Harmful Algae 99 (2020) 101925

Contents lists available at ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

Uptake of Paralytic Shellfish Toxins by Blacklip Abalone (*Haliotis rubra rubra* Leach) from direct exposure to *Alexandrium catenella* microalgal cells and toxic aquaculture feed

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ARTICLE INFO

Keywords:
PST profile
Biotoxin
Bioaccumulation
Gastropod
Epipodium

ABSTRACT

The Tasmanian abalone fishery represents the largest wild abalone resource in the world, supplying close to 25% of the annual wild-caught global harvest. Prompted by the need to manage Paralytic Shellfish Toxin (PST) contamination of Blacklip Abalone (*Haliotis rubra rubra*) from east coast Tasmania, the uptake of toxins by this species is investigated in a land-based, controlled aquaculture setting. Abalone were exposed to either live *Alexandrium catenella* microalgal cultures or PST contaminated feed pellets during a 28 day exposure period and toxins quantified in viscera, foot muscle and epipodium tissues. PST profiles of abalone foot tissues were dominated by saxitoxin and neosaxitoxin, whilst viscera more closely resembled those of the toxin source (*A. catenella* cells rich in gonyautoxin 1&4 and 2&3 or feed pellets containing *A. catenella* extracts rich in these analogues). This indicates direct uptake of PST in the viscera via browsing/grazing on the pellet and /or sedimented microalgal cells. After exposure to *A. catenella* cell culture, PST concentrations in the foot (muscle + epipodium) were on average 8 times higher than in the viscera. Higher toxicity of foot tissue was caused by higher PST content of the epipodium (up to 1,085 μg STX.2HCl equiv. kg^{-1}), which despite its small contribution to total animal weight significantly added to the overall toxin burden. Higher PST levels in the abalone foot suggest that toxin monitoring programmes may not need to routinely analyse both foot and viscera, potentially allowing for a 50% reduction of analytical costs. This option is being further investigated with continuing field studies.

1. Introduction

Risk management of algal biotoxins in aquacultured and wild-caught seafood products requires an understanding of toxin uptake and depuration mechanisms to inform monitoring and regulatory strategies that ensure consumer health and safety. Consequences of human ingestion of seafood contaminated with Paralytic Shellfish Toxins (PST) can be severe, with symptoms ranging from mild discomfort, paraesthesia, diarrhoea and vomiting to in extreme cases death (James et al., 2010). Detection of PST in seafood by importing countries can result in prolonged trade closures (multiple years in some cases) and indirectly impact sales of other seafood commodities due to reduced consumer confidence.

The most notorious shellfish poisoning syndrome, Paralytic Shellfish Poisoning, is caused by the ingestion of saxitoxin and its congeners, collectively referred to as Paralytic Shellfish Toxins (PST). The best known natural producers of these toxins belong to the microalgal genus *Alexandrium* and *Gymnodinium catenatum*. Naturally occurring blooms of these dinoflagellate species can span large coastal areas (up to 100 s of km^2 in the case of *Alexandrium*) and persist for months (Anderson et al., 2012a; Hallegraeff et al., 2012). In filter feeding bivalve molluscs, the PST produced by these microalgal species tend to rapidly (within days) build up to levels that can impact human health (Gueguen et al., 2012; Kwong et al., 2006) and have been demonstrated to subsequently

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https://doi.org/10.1016/j.hal.2020.101925
Received 22 April 2020; Received in revised form 12 October 2020; Accepted 16 October 2020
Available online 27 October 2020

accumulate in higher trophic levels. Predatory species, such as cephalopods (Robertson et al., 2004; Braid et al., 2012), crustaceans (McLeod et al., 2018; Desbiens and Cembella, 1995), certain carnivorous gastropods (Compagnon et al., 1998; Lin and Hwang, 2012) and some herbivorous gastropods, such as abalone (Pitcher et al., 2001; McLeod et al., 2017; Bravo et al., 1999), have all been documented to contain PST at concentrations exceeding the internationally agreed bivalve maximum allowable level of 800 μg saxitoxin dihydrochloride equivalents per kilogram (STX.2HCl equiv. kg^{-1}, Codex Alimentarius Commission, 2008). The resulting closures of commercial and recreational harvest areas following detection of elevated PST levels, as well as the cost of ongoing monitoring during bloom periods, provide a strong economic incentive to better understand PST uptake and depuration mechanisms in species with high market value, such as abalone.

Paralytic, diarrhoetic and Amnesic Shellfish Toxins have all been documented to accumulate in a range of wild-caught and aquacultured abalone species (Martínez et al., 1996; Pitcher et al., 2001; Malhi et al., 2014). To the best of our knowledge, no human intoxications have been confirmed to date from abalone consumption, although PST at concentrations exceeding the CODEX maximum limit (Codex Alimentarius Commission, 2013) have been reported in *Haliotis midae* from South Africa (Pitcher et al., 2001), *Haliotis tuberculata* from Spain (Bravo et al., 1999) and in *Haliotis rubra rubra* from Australia (McLeod et al., 2017; Harwood et al., 2014). The toxins have been shown to accumulate in both viscera and foot tissues, with the epipodal fringe surrounding the foot containing the highest PST concentrations in abalone from South Africa and Spain (up to 300 x higher in epipodium; Pitcher et al., 2001, Bravo et al., 1999, Bravo et al., 2001). Australian abalone, however, typically have occurred during *G. catenatum* blooms with higher PST in the viscera (up to 4 x higher in viscera; McLeod et al., 2017).

Due to their distinct feeding behaviour, abalone and other herbivorous marine gastropods have long been considered to be non-conventional vectors of PST (Shumway, 1995). As grazers, abalone predominantly feed on attached or drift macroalgae, but are also known to browse on benthic microalgae, hydrozoans, bryozoans and detritus (Shepherd, 1973). Unlike the well-established uptake of microalgal PST through direct ingestion by filter feeding molluscs (Gueguen et al., 2012; Kwong et al., 2006), the origin and uptake of PST in abalone tissues has remained elusive and largely speculative. Proposed uptake mechanisms include: browsing on sedimented phytoplankton cells and/or their cyst resting stages (Pitcher et al., 2001; McLeod et al., 2017; Bravo et al., 1996), grazing on PST containing macroalgae (Oshima et al., 1984; Etheridge et al., 2002) and/or absorption of PST through the foot epidermis (McLeod et al., 2017; Pitcher et al., 2001).

When PST were first discovered in abalone, attention immediately shifted to dinoflagellate microalgae as the source of PST. Indeed, abalone were shown to be contaminated with PST following bloom episodes, but unlike other species, such as bivalves, which readily depurate after exposure, abalone exhibited prolonged retention of high PSTconcentrations. Following bloom episodes of *A. catenella* in South Africa, toxins in the abalone were retained for up to seven months (Pitcher et al., 2001) and more than three months after *G. catenatum* blooms in Spain (Bravo et al., 1999; Martínez et al., 1996; Bravo et al., 1996). The detection of toxic abalone in the apparent absence of PST producing phytoplankton, along with either no detection of PST in mussels (Spain, Bravo et al., 1999, Martínez et al., 1996) or significantly different PST profiles between abalone and mussel tissues (South Africa, Pitcher et al., 2001), has occasionally cast some doubt on microalgae as the source of PST in these areas.

In Australian waters, however, PST concentrations in both abalone foot and viscera have been conclusively linked to seasonal phytoplankton blooms (McLeod et al., 2017). Field sampling of Blacklip Abalone over a two year period in south eastern Tasmania showed that PST levels closely followed the seasonal bloom dynamics of *G. catenatum*, with low background toxicity being retained in the foot between bloom episodes. While *Gymnodinium* blooms in Tasmanian waters have usually been confined to estuarine areas (Hallegraeff et al., 1995; Anderson et al., 2012b), recurrent large scale blooms of *A. catenella* along the east coast of Tasmania have repeatedly caused seafood harvest closures since 2012 (Hallegraeff et al., 2018). In the absence of routine monitoring for PST in abalone, precautionary Tasmanian abalone fishery closures are currently based on PST levels in bivalve shellfish farms adjacent to abalone harvest areas (Tasmanian Abalone Council, 2017). A better understanding of the uptake of PST from *Alexandrium* by abalone promises improvements to existing management and monitoring protocols (certain Tasmanian harvest areas have remained closed for more than two consecutive years). The Tasmanian abalone fishery represents the largest wild abalone resource in the world, supplying close to 25% of the annual wild-caught global abalone harvest (Tasmanian Abalone Council, 2017), providing a strong economic incentive to effectively manage the impact of PST on the fishery.

In this study, Blacklip Abalone were exposed to either live *A. catenella* microalgal cultures or a PST contaminated food source, in the form of feed pellets, with known toxin levels and profile. PST levels in viscera, epipodium and foot muscle tissues were analysed separately using liquid chromatography mass spectrometry (LC-MS) to investigate potential PST uptake by Blacklip Abalone.

2. Material and methods

2.1. Abalone stock

Wild Blacklip Abalone (*Haliotis rubra rubra*) were collected from Pelican Point near Mount Gambier, South Australia on the 12th October 2018 under ministerial exemption ME9903010. Animals were transported in seawater in 50 L containers (20 animals/container). Water temperature and dissolved oxygen (DO; Oxyguard Polaris 2) was monitored at hourly intervals during the 8 h journey to the experimental facility. Ambient air was supplied via battery operated aquarium pumps (Aqua One, 250 L h^{-1}) and supplemented with a 30–60 s burst of medical grade oxygen as required (if <85% DO).

2.1.1. Acclimation period

Upon arrival, abalone were weighed and transferred to 450 L holding tanks (50 animals/tank) to acclimate to experimental conditions and to wean abalone from their natural macroalgal diet onto a formulated diet (see below). Water in the holding tanks was constantly circulated (~1000 L h^{-1}) through canister filters (Aqua One Nautilus CF2700) loaded with preconditioned K1 media. Temperature, pH (Hach KTO sensION), DO (Oxyguard, Polaris 2), conductivity (HACH KTO sensION), ammonia (API Fishcare, LR8600) and nitrite concentrations (Hach, NitriVer® 3) were measured daily. Solid waste (faeces and uneaten feed) was removed via syphon and daily water exchanges (10–100%) were conducted in response to the recorded water parameters to maintain the following conditions: ammonia <1 ppm, nitrite <0.4 ppm, salinity 35, temperature 15.1 – 18.3 °C and pH 8–8.2.

2.1.2. Weaning of abalone

Abalone were weaned off their natural diet over a period of five weeks by slowly reducing the amount of seaweed and increasing the amount of feed pellets provided. Green (*Ulva* sp.) and red seaweed species (cf. *Platoma* and *Ploclamium* spp.) were initially harvested at the site of abalone collection. To continuously supply fresh seaweed during the weaning phase, *Ulva* was subsequently collected from Outer harbour, Adelaide. After an initial acclimatisation period of 2 days without feed, abalone were fed a diet consisting of a 50% *Ulva* and 50% red seaweed presented at 1.7% abalone body weight (BW, based on wet in-shell weight). Once abalone were feeding on the seaweed, each tank received ~1.3% BW (160 g) of seaweed and 0.08% BW (10 g) of small brood-stock pellets (circular, 3 mm diameter; Aquafeeds Australia, Mount Barker, South Australia). At the last interval of the weaning

process, abalone were fed the brood-stock pellet at 0.25% BW, supplemented with 0.4% BW *Ulva*. Prior to experiments commencing, seaweed additions were ceased and abalone weaned from the brood-stock pellet onto the non-toxic basal experimental pellets (in 25% increments).

2.2. Alexandrium cultures

Alexandrium catenella strain AT.TR/F (previously known as *A. tamarense* Group 1) was obtained from the Institute for Marine and Antarctic Studies, Hobart, Australia (originally isolated from Triabunna, Tasmania in 2012 by Chris Bolch). The microalga was cultivated in batch cultures (15 L carboys) with 120 μmol photons $m^{-2} s^{-1}$ of light supplied by a custom array of low temperature LEDs on a 12:12 h light: dark cycle. Cultures were maintained at 18 ± 1 °C and the seawater culture medium (0.22 μm sterile filtered coastal seawater) 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). The growth vessels were gently aerated (0.15 L min^{-1}) with ambient air during the dark period and 1.5–2.5% (v/v) CO_2 in the light.

2.3. Toxic feed preparation

To study the direct dietary uptake of PST by abalone, an aquaculture feed pellet was especially formulated to contain PST harvested from in-house produced *A. catenella* cultures. Feed pellets were produced using cold extrusion methods at Aquafeeds Australia.

2.3.1. Extraction of PST

Alexandrium catenella cultures were aerated with ambient air (no CO_2), but otherwise grown under the conditions described above. Extracts were prepared from these cultures as described below to determine the average PST quota at the time of harvest (9.86 pg STX.2HCl equiv. $cell^{-1}$). During, and leading up to the experimental exposure, PST concentrations in *A. catenella* (AT.TR/F) cultures were measured to contain from 3.51 - 21.23 pg STX.2HCl equiv. $cell^{-1}$ in the stationary growth phase. Based on the average PST quota, a volume of 350 L of algal culture (~5200 cells mL^{-1}, no CO_2 aeration) was harvested in batches during the stationary growth phase via centrifugation (1500 *x g* for 10 min, Beckman Avanti J-25). Based on average *Alexandrium* cell and PST yields (9.86 pg STX.2HCl equiv. $cell^{-1}$), this volume was calculated to yield sufficient total PST to reach theoretical final PST concentrations of 1200 and 12,000 μg STX.2HCl equiv. kg^{-1} for the low and high toxin diet, respectively (see below). For each batch, the supernatant was discarded, with the cell pellet resuspended in the remaining 10 mL of supernatant. This was then stored at −20 °C for up to one month to allow the required algal culture volume to be generated. One day prior to pellet manufacture, the concentrated cell suspensions were defrosted and placed in a waterbath (80 °C, 10 min) to ensure complete lysis of algal cells and release of intracellular PST. The lysed cell preparation was immediately cooled on ice before being centrifuged (3500 *x g*, 5 min, ScanSpeed 1580R) to pelletise cell fragments. The resulting cell- and fragment-free supernatant (from here on referred to as the extract) was collected and stored at 4 °C for feed pellet preparation the following day. A subsample of the extract was immediately stored at −80 °C and later shipped to the Cawthron Institute, New Zealand, for PST analysis via LC-MS (see Section 2.6).

2.3.2. Pellet preparation

Diets were formulated to contain 35% crude protein, 5% crude lipid and 17.5 MJ kg^{-1} gross energy (Bansemer et al., 2016 Table 1) and supplemented with *Ulva* meal to masque potential PST 'off-flavours' and increase palatability. Experimental diets were prepared by first mixing the required dry ingredients in a Hobart mixer (5 min; Hobart Corp., Troy, USA) before adding in the wet ingredients (water to ~30% of the total ingredient weight, fish oil, sodium alginate and calcium carbonate). The water addition was fortified with *A. catenella* PST extract to yield a control, low and high PST diet corresponding to theoretical final PST concentrations of 0, 1200 and 12,000 μg STX.2HCl equiv. kg^{-1} of diet. PST concentrations in the final pellet product were later confirmed by LC-MS to be close to the desired concentrations (1500 and 10,700 μg STX.2HCl kg^{-1} for the low and high PST pellet formulations, respectively). Combined dry and wet ingredients were mixed for an additional 5 min and the pellets manufactured using a TR110 pasta machine (Macchine Per Pasta SRL, Molina Di Malo, Italy). The resulting wet pellets were dried at 45 °C until an average moisture content of 9.5% was reached and a flat, sinking pellet produced (10 × 2 × 0.52 mm). A subsample of each diet was stored at −80 °C at the start and end of the main exposure experiment and then shipped to the Cawthron Institute, New Zealand, for PST analysis by LC-MS (Boundy et al. 2015; Turner et al. 2015; TURNER et al., in press; see Section 2.6).

Table 1
Diet formulation for the preparation of abalone feed pellets after Bansemer et al. (2016).

Ingredient	g/100 g
Salmon fish meal (fish meal 65% protein)	6.00
Soy protein concentrate	8.00
Soybean meal (solvent extracted)	28.44
Wheat flour	27.53
Fish oil	1.00
Vitamin/mineral premix	0.50
Sodium alginate	0.30
Lupins (de-hulled)	22.40
Ulva meal	5.00
Calcium carbonate	0.22
Monosodium phosphate	0.61
Total	100.00

2.4. Pilot trials

The experimental set-up was tested in two separate trials to determine the suitability of static tanks for housing abalone (i.e. no water flow, 100% water exchanges every 24 h), as well as feed palatability by examining the feed rejection behaviour of the three different experimental diets (control, low and high PST). The results for the static trial are provided in the appendix.

2.4.1. Palatability trial

To inform the choice of feed pellet for the PST treatment in the main experiment (low or high PST content), a 7-day palatability trial was conducted. Abalone were randomly allocated to 20 L static tanks to receive 2.4 g of either the control, low or high PST feed pellet (equivalent to 1% (BW) of the heaviest abalone in the trial). A fourth treatment received the control pellet in addition to 3 mL of the GSe nutrient supplement (Blackburn et al., 1989) to test the response of abalone to the *Alexandrium* culture media. Each treatment consisted of five animals housed in individual tanks, with the tank set-up identical to that described in detail for the main experiment below (0). For each experimental diet, a single 20 L tank (biofilter, no abalone) served as a feed study control. These tanks received 2.4 g of the respective diet (control, low or high PST) to estimate the loss of left-over feed to the system.

As described for the acclimation tanks above (0), temperature, DO, salinity, ammonia, nitrite and pH were recorded at the start of each day (9 am) and left-over feed collected by siphoning onto a fine filter mesh. For the duration of the experimental period (5 days), uneaten feed was collected daily from all tanks and processed as described in Section 2.5.2 below. Immediately after feed collection, each tank received a 100% water exchange.

2.5. Exposure of abalone to PST

Abalone were exposed to two different PST sources: PST containing

feed pellets or live *A. catenella* microalgal culture. Abalone were chosen at random from the two acclimation tanks and transferred to individual 25 L tanks (390 × 300 × 300 mm) containing 20 L of seawater. These static tanks (no seawater flow, 100% water exchange every 24 h) were arranged in 8 rows of 5 tanks. Each row of tanks was set in a channel through which cold water was constantly circulated (abalone race) and seawater for tank water exchanges was held in a continuously circulated, chilled reservoir (16 ± 0.5 °C, 4000 L). To buffer any potential pH fluctuations during the 24 h static period, the seawater was supplemented with sodium bicarbonate as required. Air was supplied through individual sponge biofilters (one per tank) that had previously been conditioned for 3 weeks with nitrifying *Nitrosomonas* spp. and denitrifying *Nitrobacter* spp. The tanks were randomly allocated to four different treatments: control diet (no PST; 9 replicates), toxic diet (high PST pellet; 9 replicates), microalgal exposure (control diet + *A. catenella* culture; 9 replicates), as well as a GSe nutrient control (control diet + algal culturing nutrients; 5 replicates). The respective diets (control or high PST) were fed at the end of each day (15:00–16:00) at 1% BW (2.6 ± 0.05 g per animal) of the heaviest abalone in the trial (262 g in-shell weight). To take the loss of uneaten feed to the system into account, three 25 L tanks were set up identical to the experimental tanks, although without abalone, to serve as feed study controls for each of two diets (control and toxic pellet), as well as the microalgal exposure (control diet + *A. catenella* cells).

2.5.1. Abalone husbandry

Abalone were maintained in seawater at a salinity of 35–36 and temperature of 16 ± 2 °C throughout the experimental period (4 weeks). Tanks were covered with perforated, opaque lids (~40% perforation) and light supplied at an average intensity of 13 Lux by shadecloth covered fluorescent lights on a 12:12 h light:dark cycle. All routine husbandry operations were carried out under red light. Daily operations commenced by measuring DO and temperature directly in the tank (Oxyguard, Polaris 2) and collecting a 50 mL water sample from each tank. The pH of these samples was immediately recorded (Meterlab, PHM210) and nutrient concentrations determined through colorimetric tests (ammonia, nitrite and nitrate) according to the following regime: ammonia (API Fishcare, LR8600) and nitrite (Hach, NitriVer® 3) concentrations were assayed every 3 days for each tank and nitrate (Hach, NitraVer® 5) every two weeks. Salinity was recorded daily (Amzdeal, ATC) from one of the five tanks in each row on a rotating basis (i.e. each tank was tested every 5 days).

2.5.2. Feed consumption

After routine environmental measurements were completed each day, uneaten feed was removed from the tanks by spot siphoning onto a fine filter mesh. The collected feed from each tank, including the feed study tanks, was cumulatively stored in weekly intervals to allow for later assessment of feed consumption by each animal throughout the experimental period. Immediately upon completion of feed collection, all seawater and faeces were removed by inversion of the tanks that were subsequently refilled with fresh seawater from the reservoir tank. Collected feed was dried at 105 °C for 16 h. The resulting dry weight of the unconsumed feed from the experimental tanks was subtracted from the dry weight of the feed collected from the respective feed study tanks (control and high PST) to yield the weekly pellet consumption of each individual abalone in the experiment. Feed intake was corrected for abalone wet body weight (in-shell) and expressed as g of feed consumed per kg of abalone per day (g of feed kg abalone^{-1} day^{-1}).

2.5.3. Microalgal exposure

Upon completion of the daily husbandry and feed collection, *A. catenella* culture in the late exponential/early stationary phase was added to each tank in the microalgal exposure treatment group. Algal cultures were selected based on cell density (>2 × 10^7 cells L^{-1}, 2.5% CO_2 aeration) determined by preserving a subsample in Lugol's solution (1%) and subsequent microscopic enumeration (Olympus CX31, Sedgewick-Rafter chamber). The amount of culture to be added to each tank was calculated to yield a final cell concentration of approximately 2 × 10^5 cells L^{-1} in the experimental tanks. This concentration was chosen to represent peak bloom concentrations at depth (*A. catenella* is known to reach up to 3 × 10^5 cells L^{-1} in Tasmanian waters; integrated surface samples, Condie et al. 2019). The volume of algal culture to be added to each tank was displaced prior to addition and the corresponding amount of GSe media nutrients (Blackburn et al., 1989) added to each tank in the nutrient control group.

2.5.4. Abalone dissection

Abalone were harvested and dissected into viscera, epipodium and foot muscle tissues for PST analysis. To estimate baseline PST levels in abalone (sourced from the wild), 9 individual animals were randomly selected from the two acclimation tanks when the main PST exposure experiment commenced. Shell length (measured across widest point), in-shell and shucked weight were recorded before separating the viscera from the foot. The viscera were weighed and the head and mantle discarded. The epipodium (here defined as the outer black layer of the frill and ~ 1 mm of the ventral covering of the foot) was carefully dissected from the white foot muscle tissue. The foot muscle and epipodium were weighed and the foot muscle thoroughly washed to remove all traces of mucous. All tissues (viscera, epipodium and foot muscle) were homogenised (Velp Scientifica, OV5 homogenizer) to yield a homogenous, smooth paste suitable for PST analysis.

2.6. Toxin analysis

All abalone PST samples were analysed at Cawthron Institute, New Zealand, by LC-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), with results reported in μg 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 abalone tissue (epipodium, viscera or foot muscle) into a 50 mL centrifuge tube followed by the addition of 18 mL of 1% acetic acid (v/v). 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 3200 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 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 (1000:1, v/v). Sample extracts (400 μL) were loaded onto the conditioned cartridges and then washed with 700 μL of MilliQ water. PST were then eluted using 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. (2015), Turner et al. (2015) and TURNER et al. (in press). 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 average spike recoveries observed for the different sample matrices analysed. The limit of reporting for each PST analogue differed for each matrix tested.

2.7. Statistical analysis

All data were tested for normality and homogeneity of variance with Kolmogorov-Smirnov and Levene's test, respectively. Differences between experimental groups were tested using Student's T-test or where appropriate analysis of variance (ANOVA). PST concentration data was log transformed to test for treatment effects on PST content between different abalone tissues (2-way factorial ANOVA with interaction effect). Significant differences between treatment groups detected by ANOVA were followed up with Tukey's HSD test. Unless otherwise specified, all values represent the mean ± 1 standard deviation. All analyses were performed, and graphs constructed, with the statistical package R (www.r-project.org).

3. Results

3.1. Acclimation and weaning of abalone

Abalone were transported from Mount Gambier, South Australia to the South Australian Aquatic Biosecurity Centre with minimal mortalities occurring throughout the acclimation phase (3% mortality). Within five days of arrival, abalone were feeding on an average of 1.7% body weight (BW) of seaweed. At this point, the brood-stock pellet was successfully introduced to supplement seaweed additions and animals were observed to be actively feeding on the pellets 15 days after entering the aquaculture facilities. Seaweed additions were gradually decreased over time and completely ceased on day 24. The colour of abalone faeces was subsequently observed to change from black to brown (colour of pellet). The animals readily took to the control pellet diet that was used to gradually replace the brood-stock pellet in preparation for the pilot trials. During these pilot trials, the 20 L static tanks proved suitable for housing the abalone, with water parameters remaining within their desired ranges and animals actively feeding on the pellets (see appendix for details).

3.2. Palatability trial

Water parameters throughout the palatability trial (7 days) stayed within their acceptable ranges: salinity of 35–37, temperature of 16 ± 0.6 °C, ammonia and nitrite concentrations of <0.25 ppm, and 0.13 ± 0.02 ppm respectively, and pH of 8.14 ± 0.06. Dissolved oxygen concentrations did not drop below 85% saturation except for one instance (day 2), where biofilter air supply remained disconnected overnight in one replicate exposed to the high PST pellet (53% DO). This tank was still included in further statistical analysis, as the feed consumed by this individual abalone (0.6 g of feed kg abalone^{-1} day^{-1}) was close to the average of the remaining four replicates within the same treatment (1.0 ± 0.39 g of feed kg abalone^{-1} day^{-1}).

Abalone actively fed throughout the entire experimental period, consuming significantly different amounts of feed pellets between the four diet/nutrient exposure treatments (ANOVA: $F_{(3,16)} = 9.20$, $p = <0.001$). While no significant differences in feed consumption were found amongst the control pellet (no PST), GSe nutrient exposure and high PST diet (Tukey's HSD: $p > 0.55$), animals fed the low PST pellet consumed up to twice the amount of feed over the experimental period (2.0 ± 0.6 g of feed kg abalone^{-1} day^{-1}) than in any other treatment (Tukey's HSD: $p < 0.01$, Fig. 1).

3.3. Main experiment

Abalone were fed non-toxic feed pellets to acclimate to the experimental tanks over 3 days before the commencement of the respective experimental exposures (non-toxic control pellet, non-toxic control pellet & GSe nutrients, non-toxic control pellet & Alexandrium cells and toxic pellet treatments). All animals were observed to be actively feeding during this phase (average feed intake of 1.72 ± 0.8 g of feed kg abalone^{-1} day^{-1} across all treatments). Water parameters measured throughout the trial are provided in the appendix (Table A.2). No significant differences in feed intake were observed between the individuals that constituted the subsequent treatment groups (ANOVA: $F_{(3,28)} = 0.275$, $p = 0.84$). Comparison of feed consumption by abalone exposed to the GSe nutrient treatment (1.72 ± 0.65 g of feed kg abalone^{-1} day^{-1}) with that of the five *a priori* selected control animals (2.26 ± 1.24 g of feed kg abalone^{-1} day^{-1}) revealed no significant impact of algal culturing nutrients on feed intake over the four week trial (Student's T-test: $T_{(38)} = 1.73$, $p = 0.091$, data not shown). An ANOVA investigating the effect of treatment (control, toxic, *A. catenella* cell exposure) and week (1–4) on feed consumption by abalone, revealed no significant interaction between treatment and week (ANOVA: $F_{(6,96)} = 2.1$, $p = 0.066$) and no significant effect of the treatments (ANOVA: $F_{(2,96)} = 1.8$, $p = 0.174$). However, feed consumption did not remain constant over the experimental period, with abalone in all treatments consuming marginally more feed during the first two weeks of the experiment (2.17 ± 0.84 g of feed kg abalone^{-1} day^{-1}) when compared to the final two weeks (1.36 ± 0.76 g of feed kg abalone^{-1} day^{-1}; Tukey's HSD: $p = < 0.03$).

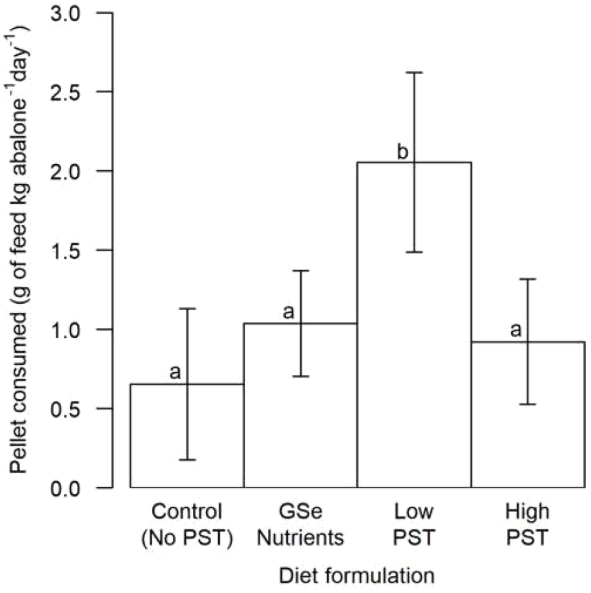

Fig. 1. Consumption by abalone of feed pellets with either no, low or high PST content (0, 1500 or 10,700 μg STX.2HCl equiv. kg^{-1} respectively) or when exposed to algal culturing media nutrients (control pellet + GSe nutrients). Error bars represent 1 standard deviation around the mean ($n = 5$) and letters denote significant differences between treatments (Tukey's HSD).

3.3.1. PST accumulation

PST levels above the limit of quantification were found in abalone collected from the South Australian coast. These abalone (acclimated for 2 months prior to dissection on day 0) presented with up to 296 μg STX.2HCl equiv. kg^{-1} in the epipodium (mean = 204 ± 72 μg STX.2HCl equiv. kg^{-1}), but had much lower PST concentrations in the foot muscle (8 ± 3 μg STX.2HCl equiv. kg^{-1}) and viscera (5 ± 2 μg STX.2HCl equiv. kg^{-1}). Total PST concentrations differed significantly between the abalone exposure treatments and tissue types at the end of the 28 day exposure period (ANOVA: $F_{(9128)} = 12.04$, $p = < 0.0001$, Table A.2).

Across all treatments, the highest PST concentrations were detected in the epipodium, followed by the viscera and foot muscle tissues (Fig. 2). When considering the contribution of individual tissues to the whole animal, only the total PST concentration in the *A. catenella* cell exposure treatment proved significantly different from the control group (Tukey's HSD: $p = < 0.0001$). After the 28-day exposure, the animals exposed to the toxic microalga accumulated up to 128 μg STX.2HCl

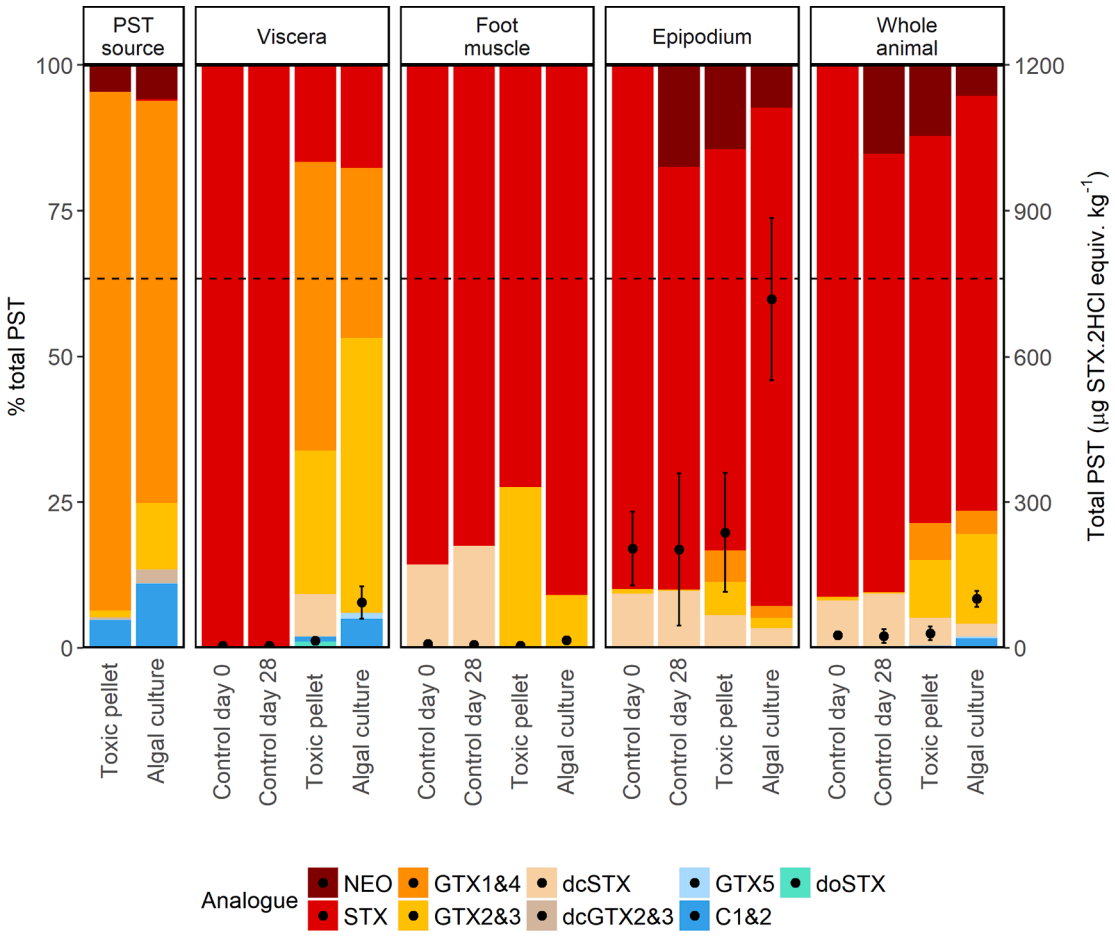

Fig. 2. PST profiles (percentage toxicity) in *A. catenella* algal culture and the toxic feed pellets prepared from algal extracts (PST source). PST profiles for epipodium, foot muscle and viscera tissues as well as the whole abalone are given for each treatment group. The control treatment is split into animals harvested on day 0 and day 28 (start and end of experiment). Total PST concentration is represented by the black points and expressed as µg STX.2HCl equiv. kg^{-1} on the secondary y-axis on the right, with error bars representing 1 standard deviation around the mean ($n = 9$). The CODEX abalone maximum level (800 µg STX.2HCl equiv. kg^{-1}) is represented by the dashed horizontal line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

equiv. kg^{-1} (mean = 101 ± 17 µg STX.2HCl equiv. kg^{-1} for whole animals). In this treatment group, 72 ± 8% of the total PST was situated in the epipodium.

3.3.1.1. Viscera. In the viscera, very low levels of PST were detected in the day 0 and day 28 control groups (5 ± 2 µg STX.2HCl equiv. kg^{-1}), with those in the toxic pellet treatment only marginally higher (15 ± 8 µg STX.2HCl equiv. kg^{-1}). The highest viscera PST concentrations were observed in animals exposed to *A. catenella* cells (93 ± 34 µg STX.2HCl equiv. kg^{-1}: Tukey's HSD: $p = < 0.001$). Only STX was detected in day 0 and day 28 control animals, whereas the PST profile in the *A. catenella* cell and toxic pellet treatments following exposure proved more complex. Here, STX made up only 17–18% of the profile, with substantial proportions of gonyautoxins (GTX1&4, 29 and 50%, in *A. catenella* cell and toxic pellet treatments, respectively) and GTX2&3 (24 and 47%, respectively) detected. The remainder of these profiles was made up of minor contributions from C1&2 and decarbamoylsaxitoxin (dcSTX), with trace levels of deoxydecarbamoyl-saxitoxin (doSTX) and GTX5 detected in the toxic pellet and *A. catenella* exposure treatments, respectively. Compared with PST profiles in epipodium or foot muscle tissues, the PST composition in the viscera of exposed abalone most closely resembles that of their respective PST sources (toxic pellets or *A. catenella*), particularly in regard to the higher GTX content (GTX1&4 and GTX2&3, Fig. 2).

3.3.1.2. Epipodium. Significantly higher PST concentrations were detected in the epipodium of animals directly exposed to the *A. catenella* cells (mean = 718 ± 167, max = 1085 µg STX.2HCl equiv. kg^{-1}) than in any of the other three treatments (Tukey's HSD: $p = < 0.00001$). The total PST concentration in the epipodium did not differ between the toxic pellet and control treatment or tissues of control animals on day 0 (Tukey's HSD: $p = 0.9994$). Indeed, PST profiles in the epipodium in control abalone at the start of the experiment (control day 0), as well as those across all three treatments at the end of the experimental period were dominated by STX (69–90% between treatments) and lower amounts of neosaxitoxin (NEO, 7–18%), dcSTX, (3–11%) and GTX1&4 and GTX2&3 (0.3–11%).

3.3.1.3. Foot muscle tissue. PST concentrations in the foot muscle tissues of the control (day 0 and day 28) and toxic pellet treatments did not exceed a maximum of 128 µg STX.2HCl equiv. kg^{-1}. Although PST concentrations in the foot muscle tissue of animals exposed to the *A. catenella* cells statistically differed from the other groups (Tukey's HSD: $p = < 0.001$), they proved only marginally higher (16 ± 4 µg STX.2HCl equiv. kg^{-1}). The PST profile was again dominated by STX (72–90%), with dcSTX (day 0 and 28 control only) and GTX2&3 (toxic pellet and *A. catenella* cell treatments) detected as the only other notable PST analogues.

4. Discussion

Historically, the source and uptake mechanisms of PST in abalone

were elusive and the subject of speculation (Bravo et al., 2001; McLeod et al., 2017; Etheridge et al., 2002; Pitcher et al., 2001). The controlled tank experiments reported on here add to the growing body of evidence that abalone are able to take up PST from direct exposure to toxigenic phytoplankton, albeit in this case, limited amounts (up to 128 μg STX.2HCl equiv. kg^{-1} in whole animals over a 28-day period at simulated *A. catenella* peak bloom concentrations of 2×10^5 cells L^{-1}).

No adverse effects of PST exposure to either the diet (toxic pellet) or *A. catenella* cells were observed on abalone behaviour (i.e. no reduced feed intake or other avoidance behaviour). Interestingly, the pilot palatability trial showed preferred feed intake of the low PST pellet over the control diet and no difference in feed intake between non-toxic control and high PST pellet (10,700 μg STX.2HCl equiv. kg^{-1}). However, it should be noted that PST levels exceeding 1000 μg STX.2HCl equiv. kg^{-1} have been known to paralyse (i.e. prevent attachment or righting) both aquacultured and wild South African *H. midae* (Pitcher et al., 2001).

The relative contribution of viscera and foot tissues to total PST contamination appears to be microalgal source specific. While Blacklip Abalone sampled during *G. catenatum* blooms in the Huon Estuary (Tasmania, Australia) presented with up to four times higher PST levels in the viscera than the foot (McLeod et al., 2017), we here detected higher PST concentrations in the combined foot tissues (epipodium + foot muscle) after exposure to *A. catenella* (on average four times higher than in the viscera). The latter agrees with the PST analysis of Blacklip Abalone collected from the east coast of Tasmania during *A. catenella* blooms, where higher toxicity was also reported in the foot tissues (SARDI, unpublished data).

In our tank experiments, the higher toxicity in the combined foot tissues (epipodium + foot muscle) was caused by the distinctly higher concentration of PST accumulated in the epipodium (up to a maximum of 1085 μg STX.2HCl equiv. kg^{-1} over 28 days of *A. catenella* exposure, contributing 70 ± 8% of total toxin burden). This accumulation of PST in the epipodial tissues of the foot has also been reported in Spanish Orma (*H. tuberculata*) and South African *H. midae* collected from the field (Bravo et al., 1999; Pitcher et al., 2001), raising the possibility of direct uptake of PST through the outer layers of the foot epidermis (McLeod et al., 2017). PST have been demonstrated to be concentrated in glandular epidermal cells of Spanish Orma and their mucilage covering, leading Bravo et al. (2001) to suggest that the epipodium may serve as a storage organ for PST and/or a PST secretory function.

Although the epipodium constitutes only a small percentage of the overall weight of the abalone, its high PST level contributes substantially to the overall toxin burden. Removal of the majority of the epipodium through scrubbing before market could therefore significantly reduce toxicity of contaminated animals and may be employed as a potential mitigation strategy. However, despite considerable PST reductions (around 70% removal; present work, Dowsett et al. 2011, Pitcher et al. 2001), scrubbing of the foot leads to a significant reduction in product value from AUD80 kg^{-1} for live, wild abalone compared to AUD40 kg^{-1} for scrubbed and canned product (beach prices, Tasmanian Abalone Council, 2017). Current risk management therefore aims to allow live harvest, implementing a closed/open status of individual harvest blocks (Tasmanian Abalone Council, 2017). Rather than canning product that could otherwise be sold at premium prices overseas, industry preference at this stage is to invest in the validation of a rapid PST screening test for abalone tissues, such as the Neogen lateral flow immunoassay (Neogen Corporation, Lansing, USA, previously validated for use in mussel and oyster tissues by Turnbull et al., 2018, Dorantes-Aranda et al., 2019). An effective pre-screening test could significantly reduce the amount and thereby cost of analytical testing required to inform management of individual abalone blocks.

Furthermore, our laboratory results and limited field data (SARDI, unpublished data) suggest that the cost of analytical testing may be halved by preferentially testing the foot and not the viscera of abalone suspected to be contaminated with PST from *A. catenella*. Currently, PST are monitored through separate analysis of the viscera and foot tissue of individual abalone, costing around AUD5000 per sampling interval of each harvest block. Collection of additional field data is currently underway to determine if the observed higher PST concentration in the foot tissue after *A. catenella* exposure holds true across multiple bloom seasons in the field. Linking of PST levels in abalone, rocklobster and bivalves to *A. catenella* bloom dynamics will allow for more targeted sample collection and promises to further improve the Tasmanian marine biotoxin management plan.

The oral uptake efficiency of PST delivered via the feed pellet appears to be very low. This is supported by previous work feeding PST contaminated pellets (150 μg STX.2HCl equiv. kg^{-1}) to Greenlip Abalone (*H. laevigata*), which showed limited uptake over a 50-day exposure period (19.8 μg STX.2HCl equiv. kg^{-1} in the foot muscle and concentrations below the limit of quantification in the viscera; Dowsett et al., 2011). Even though in this study we have fed more concentrated PST pellets by almost two orders of magnitude (10,700 μg STX.2HCl equiv. kg^{-1}, comparable daily feed intake), significant concentrations of PST (in statistical terms) were only found in the viscera (not the foot muscle or epipodium) after 28 days of exposure to the highly toxic pellet (15 ± 8 μg in viscera compared to control 4 ± 2 μg STX.2HCl equiv. kg^{-1}). This low uptake of PST indicates a low oral uptake efficiency of PST by Blacklip Abalone (at least for this particular food source).

However, PST from microalgal cell suspensions appeared to be more readily taken up, with PST concentrations in viscera on average 6 times higher than in the pellet treatment (up to a maximum of 149 μg STX.2HCl equiv. kg^{-1}), with substantially higher PST levels found in both foot muscle and epipodial tissues (up to 1085 μg STX.2HCl equiv. kg^{-1} in epipodium). In both treatments (algal culture and toxic pellet exposure), the PST profile of the viscera most closely resembled the respective PST source (>80% GTX1&4 and GTX2&3 analogues, no STX), whereas foot muscle and epipodium tissues contained almost exclusively STX and NEO. The question of biotransformation of PST was raised during investigations into the putative source of PST in Spanish and South African abalone (Bravo et al., 1999; Pitcher et al., 2001). While in these scenarios, absence of PST in bivalves in the region where PST contaminated abalone were found questioned involvement of PST producing microalgae, the laboratory results obtained in the present work closely align with PST profiles found in Blacklip Abalone from the Tasmanian east coast collected during *A. catenella* blooms (SARDI, unpublished data). Those field samples presented with higher percentages of GTX1&4 and GTX2&3 in the viscera, whereas the foot was dominated by STX & NEO. South African abalone were also found to contain almost exclusively STX in the foot, despite the *A. catenella* PST profile (putative source) consisting predominantly of C-toxins and moderate amounts of STX, GTX4 and decarbamoylgonyautoxin 3 (dcGTX3; Pitcher et al., 2001). From the tank experiments, it cannot be concluded whether the observed variation in PST profiles between abalone tissues is due to biotransformation or variable uptake and depuration rates.

Maximum PST concentrations in whole animals following experimental exposure to *A. catenella* (128 μg STX.2HCl equiv. kg^{-1}) were lower than PST levels occasionally observed in the field (>800 μg STX.2HCl equiv. kg^{-1}). These differences may be explained by the position of microalgal cells in the water column, differing lengths of exposure and variations in intra- and extracellular toxin content. Even though the Tasmanian *A. catenella* strain used in our experiments (AT. TR/F) exhibits one of the highest reported PST cell quotas (up to 21.23 pg STX.2HCl equiv. $cell^{-1}$, compare with e.g. Jester et al. 2009, Lilly et al. 2002, Montoya et al. 2010, Sekiguchi et al. 2001 and Varela et al. 2012), field observations have shown that toxin production can be up to 13 times higher in the field than in corresponding laboratory cultures (Montoya et al., 2010). However, care has to be taken when comparing PST production of laboratory cultures, as it can vary considerable, even between cultures of the same strain (3.51 – 21.23 pg STX.2HCl equiv. $cell^{-1}$ in our case). Several abiotic and biotic factors (e.g. microbiome) are known to influence PST production by *A. catenella* (e.g. Griffin et al.,

2019, Laabir et al., 2013, Hold et al., 2001) and it appears likely that intra- and extracellular toxin quotas would differ between *A. catenella* cells in the field and the laboratory cultures used in this study (Hallegraeff et al., 2018; Sekiguchi et al., 2001).

A. catenella blooms in Tasmanian waters can persist longer than our 28 day exposure period (3–6 months, Condie et al., 2019), with periods of increased exposure to sedimented algae and extracellular PST during bloom termination. Suspension of live *A. catenella* cells in the water column (as in our experimental exposures) would reduce the number of microalgal cells available for grazing and/or epipodial uptake through direct contact, while abalone would still have been exposed to extracellular PST (not quantified). Prolonged retention of PST in wild South African *H. midae* (>7 months; Pitcher et al., 2001, Etheridge et al., 2002) suggest slow depuration rates, and the potential for cumulative PST uptake over subsequent bloom seasons. This, however, does not appear to be the case for wild Tasmanian Blacklip Abalone populations exposed to bloom episodes of *G. catenatum*, which are known to depurate PST and only retain low levels between bloom episodes (McLeod et al., 2017). Comparable low background levels of PST were detected here in wild South Australian Blacklip Abalone sourced from an area where no PST monitoring currently occurs. While PST levels were low (<38 μg STX.2HCl equiv. kg^{-1}), this detection serves as a reminder for industry to consider developing biotoxin response/management plans for non-traditional PST vectors.

5. Conclusions

Effective management of abalone as non-traditional vectors of PST poses a challenge, as comparatively little is known about uptake mechanisms and depuration rates. The tank experiments for the first time proved direct PST uptake by Blacklip Abalone from PST producing dinoflagellate cells from tank experiments, along with uptake from PST rich feed pellets. While the PST source was dominated by gonyautoxins (GTX1&4 and GTX2&3 from feed pellet or *A. catenella* cells), saxitoxin and neosaxitoxin were predominantly observed in abalone foot tissues. These results closely align with field observations from the east coast of Tasmania and provide strong evidence that direct exposure to *A. catenella* is driving elevated PST levels in Blacklip Abalone on the Tasmanian east coast. While the present results newly confirm oral uptake of PST from both toxic feed pellet and microalgal cells, the potential contribution of PST transfer across the epipodium into the foot remains to be quantified. As previously suggested, higher PST levels in the epipodium offer the option of significantly reducing toxicity through scrubbing, although associated value reduction (live abalone vs. processed) is currently not an attractive option to industry.

Author declaration

This manuscript has not been published and is not under consideration for publication elsewhere. A heavily modified and preliminary version will be submitted as a chapter in the final report to the funding body (Fisheries Research and Development Corporation). We have no conflicts of interest to disclose.

The submitted version of this publication has been viewed, and is approved by all authors. Author contributions are as follows:

Author	Funding/ conception	Design	Practical	Analysis	Written
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Gustaaf Hallegraeff	X	X			X
David Stone		X	X		X
Matthew Bansemer		X	X		X
Tim Harwood	X		X		X
Alison Turnbull	X	X	X	X	X

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank Natalie Dowsett (SARDI) for helpful advice on experimental design, Marty Deveney for making the South Australian Aquatic Biosecurity Centre available for the experimental work and Chris Bolch for providing the *A. catenella* culture strain. Further thanks are due to Stephen Mayfield and Lachlan McLeay (Primary Industries and Regions South Australia) for organising collection permits, as well as Andrew Hogg and his team of divers (SARDI) to collect the animals required for the experimental work. Last but not least, we would like to thank our technicians Elliot Brown, Jan Lee and Geoff Holds, who did a marvellous job maintaining the day to day running of the biosecurity facility. This project was supported by Fisheries & Research Development Corporation, Abalone Council of Australia, and SARDI under FRDC grants no. 2017-225 and 2017-051.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hal.2020.101925.

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