the haemolymph. Thus we conclude it is not possible to use haemolymph in a non-lethal manner to indicate toxin levels in lobster.

There was no impact of large PST loads in the hepatopancreas of Southern Rock Lobster on survival of lobster and high PST concentrations were correlated with an increase in feed intake. This indicates that lobsters in the field will continue to feed during toxic blooms. Animal growth and fisheries productivity is therefore unlikely to be impacted by bloom events and associated toxin accumulation. Catchability from baited traps (as used in Australia and New Zealand by commercial and recreational fishers) is unlikely to be reduced. PST is likely to be excreted through the faeces, antennal glands and gills.

Uptake and depuration rates of PST in Southern Rock Lobster tissues varied between analogues and resulted in a reduction in the proportion of the highly potent analogues present in lobster tissues relative to those present in the toxic feed. In particular, the depuration rate of GTX1&4 was significantly higher than that of most other analogues. It is unknown whether this is caused by biotransformations, or selective retention or elimination.

Information obtained in this study supports frequent monitoring for PST in Southern Rock Lobster hepatopancreas to manage public health and market access risks during PST producing blooms, particularly where prey items able to concentrate toxins to high levels (e.g. bivalve molluscs) are available. Equally important for the fishery is that lobsters do not retain high levels of toxins for long periods. Fisheries can expect to be able to safely harvest Southern Rock Lobster relatively soon after the bloom has passed.

4. Accumulation of paralytic shellfish toxins by Southern Rock Lobster causes minimal impact on lobster health

Alison Turnbull, Navreet Malhi, Andreas Seger, Jessica Jolley, Gustaaf Hallegraeff & Quinn Fitzgibbon.

Abstract. Recurrent dinoflagellate blooms of *Alexandrium catenella* expose the economically and ecologically important Southern Rock Lobster in Tasmania to paralytic shellfish toxins (PST), and it is unknown if PST accumulation adversely affects lobster performance, health and catchability. In a controlled aquaculture setting, lobsters were fed highly contaminated mussels to accumulate toxin levels in the hepatopancreas (mean of 6.65 mg STX.2HCl equiv. kg^{-1}), comparable to those observed in nature. Physiological impact of PST accumulation was comprehensively assessed by a range of behavioural (vitality score, righting ability and reflex impairment score), health (haemocyte count, bacteriology, gill necrosis and parasite load), nutritional (hepatopancreas index and haemolymph refractive index) and haemolymph biochemical (21 parameters including electrolytes, metabolites, and enzymes) parameters during a 63 day period of uptake and depuration of toxins. Exposure to PST did not result in mortality nor significant changes in the behavioural, health, or nutritional measures suggesting limited gross impact on lobster performance. Furthermore, most haemolymph biochemical parameters measured exhibited no significant difference between control and exposed animals. However, the concentration of potassium in the haemolymph increased with PST, whilst the concentration of lactate and the sodium:potassium ratio decreased with PST. In addition, exposed lobsters showed a hyperglycaemic response to PST exposure, indicative of stress. These findings suggest that PST accumulation results in some measurable indicators of stress for lobsters. However, these changes are likely within the adaptive range for Southern Rock Lobster and do not result in a significant impairment of gross performance. Our findings support previous conclusions that crustaceans are relatively tolerant to PST and the implications for the lobster fishery are discussed.

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4.1 Introduction

The east coast of Tasmania has experienced recurrent dinoflagellate blooms of *Alexandrium catenella* (Whedon and Kofoid) Balech since 2012 (Condie et al., 2019). This alga produces paralytic shellfish toxins (PST) that are accumulated by a range of seafood (Campbell et al., 2013), including the commercially important Southern Rock Lobster (AUD 93 M production per annum in Tasmania; Mobsby, 2018). The Tasmanian *A. catenella* is highly toxic (9.9 pg STX.2HCl $cell^{-1}$ in culture; Seger et al., 2020), forms dense, widespread blooms (maximum reported cell numbers 300,000 L^{-1}) that can cover 200 km of coastline and remain in place over extended periods (Condie et al., 2019). Research conducted since 2012 has focused on understanding PST uptake and depuration in Southern Rock Lobster hepatopancreas (Madigan et al., 2018a; Turnbull et al., 2020a); quantifying the risk to human health (McLeod et al., 2018); and providing effective industry risk management options. Concern over potential impact on the valuable lobster fishery productivity and catchability led to the desire to understand other impacts on Southern Rock Lobster. Lobsters are an important keystone ecological species (Eddy et al., 2014; Power et al., 1996), so any negative impact from PST accumulation on lobster health could potentially drive system–wide shifts in marine communities on the east coast of Tasmania.

Paralytic shellfish toxins act by blocking sodium channels, disrupting ion transport and therefore nerve transmissions (Cestèle and Catterall, 2000). Some bivalve molluscs display high tolerance to PST (Twarog et al., 1972) and accumulate high levels of this toxin, thereby posing a well-documented risk to human health (Deeds et al., 2008; Shumway, 1995). Non-traditional vectors of PST, such as crustaceans and gastropods, have also been shown to accumulate PST toxins (Condie et al., 2019; Costa, 2016; Costa et al., 2017; Silva et al., 2018; Terrazas et al., 2017; Turnbull et al., 2020a). The focus of research on toxin accumulation in non-traditional vectors remains on the perspective of human health. Evidence exists, however, that PST can also have negative health impacts on the marine species accumulating them. Mass mortalities are the extreme impact; a well-documented event originating in the St Lawrence Estuary, Canada, in 2008 attributed the mortality of marine mammals (beluga, seals, porpoises), multiple bird and fish species, crabs and whelks to accumulated PST (Starr et al., 2017).

Bricelj and Shumway (1998) reviewed the differences in PST accumulation across bivalve species, finding that in general, bivalves that are insensitive to saxitoxin (STX) can accumulate high levels of toxins. Sensitivity to PST in some bivalves has also been demonstrated using neurological responses (in vitro blocking of the action potential of nerve fibres; Kvitek and Beitler, 1991); Twarog et al. (1972)); physiological responses (reduced filtration rates; Bricelj and Shumway, 1998); or behavioural responses (inhibition of siphon retraction and burrowing; Bricelj et al. (1996); Bricelj et al. (2011).

A variety of crustaceans are also known to accumulate PST to different levels (reviewed by Deeds et al., 2008). Xanthid crabs in particular display a strong tolerance to STX and can accumulate concentrations that would be fatal to other animals (Arakawa et al., 1995; Llewellyn, 1997; Llewellyn and Endean, 1989, 1991; Noguchi, 1993). Tolerance in xanthid

crabs is derived either from unresponsive neurocytes (Bricelj et al., 2005) or toxin binding proteins (Lin et al., 2015; Llewellyn, 1997) and has often been measured using in vitro nerve bioassay (Hwang et al., 1990; Twarog et al., 1972). However, discrepancies have been noted between such bioassays and whole organism responses, attributed to behavioural and physiological responses such as feeding inhibition, burrowing and shell closure in bivalves (Bricelj et al., 1991; Bricelj et al., 1996; Daigo et al., 1988b). In addition, a recent focus on the ecological impact of marine toxins has seen a more holistic approach to measuring PST impact. Studies on marine invertebrates have shown PST can affect metabolism, immune responses, scope for growth and reproductive capacity (Andrade-Villagr, 2019; Haberkorn et al., 2010; Le Goïc et al., 2013; Navarro et al., 2014; Navarro et al., 2016).

No previous studies have examined the impacts of PST levels on lobster health and performance capability. Vitality or performance capability of crustaceans is commonly measured by gross or whole body measures of performance or physiological indicators of stress which can be either adaptive responses (allowing the animal to compensate for stress and possibly return to homeostasis), and maladaptive responses (resulting in a decreased ability to compensate/react to stress (reviewed by Stoner, 2012). Whole body measures of performance (including survival, nutritional condition and health condition) are useful approaches for determining organismal performance but can be slow to react thus requiring examination over chronic timeframes. Recently, there has been a growing trend for the use of reflex or behavioural indicators of vitality which are quick and easy to measure and have been show to reliably correspond with prolonged whole body performance (James et al., 2019; Paterson et al., 2005; Stoner, 2009; Stoner, 2012; Stoner et al., 2008). Reflex actions are consistent, involuntary and nearly instantaneous, and are independent of size, strength, motivation and gender. Stoner (2012) states that when crustacean reflex responses are combined into a single score, it can provide an accurate indicator of crustacean vitality which is applicable to a variety of stressors.

Other common tools for the assessment of health and vitality of crustaceans are the analysis of haemolymph parameters related to physiological stress and health. Immune responses of crustacea to stressors such as capture, wet storage, emersion, and air exposure have included raised bacteraemia levels and changes in haemocyte counts (Day et al., 2019a; Fitzgibbon et al., 2017a; Fotedar et al., 2001; Paterson et al., 2005; Simon et al., 2016; Tsvetnenko et al., 2001). Furthermore, haemolymph concentration of ions (e.g. potassium, sodium, magnesium, calcium, bicarbonate, pH), metabolites (e.g. ammonia, urea, glucose, lactate) and hormones (e.g. crustacean hyperglycaemic hormone) have been used to study the impact stress in Southern Rock Lobster exposed to seismic noise, translocation, holding and transport pressures (Chandrapavan et al., 2011; Day et al., 2019a; Fitzgibbon et al., 2017a; Morris and Oliver, 1999; Simon et al., 2016).

The present work aimed to examine the impact of PST accumulation on lobster PST lobster health and vitality and employed a holistic approach to examine the impacts at both an organismal (survival, nutritional condition, reflex, behaviour and health) and cellular level (haemolymph immune and biochemical parameters). A laboratory tank experiment was conducted to examine PST toxicokinetics in Southern Rock Lobster following consumption

of highly toxic mussels collected during an *A. catenella* bloom on the Tasmanian east coast (Turnbull et al., 2020a). Observations of the potential impact of PST accumulation on feed intake by Southern Rock Lobster in an experimental system were previously documented by Madigan et al. (2018a), and Turnbull et al. (2020a). The present work was conducted during the same experiment described by Turnbull et al., 2020, whereby a comprehensive range of behavioural, health, nutritional and haemolymph biochemical responses were recorded to examine the impact of exposure to high, field relevant concentrations of PST on Southern Rock Lobster.

4.2 Methodology

Experimental system

The experimental system used is described in detail in Turnbull et al. (2020a). Briefly, adult rock lobsters, weighing 600 – 800 g, were sourced direct from fishing vessels in South Australia. The lobsters (n=77) were sourced from a mix of shallow and deep habitats; with an approximately equal mix of red and brindle animals, only one of which was female. Lobsters were transported to the South Australian Aquatic Biosecurity Centre at Roseworthy, where they were held in individual 30 L tanks in a flow through system. Water temperature and salinity were maintained between 13.2 and 14.8 ^{o}C, and below 37 ppt respectively. Water quality was maintained using pre-conditioned sponge biofilters, and varying flow rate between 2 – 10 L h^{-1}. The sea water supply was supplemented with bicarbonate soda to maintain pH between 7.8 - 8.2. Dissolved oxygen was 94.7% ± 3.9% saturation. Faeces and surplus food were removed daily and 10 lumens of light provided on a 12:12 h light:dark cycle.

Lobster treatments

The experimental treatments were as described in Turnbull et al. (2020a). Lobsters were held for 13 days to acclimatise, during which they were fed non-toxic in-shell blue mussels (*Mytilus galloprovincialis* Lamarck), to a maximum of 3 mussels per day. Lobsters (n = 77) were randomly divided into control and exposed groups, 7 of each to be harvested on days 7, 17, 27, 41 and 63 with an additional control group harvested on day 0. Seven replicates were used to minimise the number of experimental animals for ethical reasons whilst still allowing statistical rigour.

Control animals were fed non-toxic mussels, whilst exposed animals were fed toxic, in-shell blue mussels during the uptake phase (days 0-26), and non-toxic mussels during the depuration phase (days 27-62). Toxic mussels were sourced from an *A. catenella* bloom on the east coast of Tasmania in 2017 and contained 6.10 mg saxitoxin STX.2HCL equiv. kg^{-1}. All mussels were sourced from commercial farms that regularly monitor for biotoxins: toxins other than PST were not present at the time of collection. The PST content of toxic and non-toxic mussels were confirmed by LCMS-MS analysis at Cawthron Institute.

Lobster harvest

Harvest, assessment of behavioural responses and collection of tissues occurred in a consistent order across each harvest. On each harvest day, lobsters were first assessed for behavioural parameters. Following euthanising in an ice slurry, haemolymph samples (5-15 ml) were immediately taken from the sinus under the right 5th leg joint using a sterile pre-chilled 5 ml syringe fitted with a 26 gauge needle and aliquoted for health, nutritional and biochemical assessments (Sections 3.4.3 – 3.4.5). The animals were kept on ice over-night, then weighed, and the carapace length measured. Further health parameters were noted during dissection, and tissues collected and weighed.

Assessment of lobster behavioural response

A total of 7 reflex and two behavioural tests were measured in a consistent order and manner across all harvests by the same researcher. Each lobster was transferred to a 50 L tank and allowed to acclimatise for 5 minutes. The lobster was then removed from the water, held carefully from the carapace so as not to interfere with leg positions, and photographed for later assessment of reflexes. Photographing was performed within 10 sec for all lobsters to limit emersion stress. Eyestalk response was assessed, before placing the lobster back into the 50 L tank to assess the antennal response. Detail of each reflex test and scoring criteria is provided in Table 4.1.

A positive reflex response was scored 0, and a negative reflex response scored 1, as described by Stoner et al. (2008). A reflex impairment score (RIS) was calculated for each animal by summing all the reflexes measured, with the potential scores ranging from 0 – 7; 0 indicating maximum vigour. Vitality was visually assessed immediately prior to antennal response, on a lobster commercial operator 1-5 scale similar to that described by Spanoghe and Bourne (1997) (1 = dead; 2 = limp tail, no escape response, no response to handling; 3 = limp tail, some response to handling, i.e. leg movement; 4 = mostly alert, tail held erect; 5 = alert with vigorous escape behaviour). Finally, righting response time was measured by placing each animal ventrum-up in a tank of saltwater and recording the time taken to return to dorsum-up, following Day et al. (2019b).

Assessment of lobster health response

Haemolymph samples were preserved for haemocyte count by adding 200 µl to a centrifuge tube containing 300 µl of a chilled anticoagulant (Lillie's formol calcium, 1.3 M formalin, 126 mM calcium acetate). Haemocyte samples were stored at 4 ºC and counted in an Improved Neubauer haemocytometer 40 x magnification (Olympus CX41 RF) within 48 hours. A minimum of 100 haemocytes were counted for each sample, across a minimum of 10 squares. Bacteraemia levels were assessed by sterilely plating 100 µl of haemolymph onto Zobell's marine agar (ZMA, Thermofisher) and 100 µl onto thiosulphate-citrate-bile salts agar (TCBS, Thermofisher). Bacterial plates were incubated at 26 ºC for 48 hours before colonies were counted. During dissection lobsters were visually examined for necrosis and parasites in gill tissues (both noted as present/absent).

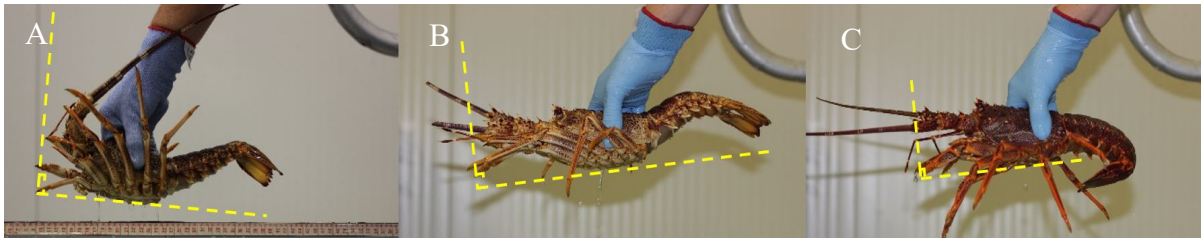

Figure 4.1 Decreasing reflex responses of Southern Rock Lobster from A. strong through to C. weaker. A: primary and secondary periopods lifted above the carapace base, tail arched, and primary and secondary antennae lifted (no reflex impairment, score = 0). B: primary periopod lifted, but periopod 4 below the base of the carapace, tail arched, and primary and secondary antennae not lifted (medium reflex impairment, score = 3).C: primary periopod lifted, but periopods 2-5 below the carapace, tail not arched, and primary and secondary antennae not lifted (high reflex impairment, score = 4).

Assessment of lobster nutritional response

Brix index was assessed by placing 100 µl of haemolymph on a Hanna Refractometer (H196801). Hepatopancreas index was calculated as the ratio of hepatopancreas wet weight to lobster wet weight.

Assessment of lobster haemolymph biochemical response

Haemolymph pH was analysed in a 500 µl sub-sample using Radiometer Analytical pH meter (PHM210) with micro-electrode (B10C162). Haemolymph was then distributed into duplicate 1.5 ml centrifuge tubes, spun at 10,000g for 5 min (Sigma Microcentrifuge 1-14), and the supernatant snap frozen in liquid nitrogen then stored at -80 ºC. Frozen haemolymph supernatant was sent to Crustipath Laboratories, Canada, for analysis using a Cobas c501 automated biochemistry analyser (Roche Diagnostics Corporation, Indianopolis, IN, USA) as described by Day et al. (2019a); Fitzgibbon et al. (2017a). The electrolytes sodium (Na), chloride (Cl), and potassium (K) were measured using an Ion-Selective Electrode (ISE). All other components were measured photometrically: electrolytes magnesium (Mg) and bicarbonate (bicarb); minerals calcium (Ca) and phosphorous (P); metabolites glucose (Gluc), lactate (Lact), cholesterol (Chol), triglyceride (Trig), total protein (TP), albumin (Alb), globulin (Glob), urea, and uric acid (UA); and enzymes lipase (Lip), amylase (Amy), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), sorbital (SDH), glutamate dehydrogenases (GD) and gamma-glutamyl transferase (GGT). Osmolality was measured on a Micro-Osmette (Precision Systems Inc., Natick, MA) via freezing point depression.

PST analysis

The hepatopancreas was homogenised using a VELP OV5, frozen at -80 ºC, and sent to Cawthron Institute, New Zealand, on dry ice for PST analysis. PST analysis was conducted by

LC-MS/MS (Waters Acquity UPLC i-Class system coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer with electrospray ionization), following Boundy et al. (2015) and Turner et al. (2015), with minor variations as detailed in Turnbull et al. (2020a). Results were reported in mg STX.2HCl equiv. kg^{-1} calculated using FAO toxicity equivalency factors (FAO/WHO, 2016).

Table 4.1 Criteria for assessing reflex impairment in Southern Rock Lobster, listed in the order tested. All criteria were assessed for the lateral side of the animal facing the camera.

Reflex	**Test**	**Positive response (scored as 0)**	**Negative response (scored as 1)**	**Method of assessment**
Primary periopod (claw) lift	Lift lobster out of water	Primary periopod raised above carapace base	Primary periopod not raised above carapace base	Photograph
Periopod 2-5 lift	Lift lobster out of water	Periopods 2-5 raised above the base of the carapace	One or more of periopods 2-5 not raised above carapace base	Photograph
Primary antennal lift	Lift lobster out of water	Last segment of primary antenna angled above the vertical relative to carapace base	Last segment of primary antenna not angled above the vertical relative to the carapace base	Photograph
Secondary antennal lift	Lift lobster out of water	Last segment of secondary antenna angled above the vertical relative to the carapace base	Last segment of the secondary antenna not angled above the vertical relative to the base of the carapace	Photograph
Tail arch	Lift lobster out of water	Abdomen held straight or arched upwards, with the point where abdomen meets uropods raised above the carapace base	Abdomen curling under with the point where abdomen meets uropods not raised above the carapace base	Photograph
Eyestalk response	When emersed, gently squeeze eyestalks inwards and release	Rapid (≤1 sec) separation of eyestalks	Delayed (>1 sec) separation of eyestalk	Stopwatch
Antennal response	When immersed place hand 5 cm in front of eyes	At least one of the primary antennae rapidly touches hand (≤1 sec)	Delayed (>1 sec) or no antennae touches hand	Stopwatch

Data analysis

All statistical analyses were performed using R Software (R Core Development Team version 3.6, April 2019). Continuous datasets were checked for homoscedasticity using Levene's test and normality using the Shapiro-Wilk test, with appropriate transformations if necessary. Analysis of variance was used to test for significant differences between groups for data with normal distributions (no transformation: hepatopancreas index, haemocyte count, brix, pH, Na, K, Na:K, Cl, Ca, TP, Glob, Alb:Glob (A:G), and measured osmolality; log transformations: time to right, Gluc, Chol, Trig, Alb, UA, GD; square root transformations: P and Lact), followed by post-hoc analysis using Tukey HSD tests. Prior to transformations, P, Lact and bicarb concentrations that were reported as less than the level of detection were replaced with 0.5* LOD (n=2, 4, and 13 respectively). Two-way random permutation tests were used to test for significant differences in continuous data that could not be transformed to a normal distribution (bacterial counts on ZMA and TCBS, Mg and bicarb).

For discrete and ordinal datasets (reflex impairment score and vitality respectively), ordinal logistic regression was used to test for significant differences between groups, with p values calculated by comparing the t-value against the standard normal distribution. Ordinal chi-squared analysis was used to test for association between vitality and RIS. Logistic regression was used to test for significant differences between groups in binary datasets (necrosis and gill parasites). Analytes where most of data were below the LOD were not tested for significant differences between groups (Creatinine, Urea, ALT, ALP, AMY, AST, GGT, and SDH). Differences were considered statistically significant when p values <0.05.

4.3 Results and Discussion

Stocking animals

There was no significant difference between lobster harvest wet weights (745 ± 70 g; p value = 0.67) or carapace lengths (118 ± 4 mm; p value = 0.90) between treatment groups. During the experiment, two lobsters successfully moulted (exposed treatment, day 41 and 63 harvest groups), and a further two were noted to be in preparation for moult during dissection (thickened inner skins and soft shells). One male lobster died (control, day 7 harvest group). The low death rate, and ability to successfully moult supports conclusions that PST accumulation had limited gross impact on lobster health as mortality or deformity of lobsters often manifest at the moult in response to environmental or physiological stress (Menu-Courey et al., 2019; Smith et al., 2017).

PST levels in Hepatopancreas

Uptake and depuration of PST in the lobsters is described in detail in Turnbull et al. (2020a). Hepatopancreas PST concentrations began at negligible levels (0.03 ± 0.03 mg STX.2HCl equiv.kg^{-1}), increased with time in lobsters fed toxic mussels, peaking at an average of 6.65 ±

1.64 mg STX.2HCl equiv.kg^{-1} at the end of the uptake phase, and returned to low levels (0.26 ± 0.17 mg STX.2HCl equiv.kg^{-1}) after several weeks of depuration (Figure 4.2).

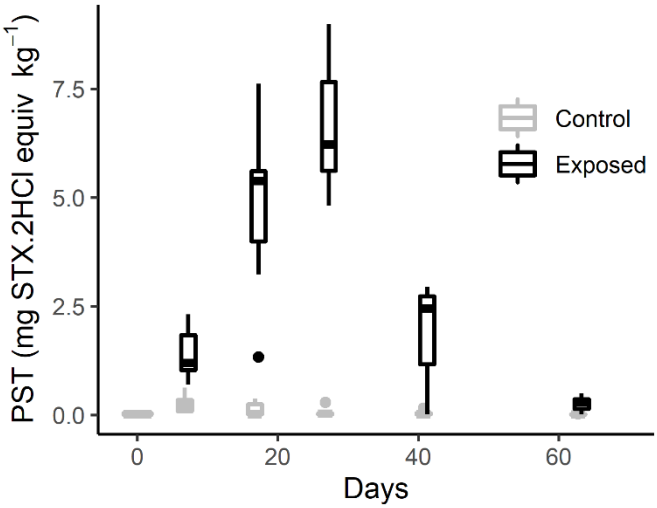

Figure 4.2 Paralytic shellfish toxin concentrations in the hepatopancreas of control (fed non-toxic mussels) and exposed (fed toxic mussels up to day 27, then fed non-toxic mussels during depuration) lobsters at harvest.

Paralytic shellfish toxins in the hepatopancreas of Southern Rock Lobster in this experiment approached levels comparable to those found in the field (maximum of 9.0 mg STX.2HCl equiv. kg^{-1} compared to a maximum 13.6 mg STX.2HCl equiv. kg^{-1} in the field), and for a period similar to exposures observed in the field (Condie et al., 2019). Thus, the impact assessments made for this study are directly relevant to populations of Southern Rock Lobster on the east coast of Tasmania. It should also be noted that the control lobsters used in this study showed low levels of PST, indicating that the animals (sourced from South Australia) were not naïve to PST challenge.

There have been no reports of LD50 for PST in lobster species, although levels as high as 32 mg STX equiv kg^{-1} have been measured in the hepatopancreas of the American spiny lobster *Homarus americanus* (Haya et al., 1994). Other crustacea show significant variation in sensitivity to PST, with LD50s determined via intra-muscular injection of PST into the carapace of xanthid crabs ranging from 1MU per 20g to 10,000 MU per 20g $^{-1}$ (approximately 0.01 - 100 mg STX kg^{-1}: Daigo et al., 1988a; Hwang et al., 1990; Koyama et al., 1983).

Behavioural response to PST accumulation

There was no significant difference in vitality, time to right or reflex impairment score between control and exposed animals (*p* values 0.44, 0.112, and 0.83 respectively), across days (*p* values 0.57, 0.33, and 0.13), nor for the interaction of treatment and days (*p* values 0.71, 0.39, and 0.80). Lobsters in all groups showed strong vitality throughout the experiment with most having a vitality of 5 (n=51) or 4 (n=22), and only 2 animals having a vitality of 3. All lobsters

righted quickly when placed dorsi-ventrum up under water (maximum 9 s, mean for each group 2.0 - 3.6 s)

Image analysis proved to be a useful tool to rapidly capture several reflexes simultaneously and allows later analysis of reflexes. This reduced the time between emersion and euthanising to less than 5 minutes, thereby limiting the stress of harvest and time for biochemical responses that otherwise could confound physiological results.

Lobster reflex impairment scores were generally low across both control and exposed treatment groups indicating limited reflex impairment of lobsters throughout the experiment (Table 4.2). In terms of individual reflexes, no lobsters had impairment of eyestalk response, whilst impairment of antennal response was relatively common, and impairment of the individual reflexes measured by image analysis was more variable.

The lack of performance/behavioural response to PST accumulation suggests that Na channels associated with nerve pathways responsible for both reflex muscle movement and more complex behavioural responses requiring spatial sensing, processing of signals and muscular responses are not being blocked by PST. High tolerance to PST could be explained by either unresponsive nerve cells (Bricelj et al., 2005) or the presence of toxin binding proteins reducing PST levels (Lin et al., 2015; Llewellyn, 1997). We found no PST in Southern Rock Lobster haemolymph at the peak of the uptake phase during this experiment (Turnbull et al., 2020a), leading weight to the latter hypothesis.

Reflex impairment score was significantly correlated with vitality (p value <0.05), with high vitality scores associated with lower reflex impairment (Table 4.3). Reflex and behavioural measures have been found to be useful indicators of vitality and mortality in the commercial crustacean species trade (James et al., 2019; Stoner, 2012). However, limited work has occurred examining objective reflex measures as a stress response in spiny lobster (Haupt et al., 2006; Simon et al., 2016; Spanoghe and Bourne, 1997). Both Haupt et al. (2006) and Simon et al. (2016) considered each reflex separately, whereas Spanoghe and Bourne (1997) combined five reflexes (tail flips, leg motion, leg retraction, maxilliped motion and righting behaviour) into one moribund and mortality score, in an experiment determining the impact of transport processes and conditions on lobster survival. The strong correlation between the reflex impairment score and vitality found in this study indicates the score provides a method of objectively measuring vitality in Southern Rock Lobster. Furthermore, the variability seen across the individual reflex measures show promise for the development of a reflex action mortality predictor (RAMP) score (Stoner, 2009; Stoner, 2012; Stoner et al., 2008), which is yet to be developed for spiny lobster.

Table 4.2 Frequency table of reflex impairment scores (RIS) for Southern Rock Lobster control and exposed treatment groups during the uptake (days 0-27) and depuration phases (days 41-63).

		Days					
RIS	**Treatment**	**0**	**7**	**17**	**27**	**41**	**63**
1	Control	1	3	2	1	0	0
	Exposed		1	2	1	2	0
2	Control	1	1	3	2	2	1
	Exposed		0	2	1	0	2
3	Control	1	0	1	0	2	3
	Exposed		4	1	3	2	1
4	Control	2	1	0	1	0	1
	Exposed		2	1	0	1	1
5	Control	1	0	1	1	1	2
	Exposed		0	0	2	1	3
6	Control	0	1	0	2	2	0
	Exposed		0	1	0	1	0

Table 4.3 Reflex impairment scores in relation to vitality in Southern Rock Lobster. All treatment groups combined.

	Reflex impairment score					
Vitality	**1**	**2**	**3**	**4**	**5**	**6**
3	0	0	0	0	1	1
4	0	1	6	3	6	6
5	13	14	12	7	5	0

Health response to PST accumulation

A change in haemocyte counts have been shown to be indicative of stress in several studies: some finding an increase with starvation, capture, storage and transport; and others finding decrease with emersion, transport and humid storage (summarised in Table 4.4). In our study, haemocyte counts increased significantly over the course of the experiment, but no significant difference was seen between control and exposed treatment groups (Figure 4.3, p value = 0.76). This increase over time may be attributed to either improved nutritional status due to regularly feeding until satiated, or stress from daily handling activities.

A review of microbiota in crustaceans by Wang and Wang (2015) showed that bacteria are common in the haemolymph of crustaceans, although this varies across species and individuals, and with environmental conditions. All lobsters harvested on day 0 in this

experiment contained bacteraemia, albeit at highly variable levels. Similarly, variable levels were described for Western Rock Lobster by (Evans, 2003), with 50% of animals contaminated, and an average colony rank of 0.5 (colony rank of 1 = 10-250 colonies/ml); lower than the level of bacteraemia in our experiment. Whilst both the prevalence of contamination and the mean concentration of bacteria decreased throughout our experiment, the high variability between individuals meant that this decrease was not significant across time (*p* values 0.05 for both ZMA and TCBS), or between control and exposed treatment groups (*p* values 0.52 and 0.76 for ZMA and TCBS estimates respectively).

The proportion of animals exhibiting necrosis or gill parasites also increased significantly with time in the experimental system (Figure 4.3). However, neither of these parameters were affected by accumulation of PST in the hepatopancreas (*p* values both 0.34).

The lack of difference between control and treatment groups in all the health measures assessed in this study (haemocyte counts, bacteraemia, necrosis and gill parasites) suggests that PST does not significantly compromise the immune health status of Southern Rock Lobster.

Table 4.4 Summary of significant differences in behavioural, reflex, immunological and blood chemistry parameters between control and exposed Southern Rock Lobster (Treatment); across days; and for the interaction between treatment and days, measured by ANOVA, ordinal logistic regression (OLR) or logistic regression (LR).

Variable	Treatment	Days	Treatment: Days	Correlation with PST (slope)	2-way ANOVA[1], OLR, or LR
Behaviour					
Vitality					NS
Time to right					NS
Reflex Impairment Score					NS
Health					
Haemocyte count		***			F = 29.6
ZMA					NS
TCBS					NS
Necrosis		*			Z=2.2
Parasites		*			Z=2.4
Nutritional					
Hepatopancreas Index		*			F = 4.7 (Days)
Brix		**			F = 0.4
Hemolymph biochemical					
pH					NS
Na			*		F = 4.2
K	*		**	*** (-ve)	F = 6.3 (Treatment) F = 9.5 (Treatment:Days)
Na:K	**		*	*** (+ve)	F = 10.2 (Treatment) F = 6.5 (Treatment:Days)
Cl			*		F = 5.0
Mg					NS
Bicarb					NS
Ca		***			F = 13.9
P					NS
Gluc	*		**	*** (+ve)	F = 5.3 (Treatment) F = 10.2 (Treatment:Days)
Lact	**		**	None	F=7.5 (Treatment) F=7.1 (Treatment:Days)
Chol					NS
Triglyc					NS
TP		**			F = 9.4
Alb					NS
Glob		**			F=9.2
A:G	*			None	F = 4.1
UA					NS
Lip					NS
GD					NS
Measured Osmolality					NS

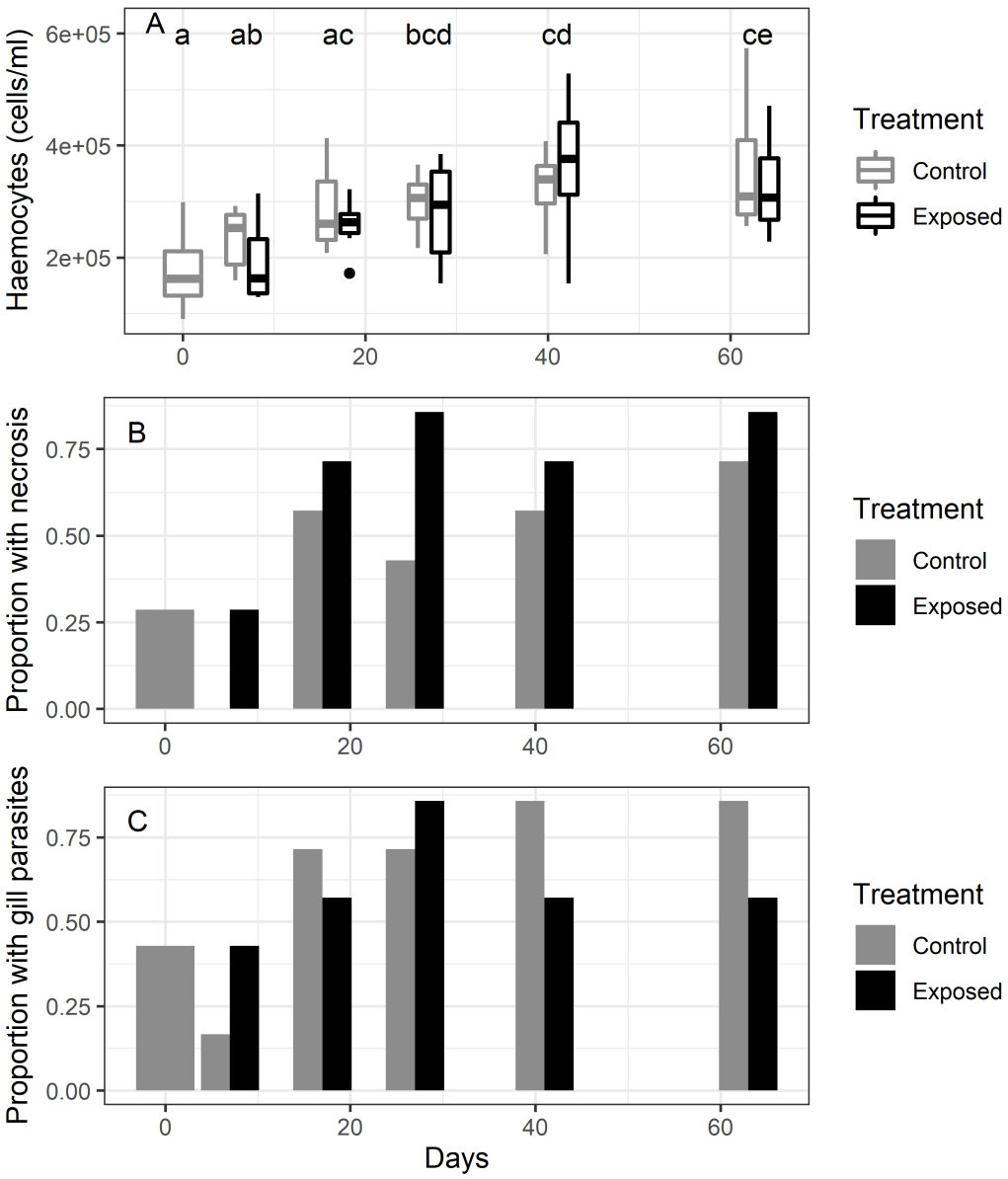

Figure 4.3 The increase in haemocyte counts in Southern Rock Lobster haemolymph (A), the proportion of lobster showing necrosis and/or blisters (B) or parasites (C) in control (grey) and PST exposed (black) treatment groups over the course of the experiment. Days on which haemocyte counts are not significantly different from each other share the same letter. There was no significant difference between control and treatment groups for these parameters.

Nutritional response to PST accumulation

Both the Brix and the hepatopancreas index showed a significant increase with time in the experimental system (Figure 4.4) This is likely to be a result of eating high quality food daily to satiation, and along with increases in TP and globulin, indicates an improvement in nutritional condition across the experiment (Day et al., 2019a; Fitzgibbon et al., 2017b; Mendo et al., 2016; Simon et al., 2015). However, neither Brix nor the hepatopancreas index differed between control and exposed treatment groups (*p* values = 0.54 and 0.70 respectively) suggesting limited impact of PST on nutritional condition of lobsters within the experimental systems examined.

Haemolymph biochemistry response to PST accumulation

Changes in Southern Rock Lobster haemolymph biochemistry have been noted as reflective of stress, as summarised in Table 4.4) Interpretation of biochemistry variations is complex however, as haemolymph constituents also vary with nutritional status, moult and reproductive cycles (Chandrapavan et al., 2011). Several studies listed in Table 4.5 found a decrease in pH associated with emersion and high temperature (Dove et al., 2005; Lorenzon et al., 2007; Simon et al., 2015; Simon et al., 2016; Whiteley and Taylor, 1992). A decrease in pH is a common stress response in lobster caused by respiratory and metabolic acidosis. In our experiment however, no significant difference was seen between PST exposed and control treatment groups (*p* value = 0.28) or across days (*p* value = 0.47).

The electrolytes in Southern Rock Lobster haemolymph showed varying responses to PST accumulation. Potassium (K) decreased during the uptake phase of exposed lobsters and increased again during depuration, whereas control lobster K levels were more stable (Figure 4.5 A). Levels of K were significantly different between treatments. Correspondingly, the ratio Na:K of exposed lobsters displayed the opposite trend to K. This ratio also showed significant differences between control and exposed lobsters (Figure 4.5 B), with exposed lobsters harvested on days 17 and 27 having significantly higher ratios than the controls harvested on the same days (*p* value of 0.03 and 0.01 respectively). Paterson and Spanoghe (1997) claim that the concentration of K in crustacean haemolymph is generally tightly regulated. However, other studies have shown an increase in the concentration of K in response to translocation, storage, and high temperature (Chandrapavan et al., 2011; Dove et al., 2005; Paterson et al., 2005), in contrast to the decrease related to PST accumulation in this study.

Decreasing levels of K in the haemolymph of exposed animals, and an increasing ratio of Na:K, were linearly related to increasing PST concentrations in the hepatopancreas (Figure 4.6 A & B, *p* values < 0.005), suggesting that PST does impact ionic regulation. This finding agrees with suggestions that PST can act to disrupt ion transport (Bricelj et al., 2005; Cestèle and Catterall, 2000).

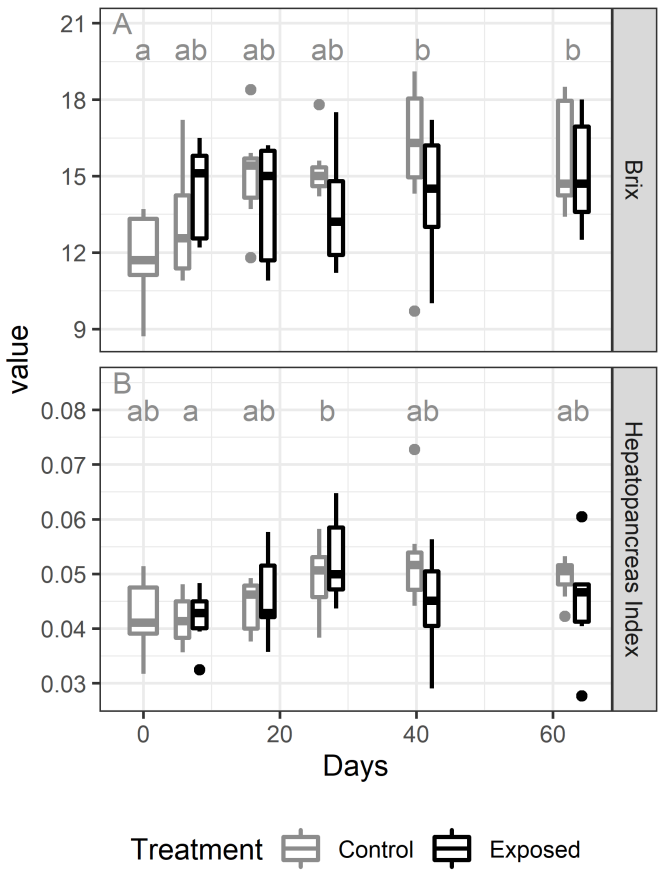

Figure 4.4 The increase in Brix (A) and the hepatopancreas index (B) in Southern Rock Lobster control (grey) and PST exposed (black) treatment groups over the course of the experiment. Days which are not significantly different from each other share the same letter. There was no significant difference between control and treatment groups for these parameters.

The concentration of Ca in the haemolymph increased with time in the system (Figure 4.5 F), showing a significant difference between days. A major proportion of Ca is bound to protein (Neufeld and Cameron, 1993) which also increased significantly over the course of the experiment (Figure 4.5 G), potentially explaining the rise in Ca over the course of the experiment.

Sodium and Cl were variable across the experiment with no significant differences shown between treatment groups (*p* values 0.18 and 0.35 respectively), or across days (*p* value = 0.16 and 0.41). Both these electrolytes showed a significant interaction between treatment and days, with the control group on day 27 being significantly different to the exposed groups on day 41 (Na and Cl) and day 63 (Na only). The other electrolytes examined (Mg, bicarb and P) showed no significant differences between treatment groups (*p* values 0.45, 0.22, and 0.80 respectively), days (*p* values 0.41, 0.05, 0.07) or the interaction of treatment and days (*p* values 0.16, 0.70, 0.79).

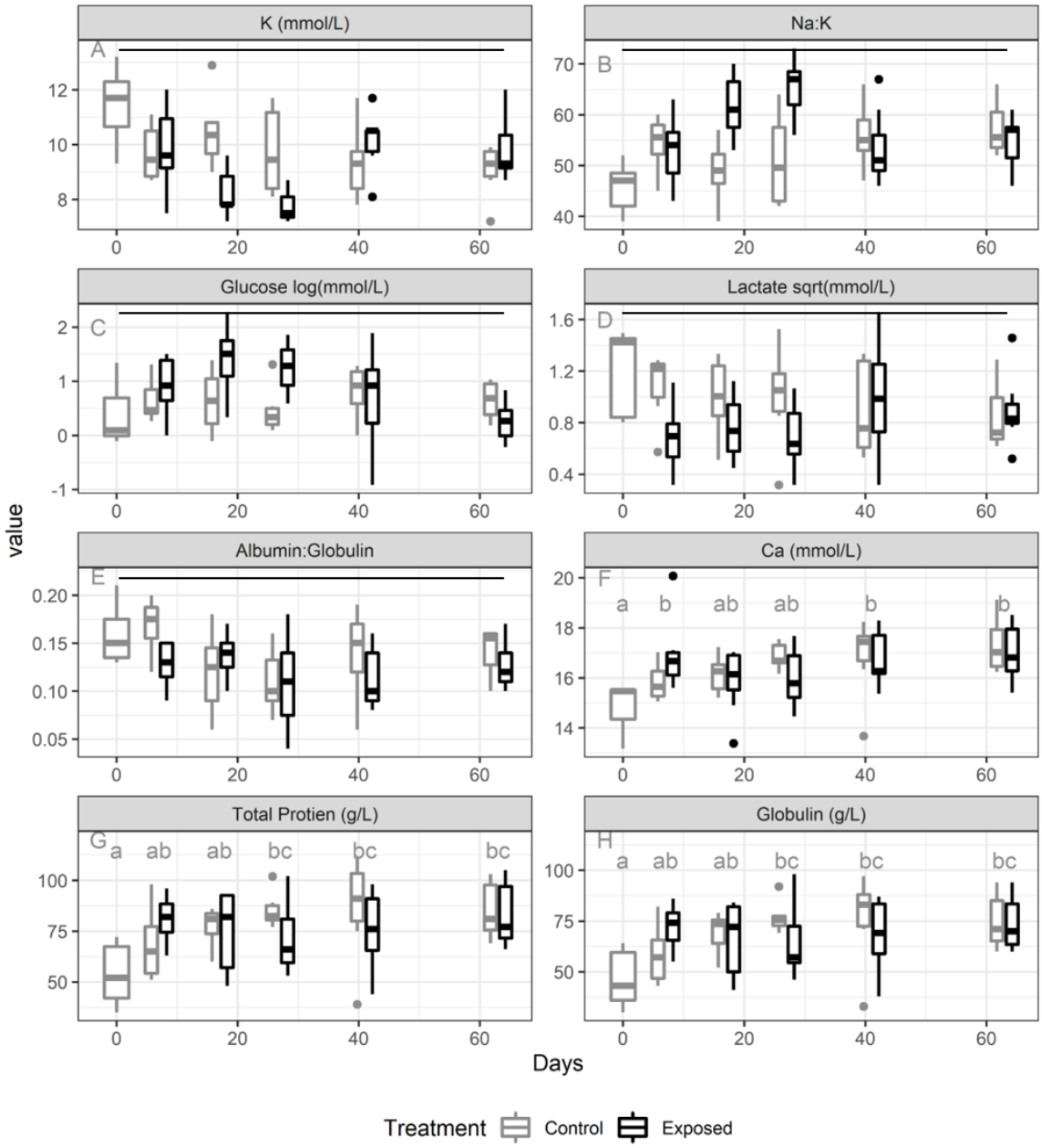

Figure 4.5 Haemolymph biochemistry in PST control (grey) and exposed (black) Southern Rock Lobster harvested on days 0, 7, 17, 27, 41 and 63. Potassium, Na:K, Gluc, Lact and A:G (A, B, C, D, and E respectively) show significant differences between exposed and control lobsters, but not between days. Calcium, Glob and TP (F, G and H respectively) show a significant difference between days (days that are not significantly different from each other share the same letter), but not between control and exposed treatment groups. Significant differences between treatments are shown by horizontal bars.

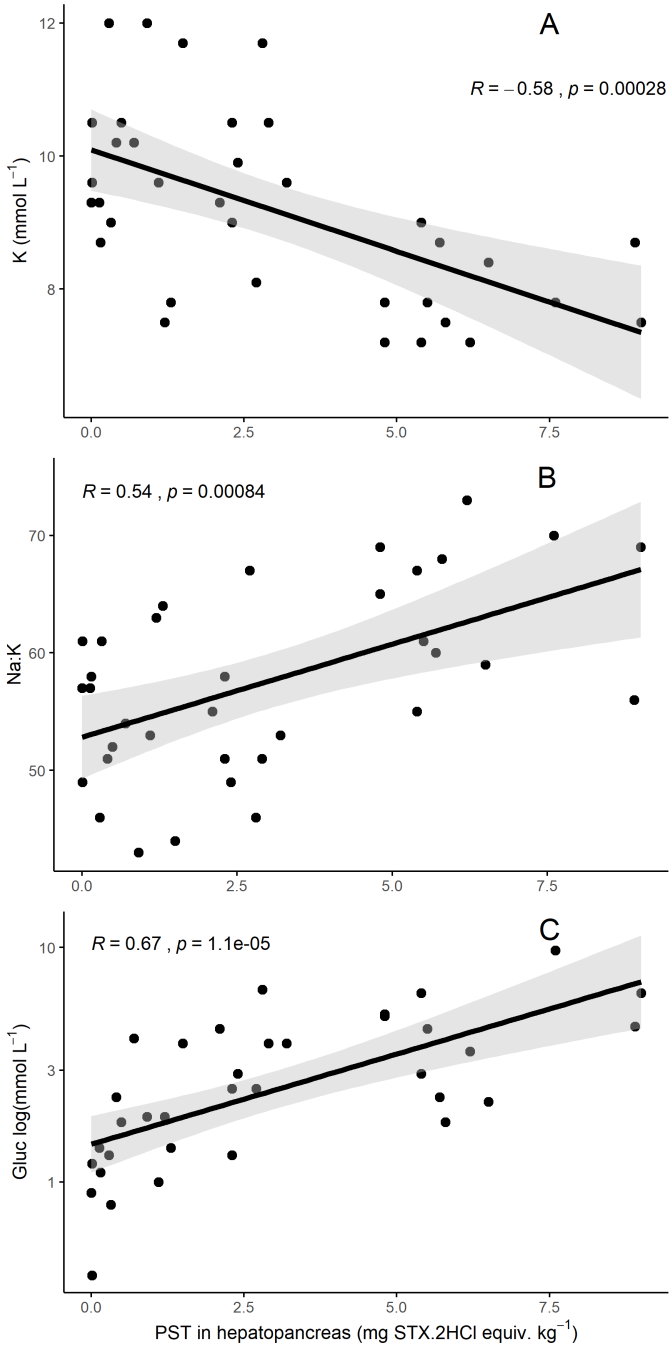

Figure 4.6 Potassium (A), Na:K (B) and glucose (C) concentration in the haemolymph of Southern Rock Lobster consuming toxic mussels in relation to increasing PST concentration in the hepatopancreas, including regression line and 95% confidence interval.

Chandrapavan et al. (2011) described a change in ion ratios in stressed translocated lobsters: Mg was normally lower than K, but in stressed animals, Mg exceeded K concentrations. This also occurred in our PST experiment: Mg exceeded K only in exposed animals during the uptake phase, that is when feeding on toxic mussels .

Of the haemolymph metabolites examined, only Gluc and Lact showed a significant difference between exposed and control lobsters (Figure 4.5 C & D). Globulin and TP differed

significantly between days, both increasing with time in the system (Figure 4.5 H & G). The ratio of A:G differed significantly between treatments (Table 4.4, Figure 4.5 E), although did not correlate with PST concentration in the hepatopancreas (*p* value 0.48), and neither Alb nor Glob showed significant differences between treatments (*p* values 0.28 and 0.54 respectively). Cholesterol, Trig and UA showed no significant difference between groups (*p* values 0.52, 0.68 and 0.42 respectively), and urea levels were all below the LOD .

Increased release of Gluc is common in lobsters exposed to a range of stressors such as emersion, high temperature, handling and heavy metals, and is related to increased release of crustacean hyperglycemic hormone (cHH: Basti et al., 2010; Chang et al., 1998; Chang et al., 1999; Dove et al., 2005; Lorenzon, 2005; Lorenzon et al., 2007; Morris and Oliver, 1999; Paterson et al., 2005; Simon et al., 2016; Whiteley and Taylor, 1992). Furthermore, the change in Gluc concentration in the haemolymph correlated with levels of PST in the hepatopancreas (Figure 4.6 C), increasing with PST accumulation and returning to control levels as PST levels decreased. This conclusively links the hyperglycaemic response to PST accumulation. As cHH also has a role in the moult cycle, gonad development, lipid metabolism and hydromineral regulation (Lorenzon, 2005), there is potential that PST accumulation will be indirectly impacting these functions.

An increase in Lact concentration due to metabolic acidosis is also a common stress response in lobsters (Paterson and Spanoghe, 1997), but we found the opposite in our study. Simon et al. (2015) claim that Gluc, Lact, UA and A:G are transient conditions that may be better indicators of stress than parameters associated with nutritional condition. In our PST study, three of these indicators differed between exposed and control lobsters.

The haemolymph concentration of most enzymes was low and could not be tested for significant differences (Amy, ALT, AST, ALP, SDH, GGT). Only Lip and GD were measurable, and neither showed a significant difference between groups (*p* values 0.74 and 0.87 respectively). Similarly, there was no significant difference detectable in the haemolymph measured osmolality between groups (*p* value 0.46).

Previous studies of crustaceans similarly found limited impact of PST exposure on physiological performance and support conclusions that crustaceans are relatively tolerant when compared to other taxa. Yentsch and Balch (1975) examined the impact of PST accumulation in American lobster, *Homarus americanus,* following consumption of one feed of clams contaminated with 0.3-22.4 mg kg^{-1} PST (STX equivalents not stated), measured over 48 -120 hours. No measurements of PST in the hepatopancreas were taken, and it is unlikely this organ accumulated high levels of PST in this time frame, but up to 1.2 mg kg^{-1} toxin was found in the gut. They found no respiratory impairment or change in oxygen consumption associated with PST exposure. Robineau et al. (1991) examined the survivability of *H. americanus* larvae (stages I – IV) exposed to PST through consumption of contaminated zooplankton (0.05 and 0.08 µg STX equiv. g^{-1} in the zooplankton; no measurement of PST in larvae). No impact on lobster larvae survivability was found, in contrast to mackerel larvae, for which daily death rates of up to 36% were recorded. Xanthid crabs similarly display a strong tolerance to STX and can accumulate extremely high levels of this toxin. This has been

related to levels of saxiphilin and other members of the transferrin family of proteins in the haemolymph of xanthid crabs (Lin et al., 2015; Llewellyn, 1997; Negri and Llewellyn, 1998). Lobster haemolymph also possesses transferrins (Goudard et al., 1991; Huebers et al., 1982; Liang et al., 1997), leading to the possibility that similar PST-binding proteins could account for the high level of tolerance to STX displayed by these crustaceans.

The ecological significance of *A. catenella* blooms on the east coast of Tasmania is yet to be determined. This alga first reached significant numbers in 2012 and has since recurred annually, resulting in PST accumulation in abalone, lobster, crab and bivalve molluscs (Campbell et al., 2013; Condie et al., 2019; Seger et al., 2020). As lobster are a keystone species (Butler et al., 2006; Eddy et al., 2014), any impact on their performance could significantly alter the ecology of this area. This study points to minimal impact from PST on Southern Rock Lobster, although the hyperglycaemic response is concerning given the role of cHH in several lobster physiological processes. Toxic algae have been shown to reduce physiological responses of bivalve molluscs to multiple climate change parameters of warming, freshening and acidification (Turner et al., 2019). The east coast of Tasmania is also a climate change hot spot (Hobday and Pecl, 2014), highlighting the importance of this type of research to understand both the direct and indirect ecological impact of the recurring *A. catenella* blooms in Tasmania.

Fisheries management of Southern Rock Lobster in south-east Australia is based around the determination of Total Allowable Commercial Catch (TACC), supported by geographic and seasonal fishing closures plus limits on lobster size and fishing gear (Linnane et al., 2019). A stock assessment model based on catch, effort and length-frequency data (Punt and Kennedy, 1997) underpins the annual development of TACC quotas. The fact that the health and catchability of adult lobster are not impacted by high levels of PST accumulation (current study plus Turnbull et al., 2020), implies that the occurrence of harmful *A. catenella* blooms should not impact fisheries management and performance, other than the need to consider the consequence of reduced catches caused by fisheries closures during large blooms. Fortunately, the highest risk of fisheries closures due to the human health and market access risks associated with PST accumulation in Southern Rock Lobster on the east coast of Tasmania is coincidental with long standing seasonal spawning closures

Table 4.5 Summary of experimental studies of stress responses in spiny lobsters examining haemolymph immunology and biochemical parameters.

	Stressor	Haemocyte count	Bacteraemia	Hepatopancreas index	Brix	pH	Electrolytes	Minerals	Metabolites	Enzymes	Others
This study	PST contaminated feed	No effect	No effect	No effect	No effect	No effect	↑Na:K ↓K	No effect	↑Gluc	No effect	Increased feed uptake; no change in behaviour, reflexes, immunology
Yentsch & Balch 1975 *Homarus americanus*	PST contaminated feed										No respiratory impairment
Stewart et al. (1967) *H. americanus*	Starvation	↓		↓							
Jussila et al. (1997) *Panulirus cygnus*	Capture and transport	↓Moribund									
Basti et al. (2010) *H. americanus*	Depth of capture, hauling rate, wet storage	↓							↑Gluc. Lact		

	Stressor	Haemocyte count	Bacteraemia	Hepatopancreas index	Brix	pH	Electrolytes	Minerals	Metabolites	Enzymes	Others
Chandrapavan et al. (2011) Southern Rock Lobster	Post-harvest stress after translocation to shallow water						↑K, Mg:K				
Day et al. (2019a) Southern Rock Lobster	Wet storage (starved)					↑	↓bicarb	↓Mg	↑TProt, Albumin, Glob, Triglyc	↑GGT, GD	
	Shell necrosis	↑				↑	↓Na, Cl		↑TProt, Albumin, Glob, Uric acid, Triglyc	↑GGT, GD	
Paterson et al. (2005) *P. cygnus*	Mortality from different types of storage	↓	↑				↑K ↓Cl, Na	↑Ca, Mg	↑ Lact, gluc ↓TProt	↑LDH	
Bernardi et al. (2015) *H. americanus*	Humid air storage	↑							↑Gluc, Lact, Urea, TProt		
	Wet storage	↓					↑Cl, Na		↓Gluc, TProt ↑ Lact, Urea		
Fotedar et al.(2006) *P. cygnus*	Wet storage after handling & emersion	No effect	No effect			↑					
Tsvetnenko et al. (2001) *P. cygnus*	Humid/wet air storage	↑	↑								
	Wet storage										

	Stressor	Haemocyte count	Bacteraemia	Hepatopancreas index	Brix	pH	Electrolytes	Minerals	Metabolites	Enzymes	Others
Simon et al. (2016) Southern Rock Lobster	Emersion	↑			↑morts	↓	↑bicarb ↓CL, Na, Na:K	↑Ca, Mg, P	↑Gluc, chol, lact urea, uric acid	↑ALT, AST, SDH ↑AMY, ALP, ALT, GGT in morts	
Morris and Oliver (1999) Southern Rock Lobster	Emersion								↑Gluc, Lact, Urate		
Whitley and Taylor (1992) *Homarus gammarus*	Air exposure					↓	↑bicarb	↑Mg in animals with poor health	↑Gluc		
Fotedar et al. (2001) *P. cygnus*	Air exposure	↓	↑								
Dove et al. (2005) *H. americanus*	High temperature	↑				↓	↑K	↑P,	↑Gluc, TProt		
Lorenzon et al. (2007) *H. americanus*	Emersion and high temperature				↑	↓	↓Cl	↑Ca	↑Gluc, TProt, Lact, chol,Triglyc		
Chang et al. (1998)	Emersion, high temperature,								↑Gluc		

	Stressor	Haemocyte count	Bacteraemia	Hepatopancreas index	Brix	pH	Electrolytes	Minerals	Metabolites	Enzymes	Others
H. americanus	and high and low salinity stress										
Fitzgibbon et al. (2017a) Southern Rock Lobster	Seismic air gun exposure	↓		No effect	↓		No effect	No effect	No effect	No effect	
Filiciotto et al. (2014) *Palinurus elephas*	Boat noise	↓							↑Gluc, TProt		

4.4 Conclusions

A comprehensive investigation of the exposure of the spiny lobster Southern Rock Lobster to PST employed a holistic approach to examine the impacts at both an organismal and cellular level. The investigation demonstrated a tolerance of high levels of PST contained in their hepatopancreas. At the organismal level, there was no major impact on survival, nutritional condition, reflex, behaviour or health observed over a 63 day exposure period. At the cellular level, a decrease in K and increase in the Na:K ratio, change in the Mg:K and A:G ratios, and in particular a hyperglycaemic response to PST exposure were indicative of stress, although the lack of change in vitality and behavioural, reflex and immunological responses in relation to PST accumulation argue that any stress incurred was within the physiological adaptive range of lobsters. This finding is directly relevant to populations of Southern Rock Lobster on the east coast of Tasmania as PST accumulation during the experiments was similar to maximum levels found during field monitoring. Impact on fisheries health and performance is likely to be minimal. However, it is acknowledged that our findings are based on studies with captive adult lobsters maintained in controlled conditions with limitless supply of nutritious food. Further studies are required to determine if the stress indicators observed here may result in a decrease in lobster performance in the wild.

5. Lobster Supply Chains Are Not at Risk from Paralytic Shellfish Toxin Accumulation during Wet Storage

Alison Turnbull, Andreas Seger, Jessica Jolley, Gustaaf Hallegraeff , Graham Knowles & Quinn Fitzgibbon

Abstract. Lobster species can accumulate paralytic shellfish toxins (PST) in their hepatopancreas following consumption of toxic prey. The Southern Rock Lobster (SRL) industry in Tasmania, Australia, and New Zealand, collectively valued at AUD 365M, actively manages PST risk based on toxin monitoring of lobsters in coastal waters. The SRL supply chain predominantly provides live lobsters which includes wet holding in fishing vessels, sea-cages or processing facilities for periods up to several months. Survival, quality and safety of this largely exported high-value product is a major consideration for the industry. In a controlled experiment, SRL were exposed to highly toxic cultures of *Alexandrium catenella* at field relevant concentrations (2×10^5 cells L^{-1}) in an experimental aquaculture facility over a period of 21 days. While significant PST accumulation in lobster hepatopancreas has been reported in parallel experiments feeding lobsters with toxic mussels, no PST toxin accumulated in this experiment from exposure to toxic algal cells, and no negative impact on lobster health was observed as assessed via a wide range of behavioural, immunological and physiological measures. We conclude there is no risk of PST accumulation nor risk to survival or quality at the point of consumption through exposure to toxic algal cells.

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5.1 Introduction

The Southern Rock Lobster fisheries in Tasmania, Australia, and New Zealand are high value live export fisheries worth AUD 97M and AUD 268 M respectively (Patterson et al., 2019; Williams et al., 2017). Lobsters are known to accumulate paralytic shellfish toxins (PST) during blooms of PST producing algal species in Tasmanian and New Zealand coastal waters (Condie et al., 2019; Hallegraeff et al., 2017a). The causative alga in Tasmania is *Alexandrium catenella* (Whedon and Kofoid) Balech, whilst New Zealand blooms may be *A. minutum* Halim*, A. pacificum* Litaker (previously identified as *A. catenella*) and *Gymnodinium catenatum* Graham (MacKenzie, 2014; Rhodes et al., 2016). The toxins accumulate in the lobster hepatopancreas via consumption of contaminated prey but are not found in the tail meat (Madigan et al., 2018a; Turnbull et al., 2020a). Whilst there is no Australia or New Zealand food standard for PST in lobster, several key export markets such as China and Hong Kong stipulate a maximum level of 0.8 mg STX equivalents kg^{-1}. Furthermore, human health risk assessment has shown a risk of illness for consumers if consuming large quantities of lobster hepatopancreas. This risk is significantly reduced if the bivalve regulatory level of 0.8 mg STX equivalents kg^{-1} is applied (McLeod et al., 2018). In both Tasmania and New Zealand, the public health and market access risks associated with PST in Southern Rock Lobster are managed during high risk periods through weekly or fortnightly biotoxin monitoring of bivalve sentinel species in coastal waters, followed by direct monitoring of lobster hepatopancreas when bivalves indicate risk.

The Southern Rock Lobster supply chain is focused on live seafood markets in Asia. Wet storage is employed to maintain animal quality and facilitate maximum price return during market fluctuations. Animals are moved into wet storage immediately after capture and remain in specialised holding facilities as they move through the supply chain (Patel et al., 2020), as depicted in Figure 5.1. Seawater used for wet storage is sourced from local coastal waters.

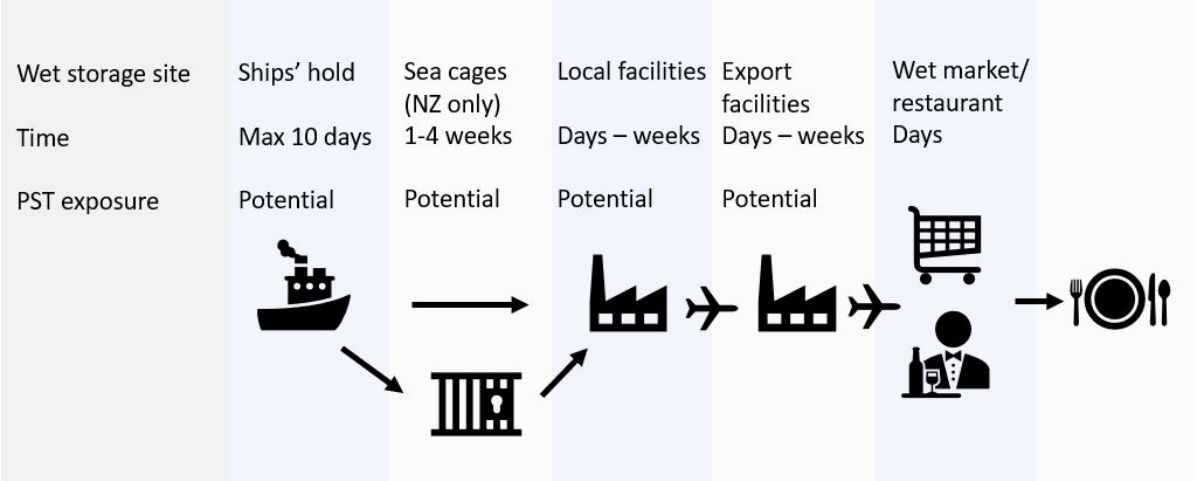

Figure 5.1 Supply chain for Southern Rock Lobster from Tasmania and New Zealand to Asian markets, showing wet storage and potential PST exposure sites. Biotoxin risk monitoring occurs in coastal waters prior to entry into the supply chain.

Wet storage times may range from a few days to several weeks depending on the time of year and market demands. If PST producing algal blooms are present in coastal waters, these algae will be inadvertently pumped into holding compartments on-vessel, in sea-cages (New Zealand only) or in local/export holding facilities. As a result, Southern Rock Lobster may be exposed to PST in the supply chain, post regulatory monitoring programs.

Crustacean gills have multiple functions; ionic transport mediating haemolymph osmoregulation; acid-base balance and ammonia excretion. Heavy metal accumulation in crustaceans also occurs via the gills (Henry et al., 2012). No studies have examined the potential for PST uptake in lobsters when directly exposed to toxic algae. Furthermore, lobsters may be in the supply chain for significant periods of time and subjected to more than one period of emersion during transport. To maximise value in the market, lobsters need to survive the rigours of international transport and thus it is integral that they start their journey in strong health (Stoner, 2012). Whilst a recent study showed no impact of PST feeding related accumulation on Southern Rock Lobster health (Turnbull et al., 2020b), no studies have so far examined the sensitivity of lobster gill cells to the superoxide radicals, exudate phycotoxins and fatty acids that are known to be produced by toxic *Alexandrium* spp and to have deleterious impact on fish gill cells (Dorantes-Aranda et al., 2015; Mardones et al., 2015).

A range of indicators have been used to assess stress and predict mortality in commercially important crustacean species (Stoner, 2012). In this study we have taken a holistic approach to determine impacts, examining both whole organism indicators (survival, nutritional condition, reflex, behaviour and health) and cellular indicators (haemolymph immunity, biochemical parameters and gill histopathology).

Commercial operators assess Southern Rock Lobster health during grading using a subjective vitality scale which is based on lobster reflex and behavioural responses (Spanoghe and Bourne, 1997). Reflex actions are consistent; involuntary; nearly instantaneous responses to

stimuli which can reliably indicate crustacean whole-body health status independently of animal size, strength, motivation and gender (James et al., 2019; Paterson et al., 2005; Stoner, 2009; Stoner, 2012; Stoner et al., 2008). Crustacean reflex scores have been previously used to provide an accurate indicator of crustacean performance in supply chain studies (Stoner, 2009; Stoner et al., 2008). Other commonly used methods to assess lobster gross or whole-body performance include survival and nutritional condition (Day et al., 2019a; Fitzgibbon et al., 2017a; Spanoghe and Bourne, 1997; Stoner et al., 2008).

Physiological indicators for the assessment of health and vitality of crustaceans commonly include immune responses (raised bacteraemia levels and changes in haemocyte counts (Day et al., 2019a; Fotedar et al., 2006; Fotedar et al., 2001; Simon et al., 2016; Tsvetnenko et al., 2001); haemolymph concentration of ions (e.g. potassium, sodium, magnesium, calcium, bicarbonate, pH), metabolites (e.g. ammonia, urea, glucose, lactate) and hormones (e.g. crustacean hyperglycaemic hormone) (Chandrapavan et al., 2011; Day et al., 2019a; Morris and Oliver, 1999; Simon et al., 2016).

The present study aimed to determine if Southern Rock Lobster could accumulate PST through exposure to PST producing algae; and to ascertain whether direct exposure to these algae could impact lobster health and vitality. In a biosecure experimental aquaculture facility, lobsters were exposed to field relevant concentrations of toxin producing *A. catenella* algae for 21 days. PST concentrations in the hepatopancreas were measured as well as a range of measures commonly employed to assess survivability and lobster health.

5.2 Materials and Methods

Experimental system

The experimental system used is described in detail in Turnbull et al. (2020a), with the exception that in this case a static system was employed, with total daily water exchange. Briefly, 450 – 600 g adult *J. edwarsdii,* (n=48 male and 1 female) were sourced direct from South Australian fishing vessels from a mix of shallow and deep habitats with no known bloom activity. The lobsters were transported to the South Australian Aquatic Biosecurity Centre at Roseworthy, where they were held in individual 30 L tanks maintained between 13.1 and 16.3 ^{o}C, salinity of less than 37 ppt, and pH between 7.7 - 8.2 (supplementing the seawater supply with bicarbonate soda as necessary). Water quality was maintained using pre-conditioned sponge biofilters. Dissolved oxygen was >90% saturation and 10 lumens of light was provided on a 12:12h light:dark cycle. Lobsters were acclimated for 7 days prior to exposure.

Algal cultures

Batch cultures of *A. catenella* strain AT.TR/F (previously known as *A. tamarense* group 1; isolated from Triabunna, Tasmania at the Institute for Marine and Antarctic Studies, Hobart, Australia) were cultivated in 15 L carboys following Seger et al. (Seger et al., 2020). Cultures were maintained in sterile filtered seawater (0.22 µm) supplemented with modified GSe nutrient concentrations (final media = 3/4 GSe nutrients, 5 mM sodium bicarbonate and 7.5 pM H_2SeO_3 to replace the soil extract in the basal recipe). Cultures were grown at 18±1 °C

under 120 µmol photons m^{-2} s^{-1} of light supplied by low temperature LEDs on a 12:12 h light:dark cycle. During the dark period the carboys were gently aerated (0.15 L min^{-1}) with ambient air, which was enriched with 1.5-2.5% (v/v) CO_2 in the light.

Cultures used for the exposure experiments were in late exponential/early stationary phase (>2 × 10^7 cells L^{-1}, 2.5% CO2 aeration) and contained 3.5 - 21.2 pg STX.2HCl equiv. $cell^{-1}$ [30]. Cell PST quotas were determined in parallel experiments by Seger et al. (2020) to be 3.5 - 21.2 pg STX.2HCl equiv. $cell^{-1}$. Briefly, suspensions of toxic cells were concentrated from four different batches of the same monoclonal source culture in late exponential/early stationary phase through centrifugation. Extracts of these suspension were produced by lysing and further centrifugation to remove cell fragments. Toxin content of the extracts were determined via LCMS-MS analysis by the Cawthron Institute, New Zealand, as described below. The average toxin profile on a molar basis was 55% C1,2, 36% GTX1,4, 3% C3,4, 2% Neo, 2% GTX2,3, 2% dcGTX2,3, and small percentages (<2% each) of STX, GTX5, GTX6, and doSTX.

Lobster treatments

Lobsters were fed to excess (3 in-shell blue mussels, *Mytilus galloprovincialis* Lamarck) at the same time each day, during the 7 days acclimation period and for the course of the experiment. The mussels were sourced from Coffin Bay, South Australian and were confirmed to be free of toxins via LCMS-MS (Boundy et al., 2015; Turner et al., 2015) at Cawthron Institute, New Zealand.

Lobsters were randomly allocated to three treatment groups: control (n=21), low exposure (n=14) and high exposure (n=14). Each treatment group was further divided into harvest groups of seven replicates each. One control group was harvested on day 0 and control, low and high exposure groups each harvested on days 7 and 21. Seven replicates were used in each group to minimise the number of experimental animals for ethical reasons whilst still allowing statistical rigour. The cell density of the *A. catenella* culture was determined daily via haemocytometer counts, and aliquots of culture added to the low and high exposed lobsters at final concentrations of 1 × 10^5 cells/L and 2 × 10^5 cells/L, respectively, immediately after the morning water exchange each day.

Specific Feed Intake

The apparent feed intake (AFI) of lobsters were measured each week following Fitzgibbon et al. (2017b). Feed control tanks for the control, low and high exposure groups were included in the random allocation, with no lobsters placed in these tanks. The feed control tanks each received 3 mussels at the same time as the lobster tanks each afternoon. Uneaten mussel meat from each tank (control and exposed animals, and feed control tanks) was collected at the beginning of each day. The shucked meat was frozen cumulatively over the period of a week. Subsequently, the uneaten mussel meat was dried at 105 °C for 24 h and weighed. The AFI of each lobster was calculated (dry weight of the uneaten food in the treatment tank subtracted from that of the respective control tank, divided by 7) and converted to SFI by dividing by the wet weight of the lobster.

Lobster harvest protocols

The harvest protocols and tests are described in detail in Turnbull et al. (2020b). Briefly, lobster behavioural responses and tissue collection were conducted in the same order by the same researchers on each harvest day. Following behavioural measurements, lobsters were euthanised in an ice slurry then haemolymph samples (5-15 mL) taken from the sinus under the right 5th leg joint. The animals were kept on ice over-night, then weighed and the carapace length measured prior to tissue dissection and collection. Gill tissue samples from each animal were immediately placed in Davidson's fixative for 24 hours, which was then replaced with 70% ethanol. Hepatopancreas were stored at -80 ^{0}C prior to PST analysis.

Behavioural responses

Lobsters were first assessed for 7 reflex responses following (Turnbull et al., 2020b): primary and secondary pereopod lift, antennae and secondary antennal lift, and tail arch via photography whilst emersed; rapid (<1 sec) eye stalk return to normal after gently squeezing together; and rapid antennal touch of hand placed directly in front of immersed animal; and two behavioural responses (righting response time measured by placing each animal ventrum-up in a tank of saltwater and recording the time taken to return to dorsum-up Day et al. (2019b); and vitality visually assessed on a lobster commercial operator 1-5 scale similar to that described by Spanoghe and Bourne (1997); 1 = dead; 2 = limp tail, no escape response, no response to handling; 3 = limp tail, some response to handling, i.e. leg movement; 4 = mostly alert, tail held erect; 5 = alert with vigorous escape behaviour). Each reflex response were scored (positive response =0, negative response =1) and summed into a reflex impairment score for each animal, as described by Stoner et al. (2008). Potential scores ranged from 0 – 7; with 0 indicating maximum vigour.

Immune health response

Haemolymph samples were preserved immediately after extraction by adding 200 to 300 µL chilled anticoagulant Lillie's formol calcium (1.3 M formalin, 126 mM calcium acetate) and haemocytes counted in an Improved Neubauer haemocytometer 40 x magnification (Olympus CX41 RF) within 48 hrs. To assess bacteraemia levels, 100 µL of haemolymph was sterilely plated onto each of Zobell's marine and thiosulphate-citrate-bile salts agars (ZMA and TCBS, Thermofisher), incubated at 26 ^{o}C for 48 hrs and colonies counted. Shell necrosis was visually noted as present/absent during dissection.

Nutritional health response

Nutritional responses were assessed via Brix index (Hanna Refractometer H196801) and hepatopancreas index (the ratio of hepatopancreas wet weight to lobster wet weight (Landman et al., 2020; Simon et al., 2015).

Haemolymph biochemical response

Haemolymph pH was measured using Radiometer Analytical pH meter PHM210 with micro-electrode B10C162, following which haemolymph was spun at 10,000*g* for 5 mins (Sigma Microcentrifuge 1-14). The supernatant was snap frozen in liquid nitrogen, stored at -80 ^{o}C and sent to Crustipath Laboratories, Canada, for analysis using a Cobas c501 automated biochemistry analyser (Roche Diagnostics Corporation, Indianopolis, IN, USA) as described by Day et al. (2019a) and Fitzgibbon et al. (2017a). Sodium (Na^{+}), chloride (Cl^{-}), and potassium (K^{+}) were measured using an Ion-Selective Electrode whilst (Mg) and bicarbonate (bicarb) were measured photometrically. The minerals calcium (Ca) and phosphorous (P); metabolites glucose (Gluc), lactate (Lact), cholesterol (Chol), triglyceride (Trig), total protein (TP), albumin (Alb), globulin (Glob), urea, and uric acid (UA); and enzymes lipase (Lip), amylase (Amy), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), sorbital (SDH), glutamate dehydrogenases (GD) and gamma-glutamyl transferase (GGT) were measured photometrically. Osmolality was measured on a Micro-Osmette (Precision Systems Inc., Natick, MA) via freezing point depression.

PST analysis

Paralytic shellfish toxins in the hepatopancreas were analysed at Cawthron Institute, New Zealand by LC-MS/MS (Waters Acquity UPLC i-Class system coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer with electrospray ionization), following Boundy et al. (2015) and (Turner et al., 2015), with minor variations as detailed in Turnbull et al. (2020a). Results were calculated using FAO toxicity equivalency factors (FAO/WHO, 2016). Results reported as part of this study were corrected based on spike recoveries observed for the different sample matrices analysed. The limit of reporting for each PST analogue differed for each matrix tested.

Gill Histology

Histopathological analysis of gill tissues was conducted at the Animal Health Laboratory, Department of Primary Industries, Parks, Water and Environment in Tasmania. Gills, stored in ethanol were embedded in paraffin, cut at 5 µm thickness, mounted and stained with haematoxylin and eosin, using standard techniques. All slides were read by the same ACVP (American College of Veterinary Pathologists) board certified veterinary pathologist.

Data analysis

Statistical analyses were performed using R Software (R Core Development Team version 3.6, April 2019). Continuous datasets were checked for normality and homoscedasticity using the Shapiro-Wilk test and Levene's test respectively, with appropriate transformations if necessary (no transformation: SFC, haemocyte count, brix, pH, Na, Cl, TP, Glob, Alb:Glob (A:G), and GD; log transformations: time to right, Ca, Gluc, Chol, and Alb; square root transformations: P, bicarb, Trig, and UA). Analysis of variance was used to test for significant differences between groups for data with normal distributions, followed by post-hoc analysis using Tukey HSD tests. Prior to transformations, P, Lact, bicarb, Trig, UA concentrations that

were reported as less than the level of detection were replaced with 0.5* LOD (n=1, 12, 1, 2, 1 respectively). Two-way random permutation tests were used to test for significant differences in continuous data that could not be transformed to a normal distribution (bacterial counts on ZMA and TCBS, K, Na:K, Mg, Lact and measured osmolality).

Ordinal logistic regression was used to test for significant differences between discrete and ordinal datasets (reflex impairment score and vitality respectively), with *p* values calculated by comparing the t-value against the standard normal distribution. Ordinal chi-squared analysis was used to test for association between vitality and RIS. Significant differences between groups in binary datasets (necrosis and gill parasites) were tested using logistic regression.

Analytes where most of data were below the LOD were not tested for significant differences between groups (Creatinine, Urea, ALT, ALP, AMY, AST, GGT, and SDH). Differences were considered statistically significant when *p* values <0.05.

5.3 Results

Stocking animals

Lobsters arrived in good condition, with vitalities on receipt ranging from 4-5 (maximum score possible is 5). There was no significant difference in the lobster harvest wet weights (514 ± 34 g; *p* value = 0.59) or carapace lengths (104 ± 3 mm; *p* value = 0.37) between each treatment group. No lobsters moulted during the experiment, but two lobsters in the high exposure treatment groups died; one on day 10 and one on day 11.

Specific feed intake

Lobsters from all treatment groups ate well during the experiment, with no significant difference in the specific feed intake (SFI) between treatment groups. Feed consumption decreased during the experiment, with SFI during weeks 2 and 3 being significantly less than that at week 1 (Figure 5.2; *p* value = 0.0009).

PST accumulation

Lobsters had low concentrations of PST in the hepatopancreas on receipt (mean 0.03 ± 0.01 mg STX.2HCl equiv. kg^{-1}). Lobsters harvested on day 21 had significantly lower PST than those harvested on day 7 (*p* value = 0.001), but not on day 0 (*p* value = 0.53; Figure 5.3). There was no significant difference between PST levels between treatment groups (*p* value = 0.64).

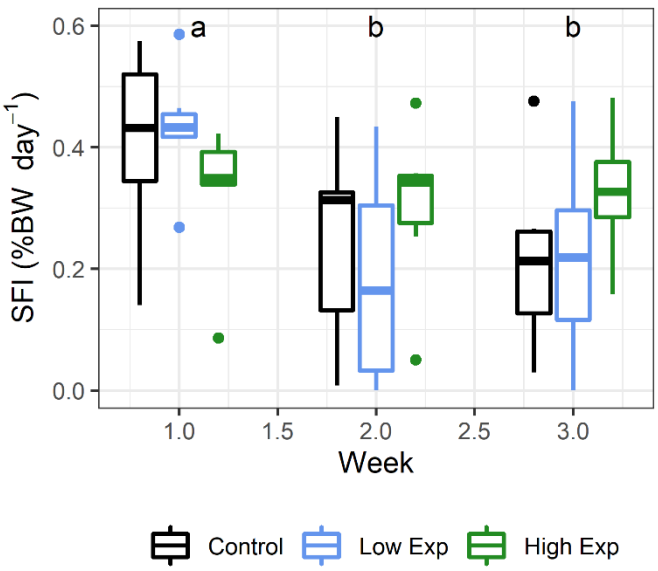

Figure 5.2 Weekly specific feed intake of lobsters, Southern Rock Lobster, exposed to 0 , 1 x 105 or 2 x 105 cells of *A. catenella* per litre of tank water (control, low or high exposure groups, respectively) across three weeks of exposure. Weeks where SFI is not significantly different share the same letter.

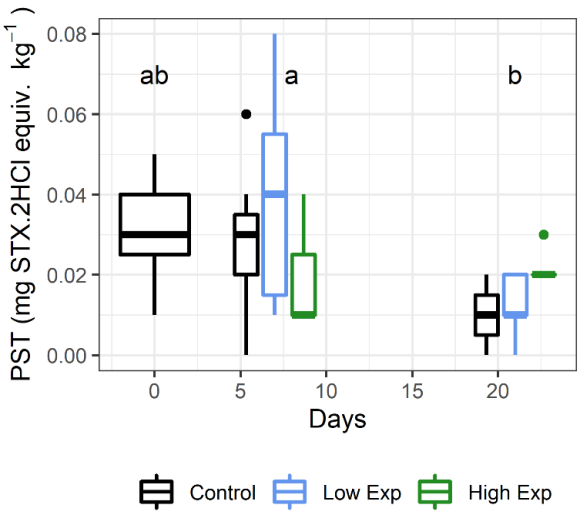

Figure 5.3 PST concentrations in the hepatopancreas of lobsters, Southern Rock Lobster, harvested on days 0, 7 and 21 after exposure to 0 , 1 x 105 or 2 x 105 cells of *A. catenella* per litre of tank water (control, low or high exposed groups respectively). Days where the PST concentration is not significantly different share the same letter.

Lobster health responses

There was minimal difference between treatment groups across a wide range of behavioural, immunological and physiological parameters measured, as summarised in Table 5.1.

Behavioural responses

No significant difference between treatments or across days in the experimental system was seen in any of the behaviour measures tested (other than SFI discussed above). Lobster vitality remained high throughout the experiment with 36 animals scoring the maximum vitality score (5), seven scoring vitality of 4 and three scoring vitality of 3. All lobsters responded quickly to being placed ventrum-up, righting within 28 seconds. Impairment of reflexes was low, with 32 lobsters showing impairment of three or less reflexes. Reflex impairment scores were significantly related to vitality scores (p value = 0.0003;).

Immune health response

There was no change to bacteraemia concentrations or the prevalence of necrosis between treatment groups or days, however haemocyte counts did increase significantly across all treatment groups at day 21 compared to days 0 and 7 (Table 6.1; p values = 0.0007 and 0.002 respectively).

Nutritional response

There were no significant differences between the nutritional indicators of Brix and heaptopancreas index across treatments or across days (Table 5.1).

Haemolymph biochemical response

Of the range of electrolytes, minerals, metabolites and enzymes examined, only pH and lipase showed any significant difference between treatments (Figure 5.4).

The low exposure treatment group showed significantly lower pH than the control group (p value = 0.02), whilst both low and high exposure groups showed significantly higher lipase concentrations than the control group (p value = 0.03 and 0.02 respectively).

No difference was observed in electrolyte, mineral or enzyme levels across the course of the experiment, but the metabolites cholesterol, total protein and triglyceride all increased significantly across the duration of the experiment (Figure 5.5).

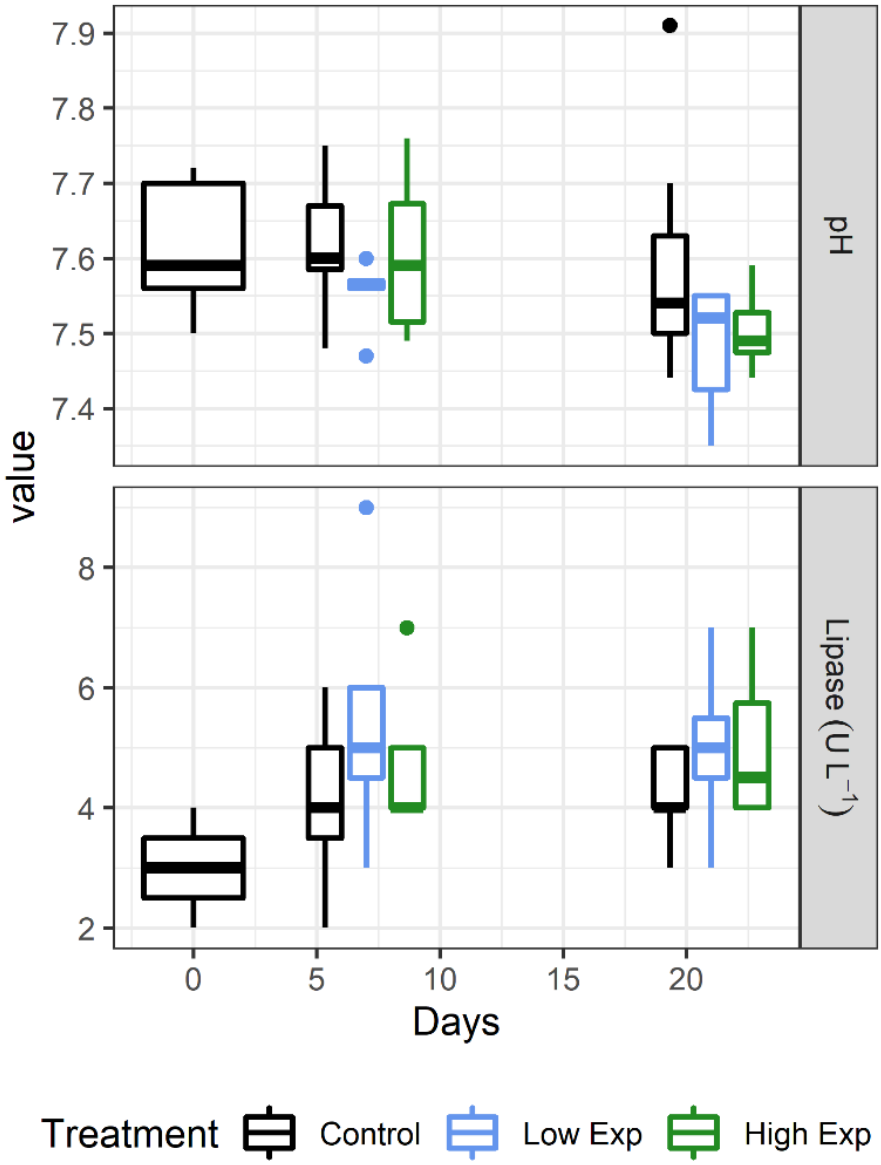

Figure 5.4 Haemolymph biochemical parameters that showed significant differences between Southern Rock Lobster treatment groups. Low exposed (dark grey) lobsters had significantly lower pH than the control group (p value = 0.02). Both high and low exposed lobster groups had significantly higher lipase than the control group (p values = 0.03 and 0.02 respectively).

Table 5.1 Summary of differences in behavioural, reflex, immunological and blood chemistry parameters between control and exposed Southern Rock Lobster (Treatment); across days; and for the interaction between treatment and days, measured by ANOVA, ordinal logistic regression (OLR) or logistic regression (LR). Significant differences are marked with asterisks (*p = 0.05-0.01, **p = 0.01-0.001, *** p <0.001).

Variable	Treatment	Days	Treatment: Days	2-way ANOVA, OLR, or LR
Behaviour				
Vitality				NS
Time to right				NS
Reflex Impairment Score				NS, Vitality and RIS sig related p<0.001
Specific Feed Intake		***		F = 12.3, week 1 higher than weeks 2 &3
Immune response				
Haemocyte count		***		F = 18.0, weeks 1 &2 lower than week 3
Bacteraemia on ZMA media				NS
Bacteraemia on TCBS media				NS
Necrosis				NS
Nutritional				
Hepatopancreas Index				NS
Brix				NS
Hemolymph biochemical				
pH	*			F=4.0, Control higher than low exp treatment
Sodium				NS
Potassium				NS
Sodium:potassium				NS
Chloride				NS
Magnesium				NS
Bicarbonate				NS
Calcium				NS
Phosphorus				NS
Glucose				NS
Lactate				NS
Cholesterol		***		F=8.8, week 1 lower than week 3
Triglyceride		*	*	F=7.2 (days), week 1 lower than week 3 F=3.5 (treatment:days)
Total protein		*		F = 4.9, week 1 lower than week 3
Albumin				NS
Globulin				NS
Albumin:globulin				NS
Uric acid				NS
Lipase	**			F = 7.4, control lower than both exposed treatments
Glutamate dehydrogenase				NS
Measured Osmolality				NS

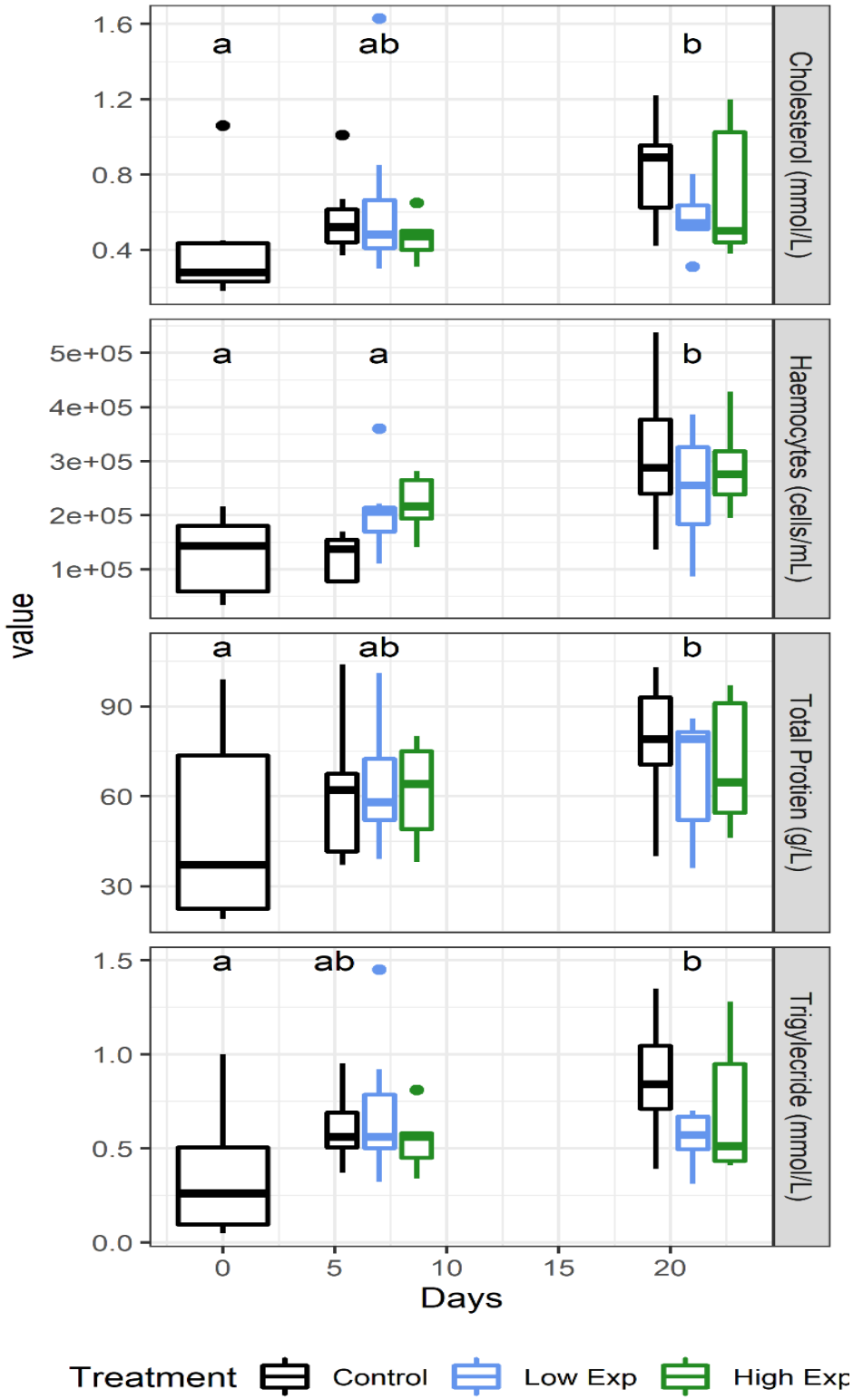

Figure 5.5 Parameters in Southern Rock Lobster haemolymph that changed significantly over the course of the experiment in control (black), low (blue) and high (red) algal exposure treatments (0, 1 x 105, 2 x 105 *A. catenella* cells/L respectively). Days which are not significantly different from each other share the same letter.

Histopathological findings in gills

Initial examination of gill filaments from the 6 high exposed and 3 of the control animals showed no differences in histopathological findings, so no further examinations of other lobster gills occurred. Diffuse pooling of haemolymph was observed in the lamellae and central axis of all gills examined, consistent with agonal change. All gills examined also showed multifocal deposits of rod and/or filamentous bacteria and low to moderate numbers of ciliated protozoa. Low numbers of free-living larval nematodes were found between lamellae on three of the six samples of gill tissues examined from exposed lobsters, and one of the three gill tissues from control lobsters. The gills of two of the high exposure lobsters and one control lobster showed microvesicles in reserve inclusion cells (Figure 5.6) likely to be storing lipid (Travis, 1954).

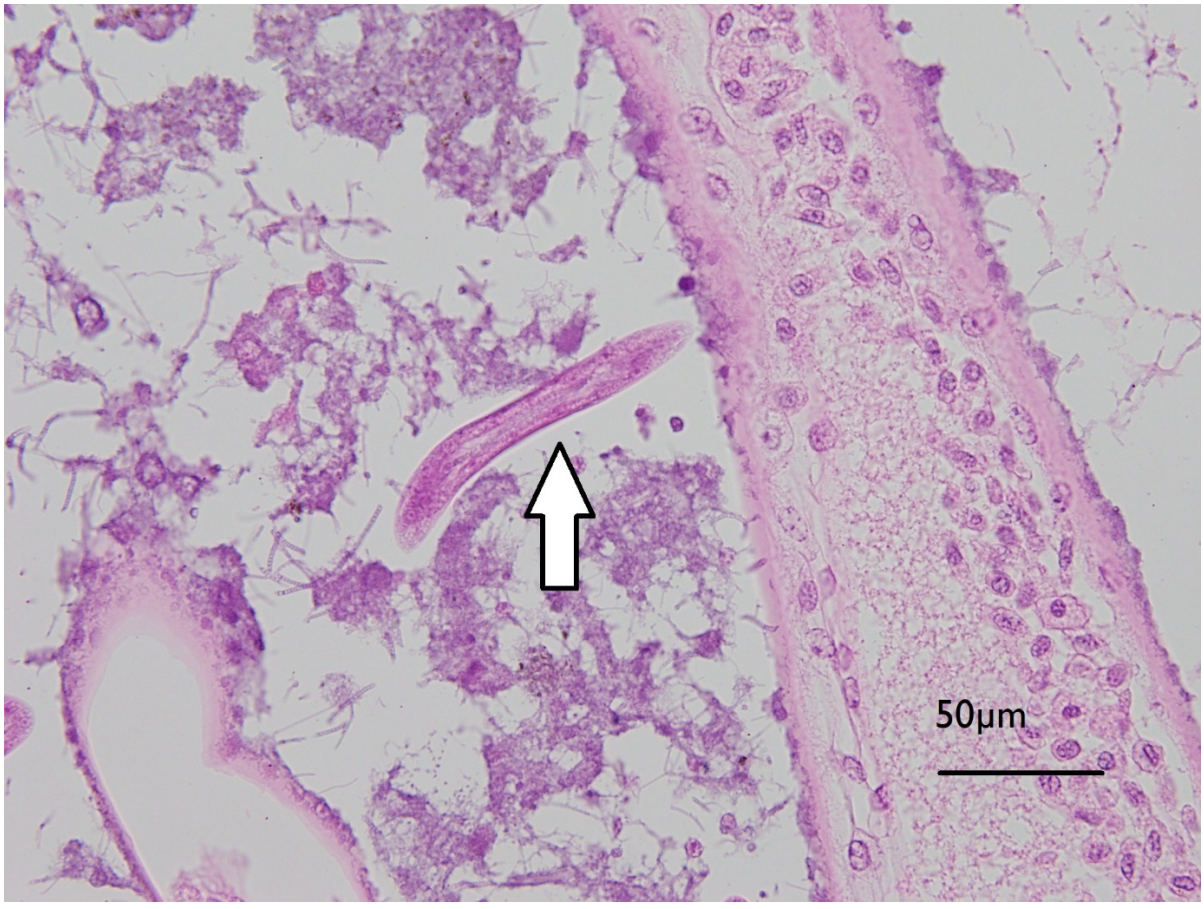

Figure 5.6 Micrograph of Southern Rock Lobster gill filament with biofouling (mixed bacterial colonies including *Leukothrix* like organisms adhered to filament) and free-living nematode between filaments as indicated by the arrow.

5.4 Discussion

No uptake of PST was detected in Southern Rock Lobster exposed to high but field relevant concentrations of *A. catenella* in an experimental setting over a three-week period. This was

in striking contrast to significant PST accumulation in lobster hepatopancreas (reaching a maximum of 9.0 mg STX.2HCl equiv. kg^{-1}) observed in parallel experiments feeding lobsters with toxic mussels (Turnbull et al., 2020a), and uptake of PST by abalone when exposed to toxic algal cells in a similar experiment to the current study (Seger et al., 2020). In the latter experiment, abalone were exposed to the same highly toxic strain of *A. catenella* (AT. TR/F) at the same level of the high exposure in this experiment (2×10^5 cells L^{-1}). Abalone were able to accumulate up to 128 µg STX.2HCl equiv. kg^{-1} in this experiment, although it is unknown if accumulation occurred across the gills, epipodium, via the viscera or a combination of these routes.

Furthermore, the minimal impact on lobster health demonstrated in this experiment across a range of organismal and cellular levels, indicates there will be no detrimental effect on survivability and vitality of these animals caused by exposure to toxic cells. Minimal whole organism and cellular responses were observed in Southern Rock Lobster following accumulation of high levels of PST in their hepatopancreas (Turnbull et al., 2020b) and when exposed to toxic cells (current experiment), indicating that lobsters are relatively resistant to the action of PST. However, this response is in contrast to significant histopathology and mortality experienced by Blue mussel and Pacific oyster larvae when exposed to extracellular exudates of the same Tasmanian *A. catenella* strain at equivalent cell concentrations of 100 to 1,000 cells mL^{-1} (Supono et al., 2020).

The present study exposed lobsters to aliquots of cultured algae and replicated environmental conditions where animals would be exposed to both cells and cell exudates. The toxic cells were presented to the algae at the highest level recorded from the Tasmanian blooms (Condie et al., 2019). It is likely toxins in wet storage would be equal to or less than those found in the field, as wet storage areas either draw directly from coastal waters in a continuous flow through systems or recirculate sea water through filtration and sedimentation systems to maintain water condition.

The cultured *A. catenella* strain (AT. TR/F) was originally isolated from a bloom on the east coast of Tasmania and contained up to 21.2 pg STX.2HCl equiv. $cell^{-1}$; a relatively high PST cell quota from cultured algae (Jester et al., 2009; Lilly et al., 2002; Sekiguchi et al., 2001; Varela et al., 2012). The toxin profiles of the *A. catenella* cells were predominantly C1,2 and GTX1,4, with minor levels of C3,4, Neo, GTX2,3, STX, dcGTX2,3, and GTX5,6 (see Section 4.2 below). These analogues are the same as those found in toxic shellfish from the east coast of Tasmania (Dorantes-Aranda et al., 2020; Turnbull et al., 2018b) and are thus considered representative of the Tasmanian *A. catenella* blooms. The same PST analogues are also found in New Zealand *A. pacificum*, *A. minutum* and *G. catenatum* isolates, although toxin proportions vary (Harwood et al., 2013; MacKenzie, 2014). In particular, the proportion of C3,4 toxins tend to be higher in *A. pacificum* isolates, Neo and STX higher in *A. minutum* isolates, and C3,4 and GTX2,3 higher in *G. catenatum* isolates. Absolute concentrations present at any time will vary with the cell number and toxin content of the cells and the amount of cell exudates.

Bioactive exudates from ichthyotoxic species have been demonstrated to have harmful impacts on the gills of adult Pacific oysters (Castrec et al., 2018) and a range of fish species (Chen and Chou, 2001; Ma et al., 2011; Mardones et al., 2015). Compromised gills show necrotising degeneration of the epithelium of the secondary lamellae and sloughing and swelling of the primary lamellar epithelium with congestion of branchial vessels (Hallegraeff et al., 2017b; Roberts et al., 1983; Shimada et al., 1983). None of these effects were seen in the lobster gills exposed to high concentrations of *A. catenella* in this experiment.

A wide range of indicators used to predict the health of lobsters throughout the supply chain were assessed in this study. The only characteristics that demonstrated significant differences between treatment groups was haemolymph pH and lipase concentration. The difference in pH was between control and low exposure groups, with control group showing higher pH. A decrease in pH is a common stress response in lobsters caused by respiratory and metabolic acidosis. Other studies found a decrease in pH associated with emersion and high temperature (Dove et al., 2005; Lorenzon et al., 2007; Simon et al., 2016; Whiteley and Taylor, 1992). Given the high exposure group was not significantly different to the control and low exposure group, it is unlikely this difference was caused by the exposure to *A. catenella*.

Lipase plays an important role in the digestion of fats. The increase seen between control and exposed groups of lobsters was influenced by the relatively low level of lipase measured in the control group of lobsters at the start of the experiment. No significant differences were seen between control and the two treatment groups if the lobsters on day 0 were excluded from the analysis (p value = 0.21). This observed difference could be related to the improved nutritional condition of the lobsters across the experiment, as shown by significant increases in cholesterol, protein and triglyceride levels. This increase in nutritional status was also detected in similar experiments feeding Southern Rock Lobster mussels to excess daily (Turnbull et al., 2020b) and was also associated with a similar decrease in feed intake during the course of the experiment as seen here.

The only immune response indicator that showed variation across the experiment was haemocyte levels, which increased with time in system in all treatment groups. Other studies looking at stress in lobster supply chains have found varying results; some finding an increase in haemocyte levels with starvation, capture, emersion, storage and transport (Day et al., 2019a; Simon et al., 2016; Tsvetnenko et al., 2001) and others finding a decrease (Basti et al., 2010; Fotedar et al., 2001; Jussila et al., 1997; Paterson et al., 2005; Stewart et al., 1967). No difference in haemocyte levels was seen in a similar experimental study looking at the impact of PST accumulation on Southern Rock Lobster health (Turnbull et al., 2020b). It is possible that the increase over time in all treatment groups may be related to the static experimental system.

In conclusion, we have conducted the first reported experiment to examine the uptake of PST by lobsters during exposure to PST producing algal cells, as would potentially occur in lobster supply chains. The algal culture used was highly toxic, produced a range of commonly found PST analogues, and was presented at relatively high concentrations. The lobsters were exposed to toxic cells for three weeks: longer than would normally be experienced in the *J edwardsii*

supply chain in Tasmania and New Zealand. From the lack of uptake of PST in lobsters during this study and the lack of impact on animal health, we conclude that wet storage of lobsters in coastal waters contaminated with the PST producing algae typically found in Tasmania and New Zealand, as occurs in the Southern Rock Lobster supply chain, does not pose a human health risk, nor to animal health. Therefore, no market access risk or risk to commercial returns through ill health exist from this practice.

6. Field validation of the Southern Rock Lobster paralytic shellfish toxin monitoring program in Tasmania, Australia

Alison Turnbull, Juan Dorantes-Aranda, Tom Madigan, Jessica Jolley, Hilary Revill, Tim Harwood & Gustaaf Hallegraeff

Abstract . Paralytic shellfish toxins (PST) are found in the hepatopancreas of Southern Rock Lobster from the east coast of Tasmania in association with blooms of the toxic dinoflagellate *Alexandrium catenella*. Tasmania's rock lobster fishery is one of the state's most important wild capture fisheries, supporting a significant commercial industry (AUD 97M) and recreational fishing sector. A comprehensive 8 years of field data collected across multiple sites has allowed continued improvements to the risk management program protecting public health and market access for the Tasmanian lobster fishery. High variability was seen in toxin levels between individuals, sites, months and years. The highest risk sites were those on the central east coast, with July to January identified as the most at-risk months. Relatively high uptake rates were observed (exponential rate of 2% per day), similar to filter-feeding mussels, and meant that lobster accumulated toxins quickly. Similarly, lobsters were relatively fast detoxifiers, losing up to 3% PST per day, following bloom demise. Mussel sentinel lines were effective in indicating a risk of elevated PST in lobster hepatopancreas, with annual baseline monitoring costing approximately 0.03% of the industry value. In addition, it was determined that if hepatopancreas PST levels in 5 individual lobster from a site were < 0.43 mg STX equiv. kg^{-1} this would result in a 97.5% confidence that any lobster from that site would be below the bivalve maximum level of 0.8 mg STX equiv. kg^{-1}. The combination of using a sentinel species to identify risk areas and sampling 5 individual lobster at a particular site provides a cost-effective strategy for managing PST risk in the Tasmanian commercial lobster fishery.

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6.1 Introduction

Recurrent blooms of the toxic dinoflagellate *A. catenella* (Whedon and Kofoid) Balech have occurred in winter on the east coast of Tasmania since 2012 (Condie et al., 2019). The species involved is highly toxic, resulting in paralytic shellfish toxins (PST) increasing rapidly in bivalve molluscs. Public health and market access risks are managed through weekly monitoring of toxin levels in shellfish flesh, with closures occurring when PST exceeds the domestic regulatory maximum level (ML) of 0.8 mg saxitoxin (STX) equiv. kg^{-1} in bivalve flesh. Closures may be for extended periods (over 20 weeks; unpublished data), with the maximum PST level detected of 150 mg STX equiv. kg^{-1} in mussels in 2017 grossly exceeding the ML (Condie et al., 2019).

During the initial bloom in 2012, PST was detected in multiple bivalve species (oysters, mussels, scallops and clams) and several non-traditional vectors (rock lobsters, abalone, giant crab) (Campbell et al., 2013). Close to 30% of the rock lobster hepatopancreas samples (n=93) taken during the bloom exceeded the domestic bivalve ML.

Following the 2012 bloom and these unexpected PST results, industry, regulators and scientific experts conducted a risk ranking for all commercial seafood species in Tasmania (Hallegraeff et al., 2018). The risk ranking process included consideration of commercial catch volumes for all seafood; edible portions of the seafood; ecological groups and trophic levels in the food web; the four main marine biotoxin groups (paralytic, diarrhetic, amnesic and neurotoxic shellfish toxins) and their history in Tasmania; and peer reviewed literature for toxin accumulation in non-traditional vectors worldwide. The review concluded the toxin:species pairing of PST in Southern Rock Lobster represented the greatest food/hazard risk, warranting a specific risk management program. Tasmania's rock lobster fishery is one of the state's most important wild capture fisheries, supporting a significant commercial industry (AUD 97M) and recreational fishing sector.

A significant body of research has occurred to underpin the Tasmanian rock lobster risk management program. Toxins were confirmed to accumulate to levels of concern only in the lobster hepatopancreas (Madigan et al., 2018a). Cooking studies demonstrated the toxicity of the hepatopancreas remained the same during steaming and boiling, with no change in the toxin profile but a reduction in the amount of hepatopancreas present for consumption (Turnbull et al., 2018a). A survey of recreational fishers in Tasmania and South Australia confirmed that the hepatopancreas was commonly consumed (Madigan et al., 2018b). Consumer exposure to PST was estimated using a 2-D Monte Carlo model (McLeod et al., 2018). The exposure assessment concluded that lobster hepatopancreas consumption during *A. catenella* blooms may be concerning for a small proportion of consumers but instigating harvesting restrictions for lobsters when levels exceed the bivalve ML reduced the probability of illness occurring.

Environmental conditions likely to trigger *A. catenella* blooms were determined to be water temperatures of 10–15 °C and stratification of coastal waters via salinity and/or temperature gradients (Condie et al., 2019). Experimental studies feeding highly toxic mussels to Southern

Rock Lobster found: exponential uptake and depuration rates of 6 and 7% per day respectively; potential excretion routes for PST are via the faeces, antennal glands and gill; there is no impact of PST on lobster health; and confirmed PST uptake is not possible via exposure to *A. catenella* cells, as would occur in boat wells, sea cages and holding facilities of the live lobster supply chain (Turnbull et al., 2020a; Turnbull et al., 2020b; Turnbull et al., 2021).

The current risk management of biotoxins in Tasmanian lobsters is described in the Rock Lobster Biotoxin Monitoring Plan (DPIPWE, 2020). The east coast of Tasmania is divided into eight rock lobster biotoxin management zones. Biotoxin sampling in lobster is triggered during high-risk seasons (winter – spring) by elevated PST levels in the common blue mussel *Mytilus galloprovincialis* Lamark from sentinel mussel sites. Management zones are closed for lobster harvest prior to sampling and remained closed until biotoxin results indicate it is safe to harvest. The closure for sampling (including research sampling) is to avoid the risk of a non-compliant lobster entering the export market chain of supply. The industry currently has little traceability capacity: if there was to be a market recall due to a non-compliant lobster PST sample, it could impact all Southern Rock Lobster harvested between the date of sampling and receipt of the PST results. Previously, the average time between sampling and receipt of toxin results was 7-10 days, however the adoption of the LC-MS/MS technique (Boundy et al., 2015; Turner et al., 2015) in Tasmania has reduced this to an average of 3 days. The sampling closures are formal legislative closures with significant penalties. Fishers are notified at least 48 hours ahead of intended sampling date. Commercial fishing cannot resume until the PST results have been received, reviewed against the decision protocols and the zone formally reopened. There is a high level of industry awareness and support for the biotoxin closure policy.

Risk management sampling involves harvesting 5 legal sized lobster from one site within each zone and individually analysing each hepatopancreas for PST. Zones are deemed safe for harvest if all hepatopancreas samples contain <0.5 mg STX equiv. kg^{-1}; unsafe for harvest if any hepatopancreas sample exceeds 0.8 mg STX equiv.kg^{-1}; and questionable if any hepatopancreas sample is between 0.5 and 0.8 mg STX equiv. kg^{-1}. In the latter case, further information is sought to allow an assessment of the risk. Management zones may also be closed for fisheries management reasons during the high-risk period, with seasonal fisheries closures usually occurring from early September to late November/early December for the upper zones, and from early September to late November/early December for the remaining zones.

Field studies of PST in Southern Rock Lobster on the east coast of Tasmania occurred concurrently with the experimental work described above from 2012 to 2020 inclusive. The studies aimed to identify field accumulation and depuration rates, and variability in PST accumulation in lobster individuals to produce data that, combined with the experimental studies, could improve risk management activities. The field studies aimed to refine information on high-risk months and sites on the east coast of Tasmania; inform sampling frequency and the minimum number of representative samples; and evaluate the use of sentinel species as a risk management option.

6.2 Materials and Methods

Monitoring of PST in Southern Rock Lobster hepatopancreas

Field sampling of Southern Rock Lobster occurred either on a regular basis through the high-risk period (research sampling; Great Oyster Bay and Maria zones) or when triggered from mussel sentinel results (risk management sampling in all zones (DPIPWE, 2020)). In both cases lobster (n=5) were caught at each site, normally by SCUBA divers in < 8 m, but occasionally from lobster traps. Lobster were kept on ice and transported back to the laboratory usually within 24 hours. Lobster where then euthanized, sexed, weighed, carapace length measured and dissected to remove the hepatopancreas. Each lobster hepatopancreas was homogenized and individually analysed for PST. In some risk management samples prior to 2017 ovaries were included in the hepatopancreas sample. Hepatopancreas samples were either transported immediately to Analytical Services Tasmania or Symbio for PST analysis as above, or frozen at -400C and transported to Cawthron Institute New Zealand for analysis.

Mussel sentinel sampling

At least one sentinel mussel site exists in each lobster biotoxin management zone. These may be either commercial marine farms in coastal areas; marine infrastructure in coastal areas (e.g. jetties); or specific mooring lines with bags of *M. galloprovincialis* attached prior to the beginning of each risk season. Pooled samples of mussels (n=15) from each site were homogenised on a weekly (bivalve commercial monitoring program) or fortnightly (lobster mussel sentinel program) basis and analysed for PST at Symbio (prior to December 2017) or Analytical Services Tasmania (post November 2017). Occasionally only PST screen analysis was undertaken (Section 4.5.10). The frequency of the sentinel sampling increased if any data from the east coast of Tasmania indicated an elevated biotoxin risk.

PST analysis

Analyses of all research samples collected prior to 2018 and all risk management samples were conducted via pre-column oxidation HPLC-FLD (Harwood et al., 2013) based on AOAC method 2005.06 (Lawrence et al., 2005) at Symbio Laboratories, Sydney; Analytical Services Tasmania; or Cawthron Institute, New Zealand. Research lobster hepatopancreas samples from 2018 on were analysed via LCMS/MS (Boundy et al., 2015; Turner et al., 2015) at Cawthron Institute, New Zealand. Total PST concentrations from all analysis are expressed in µg or mg STX equiv. kg^{-1}, calculated using the Food and Agriculture Organisation/World Health Organisation's toxicity equivalency factors (FAO/WHO, 2016).

Analysis of PST via AOAC method 2005.06 consisted of extraction of PST from the sample in 1% acetic acid in a boiling water bath for 5 min, followed by cooling, centrifuging, dilution with water, pH adjustment and SPE cleanup. Aliquots of this extract then underwent periodate oxidation prior to HPLC-FLD analysis to determine the PST screen result. The PST screen typically overestimates sample toxicity as it is assumed that chromatographic peaks generated from co-eluting oxidation products are due entirely to the more toxic analogue (a more detailed explanation of this approach can be found in Harwood et al. (Harwood et al., 2013)). Several

PST analogues are known to give multiple co-eluting fluorescent oxidation products. If proceeding to PST confirmation the initial pH-adjusted extract was separated into 3 fractions via anion exchange SPE. These were then oxidized using both periodate and peroxide, with all of the resulting fractions analysed by HPLC-FLD and the resulting spectra interpreted. PST present in samples were quantified by comparison with certified reference materials. Recovery, dilution and toxicity equivalency factors were applied to give the contribution of each analogue to sample toxicity. These were then summed to give total sample toxicity in STX equivalents.

Analysis of PST via LCMS/MS occurred as described in Turnbull et al. (Turnbull et al., 2020a). PST extraction occurred in 1% acetic acid in a boiling water bath for 5 min, followed by cooling and centrifugation to remove particulate matter. Different ratios of sample to acetic acid were used in the different laboratories. For shellfish, ratios were 2 g in 18 mL, 5 g in 10 mL or 5 g in 5mL, for lobster hepatopancreas the ratios were 2 g in 18 mL or 5 g in 10 mL. This was followed by the addition of ammonium hydroxide before manual SPE cleanup using pre-conditioned amorphous graphitised polymer carbon Supelco ENVI-Carb 250 mg/3 mL cartridges. PST were then eluted with acetonitrile/water/acetic acid (20:80:1 v/v/v) and the eluent diluted with acetonitrile. Sample analysis used HILIC-MS/MS as described by Boundy et al. (2015) and Turner et al. (2015).

Statistical analysis

All data analyses were performed using the RStudio software (R Core Development Team, version 3.3.6, 2020). All PST results below the level of reporting were designated 0.1 mg STX equiv. kg^{-1} to enable statistical analyses to be performed. Bloom years were designated as 1 May to 30 April as *A. catenella* blooms on the east coast of Tasmania tend to develop in June/July and disperse by December/January. PST results could not be transformed to a normal distribution so the impact of sex and size on PST concentration was assessed using two-way randomized permutation on the 2017-2019 data only, as some hepatopancreas samples collected prior to 2017 contained ovaries. Statistical analyses were assessed against a significance level of 0.05.

To determine the number of lobsters required to be sampled at the start and end of a bloom to give a 95% confidence that the population is below the bivalve ML, all sampling occasions where all lobster were <1.0 mg STX equiv. kg^{-1} were examined. Data from both the start and end of bloom periods were tested for normality and homogeneity of PST concentrations using Shapiro-Wilke's test and Levene's test. Total PST from the two data sets were not significantly different (*p* value 0.07) and were therefore combined for analysis. A linear model of the mean and the maximum PST concentration was created, and the upper 95% prediction interval limit interpolated to find the mean and maximum total PST concentrations when 97.5% of the population was below the ML.

Only events with four or more consecutive sampling events were used to calculate the uptake and depuration rates of total PST in lobster hepatopancreas. This only occurred for sampling in the Maria zone during 2015 and 2019 (uptake) and 2012, 2017 and 2019 (depuration). Rates

of change in total PST were calculated by fitting linear models to the log PST data. Mussel PST concentrations from the same bloom period were visually assessed to determine the appropriate time frame for the associated uptake and depuration of toxins in mussels. Uptake and depuration rates for mussels were then similarly calculated.

6.3 Results

All PST results reported herein are expressed in mg STX equiv. kg^{-1} in line with the current Australian regulatory requirement for PST in bivalve shellfish and also used for PST risk management in Southern Rock Lobster. Furthermore, this aligns with the ML for China (Turnbull et al., 2020c), the main export destination for Tasmanian lobster (Steven et al., 2020). The authors point out that the Codex reporting units are different (mg STX.2HCl equiv. kg^{-1}), and our previous research on PST in Southern Rock Lobster has been reported following Codex guidelines (producing results that are effectively 24% higher (Turnbull et al., 2020c)).

Variation in PST levels across sites and time

Recurrent blooms of *A. catenella* occurred during the 8-year study period, triggering risk monitoring and research sampling in all years. Of the 496 Southern Rock Lobster hepatopancreas samples analysed for PST between 2012 and 2020, 100 (20%) did not contain PST above the level of reporting. Toxin accumulation in lobster >0.8 mg STX equiv. kg^{-1} occurred in all years, except the 2013/14 and 2014/15 bloom years (Figure 6.1a), with 86 (17%) lobster exceeding this level . There was a significant difference between years with respect to the level of PST accumulated (p value <0.005). The length and timing of the toxin event also varied annually. The longest biotoxin related harvest closure for lobster fishing occurred in the Maria zone during 2015/6, lasting 14 weeks. With the exception of one closure in the southern zone of Storm Bay Bruny in April 2013, the earliest month that lobster hepatopancreas first exceeded the bivalve ML was July (Great Oyster Bay zone, 2016), and the latest was November (several sites, several years), although it should be noted that sampling often did not occur in October when zones were closed for fisheries management reasons.

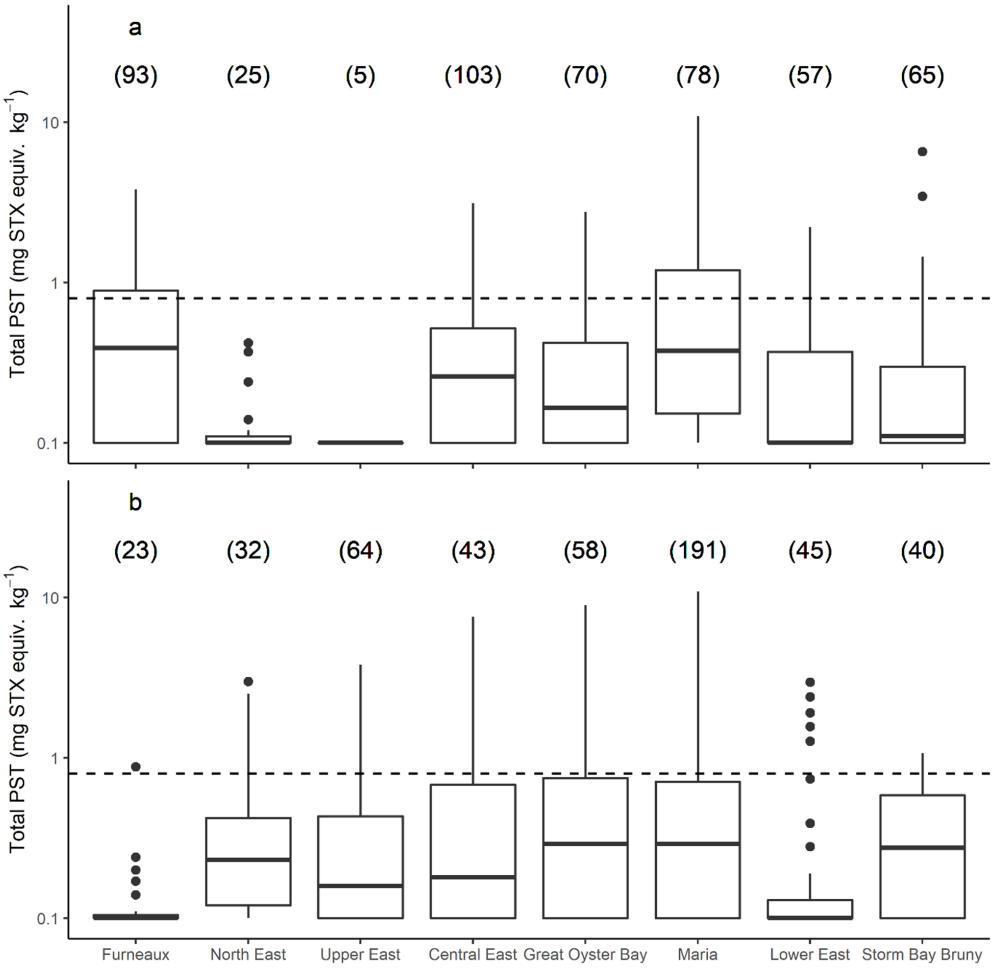

Figure 6.1 Paralytic shellfish toxins (log scale) in Southern Rock Lobster hepatopancreas across all bloom years (a) and management zones (b) on the east coast of Tasmania from 2012 to 2000. Bloom years are from May until April each year: management zones are presented (left to right) from North to South. Boxes indicate median, first and third quartiles; whiskers extend to maximum of 1.5 times the interquartile range; outlying points are plotted individually; number of samples in brackets. Bivalve maximum allowable level shown as black dotted line.

There was a significant difference in the total PST accumulated in each zone (*p* value 0.018), with the highest PST accumulation occurring in the central zones of Central East, Great Oyster Bay, and Maria (Figure 6.1b). The maximum total PST level recorded in lobster hepatopancreas was 10.9 mg STX equiv. kg^{-1} from the Maria zone in 2017. The northern zones of Furneaux and North East each only exceeded 0.8 mg STX equiv. kg^{-1} in one year late in the season (October 2015 and November 2012 respectively). Similarly, lobster hepatopancreas exceedances of the bivalve ML in the southern end of the east coast were more sporadic, with lower maximum PST levels and a shorter period of contamination.

Variation in PST accumulation within sites

There was a high degree of variability in PST accumulation in individual lobsters harvested from one site on the same date (Figure 6.2 and Figure 6.5; coefficient of variation ranged from 0.01 – 1.80, averaging 0.79 when the mean PST >0.1 mg STX equiv. kg^{-1}). Toxin accumulation was not significantly different between sexes (*p* value 0.51) and was not related to carapace length or weight (*p* values 0.78 and 0.43 respectively).

To determine if the regulatory sampling regime adequately represented PST levels across the lobster population of a site with respect to the bivalve ML, data from the start and end of the blooms (defined as periods when all samples from a site were <1 mg STX equiv. kg^{-1}) were examined. This subset of data was chosen because bloom initiation and finalization when toxin levels are below the bivalve ML is the period of most concern to the risk management program and the consistency of variability across the entire bloom was unknown. The PST concentrations in the hepatopancreas in both data sets were normal and homogeneous when transformed using a log function. Total PST levels from the two data sets were not significantly different (*p* value 0.47) and therefore the data were combined for analysis. The mean and the maximum PST concentration from each sampling event were linearly related (Figure 6.3; r= 0.95). Interpolation of the upper 95% prediction interval intersection with the bivalve ML was then used to obtain the estimates for the mean and maximum PST of a sample event below which 97.5% of the population of that site would comply with the bivalve ML. Figure 6.4 demonstrates this level of confidence would be achieved when the mean PST concentration at a site was below 0.22 mg STX equiv. kg^{-1}, or the maximum PST concentration below 0.42 mg STX equiv. kg^{-1}.

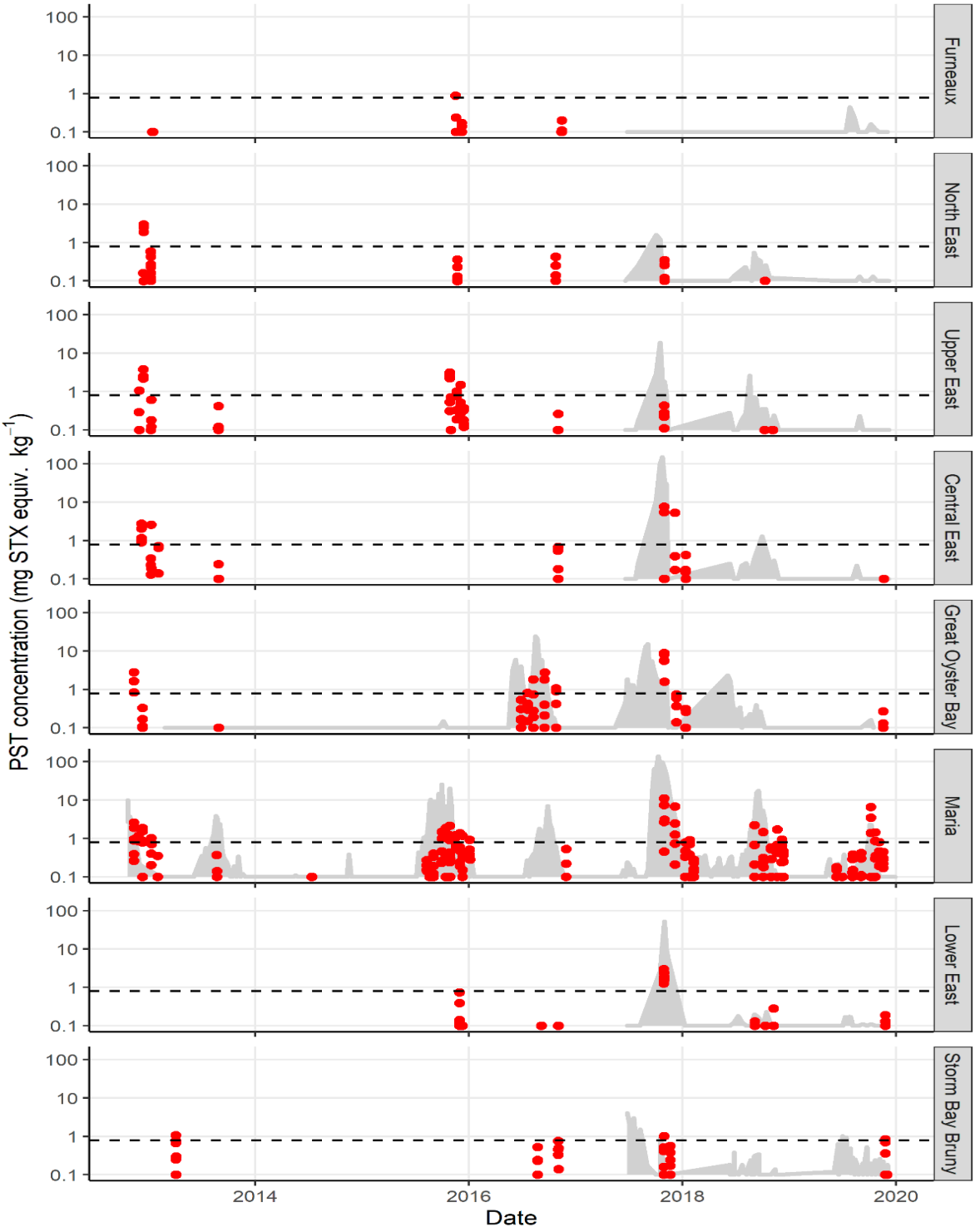

Figure 6.2 Paralytic shellfish toxins (log scale) in all management zones on the east coast of Tasmania from 2012 to 2000. Bivalve ML shown as black dotted line. PST concentration in Southern Rock Lobster hepatopancreas (red dots) exceeds the bivalve ML in every zone on at least one occasion. Prior to 2017, *M. galloprovincialis* sentinel monitoring (grey shaded area), only occurred in Great Oyster Bay and Maria zones. PST in sentinel mussels exceed the bivalve ML prior to lobster hepatopancreas on all occasions except in Storm Bay Bruny in 2019.

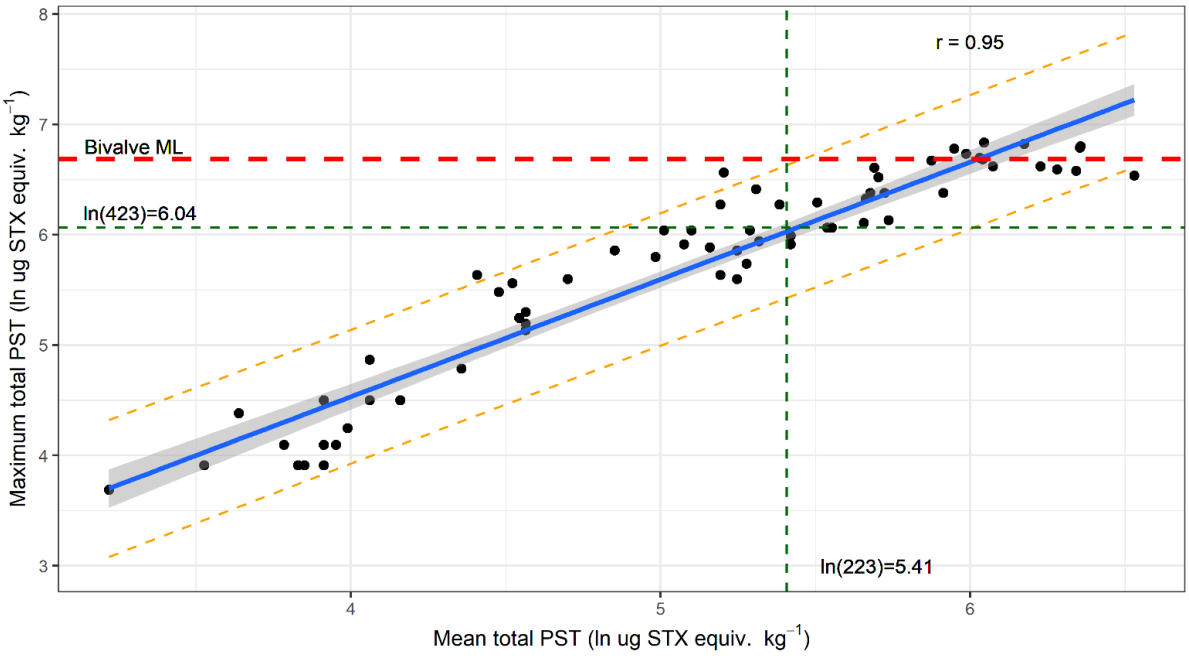

Figure 6.3 Linear model of the mean and maximum total PST concentrations found in Southern Rock Lobster hepatopancreas at each sample event at the start and end of *A. catenella* blooms on the east coast of Tasmania between 2012 and 2019 (black dots, n=68), showing the best fit regression line (blue), 95% confidence interval (dark grey shading) and 95% prediction interval (orange dotted line). The intersection of the bivalve maximum level (dashed red line) with the upper 95% prediction interval allows interpolation (green dotted lines) of the mean and maximum total PST concentration expected when 97.5% of the lobster population at a site is below the bivalve ML.

Mussels as a sentinel species

Mussels were first investigated as a sentinel species for lobster management zones during the research sampling in 2015. Mussel lines were extended to all management zones where commercial bivalve aquaculture was not occurring on the coast in 2017. Mussel accumulation of PST over a bloom season rose and fell rapidly, with high levels of PST providing warning of PST risk in lobster (Figure 6.2). For sites with matched Southern Rock Lobster and *M. galloprovincialis* data, 9 exceedances of the bivalve ML in Southern Rock Lobster hepatopancreas were recorded. All except one followed mussel exceedances. The only event that was not predicted by prior accumulation of toxins in mussels was Storm Bay Bruny in November 2019. Mussels exceeded the bivalve ML between 6 days (Maria zone October 2019) and 3 months (Great Oyster Bay zone October 2017) prior to lobster hepatopancreas exceedance, although zones that were closed for fisheries management reasons were not sampled until the fisheries closure was nearly over.

Field PST accumulation and depuration rates of PST

Field uptake and depuration rates for total PST in Southern Rock Lobster, calculated from linear models of log PST on the occasions when at least four sampling events took place at one site during the period of interest, are given in Table 6.1 Uptake and depuration rates (exponential) of total PST in Southern Rock Lobster and *M. galloprovincialis* samples in the Maria zone, 2012 – 2020. Number of samples (n) for Southern Rock Lobster represents 5 replicates at each time point, n for *M. galloprovinicalis* represents one pooled sample at each time point.. Rates could only be calculated for research sampling in the Maria zone during the 2015/16 and 2017/18 bloom years for uptake and the 2012/13, 2017/18 and 2019/20 bloom years for depuration. Corresponding rates of PST uptake and depuration for *M. galloprovincialis* at sentinel sites over the same period were similar (but lower) to those observed for lobsters.

Table 6.1 Uptake and depuration rates (exponential) of total PST in Southern Rock Lobster and *M. galloprovincialis* samples in the Maria zone, 2012 – 2020. Number of samples (n) for Southern Rock Lobster represents 5 replicates at each time point, n for *M. galloprovinicalis* represents one pooled sample at each time point.

Lobster Site & Year	**Phase**	**Southern Rock Lobster uptake/ depuration rate (mg STX. equiv. kg^{-1} day^{-1}) (*n*)**	***M. galloprovinicalis* uptake/ depuration rate in Spring Bay (mg STX equiv. kg^{-1} day^{-1}) (*n*)**
Maria Island, 2015/16	Uptake	0.009 (20)	0.075(9)
Okehampton Bay, 2019/20	Uptake	0.019 (20)	0.032 (12)
Maria Island, 2012/13	Depuration	- 0.031 (16)	- 0.043 (12)
Maria Island, 2017/18	Depuration	- 0.028 (25)	- 0.072 (23)*
Okehampton Bay, 2019/20	Depuration	- 0.017 (20)	- 0.103 (7)

Seasonal fisheries management zone closures occur on the Tasmanian east coast for reasons such as: lobster resilience at time of moulting; handling of berried females; and catch constraint and resource sharing between the recreational and commercial sectors. These closures occur in the peak biotoxin season (Figure 6.4). Mussel sentinel monitoring continues through these closures, with risk management sampling of lobsters occurring prior to re-opening if any biotoxin activity has been detected. At the highest PST risk zone, Maria, every lobster exceedance since 2015 has begun during a fisheries closure.

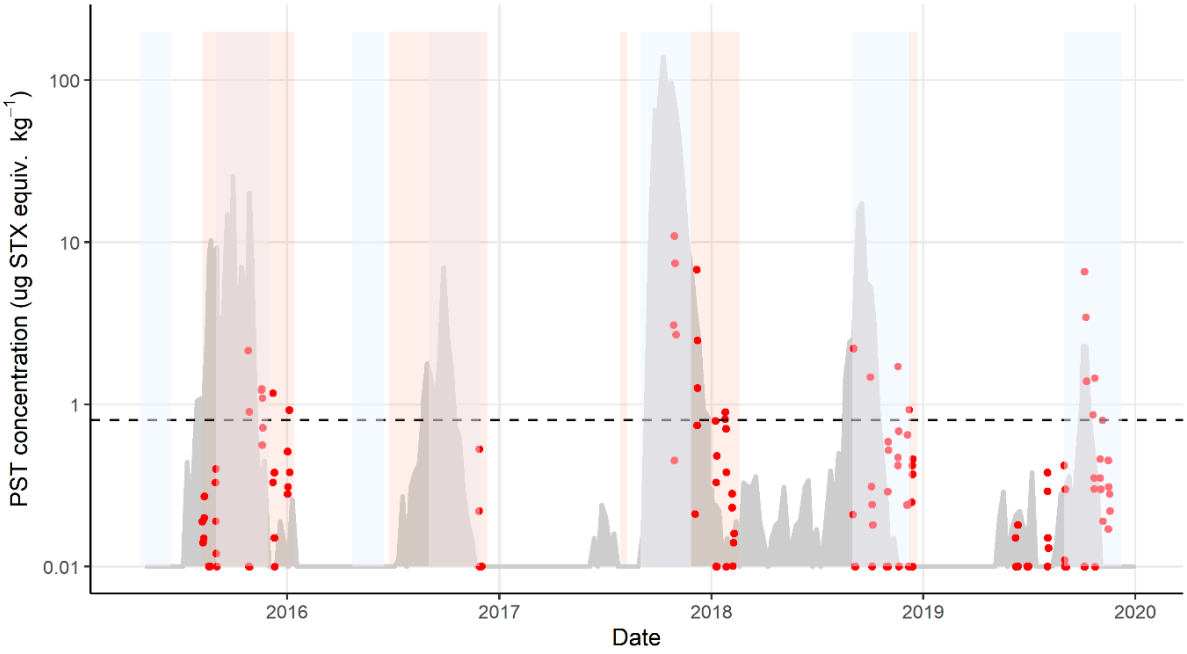

Figure 6.4 Paralytic shellfish toxins concentrations in Southern Rock Lobster and *M. galloprovincialis*, Maria zone, from 2015 – 2020: Southern Rock Lobster hepatopancreas (red dots); *M. galloprovincialis* (grey shaded area, screen and confirmed data); fisheries management closures shaded blue and lobster biotoxin closures shaded orange. Horizontal dotted line represents the bivalve ML.

PST profiles

The molar PST profile of *J. edswardsii* hepatopancreas was generally dominated by gonyautoxins (GTX)1&4; GTX2&3; and the N-sulfocarbmoyl toxins C1&2, with minor percentages of STX; C3&4; and decarbomoyl saxitoxin (dcSTX). Occasionally neosaxitoxin (NEO) or decarbomoyl gonyautoxin (dcGTX)2&3 were present above their respective reporting limits. The same analogues were found in *M. galloprovinciallis*, with GTX1&4; GTX2&3 and C1&2 also dominant in this shellfish species. The molar percentage contribution of analogues from samples taken from the same site at the same time were variable, particularly with respect to the amount of STX present, which ranged from 0-100% during some events. To examine changes in PST profiles during uptake and depuration across one bloom, the average molar percent profiles of each sample event from Maria 2019/20 bloom were investigated (Figure 6.5). This was the only bloom period where both uptake and depuration rates were able to be calculated. PST levels in lobster on the first sample event were low (<0.2 mg STX equiv kg^{-1}). On the second sample event, close to 90% of the PST analogues present were C toxins, the majority of which were C1&2. During both the uptake and depuration phases, the mean percentage of C toxins consistently decreased (Figure 6.5), whilst GTX2&3 increased. There was a decrease in GTX1&4 and a concomitant increase in STX during depuration.

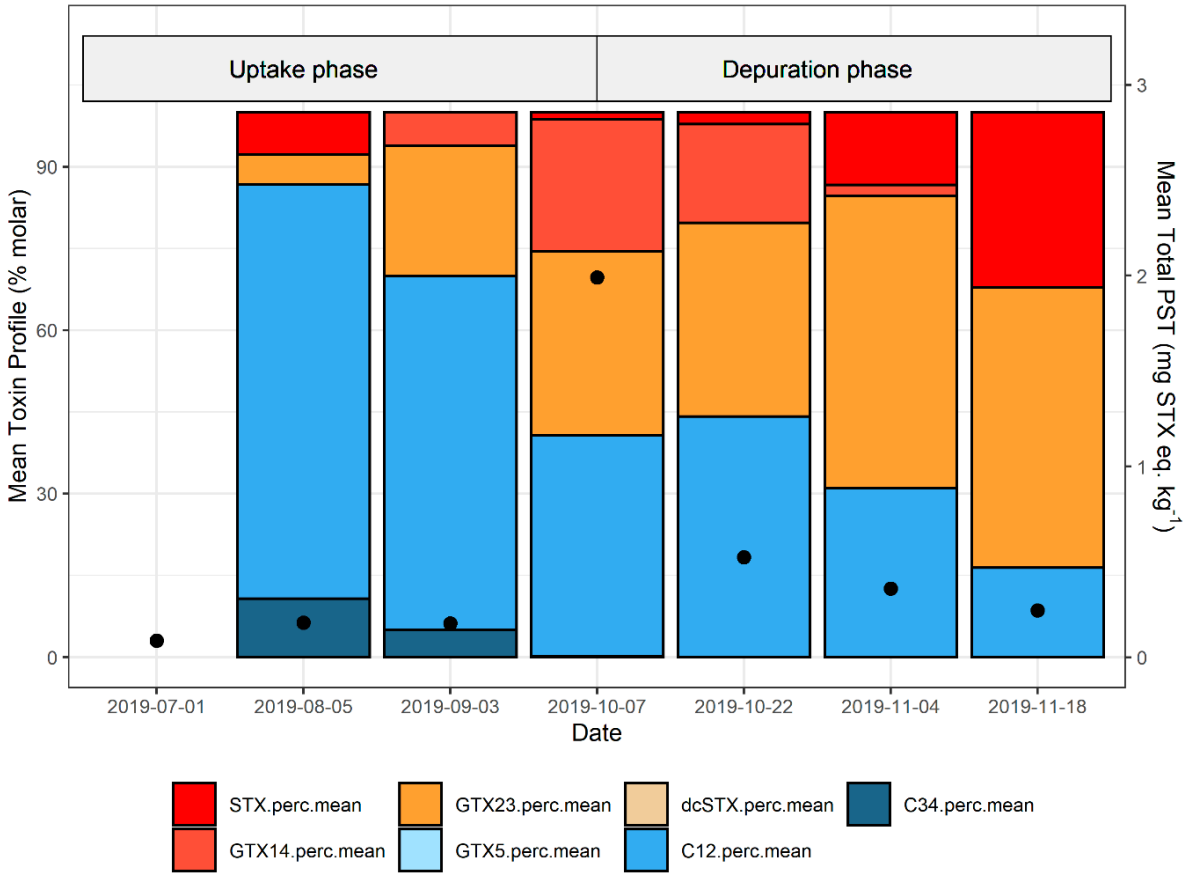

Figure 6.5 Mean paralytic shellfish toxin profiles (molar percentage) and mean total PST (toxicity equivalents) in Southern Rock Lobster hepatopancreas from Maria Island during the 2019/2020 bloom. Toxin profile data only includes lobster with mean total PST > 0.1 mg STX equiv. kg $^{-1}$, whilst total PST data includes all samples taken on that day. Mean total PST shown as black dots.

6.4 Discussion

Risk management and research sampling of Southern Rock Lobster hepatopancreas from the east coast of Tasmania only occurred in association with blooms of *A. catenella.* As a result, hepatopancreas samples frequently contained PST, often at concentrations above bivalve ML. A high level of variability was seen between bloom years with respect to the site, timing, and level of PST accumulation. Toxicity was greatest in the centre of the east coast with exceedances of the bivalve ML occurring during the months of July to January. The one exception to this was an exceedance in the Storm Bay Bruny zone in April 2013. This management zone includes the mouth of the Derwent Estuary, where *Gymnodinium catenatum* frequently blooms, particularly during Spring and Autumn (Hallegraeff et al., 2012; Hallegraeff et al., 1995). Whilst no data is available for phytoplankton assemblages in the area during April 2013, this is a plausible PST source.

The high variability between individuals concurrently collected from one site creates difficulties in risk management decisions for the total lobster population within each zone. Risk management

procedures need to be practical and affordable. The significant data set collected here has allowed an estimate of population risk of exceedance of the bivalve ML associated with the collection of 5 animals. Five animals represent an achievable sampling effort at an analytical cost of approximately AUD1250-3000, depending on the PST analytical technique used, the analysing laboratory and the turn-around time requested. In Tasmania, risk managers have chosen to review and potentially restrict harvesting when any animal exceeds the level of 0.5 mg STX equiv. kg^{-1}, which represents 63% of the ML applied for bivalves. When the maximum PST concentration of any lobster from a site is less than 0.42 mg STX equiv. kg^{-1}, we have confidence that 97.5% the population will be below the bivalve ML when harvesting is occurring. A less conservative approach could be taken if more lobsters were sampled on each occasion, but this would require a greater sampling effort and analytic expense to achieve the same level of confidence.

Mussels were first investigated as a sentinel species by Madigan et al. in the initial research project following the 2012 bloom (Madigan et al., 2017). Mussels make ideal sentinels as their high feeding filtration rates result in rapid toxin accumulation, analytical methods are well developed for this matrix, and they are easy to collect off existing marine infrastructure, or lines are easy to deploy where no infrastructure exists. Sentinel mussel sites have since been installed in all zones where commercial bivalve aquaculture is not occurring on the coast.

There have been 9 harvest closures instigated for *Southern Rock Lobster* in areas where mussel sites were concurrently sampled with lobster. All except one of these closures was preceded by elevated toxicity in the mussels. The exception was Storm Bay Bruny during November 2019. In this case the sample sites for mussels and lobsters were not in proximity. This raises the issue of variability at different sites within zones: variability is acknowledged to be likely but has not yet been investigated in detail. Experimental data has shown that Southern Rock Lobster can accumulate PST in the hepatopancreas to over the bivalve ML within 4 days if fed highly toxic mussels in large quantities (Turnbull et al., 2020a). The exponential uptake rate for that study over a period of 27 days was 6% day-1. Field rates of PST accumulation in Southern Rock Lobster hepatopancreas in this study were similar (1 and 2% per day), indicating lobster had access to PST-rich prey, and highlighting the need for a rapid sampling response during bloom inception.

The depuration rates determined for lobster in this field study varied between 1.7 and 3.1% per day, similar to the 2 and 7% found in experimental studies of PST depuration from fed Southern Rock Lobster (Madigan et al., 2018a; Turnbull et al., 2020a). In all the field events studied, depuration to the bivalve ML occurred in mussels prior to lobsters, demonstrating the usefulness of the mussel sentinel lines is valid for both uptake and depuration. Bricelj and Shumway compared depuration rates of bivalves from a variety of studies and found rates similar to those described here, varying from 2 to 15% per day (Bricelj and Shumway, 1998). They classified these as “fast detoxifiers”. Depuration rates for Southern Rock Lobster seen during this study place these lobsters on the cusp between fast and slow detoxifiers. The data confirms that Southern Rock Lobster clear PST from their hepatopancreas relatively quickly once a toxin event has passed. From the field depuration rates measured here, it would take lobsters 15-27 days to depuration from twice the bivalve regulatory level to below the bivalve regulatory level.

The PST analogues present in Southern Rock Lobster hepatopancreas were the same as those seen in experimental studies using mussels from Tasmanian east coast events as the prey source (Madigan et al., 2018a; Turnbull et al., 2020a). In the Maria zone in 2019, the proportion of C1&2 and C3&4 were higher than seen in the experimental studies, likely due to the prey sources available in the field. All C toxins decreased as the bloom progressed through uptake and depuration phases. In the experimental studies, this decrease was only seen during depuration. In both the field and experimental studies GTX1&4 reduced during depuration whilst the proportion of GTX2&3 increased. A similar pattern was observed with the field samples, with STX also increasing during depuration. A reduction of GTX1&4 relative to GTX2&3 was also described in the spiny lobster *Panulirus stimpsoni* during depuration (Jiang et al., 2006).

The risk management of PST in Tasmanian Southern Rock Lobster has evolved since the first detection of PST associated with an *A. catenella* bloom in 2012, in response to research outputs that have improved the risk-based response. A key objective of the rock lobster biotoxin monitoring program is to ensure that the risk of a Tasmanian rock lobster exceeding any import standard is as low as possible. China is an important market destination for this high value fishery and has a PST standard that applies to all seafood. Thus using the bivalve regulatory ML, which is the same as the Chinese regulatory ML, serves the dual purpose of protecting both public health (McLeod et al., 2018) and market access. There have been no illnesses reported in association with PST in Tasmanian lobster hepatopancreas.

The use of mussel lines to provide an easy to access source of bivalve shellfish for PST testing in zones where wild or aquaculture shellfish are not abundant or accessible has been a significant improvement to extend the capacity to sample areas of relevance/ importance to the rock lobster fishery. These sentinel bivalve shellfish act as an early warning system, triggering PST testing in lobsters. The two-tier monitoring program reduces both the expense of lobster sample collection and analysis, and the cost to industry of lost fishing opportunity through zone closures enacted prior to sampling.

Overlaying the biotoxin closures are regional seasonal management closures. These are implemented each year for a variety of reasons, including: lobster resilience at time of moulting; handling of berried females; and catch constraint and resource sharing between the recreational and commercial sectors. The majority of the east coast is under a regional seasonal closure between 1 September and early December. Bivalve sentinel PST testing continues throughout this seasonal closure period to inform whether lobster sampling is required prior to the scheduled reopening of the east coast fishery. For this reason, the first lobster biotoxin samples are often not taken until mid/late November when a bloom event may have reached its peak.

Biotoxin zone closures, particularly if multiple zones are closed for an extended period of time, can have significant fishery management impacts through the concentration of fishing effort in remaining open part of the fishery. To reduce the risk of localised stock depletion, a biotoxin zone may remain closed even though the lobster PST results meet the re-opening decision criteria.

The cost of the monitoring program (sample collection and analysis) is shared between rock lobster licence holders and the Tasmanian Government. The baseline monitoring of PST in sentinel bivalves costs approximately AUD 30k per annum (0.03% of Tasmanian lobster industry value), with monitoring costs increasing when bloom activity is detected, triggering increased sampling frequencies and sampling and analysis of lobster. The relatively low cost of monitoring bivalve sentinel PST data to provide an early warning of bloom activity along the whole eastern region of Tasmanian is a critical component for a cost-effective program, given the unpredictable spatial and temporal distribution/occurrence of *A. catenella* blooms.

This study represents the first comprehensive field study published on PST accumulation in lobster. Risk management programs addressing PST in lobster exist in other countries (Etheridge, 2010), supported by experimental work (Cembella and Desbiens, 1994; Desbiens and Cembella, 1995, 1997; Haya et al., 1994; Haya et al., 1992; Jiang et al., 2006). However, no field studies have been published. For the Tasmanian rock lobster fishery, the combination of previously published experimental work (Dorantes-Aranda et al., 2020; Madigan et al., 2018a; Madigan et al., 2018b; McLeod et al., 2018; Turnbull et al., 2020a; Turnbull et al., 2020b; Turnbull et al., 2018a; Turnbull et al., 2021) and the field studies reported herein have provided a strong evidence base for continued progressive improvements to the Tasmanian risk management program.

7. Detection of Paralytic Shellfish Toxins in Southern Rock Lobster Using the Qualitative Neogen™ Lateral Flow Immunoassay: Single-Laboratory Validation

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Abstract. Paralytic shellfish toxins (PST) are a significant problem for the Tasmanian shellfish and Southern Rock Lobster industries, and the introduction of rapid screening tests in monitoring programs could save time and money. We previously demonstrated that the Neogen rapid kit was the most suitable test for Pacific oysters and mussels contaminated by *Alexandrium catenella* blooms. Validation studies showed that the Neogen test returned satisfactory results for use in risk management, with low errors at the regulatory level, particularly in oysters. The single-laboratory validation protocol was applied to the hepatopancreas of Southern Rock Lobster contaminated with three distinct PST profiles, of which two mixtures were common (high in gonyautoxin 2&3 and saxitoxin, respectively), and the third mixture was observed only in a few select animals (high in gonyautoxin 1&4). The Neogen test consistently returned negative results for non-target toxins. The probability of detection (POD) of PST in the lobster hepatopancreas using the Neogen test increased with increasing PST concentrations. POD values of 1.0 were obtained at ≥0.57 mg STX.2HCl equiv. kg^{-1} in mixtures 1 and 2, and 0.95 and 1.0 for mixture 3 at 0.79 and 1.21 mg STX.2HCl equiv. kg^{-1}, respectively, with a fitted POD of 0.98 for 0.80 mg STX.2HCl equiv. kg^{-1}. The performance of the Neogen test when using four different production lots (ruggedness) showed no significant differences. The results of the present validation study were satisfactory, and we are now trialing incorporation of the Neogen test within the PST monitoring program of Southern Rock Lobster in Tasmania (see Chapter 8).

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7.1 Introduction

Paralytic Shellfish Toxins (PST) have emerged as a significant problem for the Tasmanian shellfish industry in Australia since October 2012, due to the winter-blooms of the dinoflagellate *Alexandrium catenella* (group 1, previously called *A. tamarense* or *A. fundyense*) (John et al. 2014; Fraga et al. 2015). Since then, *A. catenella* blooms have caused annual mussel farm closures for up to 4 months. Considering that the regulatory limit of PST is 0.8 mg of saxitoxin equivalents per kilogram of shellfish meat (=0.8 mg STX.2HCl equiv. kg^{-1}), Tasmanian mussels were found to contain up to 150 mg STX.2HCl equiv. kg^{-1} in 2017, almost 190 times above the regulatory limit (Condie et al. 2019). Not only mussels and oysters, but also the Southern Rock Lobster *Jasus edwardsii* (SRL) industry, a high value fishery in Australia valued at AUD$93M per year, has been affected by these bloom phenomena (Campbell et al. 2013; McLeod et al. 2012).

Accumulation of PST by various molluscs has been widely demonstrated (Bricelj and Shumway 1998;Shumway 1995; McLeod et al. 2017), thus making them potential vectors for PST in lobsters. Although the diet of SRL remains to be fully elucidated, it has been observed that other rock lobsters mainly feed on coralline algae, mussels, gastropods, barnacles, crabs, sea urchins, fish, and other lobsters; consumption of abalone has been demonstrated experimentally, while most studies reported a major preference for mussels (Joll and Phillips 1984; Mayfield et al. 2000; Dumas et al. 2013). Controlled feeding experiments did not generate significant accumulation of PST in lobster tail or white meat, but considerable accumulation of PST was demonstrated in the hepatopancreas (also called tomalley or lobster mustard) (Madigan et al. 2018; Haya et al. 1992). Although most human consumers only eat the lobster tail, 15% of Tasmanian and South Australian recreational harvesters do consume the hepatopancreas of SRL (Madigan et al. 2018). The highest PST concentration in lobster hepatopancreas in Tasmania reached 4.0 mg STX.2HCl equiv. kg^{-1} in 2012; however, in 2017 concentrations up to 13.6 mg STX.2HCl equiv. kg^{-1} were quantified in lobster hepatopancreas (DPIPWE monitoring results).

Given the transfer of PST through the food web and reports of intoxications associated with the consumption of lobsters, lobsters became of concern as a risk to public health in the early 1990s (Todd 1997) and countries such as Canada and the United States have recommended that consumers do not eat more than two hepatopancreas at any one meal, and an increase in efforts to test PST in lobsters (Todd 1997, Etheridge 2010). Tasmania initiated the monitoring of PST in SRL in 2012 after high PST levels were found in mussels,and levels above the regulatory limit were confirmed in SRL hepatopancreas (McLeod et al. 2019).

Analytical methods for PST testing approved by the AOAC include the precolumn oxidation (Pre-COX) liquid chromatography method with fluorescence detection (LC-FLD; AOAC Official Method 2005.06; 18), the postcolumn oxidation (PCOX) LC-FLD method (AOAC Official Method 2011.02; Official Methods of Analysis 2005), and the receptor binding assay (RBA; AOAC Official Method 2011.27; Official Methods of Analysis 2011), with the hydrophilic interaction LC (HILIC)-tandem Mass Spectrometry (MS/MS) being the most recently validated method (Turner et al. 2020).

Before 2017, there were no laboratories performing PST analysis in Tasmania using an official AOAC method, and all samples had to be sent to Sydney. A Tasmanian analytical laboratory was finally accredited for mussel and oyster PST testing in late 2017), and for lobster and abalone in May 2019. Previously, the application of rapid tests for the detection of PST in Tasmanian mussels and oysters was successfully explored through a single laboratory and an interlaboratory validation studies of the Neogen rapid kit for PST (Dorantes-Aranda et al. 2017; Turnbull et al. 2018, Dorantes-Aranda et al. 2018). Given the high value of the SRL industry in Tasmania, the single-laboratory validation of the Neogen test for PST was applied here to Southern Rock Lobster hepatopancreas to reduce turnaround times and costs associated with monitoring.

The Neogen rapid kit for PST is a qualitative lateral flow immunoassay that involves a manual protocol, and returns a positive or negative result using a strip reader (as opposed to a visual result) (Jawaid et al. 2015). It is a sensitive, simple and rapid test, that commonly takes no more than 10 minutes once the sample has been processed (i.e. dissect animals and homogenise). Since its development by Jawaid et al. (2015), the Neogen kit for PST has been studied and compared with other commercially available rapid tests, including quantitative ELISA kits and other qualitative kits (Dorantes-Aranda et al. 2017; Harrison et al. 2016). Previous studies on the validation of the Neogen kit for PST detection in mussels and oysters, showed that this rapid test may be adopted for official use for oysters but not for mussels. The limit of detection (LOD) was variable for these two shellfish matrices: 0.32 mg STX.2HCl equiv. kg^{-1} for mussels, although 95% of laboratories were predicted to have an estimated LOD in mussels of less than 1.11 mg STX.2HCl equiv. kg^{-1} (that is above the regulatory limit). It is not desired to obtain a LOD much lower than the regulatory limit since it could result in many precautionary closures; while at the same time, the LOD should always be below the regulatory limit to avoid false negatives. Better results were observed for oysters, the LOD calculated was 0.71 mg STX.2HCl equiv. kg^{-1}, and 0.73 mg STX.2HCl equiv. kg^{-1} for 95% of laboratories, with both values just below the regulatory limit (Dorantes-Aranda et al. 2018). These results demonstrate the importance of understanding the performance of the screening test in each matrix, to both validate the method and use the tool effectively.

In the present study we report the results of a single-laboratory validation of the Neogen test for the detection of PST in Southern Rock Lobster hepatopancreas.

7.2 Methods

Southern Rock Lobster collection and sample preparation

Collection

Lobsters were collected monthly between September and December 2018 from three areas on Tasmania's East Coast: Pirates Bay, Okehampton Bay and Binalong Bay (Figure 7.1). Five individuals were collected on each site and date. Other archived samples that had been maintained at -20°C were available from previous years (2015-2016); this included both PST-contaminated and toxin-free lobsters. An additional batch of samples was obtained from a tank experiment where lobsters were fed with contaminated and toxin-free mussels that originated from Tasmania.

Processing

Lobsters were thawed and dissected by cutting the carapace and removing the hepatopancreas, which was cut into small pieces and homogenised using a 50-mL syringe. The hepatopancreas was stored at -20°C until further use. The shell was removed, and the tail stored at -20°C.

Preparation of stock samples

Hepatopancreas samples from 2015-2016 originated from the PST monitoring program performed by government regulators, and were analysed using the AOAC.2005.06 official method (or Lawrence method; AOAC International, 2005). PST results of these samples were available. PST analyses of hepatopancreas from animals collected in 2018 and those from the feeding experiment were performed using the LC-MS method developed by Boundy et al. (24,25). All results were harmonised using TEFs recommended by FAO (26) and expressed as mg STX.2HCl equiv. kg^{-1}. Samples with similar toxin profile and with at least 0.3 mg STX.2HCl equiv. kg^{-1} were mixed to obtain stock mixtures. Three profiles were identified, plus a blank mixture, and used for the validation study (Table 7.1). Mixtures 2 and 3 did not have toxin levels ≥1.0 mg STX.2HCl equiv. kg^{-1} and were fortified with toxin standards, purchased from the National Research Council of Canada. Once homogenised, the three stock mixtures were analysed for PST using the LC-MS method to confirm the total concentration and toxin profile.

Neogen kits

The rapid kits Reveal 2.0 for PSP from Neogen were purchased through the Australian supplier Cell BioSciences. Different production lots were obtained (9562-26, 27, 28 & 29) for the ruggedness component of the validation study (see details below). Kits were maintained at room temperature (~21°C) until analysis and used before the expiry date.

Protocol of the Neogen test

The protocol was followed according to the manufacturer: 1) 1±0.05 g of the hepatopancreas homogenate was weighed out in a 70-mL plastic sample container; 2) 30 mL of distilled water were added to the sample container and mixed by hand for 30 s; 3) the mixture was transferred into side 1 of the extraction bag (previously labelled) and sealed; 4) the roller was pressed and pushed back and forth for 30 s to obtain a homogenous sample extract; 5) the mixture was poured back in the sample container from side 2 of the extraction bag; 6) the mixture was mixed again by hand for an extra 30 s; 7) a subsample of 100 µL was taken with the provided disposable pipettor and transferred into the Neogen PSP buffer vial and mixed by hand for 30 s; 8) 100 µL of this mixture was taken using a new disposable pipettor and transferred into a microwell strip (provided too); 9) a Neogen test strip was placed in the microwell containing the sample, and incubated for exactly 5 min, after which it was placed in the strip cartridge and immediately inserted in the Accuscan Pro reader to obtain the positive or negative result.

Single laboratory validation

This single laboratory validation followed the AOAC International guidelines for the validation of qualitative binary chemistry methods, which involved the following components: selectivity and probability of detection (AOAC International 2014). Ruggedness was another component included to confirm that different production lots of the Neogen kit returned the same result for replicates of the same lobster samples. A single analyst prepared all samples, and a different analyst blind-analysed all samples.

Selectivity

To confirm the specificity of the Neogen test for the target PST in SRL hepatopancreas, the PST analogues: saxitoxin (STX), gonyautoxin 1&4 (GTX1&4), gonyautoxin 2&3 (GTX2&3), N-sulfocarbamoyl gonyautoxin 2&3 (C1&2), neosaxitoxin (NEO), decarbamoyl gonyautoxin 2&3 (dcGTX2&3) and decarbamoyl saxitoxin (dcSTX) were tested. Three non-PST toxins were also included, such as okadaic acid (causative of Diarrhetic Shellfish Poisoning), domoic acid (causative of Amnesic Shellfish Poisoning) and tetrodotoxin. All PST analogues, together with okadaic and domoic acid, were obtained from the National Research Council of Canada, which were supplied in solution. Tetrodotoxin was obtained from Abcam plc (ab120054) and dissolved in acetate buffer 100 mM (pH 4.8). Subsamples of blank lobster hepatopancreas were spiked with each toxin individually and tested in triplicate using Neogen kits of production lot 9562-29.

Probability of Detection

Stock mixtures with the three toxin profiles observed in Tasmania were diluted using blank lobster samples to prepare a dilution series to cover a range of PST concentrations under and above the regulatory limit. This included target concentrations of 0.0, 0.25, 0.40, 0.60, 0.80 and 1.20 mg STX.2HCl equiv. kg^{-1} (Table 7.1), which comprised of 4, 10, 10, 38, 38 and 10 replicates, respectively. All samples were tested using Neogen kits of production lot 9562-29.

Since the validation exercise involved multiple replicates for each concentration, all mixtures prepared at each targeted dilution (Table 7.1) were homogenised and two replicates were taken to be tested for homogeneity purposes. One replicate was taken as soon as the mixture was prepared and homogenised, and the second replicate was taken after dispensing all replicates to individual sample containers for the Neogen tests. Both replicates at each concentration of the three mixtures were tested using the LC-MS method.

Ruggedness

This component considered that any possible small variation or the influence of environmental factors during the testing or the production of the different Neogen kit lots did not affect the performance of the tests. Thus, the ruggedness was to confirm that different production lots of the Neogen kits returned the same result for replicates of the same sample at each PST concentration. Samples with toxin profile 1 (Table 7.1) were used to test the performance of the four production lots

9562-26, 27, 28 and 29. Lobster samples with varying PST concentrations (0.0-1.20 mg STX.2HCl equiv. kg^{-1}, as above) were tested using six replicates for each concentration. The tests were repeated on a different day for all the Neogen kit production lots.

Analytical method for PST quantification

PST were extracted from 5.0 ± 0.1 g of lobster hepatopancreas homogenate. A volume of 3 mL of 1% acetic acid was added and the mixture vortex mixed for 90 s. The samples were placed in a boiling water bath for 5 min, then cooled in running cold water for another 5 min, before vortex mixing for 90 s and centrifugation at 3200 ×*g* for 10 min. The supernatant was recovered, the pellet was resuspended in 3 mL 1% acetic acid, and this solution was vortex mixed and centrifuged again. Both supernatants were combined and made up to 10 mL with deionized water. Sample extracts were cleaned-up using Phenomenex polymeric Strata-X SPE cartridges. The UPLC method was carried out on a Waters Acquity instrument coupled with a fluorescence detector (Excitation 340 nm; Emission 395 nm). Chromatographic separation used a Phenomenex Kinetex C18 reversed-phase column (100 × 2.1 mm i.d., 1.7 µm particle size) using gradient elution at a flow rate of 0.35 mL/min. Mobile phase A consisted of 0.1 M ammonium formate (adjusted to pH 6.0 with 1% acetic acid), and mobile phase B consisted of 90% solvent A with 10% methanol. Solvent gradient was: 100% solvent A increasing to 5% solvent B at 2 min, and then to 60% solvent B at 4.5 min before returning to 100% solvent A at 4.55 min with column re-equilibration through to 6 min.

Data analysis

Statistical analysis was performed according to methods outlined in LaBudde & Harnly (2012), Macarthur & von Holst (2012), and Wehling et al. (2011), as recommended by AOAC guidelines for the validation of qualitative binary chemistry methods. In addition to the POD for each experimental series, 95% confidence intervals were also calculated. A binomial logistic regression model was also fitted to the data in order to relate observed PODs across the range of tested concentrations and interpolate for specified concentrations. Analysis of variance was applied to test for variation between production lots and days within the ruggedness study. Results from all statistical analyses were assessed against a significance level of 0.05, and all statistical analyses were performed using R software (R Core Development Team, Version 3.5.2; 2018).

7.3 Results and Discussion

Selectivity

The Neogen test returned negative results for the non-target toxins okadaic acid, domoic acid and tetrodotoxin. The test returned positive results for the PST analogues STX, GTX2&3, C1&2, NEO, dcGTX2&3 and dcSTX when tested individually (spiked in blank SRL hepatopancreas), but not for the analogue GTX1&4. The same result was always obtained for the three replicates of each toxin. It was not surprising to observe negative results for GTX1&4 given the low cross-reactivity of the Neogen test for these combined epimers (6%). This was in line with previous findings when GTX1&4 was tested at a concentration range of 0.2-1.2 mg STX.2HCl equiv. kg^{-1}, and the Neogen kit always

returned negative results (Dorantes-Aranda et al. 2017). Positive results were observed for C1&2 at a concentration of 0.41 mg STX.2HCl equiv. kg^{-1} (Table 7.2), this also was in line with previous results where a positive response was returned by the Neogen test at ≥0.40 mg STX.2HCl equiv. kg^{-1} of C1&2 (Dorantes-Aranda et al. 2017). This last observation suggests that the cross-reactivity of the Neogen test for C1&2 is greater than 3% as claimed by the manufacturer. Obtaining positive results for C1&2 at ≥0.40 mg STX.2HCl equiv. kg^{-1} is convenient since PST-contaminated Tasmanian shellfish commonly contain these analogues (mixture 1 of Table 7.1). Additionally, 0.40 mg STX.2HCl equiv. kg^{-1} is the desired non-compliance detection limit for regulation purposes.

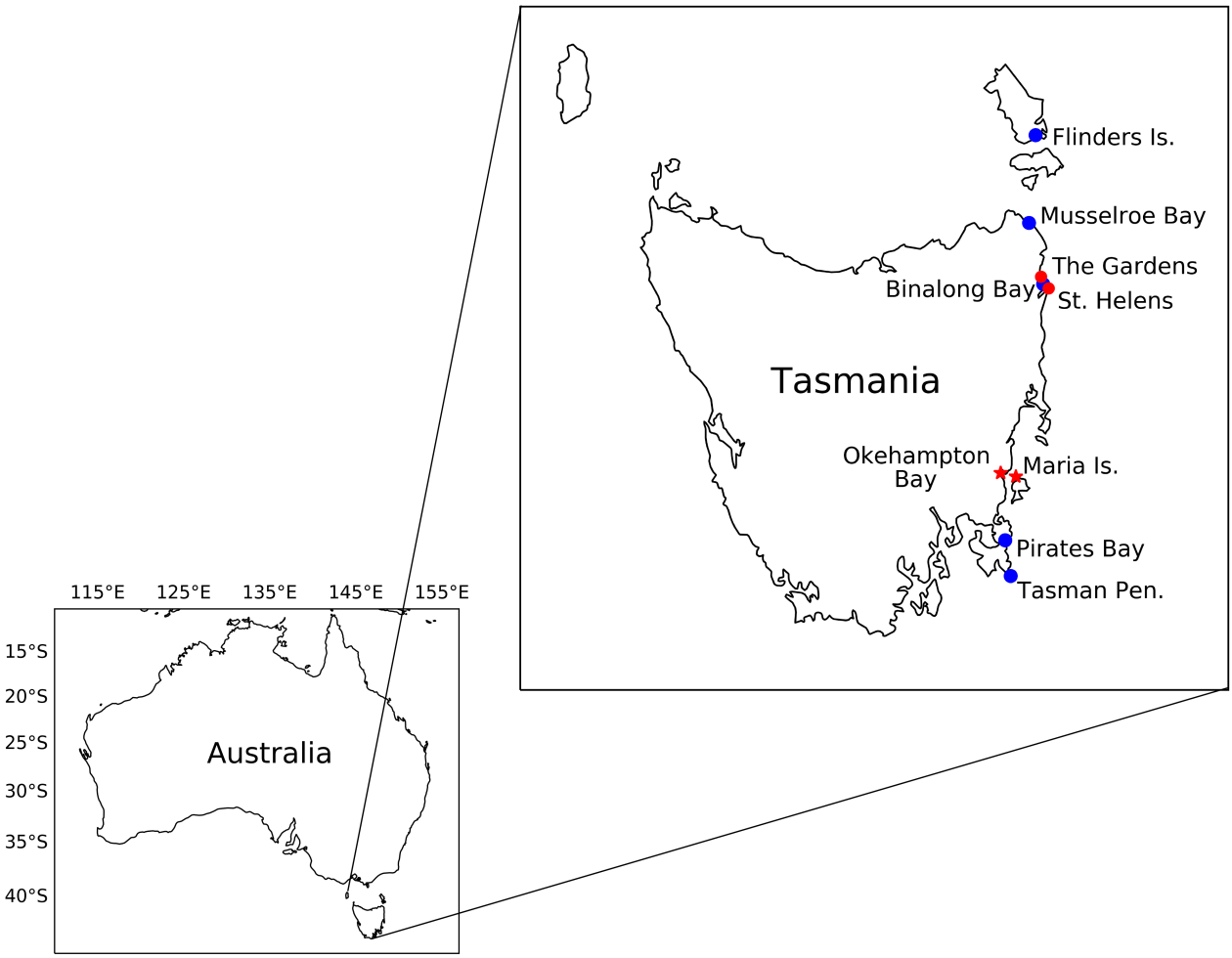

Figure 7.1 Areas from where Southern Rock Lobster were collected from Tasmania's East Coast, Australia. They were collected as part of the PST monitoring program by government regulators (2015–2016) or collected for the present study (2018). Red symbols show areas with lobsters above the regulatory limit, and star symbols show the area with the highest number of contaminated lobsters due to the proximity to the dinoflagellate bloom area.

The present results are comparable with the single-laboratory validation of the Neogen test for mussels and oysters (Turnbull et al. 2018), which used a modified protocol in order to increase the sensitivity of the Neogen kit towards GTX1&4 in mussels and oysters. In the validation study for

mussels and oysters, the Neogen test returned a positive result for GTX1&4 when an extra step was used to convert GTX1&4 to NEO (and also increased the dilution during the extraction step), and so the cross-reactivity of the Neogen test increased from 6% to 129%. Since the subsequent interlaboratory validation of the Neogen kit for mussels and oysters did not return a probability of detection lower than the regulatory limit (user errors calculated across all laboratories), the modified protocol was not recommended above the standard protocol (Turnbull et al. 2018; Dorantes-Aranda et al. 2018). Both studies demonstrated the selectivity of the Neogen test for PST analogues, and given that the present study followed the standard protocol, false negatives were obtained for GTX1&4, which confirmed the low cross-reactivity of the Neogen test for these epimers when tested individually. However, naturally contaminated organisms commonly include a combination of PST analogues, dominated by one or two of them.

Table 7.1 Profiles and concentrations of PST-contaminated Southern Rock Lobster hepatopancreas used for the single-laboratory validation of the Neogen rapid test for the detection of Paralytic Shellfish Toxins. All lobsters were collected from the East Coast of Tasmania (Figure 7.1)

	Origin **Area (date of collection)**	**Toxin profile** **(% of total PST)**	**Target concentration[e]** **(mg STX.2HCl equiv. kg^{-1})**
Blank	Tasman Peninsula (Nov 2015) Flinders Island (Nov & Dec 2015) Pirates Bay (Dec 2015) Binalong Bay (Nov 2016) Feeding experiments[a]	-	0.00
Mixture 1	The Gardens (Oct 2015) Maria Island (Nov & Dec 2015) Okehampton Bay (Nov 2018) Feeding experiments[b]	71.79% GTX2&3 15.65% STX 7.22% GTX1&4 2.94% C1&2 1.27% dcSTX 0.77% dcGTX2&3	0.25 0.40 0.60 0.80 1.20
Mixture 2[c]	Flinders Island (Nov 2015) St. Helens (Nov 2015) Musselroe Bay (Nov 2015) Maria Island (Dec 2015) The Gardens (Dec 2015) Okehampton Bay (Nov 2018) Feeding experiments[b]	42.16% STX 40.78% GTX2&3 13.10% dcSTX 3.66% dcGTX2&3 0.19% C1&2	0.25 0.40 0.60 0.80 1.20
Mixture 3[d]	Maria Island (Aug 2015) St. Helens (Nov 2015) Musselroe Bay (Nov 2015) Feeding experiments[b]	56.40% GTX1&4 35.24% GTX2&3 7.69% STX 0.63% dcSTX 0.13% C1&2	0.25 0.40 0.60 0.80 1.20

[a] Lobsters fed with toxin-free mussels (controls)
[b] Lobsters fed with PST-contaminated mussels
[c] Spiked with GTX2&3, STX, dcSTX, dcGTX2&3 standards
[d] Spiked with GTX2&3, STX, GTX1&4 standards
[e] Targeted concentrations prepared by mixing stock mixtures with blank lobster samples

STX=Saxitoxin, GTX1&4=gonyautoxin 1&4, GTX2&3=gonyautoxin 2&3, C1&2=N-sulfocarbamoyl gonyautoxin 2&3, dcGTX2&3=decarbamoyl gonyautoxin 2&3 and dcSTX=decarbamoyl saxitoxin

Table 7.2 PST and non-PST toxins tested with the Neogen kit to determine the selectivity of the kit. The concentrations used were at least half the regulatory limit, and at a concentration where a positive result was expected (for PST analogues). Triplicates of each toxin were tested, and the result was always the same. The cross-reactivity of the Neogen test reported by the manufacturer is also shown.

Toxin	**Cross reactivity of the Neogen test**	**Test concentration (mg/kg)**	**Neogen test result**
STX[a]	100%	0.55[e]	+
GTX1&4[a]	6%	0.58[e]	-
GTX2&3[a]	23%	1.15[e]	+
C1&2[a]	3%	0.41[e]	+
NEO[a]	129%	1.16[e]	+
dcGTX2&3[a]	8%	1.35[e]	+
dcSTX[a]	56%	0.58[e]	+
Okadaic acid[b]	N/A	0.11	-
Domoic acid[c]	N/A	11.60	-
Tetrodotoxin[d]	N/A	1.30	-

[a] Paralytic Shellfish Toxin analogue
[b] Diarrhetic Shellfish Toxin analogue (DST), non-PST
[c] Amnesic Shellfish Toxin (AST), non-PST
[d] This toxin is not included within the PST group (saxitoxin derivate) but has the same toxic mechanism
[e] As mg STX.2HCl equiv. kg^{-1}
N/A=not applicable

Sample homogeneity

The similarities in total PST between each pair of replicates of the three SRL hepatopancreas mixtures at different PST levels was high when confirmed with the analytical method, showing low standard deviation values (0.00-0.08, Table 7.3). Additionally, the toxin profile observed between replicates and amongst all PST levels of the same mixture was very similar, with no standard deviation values greater than 2.3% (Table 7.3). These results confirmed good sample homogeneity during preparation across the different PST levels for the three mixtures.

Moreover, all dilution preparations (mixing blank lobster hepatopancreas with each of the stock mixtures) were very close to the targeted concentrations. This was supported by obtaining correlation coefficients (r^2) between the target and quantified concentrations greater than 0.99 (data not shown). Sample homogeneity is of great importance since this assures that sample replicates contain the same PST concentration and toxin profile, challenging only the reliability of the Neogen kit.

Table 7.3 Total PST quantified for two replicates of each concentration of the three mixtures used in the present study to confirm homogeneity and comparison against the targeted concentrations (0.25, 0.40, 0.60, 0.80, 1.20 mg STX.2HCl equiv. kg^{-1}). The two replicates were sampled from the whole homogenate at the start and end of dispensing the subsamples into sample containers to perform the Neogen tests.

	Concentration (mg STX.2HCl equiv. kg^{-1})				**Average ± SD of the toxin profile (% of total PST)**
	Replicate 1	**Replicate 2**	**Average**	**SD**	
Mixture 1	0.26	0.25	0.26	0.01	71.79 ± 0.43% GTX2&3
	0.36	0.40	0.38	0.03	15.65 ± 1.52% STX
	0.52	0.61	0.57	0.06	7.22 ± 1.16% GTX1&4
	0.71	0.79	0.75	0.06	2.94 ± 0.23% C1&2
	1.08	1.06	1.07	0.01	1.27 ± 0.27% dcSTX 0.77 ± 0.71% dcGTX2&3
Mixture 2	0.30	0.26	0.28	0.03	42.16 ± 1.22% STX
	0.39	0.38	0.39	0.01	40.78 ± 1.11% GTX2&3
	0.54	0.65	0.60	0.08	13.10 ± 0.87% dcSTX
	0.78	0.76	0.77	0.01	3.66 ± 0.79% dcGTX2&3
	1.17	1.11	1.14	0.04	0.19 ± 0.16% C1&2
Mixture 3	0.20	0.24	0.22	0.03	56.40 ± 1.45% GTX1&4
	0.38	0.38	0.38	0.00	35.24 ± 1.57% GTX2&3
	0.61	0.61	0.61	0.00	7.69 ± 2.22% STX
	0.79	0.78	0.79	0.01	0.63 ± 0.25% dcSTX
	1.19	1.22	1.21	0.02	0.13 ± 0.12% C1&2

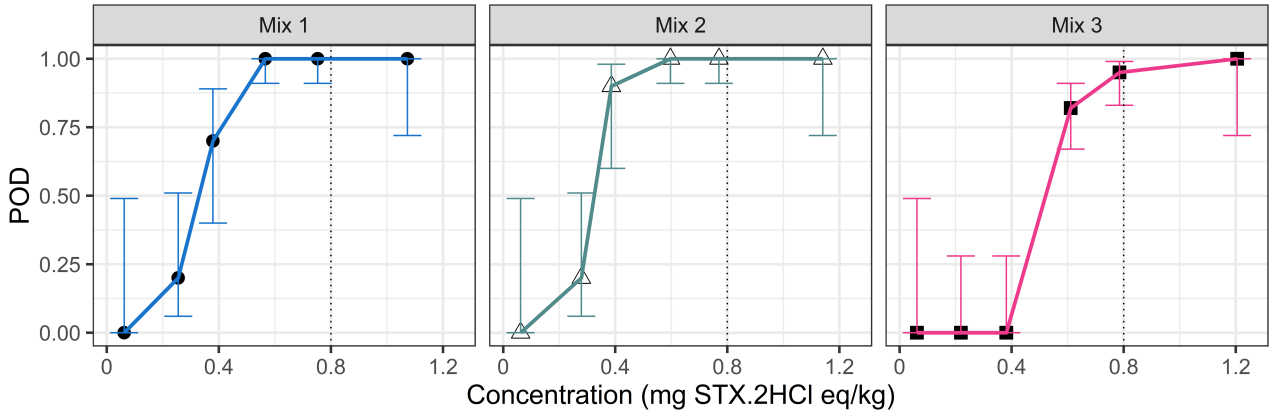

Figure 7.2 Probability of Detection (POD) of PST by the LFIA Neogen kit at various PST concentrations in the experimental series created from three different toxin mixes. validated against LC-MS analyses on the same samples by Cawthron Institute.

Probability of detection

The toxin profiles used for the probability of detection study aligned with those observed in naturally contaminated Southern Rock Lobsters. Mixture 1 was prepared by combining hepatopancreas samples with the same toxin profile (dominated by GTX2&3), and a highly contaminated stock was achieved (2.04 mg STX.2HCl equiv. kg^{-1}). Mixture 2 was prepared in a similar manner; however, the highest concentration after combining the samples was ~1.0 mg STX.2HCl equiv. kg^{-1}. The mixture was further fortified with PST standards to increase the total concentration to 1.94 mg STX.2HCl equiv. kg^{-1} while maintaining the same toxin profile (dominated by STX and GTX2&3). The profile of mixture 3 (dominated by GTX1&4) was observed less commonly in contaminated lobsters compared to mixtures 1 and 2; however, it was of interest given that the Neogen test has a low cross-reactivity for GTX1&4 (6%). For instance, amongst all the lobster samples naturally contaminated with 53-100% of GTX1&4, the total PST was between 0.15-0.66 mg STX.2HCl equiv. kg^{-1}, except for one sample that had 77% of this analogue and had a total concentration of 1.94 mg STX.2HCl equiv. kg^{-1}. Therefore, it was appropriate to include the profile of mixture 3 to investigate the potential risk that this possesses for the SRL industry.

The POD of all toxin mixtures of SRL hepatopancreas increased with increasing PST concentrations (Figure 7.2). The POD was 0.0 for samples with 0.06 mg STX.2HCl equiv. kg^{-1}, and it increased to 0.2 and 0.1 for mixtures 1 and 2 with 0.26 and 0.28 mg STX.2HCl equiv. kg^{-1}, respectively. The POD calculated for concentrations of ≥0.57 mg STX.2HCl equiv. kg^{-1} was 1.0 for mixtures 1 and 2. In contrast, POD values were 0.0 for PST concentrations of 0.06-0.38 mg STX.2HCl equiv. kg^{-1} of mixture 3, increasing to 0.82 at 0.61 mg STX.2HCl equiv. kg^{-1}, 0.95 at 0.79 mg STX.2HCl equiv. kg^{-1}, and finally a POD of 1.0 was obtained at 1.21 mg STX.2HCl equiv. kg^{-1} (Table 7.4).

The difference in POD values obtained for the three mixtures in SRL hepatopancreas can be explained due to mixtures 1 and 2 containing mostly GTX2&3 and STX + GTX2&3, respectively, for which the Neogen test has higher cross-reactivities compared to GTX1&4, the main analogue in mixture 3.

A more conservative and desirable pattern was observed for mixtures 1 and 2, whereas the probability of a positive response for mixture 3 was 0.95 at 0.79 mg STX.2HCl equiv. kg^{-1}, which was just under the regulatory limit. However, when the model was extrapolated to specific PST concentrations, a POD of 0.98 was obtained for mixture 3 at 0.8 mg STX.2HCl equiv. kg^{-1}, meaning there was only a 2% chance of obtaining a false negative in samples with this toxin profile. Despite this observation, the most common toxin profiles observed in Tasmanian SRL so far are those of mixtures 1 and 2.

Table 7.4 Probability of detection of PST in SRL hepatopancreas calculated by binomial logistic regression for all PST levels tested with the Neogen kit.

	Concentration (mg STX.2HCl equiv. kg^{-1})	**No. of replicates**	**No. of detections**	**POD**	**95% CI**
Mixture 1	0.06	4	0	0.00	(0.00, 0.49)
	0.26	10	2	0.20	(0.06, 0.51)
	0.38	10	7	0.70	(0.40, 0.89)
	0.57	38	38	1.00	(0.91, 1.00)
	0.75	38	38	1.00	(0.91, 1.00)
	1.07	10	10	1.00	(0.72, 1.00)
Mixture 2	0.06	4	0	0.00	(0.00, 0.49)
	0.28	10	2	0.10	(0.06, 0.51)
	0.39	10	9	0.90	(0.60, 0.98)
	0.60	38	38	1.00	(0.91, 1.00)
	0.77	38	38	1.00	(0.91, 1.00)
	1.14	10	10	1.00	(0.72, 1.00)
Mixture 3	0.06	4	0	0.00	(0.00, 0.49)
	0.22	10	0	0.00	(0.00, 0.28)
	0.38	10	0	0.00	(0.00, 0.28)
	0.61	38	31	0.82	(0.67, 0.91)
	0.79	38	36	0.95	(0.83, 0.99)
	1.21	10	10	1.00	(0.72, 1.00)

Ruggedness

Differences in performance among the four production lots of the Neogen test were observed when tested at PST concentrations of 0.26 and 0.38 mg STX.2HCl equiv. kg^{-1} in SRL hepatopancreas. However, ANOVA results showed that these differences were not significant (p=0.2 for 0.26 mg STX.2HCl equiv. kg^{-1}; p=0.15 for 0.38 mg STX.2HCl equiv. kg^{-1}). No differences occurred among the four production lots at PST concentrations of ≥0.57 mg STX.2HCl equiv. kg^{-1}, for which all Neogen tests always returned the same result (Table 7.6).

Table 7.5 Fitted probability of detection of PST in SRL hepatopancreas when extrapolating to specific PST concentrations using the binomial logistic regression model.

	Concentration (mg STX.2HCl equiv. kg^{-1})	**Fitted POD**
Mixture 1	0.00	0.00
	0.20	0.06
	0.40	0.83
	0.60	1.00
	0.80	1.00
Mixture 2	0.00	0.00
	0.20	0.02
	0.40	0.94
	0.60	1.00
	0.80	1.00
Mixture 3	0.00	0.00
	0.20	0.01
	0.40	0.11
	0.60	0.73
	0.80	0.98

Table 7.6 Repeatability results of the Neogen kit using four production lots and tested on mixture 1.

Concentration (mg STX.2HCl equiv. kg^{-1})	**Expected Neogen result**	**Number of detections (positive results)**							
		9562-26		**9562-27**		**9562-28**		**9562-29**	
		Rep 1	**Rep 2**	**Rep 1**	**Rep 2**	**Rep 1**	**Rep 2**	**Rep 1**	**Rep 2**
0.06	-	0	0	0	0	0	0	0	0
0.26	-	0	1	1	0	0	1	3	2
0.38	borderline	5	4	5	4	2	4	6	6
0.57	+	6	6	6	6	6	6	6	6
0.75	+	6	6	6	6	6	6	6	6
1.07	+	6	6	6	6	6	6	6	6

7.4 Conclusions

The Neogen kit proved to be selective for PST analogues in Southern Rock Lobster hepatopancreas, except for GTX1&4, for which it has a low cross-reactivity. This does not appear to be an issue so far since only a few lobster hepatopancreas samples have been found to contain this analogue at high levels, and when they contained 80-100% of this analogue, the total PST concentration was 0.43≤ mg STX.2HCl equiv. kg^{-1}. From the probability of detection study, the Neogen test returned positive results in 100% of hepatopancreas samples with concentrations ≥0.57 mg STX.2HCl equiv. kg^{-1} containing toxin profiles dominated by GTX2&3 (mixture 1) and STX + GTX2&3 (mixture 2), the two profiles that have been most commonly observed since PST started to impact on Tasmanian lobsters. The fitted POD for mixture 3 (dominated by GTX1&4) showed that the Neogen test would return positive results in 98% of samples with 0.80 mg STX.2HCl equiv. kg^{-1}, and 100% in samples with 1.21 mg STX.2HCl equiv. kg^{-1}. From the ruggedness study, it was observed that different production lots returned the same result with no significant differences, particularly at concentrations ≥0.57 mg STX.2HCl equiv. kg^{-1}. These results demonstrated that the Neogen test could be suitable for PST monitoring and regulation purposes of Southern Rock Lobster fisheries affected by *Alexandrium catenella* blooms in Tasmania. Caution should be exercised when suspected high proportions of GTX1&4 are present.

An evaluation of the Neogen test for monitoring purposes, based on the analysis of field samples from the Tasmanian lobster monitoring program, is presented in the following chapter (Chapter 4).

8. Evaluation of the qualitative Neogen™ lateral flow immunoassay for paralytic shellfish toxin monitoring in Southern Rock Lobster hepatopancreas tissues.

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Abstract. Following from the successful single laboratory validation of the Neogen lateral immunoassay in the previous chapter (Chapter 7), this chapter evaluates the use of the Neogen test for biotoxin monitoring purposes in Southern Rock Lobster hepatopancreas. The test kit was challenged against 128 samples containing various toxin profiles and concentrations representing those detected during past Tasmanian biotoxin seasons (2016-2019). The Neogen test kit provided a high confidence in detecting PST (probability of detection of 1) in high-risk samples (PST concentrations ≥0.4 mg STX.2HCl equiv. kg^{-1}). All samples containing PST above the CODEX bivalve regulatory level (0.8 mg STX.2HCl equiv. kg^{-1}) returned a positive result. The probability of detection of PST decreased with PST concentration in samples containing <0.4 mg STX.2HCl equiv. kg^{-1}. The results suggest that the Neogen test kit could be best employed as a screening assay at the start and end of the biotoxin season, when samples are most likely to contain PST below this level. At the height of the bloom, when PST levels are expected to exceed 0.4 mg STX.2HCl equiv. kg^{-1}, the Neogen test is not expected to be cost-effective, as positive results would have to be followed up with chemical analysis to confirm PST concentrations and inform management decisions Based on historic Tasmanian field monitoring of PST in Southern Rock Lobster (2012-2022, 503 observations) and the PODs obtained in this study, the Neogen test kit would have provided negative results for 179 out of the 311 samples that had PST concentrations <0.4 mg STX.2HCl equiv. kg^{-1}. Opportunities for reducing analytical costs and increasing sample turnaround time by including the Neogen assay in the Southern Rock Lobster PST monitoring program are discussed.

8.1 Introduction

The Neogen lateral flow immunoassay provides a cost-effective, rapid means (< 20 min per sample) for detecting the presence/absence of paralytic shellfish toxins (PST) in various seafood tissues (Dorantes-Aranda et al., 2018; Turnbull et al., 2018). A single laboratory validation of this assay to detect PST in Southern Rock Lobster hepatopancreas was successfully completed in Chapter 7 (Dorantes-Aranda t al. 2020). The Neogen test kit showed high selectivity (no interference from non-PST toxins), performed consistently across production lots, and reliably detected PST in Southern Rock Lobster hepatopancreas tissues contaminated with three different PST profiles. These PST profiles consisted of two of the most commonly encountered profiles in Southern Rock Lobster hepatopancreas during *Alexandrium* blooms on the east coast of Tasmania (high STX or high GTX2&3) and one rarer profile containing higher concentrations of GTX1&4. The concentrations of the individual PST analogues that make up these profiles are critically important, as the detection antibodies in the Neogen test strip have different cross-reactivities for different PST analogues (Jawaid et al., 2015). The PST profile of a given sample therefore directly influences the likelihood of obtaining a positive result at a given PST concentration.

The likelihood that the Neogen test kit will return a positive result for any given PST level is called the probability of detection (POD). For the kit to be useful as a risk management tool, the POD at the regulatory level should be 1, i.e. 100%. The SRL biotoxin protocol requires management actions at PST levels above 0.4 mg STX.2HCl equiv. kg^{-1}. Therefore, ideally the POD at 0.4 mg STX.2HCl equiv. kg^{-1} should also be high, i.e. close to 1. However, for the test kit to indicate when the risk is low, the POD should decrease rapidly at levels below 0.4 mg/kg. During the single laboratory validation, the Neogen test was able to reliably detect PST above 0.57 mg STX.2HCl equiv. kg^{-1} in STX rich samples (100% cross-reactivity, POD = 1.0), while this same level of detection (POD = 1.0) in GTX1&4 rich samples (only 5.7% cross-reactivity relative to STX) was not reached until a total PST concentration of ≥1.21 mg STX.2HCl equiv. kg^{-1}.

In nature, the PST profile of individual samples is influenced by several different factors, such as the PST profile of the algal species present (e.g. both *Gymnodinium* and *Alexandrium* bloom in Tasmanian waters), the toxin profile of the prey, and the stage of the algal bloom (toxin uptake vs. depuration phase). The single laboratory evaluation tested a high number of repetitions of three distinct PST profiles tested. However, naturally contaminated field samples present unique combinations of various PST analogues and are considerably more variable. Following on from the successful single laboratory validation, this chapter evaluates the suitability of the Neogen test kit for monitoring purposes, by challenging the test kit against 128 Southern Rock Lobster hepatopancreas samples representing a wide range of PST profiles and total PST concentrations that may be encountered in the field. These include samples collected between 2016-2019 by the Tasmanian Southern Rock Lobsters biotoxin monitoring program (n=64), the aquaculture feeding trial (n=36; see Chapter 3) and New Zealand *Gymnodinium/A. minutum* blooms (n=13).

8.2 Materials and Methods

Neogen protocol

The Neogen qualitative lateral flow immunoassay was conducted as described in detail in the previous chapter (see section 0). The test kits were purchased from Cell Science Australia and as recommended by the manufacturer kept at room temperature until analysis. Four different test kit production lots (#9562-29, 9562-43, 9562-46 and 9562-48) were used in this study and Southern Rock Lobster hepatopancreas samples analysed by three different operators. The initial analysis was conducted using the Neogen AccuScan® reader. Follow up investigative analyses (see section on new analytical platform & test strip components below) where conducted using test kit production lots lot 9562-49 or 9562-51, and the AccuScan® and Raptor® readers.

Analytical method for PST quantification.

Confirmatory chemical analysis was predominantly conducted with the LC-MS Boundy method as described in detail in section 0 (Boundy et al. 2015). All lobster hepatopancreas samples that had been collected before 2019 (before the Boundy LC-MS analysis was established at Analytical Services Tasmania), were initially analysed with the Lawrence LC-FLD method (Lawrence et al., 2005, 2011). Where PST had been detected, samples were reanalysed with the Boundy LC-MS method to account for potential shifts in total PST concentration/PST analogues during prolonged sample storage (2016-2019). Samples where Lawrence LC-FLD screening did not detect the presence of PST were not re-analysed. All samples collected from 2019 and onwards were directly analysed with the LC-MS Boundy method.

Southern Rock Lobster hepatopancreas samples

Southern Rock Lobster were collected by divers as part of ongoing biotoxin monitoring during *Alexandrium* microalgal blooms on the Tasmanian east coast (between 2016-2019). Individual lobster were processed in the laboratory to extract the hepatopancreas, which was homogenised and stored frozen (-20ºC) until analysis with the Neogen rapid test kit and confirmatory analytical methods. The samples were collected over a 4 year period (2016-2019) and represent the Tasmanian PST profiles as described in detail below. PST contaminated samples collected during the lobster aquaculture tank trials described in Chapter 7 were used to bolster sample numbers and challenge the Neogen test against different PST profiles with high total PST concentrations. Additional samples were obtained from New Zealand waters to provide an initial assessment of the suitability of the Neogen assay for use during *Gymnodinium/A. minutum* blooms.

Tasmanian biotoxin monitoring program samples

The Southern Rock Lobster hepatopancreas samples collected during the 2016-2019 biotoxin seasons were split into different sample sets that included historic samples that had been stored for multiple years since collection (n=17), as well as samples tested with the Neogen during the 2019 Tasmanian biotoxin season (n=64). The stored samples were originally collected from the Maria and Schouten

Island regions in 2016 (n=1), 2017 (n=4), and 2018 (n=12) and reanalysed with the Boundy method prior to Neogen analysis to confirm concentrations of individual PST analogues after prolonged storage at -20ºC. Samples originating from the 2019 biotoxin season (August-November) were collected from the Okehampton Bay (n=39), Wedge Island (n=10), Schouten Island (n=5), Bicheno (n=5) and Pirates Bay (n=5) regions and stored frozen at -20ºC. These samples were analysed with the Neogen test kit within months of collection.

Aquaculture feeding trial samples

Thirty-four hepatopancreas samples collected during aquaculture feeding trials of Southern Rock Lobster contaminated with a wide range of PST concentrations (0.002-9.0 mg STX.2HCl equiv. kg^{-1}) were selected to be tested with the Neogen assay. As described in detail in Chapter 3, lobster were fed naturally PST contaminated mussels representative of Tasmanian conditions (mussels were collected during the 2012 *Alexandrium* bloom on the east coast of Tasmania).

New Zealand Gymnodinium/Alexandrium minutum bloom samples

Southern Rock Lobster hepatopancreas samples (n=13) were collected during New Zealand *Gymnodinium catenatum/Alexandrium minutum mixed* blooms in 2018-19. These samples were processed as described above and stored frozen until Neogen analysis in 2022. To account for potential shifts in total PST concentration/PST analogues during storage these samples were re-analysed with the LC-MS Boundy method and PST profiles and their individual concentrations confirmed prior to Neogen analysis.

New analytical platform & test strip components

At the end of the field validation, the company producing the PSP Reveal 2.0 test strips advised of a reduced shelf-life (12 months instead of previously 18 months). At the same time, the original test strip reader (AccuScan Pro®) had been replaced by the Raptor® Solo integrated analysis platform. To determine if the new analytical platform impacted the performance of the Neogen test kit, a subset of samples were re-analysed on both the old and new Neogen readers. This subset represented a number of different PST profiles and total PST concentrations (ranging from below the level of detection to 0.65 mg STX.2HCl equiv./kg), including 52 samples from the Tasmanian biotoxin monitoring program (2018-2019), 8 aquaculture feeding trial samples and 7 samples from mixed NZ blooms (*A. minutum/G. catenatum*).

Further investigation was undertaken to determine the difference in the POD of the Accuscan Pro and the Raptor Solo. A dilution series of PST contaminated lobster hepatopancreas obtained from the aquaculture experiments described above was prepared and analysed with two different test kit production lots (lot 9562-49 and lot 9562-51). To obtain enough volume of the toxic master-mix prior to dilution, the hepatopancreas of 8 lobsters was pooled, well mixed until homogenous and a subsample analysed for PST. The toxic master-mix was confirmed to contain 3.84 mg STX.2HCl equiv./kg, made up of 85% GTX2&3, 8% C1&2, 5% STX, 2% GTX1&4, 2% GTX6 and 1% GTX5. This toxic master-mix was diluted to 0.1, 0.2, 0.3 and 0.4 mg STX.2HCl equiv./kg using Southern

Rock Lobster hepatopancreas samples where LC-MS analysis had not detected any PST. These dilutions were well-mixed and divided into 20 replicates of 1±0.05 g in 70 mL jars. Ten replicates for reach PST concentration were then combined in one bag and the other 10 in another bag to randomly mix. The samples were then frozen until analysis. A second analysist conducted the analysis blind (i.e. no knowledge of the concentrations being analysed). Each bag (10 replicates x 5 PST concentrations: 0, 0.1, 0.2, 0.3, 0.4 STX.2HCl equiv./kg) was analysed with a different production lot of Neogen PST test strips (either lot 9562-49 or 9562-52). PST in each sample were extracted until the buffer stage, following the Neogen protocol described in detail above. From the buffer, 400 µL of extract were used for analysis with the Raptor Solo and another 100 uL for analysis with the AccuScan Pro, using separate test strips for each reader (i.e. the same sample extract was tested across both readers). All analyses were conducted over 2 days.

Data analysis

Statistical analysis of the PST detection of field data was performed according to methods outlined in LaBudde & Harnly (2012), Macarthur & von Holst (2012), and Wehling et al. (2011), as recommended by AOAC guidelines for the validation of qualitative binary chemistry methods. Samples were aggregated based on their total toxicity in groups of 0.2 mg STX.2HCl equiv. kg^{-1} (i.e. <LOD, >LOD to 0.2,0.2 to 0.4 and so forth). Using the average PST concentration of samples within each bin (0, 0.13, 0.32, 0.49, 0.69 and >1.0 mg STX.2HCl equiv. kg^{-1}), a binomial logistic regression model was fitted to the data to relate observed probabilities of detection across the range of tested concentrations. To focus on use of the Neogen test kit for the Tasmanian biotoxin monitoring program, the limited number of NZ samples available for this work were excluded from this POD analysis.

The cost effectiveness of using the Neogen rapid test kits in biotoxin monitoring was calculated by firstly estimating the number of positive Neogen results that would have been returned from the 503 individual lobsters analysed during the Tasmanian Biotoxin Monitoring Program between 2012-2022, (i.e. multiplying the frequency of individual PST concentrations since the start of the monitoring program in the concentration ranges of <LOD; >LOD-0.2; 0.2-0.4; and >0.4 mg STX.2HCl equiv./kg, by the corresponding PODs determined for the Neogen test kit), then calculating total costs for analysis of 503 samples by LCMS/MS and comparing it to the projected costs of 503 samples by Neogen plus confirmation of all positive Neogen samples by LCMS/MS.

All PST results are expressed as STX.2HCl equiv. kg^{-1} using FAO TEFs (FAO, 2016). All statistical analyses were performed using R software (R Core Development Team, Version 4.1.3; 2022).

8.3 Results

Detection of PST in samples with different biotoxin profiles (AccuScan Pro® reader)

The Neogen PST test was conducted on 128 Southern Rock Lobster hepatopancreas samples containing a wide variety of PST analogues collected during biotoxin monitoring in the field (94 samples) and aquaculture tank trials (lobster were fed mussels naturally contaminated with PST; 34 samples). PST were not detected by confirmatory analysis (either Lawrence or Boundy method) in 28 of these samples (PST below the limit of detection). Consistent with this analysis, all of these samples also yielded a negative result when analysed with the Neogen AccuScan Pro® reader (i.e. no PST detected; data not shown).

Samples collected during PST monitoring in Southern Rock Lobster hepatopancreas on the Tasmanian east coast (2016-2018), presented with higher concentrations of STX, GTX2&3 and in some cases smaller amounts (<15% toxicity) of dcSTX and GTX1&4 after long-term storage (Figure 8.1 A). With the exception of a single sample at 0.85 mg STX.2HCl equiv. kg^{-1} and two samples at 0.65 and 0.59 mg STX.2HCl equiv. kg^{-1}, the majority of samples contained total PST concentrations below 0.5 mg STX.2HCl equiv. kg^{-1} (14 samples). The Neogen test kit returned a positive result for all 17 of these samples.

Field monitoring of PST in Southern Rock Lobster hepatopancreas during the 2019 biotoxin season on the east coast of Tasmania provided a wide range of PST profiles to challenge the Neogen test kit against (Figure 8.1 B). Successful detection of PST with the Neogen test kit varied between the total PST concentration and the individual analogues making up the PST profile. All eight samples above the CODEX bivalve regulatory level (CODEX Stan 292-2008) tested positive on the Neogen test (0.8 – 8.14 mg STX.2HCl equiv. kg^{-1} total PST; samples with high levels of GTX5, dcSTX and minor contributions of C1&2, as well as Neo). Of the 28 samples with total PST concentrations below 0.8 mg STX.2HCl equiv. kg^{-1}, 11 samples tested negative and 17 samples positive. The two highest total PST values at which the Neogen test returned a negative result occurred at 0.28 and 0.35 mg STX.2HCl equiv. kg^{-1}. The profiles of these two samples exclusively contained the PST analogues dcSTX (38 and 61%) and Neo (62% and 39%, respectively).

Feeding of Southern Rock Lobster with naturally PST contaminated mussels during aquaculture trials yielded a wide range of total PST concentrations in the hepatopancreas to challenge the Neogen test against (0.002 – 9 mg STX.2HCl equiv. kg^{-1}, Figure 8.1 C). These samples contained high amounts of GTX2&3, GTX1&4 and STX, with minor contributions of GTX5, dcGTX2&3, Neo, dcSTX and C1&2 (the exact PST profiles are discussed in detail in Chapter 3). All 24 samples with total PST concentrations above the CODEX bivalve regulatory level tested positive on the Neogen test. Of the 10 samples with total PST concentrations below this level, the Neogen test returned a negative result for three samples at the lower end of the concentration range (total PST of 0.002, 0.06 and 0.15 mg STX.2HCl equiv. kg^{-1}).

Confirmatory PST analysis (Boundy LC-MS method) of Southern Rock Lobster samples collected from the field during *Gymnodinium catenatum/Alexandrium minutum* blooms in New Zealand waters

revealed PST profiles containing higher levels (>20% of total toxicity) of C1&C2, dcSTX, GTX2&3 and dcGTX2&3 (Figure 8.1 D). The exact contribution of individual PST analogues differed between the samples, with some containing the additional analogues GTX1&4, GTX5 and GTX6. Most of these samples contained total PST concentrations at or below 0.5 mg STX.2HCl equiv. kg^{-1} (11 samples), with one sample at the CODEX bivalve regulatory level (0.8 mg STX.2HCl equiv. kg^{-1}) and one above (1.4 mg STX.2HCl equiv. kg^{-1}). Regardless of the total PST concentration, the Neogen test returned positive results for all 13 of these samples.

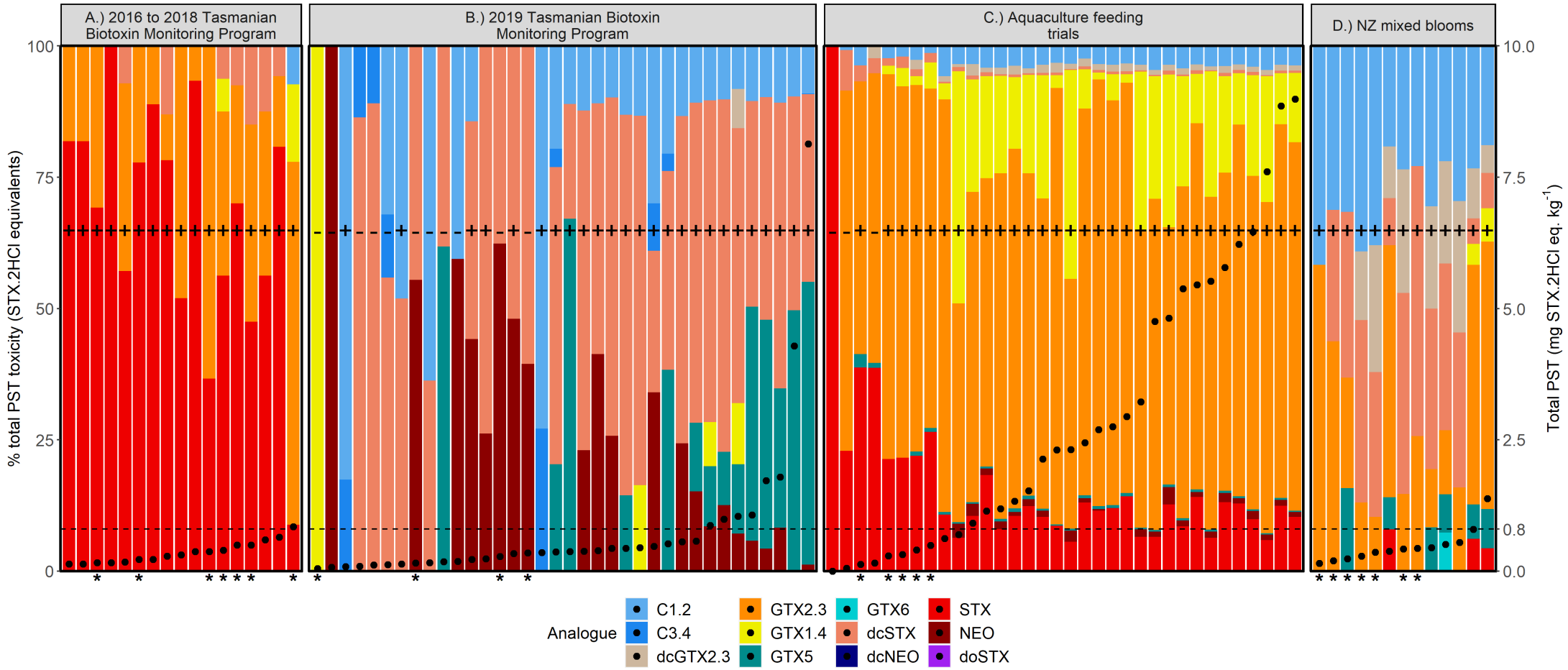

Figure 8.1 Neogen analysis of Southern Rock Lobster hepatopancreas samples collected as part of the Tasmanian Biotoxin Monitoring program, including historic samples from the 2016-2018 (A, stored long-term & reanalysed) and the 2019 biotoxin seasons (B, stored short-term), as well as samples collected during aquaculture feeding trials (C) and a 2019 *Gymnodinium/A. minutum* mixed bloom in New Zealand waters (D). Coloured bars indicate the contribution of individual toxin analogues to total sample toxicity (primary y-axis as percentage) and black points are representative of the total PST concentration in the identical sample (secondary y-axis as mg STX.2HCl equiv. kg^{-1}). The Neogen result is indicated by the positive (+) and minus (-) symbols and the broken line indicates the CODEX bivalve regulatory level (0.8 mg STX.2HCl equiv. kg^{-1}). The asterisks indicate samples that were re-analysed with the new Raptor® analytical platform.

Probability of PST detection in field samples (AccuScan Pro® reader)

The 115 Tasmanian Southern Rock Lobster hepatopancreas samples described in the previous section (0) were used to estimate the POD of PST at different PST concentrations (Table 8.1). Based on this analysis, the Neogen test exhibited a POD of zero for samples where PST were not detected by either the Boundy or Lawrence confirmatory analytical method (i.e. no false positives were obtained with the Neogen test). At total PST concentrations above the limit of detection of confirmatory analysis and below 0.2 mg STX.2HCl equiv. kg^{-1}, the Neogen test kit is expected to provide a positive result for approximately half of the samples tested. This POD increases with increasing total PST concentration, with 4 out of 5 lobster samples expected to test positive at PST concentrations of 0.2-0.4 mg STX.2HCl equiv. kg^{-1} (POD = 0.79). At total PST concentrations greater than 0.4 mg STX.2HCl equiv. kg^{-1}, the Neogen test kit is expected to return positive results for all samples (POD = 1.0).

Table 8.1 Probability of obtaining a positive result (POD) on the Neogen test using the AccuScan Pro® reader at given concentration ranges of PST (determined via confirmatory PST analysis). The POD is based on the Neogen analysis of 115 individual Tasmanian Southern Rock Lobster hepatopancreas samples contaminated with naturally occurring PST. The number of field samples used to calculate the POD are provided for each PST concentration range.

PST range (mg STX.2HCl equiv. kg^{-1})	Number of observations	Probability of detection (POD)	Interpretation
<LOD	28	0	All lobsters expected to test negative
>LOD-0.2	20	0.45	Half the lobsters expected to test positive
0.2-0.4	19	0.79	4/5 lobsters expected to test positive
0.4-0.6	12	1	All lobsters expected to test positive
0.6-0.8	3	1	
0.8-1.0	4	1	
>1.0	30	1	

*LOD = limit of detection of confirmatory PST analysis (either Lawrence or Boundy method)

New analytical platform

A total of 49 hepatopancreas samples were re-analysed using the Raptor® Solo test strip reader, which replaced the AccuScan Pro® reader in 2022. The AccuScan Pro® reader had previously been employed in the initial laboratory study (Chapter 7) and the present field sample validation. Re-analysed samples with detectable levels of PST are indicated by an asterisk in Figure 8.1. Both readers returned positive results for all samples with total PST concentrations above 0.4 mg STX.2HCl equiv. kg^{-1} and performed similarly in the >LOD-0.2 and 0.2-0.4 mg STX.2HCl equiv. kg^{-1} ranges (Table 8.2). Of the 22 samples where Lawrence screen or confirmatory analysis had not detected the presence

of PST previous to Neogen analysis, no detections were recorded with the AccuScan Pro® reader (i.e. no false positives), whereas the Raptor® detected PST on 5 occasions. All 7 NZ hepatopancreas tissues obtained from mixed *Gymnodinium/A. minutum* blooms re-rested positive with the Raptor®.

Table 8.2 Frequency of PST detection differences between the AccuScan Pro® and Raptor® readers in a subset of field and experimental samples.

		Number of detections		Frequency of detection (%)	
PST range (mg STX.2HCl equiv. kg^{-1})	**Number of observations**	**AccuScan Pro®**	**Raptor®**	**AccuScan Pro®**	**Raptor®**
<LOD	22	0	5	0	23
>LOD-0.2	7	2	2	29	29
0.2-0.4	7	5	6	71	86
0.4-0.6	5	5	5	100	100
0.85	1	1	1	100	100
NZ samples (0.12-0.4)	7	7	7	100	100

The observed differences between the AccuScan Pro® and Raptor ® reader were explored further using a dilution series of lobster hepatopancreas from the aquaculture feed trials. The results were highly variable between production test kit lots. Lot 9562-49 results were relatively similar for the two readers, varying by ± 20% between readers at each concentration analysed, whereas lot 9562-51 results varied by ± 100% between readers, with the Raptor ® recording 100% positive results at all concentrations except 0 mg STX.2HCl equiv./kg (Table 8.3).

Table 8.3 Frequency of PST detection (%) from a dilution series of a mixed hepatopancreas sample derived from aquaculture feeding trials using two Neogen PST test strip production lots with both the AccuScan Pro® and the new Raptor ® readers. Each concentration was measured in 10 replicates for each Neogen test kit production lot.

	Frequency of detection (%)			
	AccuScan Pro		Raptor®	
Concentration (mg STX.2HCl equiv./kg)	**Lot1**	**Lot2**	**Lot1**	**Lot2**
0	0	10	0	10
0.1	20	20	0	100
0.2	70	80	60	100
0.3	100	90	70	100
0.4	80	100	100	100

Test kit production lots: Lot 1 = 9562-49, Lot 2 = 9562-51

A POD table for the Raptor ® reader (Table 8.4) was created by combining the results in Table 8.2 and Table 8.3. Combining the results provided a greater number of different PST profiles from the field samples, as well as variety in test kit production lots (lot 9562-48 for field samples and lots -49 and -51 from the dilution series).

Table 8.4 The probability of detecting PST in lobster hepatopancreas using the Neogen Raptor ® reader. Data drawn from combining results of analysis of field samples and a dilution series of samples obtained from an aquaculture feeding trial.

	Number samples	Number detects	POD
<LOD	42	6	0.14
>LOD-0.2	27	12	0.44
0.2-0.4	47	39	0.83
>0.4	26	26	1.00

Table 8.5 Estimated number of Neogen PST detections based on the the AccuScan Pro® and Raptor ® probabilities of detection and aggregated monitoring data of PST in Southern Rock Lobster hepatopancreas samples collected from Tasmania waters (2012-2019 and 2022).

PST range (mg STX.2HCl equiv. kg^{-1})	**Number of field observations (biotoxin monitoring field data)**	**AccuScan Pro®**		**Raptor ®**	
		POD	**Number expected PST detections**	**POD**	**Number expected PST detections**
<LOD	102	0	0	0.14	15
>LOD-0.2	126	0.45	57	0.44	56
0.2-0.4	83	0.79	66	0.83	69
0.4-0.6	54	1.00	54	1.00	54
0.6-0.8	23	1.00	23	1.00	32
0.8-1.0	29	1.00	29	1.00	29
>1.0	86	1.00	86	1.00	86
Total	503		315		331

Cost effectiveness of using Neogen rapid test kits for PST detection in routine lobster biotoxin monitoring

The historic data of the Tasmanian Southern Rock Lobster Biotoxin Management Program (2012-2022; total of 503 individual samples) was divided into concentration ranges of <LOD; >LOD-0.2; 0.2-0.4; and >0.4 mg STX.2HCl equiv./kg. The retrospective estimated frequency of detection of PST in lobster in each concentration range during bloom sampling was calculated for the AccuScan Pro® and Raptor ® readers by multiplying each frequency by the respective PODs (Table 8.5). The

total number of positive detections expected from the 503 samples was 314 and 331 respectively, with negative results only occurring at concentrations below 0.4 mg STX.2HCl equiv. kg^{-1} total PST).

In 2020 the cost of lobster LCMS/MS analysis was $508 per lobster hepatopancreas sample (includes sample processing cost). At this price, the analytical costs for analyzing 503 lobster would be $255,524.

In the scenario where Neogen test kits are integrated into biotoxin management, all lobster hepatopancreas samples would be analysed by Neogen first, then only the positive hepatopancreas samples would be analysed by LCMS/MS. The cost of the Neogen assay is $92 per lobster ($460 for 5 lobsters, see **Error! Reference source not found.** 8.6 for breakdown of costs). Thus, the cost of a nalyzing 503 lobster using the Neogen test kits would be $46,276.

- As 315 of these samples would be positive if using the AccuScan Pro® reader, a further $160,020 (315 x $508) would be spent on LCMS/MS analysis, totaling $206,296
- As 331 of these samples would be positive if using the and Raptor ® reader, a further $168,148 (331 x $508) would be spent on LCMS/MS analysis, totaling $214,424.

Table 8.6 Estimated cost of Neogen analysis for 5 lobster hepatopancreas samples (includes labour for sample processing).

Materials	**Cost**
Cost Neogen test kit & shipping (24 tests/kit @ $960 = $40/test)	200
Consumables	50
Labour	
Dissection (2 technician hours @ $60/hour)	120
Neogen analysis & reporting (1.5 technician hours @ $60/hour)	90
Total cost for 5 Lobster (1 sample occasion)	**460.00**

The cost savings in analysis from using Neogen combined with LCMS/MS instead of LCMS/MS alone is therefore $49,228 or $41,100 using the AccuScan Pro® or Raptor ® readers respectively. Across the eight biotoxin seasons, this equates to approximately $6,154 or $5,138 per annum respectively. The initial set-up cost for a Neogen test strip reader and materials required for analysis could be purchased for around $5,000.

8.4 Discussion

Evaluation of Neogen test kit performance

The application of the Neogen lateral flow immunoassay for the detection of PST in Southern Rock Lobster hepatopancreas was successfully extended to field and aquaculture feeding trial samples following the initial single laboratory method validation by Dorantes-Aranda et al. (2020, Chapter 7). Challenged against a wide variety of PST profiles and total PST concentrations (128 samples), the Neogen test exhibited a high probability of PST detection at concentrations above 0.4 mg STX.2HCl equiv. kg^{-1} (POD = 1.0).

The probability of obtaining a positive result on the Neogen test is directly dependent upon the PST analogues present in a sample and their individual concentrations (Dorantes-Aranda et al., 2020; Jawaid et al., 2015). Across all PST profiles examined here, the Neogen test kit detected PST in 86 of 100 samples where the presence of PST had previously been confirmed by chemical analysis (either Boundy or Lawrence method). Non-detection of PST by the Neogen test in the other 14 samples can be explained by the lower total PST concentration (<0.35 mg STX.2HCl equiv. kg^{-1}, as confirmed with chemical analysis), and the varying cross-reactivities of the Neogen detection antibodies for different PST analogues (Jawaid et al, 2015). Comparative analysis of samples with the new analytical platform (Raptor®) indicates that this reader performs similar to the AccuScan Pro® reader employed previously in the single laboratory validation study (Chapter 7) and employed in the current field validation. Our investigation showed that the Raptor® reader has a higher probability of detection of PST at lower PST concentrations (14% of samples tested positive where no PST was detected via confirmatory analysis c.f. 0% with the Accuscan Pro® reader).

The samples for which the Neogen test returned negative results contained high percentages of C-toxins, dcSTX, GTX2&3 and GTX5, which exhibit much lower cross-reactivities and therefore a much lower likelihood of detection than for samples containing analogues with high cross-reactivities at the same concentration (e.g. STX or Neo, 100% and 128% cross-reactivity, respectively, see Table 8.7). However, these results cannot solely be attributed to the analogues present, as the Neogen test returned positive results for all the New Zealand samples analysed here (high C-toxin, GTX2&3 and dcSTX). It appears likely that the challenge of detecting PST in these samples was further increased by their lower total PST content (<0.35 mg STX.2HCl equiv. kg^{-1}), particularly in light of the changes in POD with the new Raptor reader.

Of critical importance for test kit performance and suitability for regulatory applications, is the detection of high toxicity analogues (as exemplified by higher TEF) that exhibit lower cross-reactivities, such as GTX1&4, GTX2&3, as well as dcSTX. While the Neogen test kit successfully detected PST in samples containing these analogues at higher concentrations in Tasmanian and New Zealand field samples (>0.4 mg STX.2HCl equiv. kg^{-1}), validation of this test in other localities outside of Tasmania or for use in other species (e.g. abalone), will need to carefully take into account both the local variation in PST profiles and matrix effects to avoid false negative results. Comparative analysis of samples with the new analytical platform (Raptor®) indicates that this reader performs

similar to the AccuScan Pro® reader employed previously in the single laboratory validation study (Chapter 7) and employed in the current field validation. Our investigation showed that the Raptor® reader has a higher probability of detection of PST at lower PST concentrations (14% of samples tested positive where no PST was detected via confirmatory analysis c.f. 0% with the Accuscan Pro® reader).

Table 8.7 Cross-reactivities of the Neogen lateral flow immunoassay against individual PST analogues in relation to the STX parent molecule, expressed as mean ± 1 standard deviation. The toxin equivalency factor relating the toxicity of each individual PST analogue to STX is also provided in order of toxicity (a higher TEF indicates higher toxicity analogues).

PST Analogue	**Neogen cross-reactivity***	**TEF****
NEO	128.9 ± 29%	**2.0**
STX	100%	**1.0**
GTX1&4	5.7 ± 1.5%	**1.0**
GTX2&3	23.4 ± 10.4%	**0.6**
dcSTX	55.6 ± 10.9%	**0.5**
dcGTX2&3	8.3 ± 2.7%	0.4
dcNEO	28.0 ± 8.9%	0.2
C1&2	3.1 ± 1.2%	0.1
GTX5	23.3 ± 14.4%	0.1

* Jawaid et al. (2015)
** FAO/WHO (2016).

In the Tasmanian context, based on the field samples analysed here, the Neogen test was able to reliably detect the presence of PST across a range of PST profiles and varying PST concentrations ranging from 0 to 9.0 mg STX.2HCl equiv. kg^{-1}, with similar POD values to those observed in the single laboratory validation of the method for lobster hepatopancreas (Chapter 7). As observed in the field (Turnbull et al., 2021, Chapter 6) and shown in aquaculture feeding trials (Madigan et al., 2018; Turnbull et al., 2020; Chapter 3), Southern Rock Lobster hepatopancreas typically contain higher concentrations of C-toxins, dcSTX, GTX2&3 and GTX1&4 when PST are actively taken up from PST contaminated prey items during *Alexandrium catenella* blooms. At the end of the uptake phase, these studies have shown that GTX2&3 and STX generally are more prevalent, accompanied by a decrease in dcSTX and a more pronounced shift towards STX in the depuration phase. All of these analogue combinations were represented in the sample sets used for this field validation and the Neogen test were able to reliably detect PST (POD = 1.0) at concentrations above 0.4 mg STX.2HCl equiv. kg^{-1}. It should be noted that this probability of detection is specific to the PST profiles of the Tasmanian samples analysed here. The samples used for this field validation were collected during blooms on the Tasmanian east coast (including Storm Bay), an area where both *A. catenella* and *Gymnodinium catenatum* may be present. The causative alga was often not identified although the broad range of profiles in lobster hepatopancreas shown in Figure 8.1 are indicative of multiple source algae. Future lobster collection in this area should be accompanied by phytoplankton sampling to confirm the source of PST in lobster hepatopancreas in this area.

Given the different cross-reactivities of the Neogen detection antibodies for individual PST analogues, the likelihood of PST detection is expected to vary if different PST vectors should be present (i.e. lobster consuming prey with different PST profiles) or if bloom conditions should suddenly change (e.g. blooms of a new algal species producing a different PST profile). Further work testing samples contaminated with the *Gymnodinium/A. minutum* derived PST is required to ascertain how applicable the Neogen assay is to these blooms (positive Neogen results at lower PST concentrations may limit the cost-effectiveness of the assay, see discussion below). The high likelihood of detection at PST concentrations half the CODEX bivalve regulatory level, combined with positive detections in hepatopancreas samples with PST profiles containing harder to detect PST analogues (e.g. GTX5, dcSTX and GTX1&4), provides additional confidence that minor shifts in PST profiles would be detected at concentrations relevant to biotoxin risk management.

Suitability for field monitoring

The high probability of detection of PST in lobster hepatopancreas above 0.4 mg STX.2HCl equiv. kg^{-1} (POD =1.0) makes the Neogen assay attractive for use in routine field monitoring, as it gives high confidence that the presence of PST will be detected in these higher risk samples. However, the probability of detection at lower PST concentrations needs to be considered when evaluating the use of the test kit, as the kit will be most useful if it returns a low rate of positive results for concentrations below 0.4 mg STX.2HCl equiv. kg^{-1}. At PST concentrations between 0.2-0.4 mg STX.2HCl equiv. kg^{-1}, four out of five lobsters would be expected to test positive (POD = 0.79). As per the current Southern Rock Lobster Biotoxin Management Plan, a decision to close or open the fishery is typically made when PST concentrations in lobster hepatopancreas reach between 0.4-0.65 mg STX.2HCl equiv. kg^{-1}. Solely relying on the Neogen test would therefore mean that positive detections at PST concentrations below this "decision range" may result in too many prolonged closures. Instead, a much more feasible approach appears to be using the Neogen test to pre-screen samples and following up any positive detections with confirmatory chemical analysis.

How best to incorporate the Neogen test kit into ongoing biotoxin monitoring in Tasmanian waters requires careful evaluation of its cost-effectiveness at different stages of the bloom. Sampling of Southern Rock Lobster in Tasmania is triggered when PST are detected at increasing levels in mussels. As filter feeding bivalves, mussels tend to rapidly accumulate PST and are therefore employed as sentinel species to monitor PST activity and inform lobster sampling (see Chapter 6 for the confirmation of mussels as sentinel species). Although the Neogen test has been successfully validated for use in mussels, its qualitative nature makes it unsuitable to identify PST concentration trends, which require confirmatory PST analysis. Instead, the Neogen test kit could best be employed once lobster sampling has been initiated. At that stage of the biotoxin season (i.e. PST uptake phase), PST levels in SRL hepatopancreas would be expected to be lower and contain PST analogues with lower Neogen antibody cross-reactivities (i.e. harder to detect). Negative Neogen results at this point of the bloom would negate the need for confirmatory analysis, as we have a high level of confidence that a negative result is below 0.4 mg STX.2HCl equiv. kg^{-1}. Any positive Neogen detections at this stage could then be followed up with chemical analysis to confirm PST levels for management decisions. This would mean negative results would result in re-opening the fishery (which is closed

for sampling) on the same day as processing, positive results would result in no change to the current time frame (i.e. opening decisions would be dependent on confirmatory analysis). Employing the Neogen test at the height of the bloom would not be cost-effective, as PST concentrations in the lobster hepatopancreas would be expected to commonly exceed 0.4 mg STX.2HCl equiv. kg^{-1} and therefore return positive results that would require confirmatory analysis. However, towards the end of the bloom, when confirmatory analysis indicates a downward trend in PST levels (i.e. depuration phase), pre-screening of samples with the Neogen assay would again be valuable, since non-detects with the test kit would indicate PST levels below 0.4 mg STX.2HCl equiv. kg^{-1} and the fishery could open again. Table 8.8 summarises the applicability of the Neogen test kit at different stages of the bloom.

Table 8.8 Value of the qualitative Neogen rapid test kit for monitoring of PST in Tasmanian Southern Rock Lobster hepatopancreas.

Bloom stage	**Value of Neogen rapid test kit**	**Explanation**
Mussel sentinel monitoring	Low	Quantification of risk needed in order to assess trends and trigger additional management plans
Lobster sampling during initial bloom phase	High	Negative tests expected to be frequent. LC-MS testing not required if test is negative, reducing costs and turnaround time
Lobster sampling during height of bloom	Low	Majority of tests expected to be positive, therefore majority of samples will need LC-MS testing, which will give a quantitative result and allow tracking of PST concentrations to determine the onset of toxicity demise
Lobster sampling at bloom demise	High	Negative tests expected to be frequent. LC-MS testing not required if test is negative, reducing costs and turnaround time

To determine if this approach would be cost-effective in Tasmania, the probability of detection at these PST levels was assessed against the frequency of which they occurred in the field (2012-2019 and 2022 Southern Rock Lobster biotoxin monitoring data). To provide a "worst case scenario" for cost effectiveness, this analysis assumed Neogen test kits would be used consistently throughout the bloom, which is not a recommendation made in Table 8.8. Based on the analysis of the available data, the Neogen test would have been expected to translate to a total cost saving of between $41,100 - $49,228 across the eight biotoxin seasons monitored; approximately $5138 - $6,154 or per annum. The initial set-up cost for a Neogen test strip reader and materials required for analysis is approximately $5,000, and an annual cost of $1000 for a box of Neogen test kits would be needed, whether or not a bloom developed due to slow kit delivery times and the 12-month shelf-life of the

kits. The cost effectiveness of kit use would be increased substantially by targeted use during only the bloom initiation and bloom demise phase.

In addition to the reduced analytical cost, a key advantage of the Neogen rapid test over currently employed confirmatory analysis is the shorter analytical time. Considering time taken for collection of the animals in the field, transport to the laboratory, processing of samples and analysis, current turn-around for LC-MS analysis via the Boundy method is 2-3 days and 1-1.5 days for the Neogen test kit. This is particularly advantageous, as the Southern Rock Lobster fishery will remain closed once sampling has been initiated until a negative result has been obtained or PST concentrations have been confirmed.

8.5 Conclusions

Qualitative screening tools can be a cost effective and rapid tool for identifying samples of public health concern. When positive screen results are returned, samples are sent for more expensive confirmatory analysis. Negative screens results do not need to be analysed any further, thus saving analytical costs and time.

For the Neogen rapid test kits for PST to be useful as screening tools in routine biotoxin monitoring the test kits need to provide:

1. A high level of confidence that samples containing PST above the level of concern (in this case 0.4 mg STX equiv./kg as per the lobster biotoxin management plan) will be positively identified and
2. A low level of PST detection below the level of concern.

The Neogen PST test kit reliably detected PST across various toxin profiles and concentrations representing PST uptake and depuration phases of past Tasmanian biotoxin seasons, as well as unique PST analogue combinations.

Based on the 128 samples analysed in this study, the Neogen test kit provided high confidence in successfully detecting PST in high-risk samples (PST concentrations ≥0.4 mg STX.2HCl equiv. kg^{-1}, POD = 1.0). No false negatives were detected with the original reader (AccuScan Pro) used for the field validation and a low percentage of false negatives were detected with the Raptor® reader. Based on these results we conclude that the Neogen test kit would be best employed as a screening assay at the start and end of the biotoxin season to facilitate monitoring programs by making faster decisions when PST levels are expected to be less than half the regulatory limit. Conservative estimate of cost savings by using the test kits are around $5138 - $6,154 per annum, however this value could be improved through a targeted use of the kit during bloom initiation and bloom demise only.

It is recommended that any positive detections with the Neogen test are followed up with confirmatory chemical analysis to inform risk management decisions (i.e. a combination of analytical methods should be used in the monitoring program).

It should be noted that the probability of detection is specific to the PST profiles of the samples analysed here. Given the different cross-reactivities of the Neogen detection antibodies for individual PST analogues, the likelihood of PST detection is expected to vary if different PST vectors are present (i.e. lobster consuming prey with different PST profiles) or if bloom conditions should suddenly change (e.g. blooms of a new algal species producing a different PST profile) and analytical platforms continuously evolve (e.g. recent introduction of the Raptor® analytical platform). Further work testing the POD of PST in lower toxicity samples contaminated with PST derived from *Gymnodinium castenatum*/*Alexandrium minutum* blooms is required to ascertain how cost-effective use of the Neogen assay would be during these blooms.

9. Conclusions

The knowledge created during this research project has direct application to improved risk management of PST in Tasmanian Southern Rock Lobster. Through a combination of field and laboratory studies, we have been able to improve our understanding of uptake and depuration rates, variability between individuals, mechanisms of uptake and depuration, the resilience of adult lobster to PST accumulation, and the potential application of rapid test methods.

Feeding of captive lobsters in aquaculture tank trials (Chapter 3) showed that toxins rapidly accumulated in the hepatopancreas (2% per day), reaching a maximum of 9.0 mg STX.2HCl equiv. kg^{-1} and then depurated at a rate of 7% per day once toxic fed was removed. No impact of PST on lobster health and behaviour were observed (Chapter 4). This rapid PST uptake and relatively fast depuration were confirmed by field observations on the Tasmanian east coast (Chapter 6), indicating that lobster can become toxic to human consumers in a period as short as 1 week, but may detoxify faster than initially assumed.

No negative impacts on animal health nor PST accumulation were observed when adult lobsters were exposed to toxic *Alexandrium* microalgae during aquaculture tank trials (Chapter 5). Lobsters exposed to toxic algae during wet storage in long supply chains (on vessel, in sea-cages or at processing facilities) therefore are not expected to take up PST. Furthermore, survival, quality, and safety of this high-value product are not impacted by accumulation of PST nor by exposure to toxic algal cells in the water.

Examination of 8 years of Tasmanian data on PST in lobster hepatopancreas PST and in abalone (foot, viscera), oysters and sea urchins confirmed that mussels are the most reliable early warning sentinel species (Chapter 6). The central east coast presented the highest risk sites, with July to January identified as the most at-risk months. Based on current data, there is no scientific justification to suggest any modifications to existing lobster management zones. However, it is noted that this project did not have an opportunity to look at PST variability within specific zones due to the lack of bloom activity over the past 2 years. The combination of using mussels as a sentinel species to identify risk areas, sampling 5 individual lobster at a particular site and using a conservative PST for closure was determined to provide a cost-effective strategy for managing PST risk in the Tasmanian commercial lobster fishery.

Following the single laboratory validation of the Neogen test kit (Chapter 7), its use for monitoring purposes was challenged against a range of toxin profiles and concentrations representing PST profiles encountered during previous Tasmanian biotoxin seasons (Chapter 8). Based on the analysed toxin profiles, the test kit provides high confidence in detecting PST at levels above 0.4 mg STX.2HCl equiv. kg^{-1} in Tasmanian lobster hepatopancreas tissues. Initial results for typical NZ lobster profiles during *Gymnodinium catenatum/A. minutum* blooms show the Neogen test kits does return positive results for samples where PST is detectable by LC-MS analysis. More work is needed however to determine the POD of PST at low levels for these profiles, as all of our initial results returned positive results.

An analysis of the cost efficiency of integrating Neogen into lobster PST risk management as a screening tool to identify samples that need to be analysed by LCMS/MS showed that average annual cost savings of $5,138 - $9,154 could be made. The initial set-up cost for a Neogen test strip reader and materials required for analysis is approximately $5,000, and an annual cost of $1000 for a box of Neogen test kits would be needed, whether or not a bloom developed due to slow kit delivery times and the 12 month shelf-life of the kits. The cost effectiveness of kit use would be increased substantially by targeted use during only the bloom initiation and bloom demise phase. In addition the kit allows for increased sample turn-around (1-1.5 days vs. 2-3 days for confirmatory LC-MS analysis), which translates to shorter fishery closures (should no PST be detected).

Southern Rock Lobster is a common species to Australian and New Zealand fisheries. Collaborations between NZ and Australia proved successful during this project to provide additional research support to both countries (samples and expertise), share the knowledge gained and successful risk management approaches.

10. Implications

Implications for biotoxin risk monitoring in Southern Rock Lobster are:

1. sentinel sites in the high risk zones of the central east coast should be maintained;
2. animal toxin monitoring should be frequent at the start of a bloom;
3. mussel sentinel lines are a cost-effective early warning system for toxin accumulation and should be in use during the high risk periods of July to January;
4. it is adequate to sample 5 individuals per site provided a reduced trigger level of closure of harvest is employed;
5. depuration is relatively quick so that sampling to confirm re-opening should occur soon after bloom collapse (as indicated by mussel PST levels);
6. non-lethal sampling is not possible as haemolymph PST levels do not reflect levels in the hepatopancreas.
7. Based on the analysed toxin profiles, the test kit provides high confidence in detecting PST at levels above 0.4 mg STX.2HCl equiv. kg^{-1} in Tasmanian lobster hepatopancreas tissues. That means that during years with dense widespread blooms the Neogen rapid test could facilitate monitoring programs by making faster decisions when PST levels are less than half the regulatory limit (negative Neogen results). Negative Neogen results would not need to be followed up with more expensive LC-MS analysis. Based on the initial validation with the old reader and test strips, this would have presented average annual cost savings of $5,138 - $6,154 ($41,100 - $49,228 across the eight biotoxin seasons monitored) and increased sample turn-around (1-1.5 days vs. 2-3 days for confirmatory LC-MS analysis), which in turn translates to shorter fishery closures (should no PST be detected).
8. The Neogen rapid test kit is also likely to be an effective tool for risk management in Southern Rock Lobster fisheries affected by *G. catenatum/A. minutum* blooms, however more samples would need to be analysed to determine how cost effective this could be.

11. Recommendations

1. The Tasmanian Biotoxin Monitoring Plan for Southern Rock Lobster is reviewed in light of the findings of this report and appropriate changes made.
2. Particular attention is to be paid to maintaining the high-risk sentinel sites in Great Oyster Bay and Maria Island as these sites tend to display PST before other sites.
3. References to the publications here-in are added to the lobster biotoxin management plan to provide regulatory authorities with confidence in the protocols supported by this work.
4. Neogen rapid test kit are used at the start and end of Tasmanian PST biotoxin seasons to rapidly and more cost-effectively screen lobster hepatopancreas samples for PST.
5. Additional lobster samples containing low concentrations of PST from *Gymnodinium catenatum* blooms are analysed to determine the value of the rapid test kit for use in New Zealand and in Tasmania when *G. catenatum* is present.

12. Further developments

1. The Neogen test kit returned positive results for all of the NZ mixed *A. minutum* and *G. catenatum* samples analysed. Further samples are needed at low PST results to determine how conservative this tool would be if used in NZ and Tasmania during blooms of these toxic species. Once the POD curve for rock lobster samples contaminated with PST from *G. catenatum/A. minutum* has been determined, a similar cost/benefit analysis could be undertaken for the NZ fishery.
2. We found no impact of PST on animal health in this study, although we only used adult lobsters. Further work is needed to understand potential impacts on juveniles and fecundity.
3. Earlier sampling in Tasmania pooled ovary tissue with the hepatopancreas. Ovaries are consumed in some cultures but at this point we have no understanding if ovaries accumulate PST. As male lobsters were used in experimental studies to reduce variability in results due to sex, we were unable to analyse ovaries for PST in this study and thus recommend some analysis be undertaken in future blooms.
4. An IMAS PhD project is currently investigating the dietary preferences of Tasmanian lobsters through faecal DNA analysis. The same technique could be employed to lobster sampled as part of routine biotoxin monitoring to ascertain the dietary vectors of PST to lobster.
5. Now that the Neogen test kit has been shown to be cost effective during bloom development it could be used to study the geographic variability of PST within zones. This study would need to be undertaken in consultation with regulators and fishers to determine whether the aim is to evaluate the current divisions or determine options for new geographic divisions at a finer scale.
6. We continue to support the proposal put forward by SafeFish to Food Standards Australia New Zealand to adopt the Codex standard for PST expression (mg STX.2HCl equiv. kg^{-1}).
7. With the closure of Spring Bay mussels, the critically important Spring Bay mussel monitoring station should be continued.

13. Extension and Adoption

A project Steering Committee meeting was held in Hobart on 23 July 2018, with Chair Chris Izzo (FRDC) and members Grant Pullen, Hilary Revill, Matt Bradshaw, Owen Hunt (DPIPWE), Shelly Alderman (DAWE), Colin Buxton, Tom Consentino (SRL), John Sansom (TRLFA), Daryl Sykes (NZ RLIC), and Dean Lisson (ACA). The steering committee was not able to resolve the issue of how to collect scientific samples during open lobster season.

Gustaaf Hallegraeff reported on project progress at the TransTasman SRL meeting on 21 August 2018 at Melbourne airport. Both Ali Turnbull and Gustaaf Hallegraeff presented at the Australian Marine Science (AMSA) Conference in Adelaide in July 2018. Ali Turnbull presented in Wellington, at the Seafood New Zealand annual conference on 5th November 2018, and both Ali Turnbull and RA Juan Dorantes-Aranda presented on this work at the Australian Shellfish Quality Assurance Program (ASQAP) science day in Sydney on 11 September 2018.

A TransTasman SRL biotoxin risk management plan workshop (20+ participants, incl. New Zealand Daryl Sykes, Cathy Webb, Tim Harwood, DPIPWE Hilary Revill) to report and discuss these results was held at IMAS Hobart on 29 -30 April 2019. This workshop provided a valuable opportunity to discuss the research findings determined thus far and guide the future research needed. The consultation between Australia and New Zealand was particularly useful in allowing the sharing of successful strategies for risk management. Following the workshop the lobster biotoxin management plan in both countries was updated.

Ali Turnbull, Gustaaf Hallegraeff and Cathy Webb also presented results of this project at the TransTasman Rock Lobster Congress in Queenstown, New Zealand, 11-13 Aug. 2019.

On 8 May 2020 we held a Zoom meeting to report our results to the Tasmanian SRL industry representatives, attended by Hilary Revill Chair: DPIPWE/WFM, Alison Turnbull IMAS/project leader, Gustaaf Hallegraeff IMAS, Owen Hunt DPIPWE BT, Shelley Alderman DAFF, Julian Harrington CEO TSIC, John Sansom CEO TRLFA, Stewart Quin DH, Megan Burgoyne DPIPWE/Shellmap, Grant Pullen DPIPWE/WFM.

On 20 May 2020 we held a follow-up Zoom meeting with the Biotoxin Steering Committee attended by Hilary Revill, Owen Hunt, Shelly Alderman, Tom Cosentino (SRL), John Sansom, Gustaaf Hallegraeff, Alison Turnbull, Daryl Sykes (NZ).

The results of the experimental tank studies and field work were presented (remotely) by Alison Turnbull at the International Conference of Harmful Algae, Mexico in October 2021 (Appendix 14.4).

The results of the experimental tanks studies and field work from this study were included in a PhD thesis by Alison Turnbull, submitted in August 2021.

Throughout the project and particularly during COVID, the researchers have been discussing results with DPIPWE/NRET staff and supporting risk monitoring during bloom periods.

14. Project coverage

https://www.imas.utas.edu.au/news/news-items/improved-risk-management-of-paralytic-shellfish-toxins-in-lobster-fishery

https://www.islandinstitute.org/working-waterfront/how-tasmanias-lobster-fishery-responded-to-algal-blooms/

https://www.examiner.com.au/story/5544294/biotoxin-rock-lobster-risk-reduced/ [24 July 2018)

15. Project materials developed

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Turnbull, A. 2021. Paralytic shellfish toxins in Southern Rock Lobster: physiological impact and improving public health risk management. PhD. thesis, University of Tasmania

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17. Appendix

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17.3 Paralytic shellfish toxins - Call for uniform reporting units

Alison R. Turnbull, D. Tim Harwood, Michael J. Boundy, Patrick T. Holland, Gustaaf Hallegraeff, Navreet Malhi, & Michael A. Quilliam

Paralytic shellfish toxins (PSTs) are a group of over 50 closely related naturally occurring tetrahydropurine toxins with saxitoxin (STX) as one of the most common and potent. Saxitoxin was first isolated and characterised from the butter clam *Saxidomus giganteus* as the dihydrochloride salt (Schantz et al., 1975). It is traded as a stand-alone reference material, or included in diagnostic test kits, mainly as the dihydrochloride salt or diacetate salt. This is primarily due to the saxitoxin hydrate (free base) form having poor stability. However, the certificate of analysis for various reference materials may state the concentration as either the salt or free base equivalent, and care must be taken to ensure the value is traceable and used correctly in subsequent calculations.

PSTs continue to be the focus for pure and applied research into their origins, distributions, toxicity, ecology, biochemistry, and test methods. A survey of the scientific literature revealed over 1500 publications on PSTs since 2000. This interest in PSTs is spurred by the widespread occurrence of harmful algal blooms of dinoflagellates and cyanobacteria, which can lead to accumulation of PSTs in seafood and sometimes drinking water. Due to their high oral toxicities, levels of PSTs in shellfish are regulated by most countries and test methods have been established and continue to be developed to enforce regulatory limits.

However, there are inconsistencies in how PST concentrations are calculated and expressed in different areas of research, testing and national regulation. This is of great concern because of the potential for errors and misunderstandings that could result in adverse human health and trade implications. The situation is made more complex by PST analogues having a diverse range of toxicities that require application of toxicity equivalence factors (TEFs) to express the overall toxicity of naturally occurring mixtures. PST test methods have evolved from mouse bioassays developed over 80 years ago (expressing results as total toxicity in Mouse Units or STX equivalents) to modern chemical analytical methods such as HPLC and LC-MS. Chemical analytical methods enabled quantification of individual PST analogues and forced a careful evaluation of toxic potency of different STX analogues, both by intraperitoneal mouse injection (Oshima, 1995; Quilliam, 2007) and oral routes of administration (Munday et al., 2013), and were re-evaluated by a FAO/WHO Expert Group in 2016 (FAO/WHO, 2016).

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Toxic potencies vary considerably between analogues (see examples in Table 17.1). Thus differences can be found between results using testing methods such as HPLC and LC-MS, designed to measure individual analogues, compared to antibody based ELISA and LFIA methods, according to the antibodies used and the analogue profiles present. Different preparation/extraction and LC detection protocols also influence the final determination of PSTs. These differences however, are exacerbated by the variety of reporting units currently in use.

The current Codex Alimentarius standard (2015) for PSTs in bivalve molluscs has a limit of 0.8 milligrams STX.2HCl equivalents per kg tissue. The Codex standard also states that *'Total toxicity is estimated as the sum of the molar concentrations of detected analogues multiplied by the relevant specific TEFs'.*

This standard is clear and unambiguous. There is the dichotomy that the limit is expressed in mass units but TEFs are ratios of toxicities in molar units. Therefore, conversions are required. The following equation sums analytical data for the concentrations of individual toxins in a sample extract in µmole/L to a total toxicity that can be compared to a regulatory limit:

$$Conc_{shellfish} = 372.2 \times DF \times \sum(Conc_{extract} \times TEF)$$

Where:

$Conc_{shellfish}$ = Total concentration of PSTs in shellfish homogenate in mg STX.2HCl equiv. kg^{-1}

372.2 = molecular weight of STX.2HCl (g/mol)

DF = factor to account for dilution in the sample extract

$Conc_{extract}$ = concentration of the congener measured in the sample extract in µmol/L

TEF = molar toxicity equivalency factor

If the individual concentrations of PSTs in the extract are expressed in mass units then this equation must incorporate the molecular weights for each toxin in the sum. If the total toxicity is required in free STX units (for example, if regulation in a particular jurisdiction requires free STX eq), then the molecular weight factor is 299.3 instead of 372.2 g/mol, resulting in a final concentration 24% less than that expressed as STX.2HCl equivalent.

Given the above, large variations in the expression of a PST concentration can occur. We highlight the following areas for concern:

Research publications

The basis for reported concentrations of PSTs is not always clear or consistent. Molar units are preferable where toxicities are being reported and compared. Analytical data must clearly

express the concentration units: molar or mass, and state whether they are calculated as the dihydrochloride salt or free base. Any use of TEFs must reference the source of the values. The TEFs proposed by the FAO working group (2016)are recommended as new research has resulted in some changes from earlier TEFs (EFSA, 2009; Oshima, 1995; Quilliam, 2007). A survey of the most recent 120 publications on PSTs (Scopus, July 2019) showed that where total STXs were reported 63% used STX equivalents (with the assumption that this is as the free base), 32% used STX.2HCl equivalents and 5% did not state the units. The TEFs used were derived from the Oshima publication or EFSA (30% each) with less than 10% using the FAO values and 30% not reporting the source.

Laboratory test methods

All test methods for PSTs must specify the units for calibration standards, raw concentrations and final results. The calculations should also be explicitly described and the source of TEFs referenced. It is noted that some published methods lack clarity in the above areas including the widely used Lawrence pre-column oxidation method (AOAC 2005.06). This has resulted in documented systematic errors in reporting of results for PSTs by some laboratories. Incorrect guidance is provided in the example given in section 5.1 of the joint FAO/WHO technical paper, where molar TEF values were directly applied to gravimetric concentrations (μg/100g) of PSTs to give STX equivalents (FAO/WHO, 2016). While calculation errors cannot be avoided completely, this incorrect guidance from FAO must be corrected so that serious errors in the estimation of PSTs can be avoided. Table 17.1 uses the PST levels in the example used by FAO/WHO (2016) and carries through the calculations using the above equation, to provide the correct total in mg STX.2HCl equivalent per kg shellfish per the Codex (2008) standard.

National regulations

Regulatory limits set nationally for PSTs in shellfish vary. Some countries follow the Codex standard and use 0.8 mg/kg STX.2HCl equivalents (e.g. New Zealand SR 2006/38), whereas others state 0.8 mg/kg ‘saxitoxin equivalents’, which may be taken as STX free base equivalents (e.g. China GB 5009.213-2016, Australia Food Standards Code Schedule 19, Health Canada Maximum Levels for Chemical Contaminants in Foods). When this is the case, 0.8 mg/kg is effectively a 24% higher limit than Codex. Several jurisdictions, such as the European Union, and the USA (Food regulation (EC) No. 853/2004, NSSP Model Ordinance 2017), state regulatory limits equivalent to 0.8 mg/kg, without referencing whether it should be expressed as STX dihydrochloride salt or free base. Clearly these standards are not equivalent, and furthermore, some are ambiguous. Serious consequences can result from confusion about measurement results if units are not used properly. This is especially the case in international trade, which is regulated by reciprocal agreements between countries regarding the monitoring and allowable limits on contaminants such as toxins in food. A mismatch between measurement results in two countries can result in the embargo and destruction of very expensive product shipments.

Table 17.1 Example from FAO/WHO (2016) with conversion to recommended units and correct application of TEFs.

Toxin analogue	**Extract Level (µg/100g)**	**Free Base MW**	**Molar concentration (µmol/kg)**	**TEF**	**Shellfish concentration (mg STX.2HCl equiv. kg^{-1})**
STX	10	299.3	0.334	1	0.124
NEO	5	315.3	0.159	2	0.118
GTX1	15	411.4	0.365	1	0.136
GTX4	15	411.4	0.365	0.7	0.095
C1	5	475.4	0.105	0.01	0.000
C2	5	475.4	0.105	0.1	0.004
Total					**0.477**

This lack of consistency with PST units creates problems for the interpretation of data from testing laboratories and in the interpretation of research findings. It confuses the translation of risk assessment advice into regulatory limits, and causes diverse, often ambiguous, regulatory limits for the purposes of managing consumer safety and trading in seafood. Furthermore, recent epidemiological research suggests that the current acceptable daily intakes for PSTs on which these limits are based may be underestimating the potential for human illness from consumption of contaminated shellfish (Arnich and Thebault, 2018). As such a 24% difference is significant!

A major problem for end-users of the data is the lack of clarity as to what was done. Authors, editors and reviewers must do more to ensure published papers on PST are consistent and accurate with the units used. More effort also is required to achieve clarity, consistency and international harmonisation in regulation of PSTs in seafood. We recommend in all cases that the format in the Codex standard is followed, i.e. PST is expressed in mg STX.2HCl equivalents kg^{-1}, calculated using the FAO reported TEFs.

17.4 Monitoring and Management of Paralytic Shellfish Toxins in Southern Rock Lobster, Tasmania, Australia

Alison Turnbull, Andreas Seger, Navreet Malhi, Jessica Jolley, Tim Harwood, Juan Dorantes-Aranda, Tom Madigan, Graeme Knowles, Hilary Revill, Quinn Fitzgibbon & Gustaaf Hallegraeff

Abstract. The Tasmanian Southern Rock Lobster fishery has been challenged by recurrent dinoflagellate blooms of *Alexandrium catenella* since 2012. The initial bloom resulted in the first ever closure of an Australian lobster fishery due to marine biotoxins and exposed several key knowledge gaps for managing food safety and market access risk. To fill these gaps, experimental studies were conducted to determine paralytic shellfish toxin (PST) toxicokinetics. Adult male lobsters fed highly toxic mussels (6 mg STX.2HCl equiv. kg^{-1}) accumulated PST in the hepatopancreas at an exponential rate of 6% per day, reaching a maximum level of 9 mg STX.2HCl equiv. kg^{-1} in three weeks. However, lobsters exposed to toxic algae culture suspensions at 2 x 10^5 cells L^{-1} did not accumulate any toxin. Neither accumulation of PST nor exposure to toxic cells resulted in any gross impact on the health of lobsters, as assessed by a comprehensive range of behavioural, immune, nutritional and biochemical indicators. Field studies over a period of 8 years confirmed the ability of lobster hepatopancreas to rapidly accumulate and depurate toxins in the wild, with a high degree of variability. Analysis of 496 hepatopancreas samples collected during *A. catenella* blooms identified high risk sites and seasons; demonstrated the usefulness of mussels as sentinel species for indicating PST risk; and enabled quantification of the confidence level associated with current risk management sampling practices. The combined experimental and field results have led to improved risk management for this AUD 97M wild fishery.

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Introduction

An extensive dinoflagellate bloom of *Alexandrium catenella* occurred on the east coast of Tasmania in 2012, causing the first ever Australian lobster fishery harvest closure due to marine biotoxins (Campbell et al., 2013). During the bloom, Southern Rock Lobster accumulated PST to 3.9 mg STX.2HCl equiv. kg^{-1}. Since 2012, recurrent blooms of *A. catenella* have occurred during winter and spring months when water temperatures are between 10–15°C and coastal waters may stratify (Condie et al., 2019). These blooms have had an on-going impact on both the commercial lobster fishery in Tasmania, valued at AUD 97 M, and the significant recreational fishing sector. In order to better manage the associated public health and market access risks, a series of experimental and field studies was undertaken. Initial work focused on the risks to human health from PST accumulation in Southern Rock Lobster, looking at the concentration of PST in the hepatopancreas, assessing the fate of PST during cooking, and consumer exposure levels (Madigan et al., 2018a; Madigan et al., 2018b; McLeod et al., 2018; Turnbull et al., 2018).

Risk management of PST in lobster in Tasmania has adopted the bivalve PST maximum level (ML) as the regulatory level (DPIPWE 2020), however, lobster sampling strategies are necessarily different from those for molluscs due to the geographical spread of the wild fishery, the different way the animals are consumed, and the high level of variability among individual animals. Lobsters are keystone marine species, so concern was also raised over potential impacts on lobster health.

Further experimental and field studies were undertaken, seeking knowledge of the toxicokinetics of PST accumulation and depuration; of supply chain risks of exposure to toxic algal cells; impacts on lobster health; and effective methods to monitor PST levels in the field.

Material and Methods

Experimental studies

Two controlled experiments took place in a biosecure facility using adult male lobsters housed in individual tanks in a flow-through aquaculture system (Fig.17.1). In the first experiment, lobsters were fed mussels containing 6 mg STX.2HCl equiv. kg^{-1} for 27 days then moved to a non-toxic diet for a further 36 days (Turnbull et al., 2020a). PST in the hepatopancreas was examined at regular intervals during uptake and depuration using LC-MS/MS (Boundy et al., 2015; Turner et al., 2015). Exponential uptake and depuration rates were calculated and changes in the toxin profile noted. At the peak of uptake, PST was also analysed in the hindgut, antennal glands, gills and haemolymph.

In the second experiment, lobsters were exposed to toxic cultures of *A. catenella* at field relevant concentrations of 2×10^5 cells L^{-1} for three weeks (Turnbull et al., 2021b), replicating potential exposure in the supply chain. PST was measured in the hepatopancreas at three time points.

Figure 17.1 Biosecure experimental facility where lobster housed in individual tanks were exposed to PST through either food or toxic algal cultures.

During both experiments, lobster health was assessed at each harvest point via a comprehensive set of behavioural, immune, nutritional and biochemical responses, measured by the same operator in the same order on each harvest day (Turnbull et al., 2020a). Histological analysis of the toxic algae exposed lobsters was conducted using formalin fixed paraffin embedded gill tissues samples cut into 3 micron thickness and stained with haemotoxylin and eosin.

Field studies

Lobster sampling (n=496) occurred on a regular basis from 2012 to 2020 in eight lobster biotoxin management zones on the east coast of Tasmania during *A. catenella* blooms (Turnbull et al., 2021a: Figure 17.2 PST lobster management zones on the east coast of Tasmania, Australia. Sentinel mussel sample locations are indicated with an asterisk. Source: DPIPWE 2020.).

On each sample occasion, lobster hepatopancreas from each site were analysed individually for PST (n=5 animals). Blue mussels, *Mytilus galloprovincialis,* from adjacent aquaculture farms or specifically installed mussel lines were sampled over the same time period as potential sentinel species. Samples were analysed for PST using either HPLC-FLD (Lawrence et al., 2005) or LC-MS/MS (Boundy et al., 2015; Turner et al., 2015).

Uptake and depuration rates for lobster were calculated for events with four or more consecutive sampling occasions at the same site and compared to concurrent mussel uptake and depuration rates. Data from the start and end of blooms were examined to determine the number of lobsters required to give a 95% confidence that the population is below the bivalve maximum level.

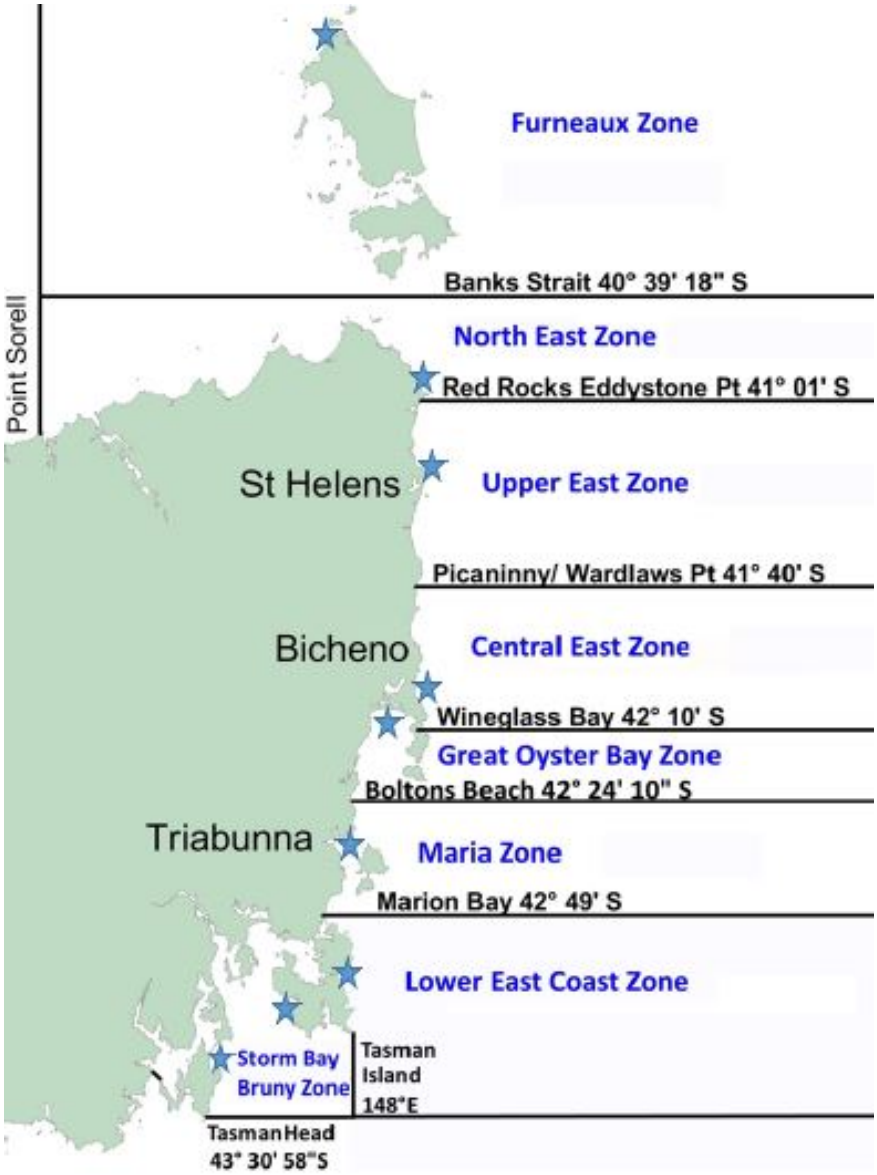

Figure 17.2 PST lobster management zones on the east coast of Tasmania, Australia. Sentinel mussel sample locations are indicated with an asterisk. Source: DPIPWE 2020.

Results and Discussion

When lobsters were fed toxic mussels, PST accumulated rapidly in the hepatopancreas at an exponential rate of 6% per day, reaching a mean of 6.7 mg STX.2HCl equiv. kg^{-1} after 27 days (Turnbull et al., 2020a). The majority of toxins in the hepatopancreas during uptake were GTX2,3 C1,2, and GTX1,4, with the proportion of the latter decreasing as uptake continued (Figure 17.3 PST analogue mean molar content in hepatopancreas of exposed Southern Rock Lobster during 27 days of uptake and 35 days of depuration). In comparison the mussel feed contained mostly GTX1,4 and GTX 2,3. The lobsters depurated at a rate of 7% per day once toxic feed was removed. PST was detected in lobster antennal glands and gills, (possible excretion routes for PST), however, it was not detected at significant levels in lobster

haemolymph. The majority of toxin in the antennal glands and gills were GTX2,3 and dcGTX2,3.

Exposure to PST did not result in mortality nor significant changes in any of the behavioural, health, nutritional and haemolymph biochemical parameters measures suggesting limited gross impact on lobster performance and indicating adult lobster are relatively tolerant to PST (Turnbull et al., 2020b).

Lobsters exposed to highly toxic algal cultures of *A. catenella* did not accumulate PST and no negative impact on lobster health or gill tissue was observed (Turnbull et al., 2021b). The results indicate that PST uptake can only occur through the consumption of toxic prey and hence that there are no food safety or quality risks from exposure to toxic cells during wet storage in boat wells, sea cages or specialized wet storage facilities in the supply chain.

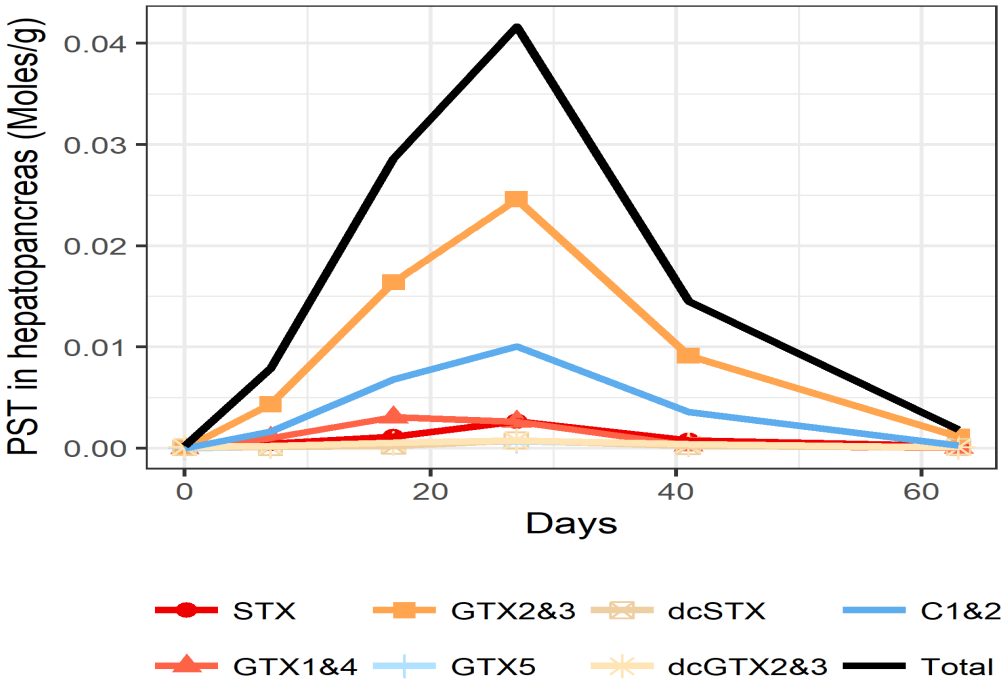

Figure 17.3 PST analogue mean molar content in hepatopancreas of exposed Southern Rock Lobster during 27 days of uptake and 35 days of depuration

Field studies and regulatory monitoring over the 8 year period showed high variability in toxin levels between individuals, sites, months and years. The central Tasmanian coast was identified as the greatest risk site, but confined to the months of July to January (Turnbull et al., 2021b; Figure 17.4). Relatively high PST uptake rates were observed in lobster hepatopancreas (exponential rate of 2% per day), similar to but consistently less than rates seen in filter-feeding mussels. Lobsters were relatively fast detoxifiers following bloom demise, losing up to 3% PST per day. Mussel sentinel lines were a cost-effective means of indicating PST risk in lobsters, with an annual baseline monitoring cost of <0.1% of the industry value. The current practice of analysing multiple lobster from a site and closing on a conservative trigger level provides a

97.5% confidence level that any lobster from that site would be below the bivalve maximum level of 0.8 mg STX equiv. kg^{-1}.

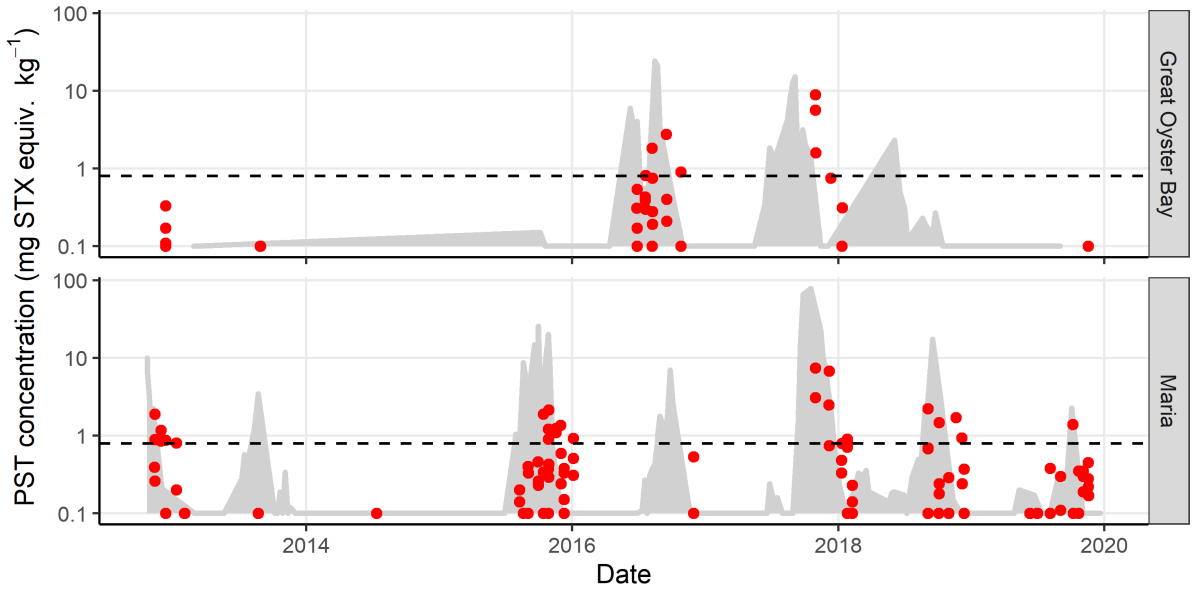

Figure 17.4 PST levels in lobster hepatopancreas (red dots) and mussels (grey areas) from the central east coast of Tasmania from 2012 to 2020 inclusive. Modified after Turnbull et al. 2021b