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Uptake, distribution and depuration of paralytic shellfish toxins from *Alexandrium minutum* in Australian greenlip abalone, *Haliotis laevigata*

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ABSTRACT

Farmed greenlip abalone *Haliotis laevigata* were fed commercial seaweed-based food pellets or feed pellets supplemented with 8×10^5 *Alexandrium minutum* dinoflagellate cells g^{-1} (containing 12 ± 3.0 μg STX-equivalent 100 g^{-1}, which was mainly GTX-1,4) every second day for 50 days. Exposure of abalone to PST supplemented feed for 50 days did not affect behaviour or survival but saw accumulation of up to 1.6 μg STX-equivalent 100 g^{-1} in the abalone foot tissue (muscle, mouth without oesophagus and epipodial fringe), which is ~50 times lower than the maximum permissible limit (80 μg 100 g^{-1} tissue) for PSTs in molluscan shellfish. The PST levels in the foot were reduced to 0.48 μg STX-equivalent 100 g^{-1} after scrubbing and removal of the pigment surrounding the epithelium of the epipodial fringe (confirmed by both HPLC and LC-MS/MS). Thus, scrubbing the epipodial fringe, a common procedure during commercial abalone canning, reduced PST levels by ~70%. Only trace levels of PSTs were detected in the viscera (stomach, gut, heart, gonad, gills and mantle) of the abalone. A toxin reduction of approximately 73% was observed in STX-contaminated abalone held in clean water and fed uncontaminated food over 50 days. The low level of PST uptake when abalone were exposed to high numbers of *A. minutum* cells over a prolonged period may indicate a low risk of PSP poisoning to humans from the consumption of *H. laevigata* that has been exposed to a bloom of potentially toxic *A. minutum* in Australia. Further research is required to establish if non-dietary accumulation can result in significant levels of PSTs in abalone.

1. Introduction

Paralytic shellfish toxins (PSTs) are the most common and widespread marine biotoxins detected globally (Whittle and Gallacher, 2000). Currently more than 30 closely related analogues of PSTs have been identified, with saxitoxin (STX), neosaxitoxin (NEO) and gonyautoxins (GTX1, GTX2, GTX3, GTX4) considered to be the most potent to marine animals and humans (Llewellyn et al., 2006; Oshima, 1995). These toxins are water soluble and heat stable and have been confirmed as causing the toxic syndrome known as paralytic shellfish poisoning (PSP) in humans, primarily via the consumption of bivalve shellfish that have accumulated PSTs from the marine environment in which they live (Llewellyn et al., 2006; Fonfria et al., 2007; Garcia et al., 2004).

PSTs have been identified as being produced by a number of toxic dinoflagellate species of microalgae

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(Arnott, 1998; Negri et al., 2003; Oshima et al., 1989). To date, nine species of *Alexandrium* have been identified in the south-eastern waters of Australia (*Alexandrium affine, Alexandrium catenella, Alexandrium concavum, Alexandrium minutum, Alexandrium margalefi, Alexandrium ostenfeldii, Alexandrium pseudogonyaulax, Alexandrium peruvianum, Alexandrium tamarense*), with seven having been cultured and tested for toxicity (Hallegraeff et al., 1991). Only *A. minutum* and *A. catenella* have been found to produce PSTs (mainly STX and its analogues) in Australia. The other key potential PST-producing species of marine dinoflagellate detected in Australian waters is *Gymnodinium catenatum*, which has been found in Tasmania, South Australia, Victoria and New South Wales (Hallegraeff et al., 1995; Negri et al., 2003). *Pyrodinium bahamense* var. *compressum* also produces PST and is found in tropical waters to the north of Australia in the Philippines, Indonesia and Papua New Guinea (Alexander et al., 2009; Hallegraeff et al., 1991; Hallegraeff and Jeffrey, 1984; Van Egmond et al., 2004).

Abalone are marine gastropod molluscs in the Haliotidae. There are approximately 100 species of abalone worldwide and the majority are found in colder waters (Gilroy and Edwards, 1998). In Australia, *Haliotis roei* (Roes), *Haliotis laevigata* (greenlip) and *Haliotis rubra* (blacklip) are the main abalone species harvested commercially (Freeman, 2001). The industry in Australia consists of both wild-caught and aquaculture sectors. The farmed industry mainly cultivates greenlip abalone as they are generally more tolerant of shallow, warm and turbid waters and are easily grown on commercial feed mixtures. Blacklip are the most important species of wild-caught abalone, including for export. These animals typically live around rocky inshore areas, of approximately 20–40 m depth from the Australian coastline (Gilroy and Edwards, 1998) which falls within the United Nations Food and Agricultural Organisation (FAO) fishing zone 57.

Abalone preferentially feed on kelp and other species of macroalgae by capturing them beneath their foot, then grazing on them using their radula (Freeman, 2001; Pitcher et al., 2001; Shepherd, 1973). In contrast to Australia, abalone harvested from South Africa and Spain have been found to contain significant levels of PSTs (up to 1609 μg STX-equivalent 100 g^{-1} in South Africa and 443 μg STX-equivalent 100 g^{-1} in Spain), but the source and the mechanism of uptake of the toxins by abalone has not been determined (Bravo et al., 1996, 1999; Etheridge et al., 2004; Nagashima et al., 1995; Pitcher et al., 2001). Several modes of PST uptake by abalone are possible, including:

(a) uptake of PST-producing bacterial cells, though bacterial PST production remains unproven (Baker et al., 2003; Bravo et al., 1996);
(b) PST production by macroalgae that the abalone feed on (Etheridge et al., 2004; Kotaki et al., 1983; Llewellyn et al., 2006); and
(c) uptake of toxic dinoflagellate and cyanobacterial cells or cysts that are trapped in the mucosal sheath surrounding the macroalgae that the abalone feed on (Bravo et al., 1996; Pitcher et al., 2001).

In Australia, the Food Standards Code and the Australian Shellfish Quality Assurance Program (ASQAP) define shellfish as 'all edible molluscan bivalves' (Anon, 2010; PIRSA, 2009). For these animals, it is an Australian standard that they are sourced from classified areas that have routine marine biotoxin monitoring programs in place. Abalone, as gastropods, are not subject to these regulations and are considered to be at lower risk of biotoxin and microbial contamination than bivalves. Consistent with this, there have been no recorded instances of human illness relating to marine biotoxin contamination of abalone compared with many outbreaks related to bivalves internationally.

Prior to July 2010, Australian abalone exported to the European Union (EU) were required to be sourced from production areas that met the requirements of European Commission (EC) regulation 854/2004 (Anon, 2004). This regulation stipulated that abalone must be obtained from production areas that were classified and had biotoxin monitoring programs in place (with a baseline of weekly monitoring). Due to the vast size of the FAO fishing zone in which Australian abalone fishers operate and the variability in the areas fished, meeting this requirement was problematic for the Australian abalone industry. Export of abalone to the EU therefore ceased between March 2007 and July 2010, as Australia did not satisfy EC requirements.

As a result of the EU market closure, preliminary experiments were undertaken to assist the wild-caught abalone industry regain market access to the EU. Production of PSTs by bacteria and macroalgae consumed by abalone remains largely unproven, and it was considered that the risk of potential uptake of PSTs by abalone is most likely related to the consumption of toxic dinoflagellate cells and cysts. Therefore, this research was undertaken to investigate if uptake of PST-producing dinoflagellate cells could result in retention of PSTs in abalone tissues. The specific aims of the experiments were to ascertain if:

1. Australian abalone are able to accumulate toxins when exposed to PSTs from *A. minutum* cells incorporated in feed;
2. Distribution of PSTs differs between the edible and non-edible portions of abalone;
3. Commercial processing reduces the levels of PST in the edible portions of abalone.

Coincidentally our experimental results also allowed us to investigate whether abalone depurate PSTs when placed in uncontaminated seawater and fed uncontaminated feed.

2. Materials and methods

2.1. A. minutum *culture growth and maintenance*

A. minutum was chosen for this work because it is a PST-producing species of microalgae that has been detected in abalone harvesting areas around Australia and is relatively easy to culture in the laboratory. The strain of *A. minutum* used was obtained from the CSIRO Collection of Living Microalgae, code CS-323/06 (also known as AMAD 06) and

was isolated in October 1987 by J. Cannon and S. Blackburn from the Port River in Adelaide, South Australia (Hallegraeff et al., 1991). The predominant toxins produced by this strain of *A. minutum* are GTX-1,4, however low levels of GTX-2,3 have also been reported (Lippemeier et al., 2003; Negri et al., 2003). This strain was first confirmed to produce PSTs in 1989 (Oshima et al., 1989) and was fully characterised in 1991 (Hallegraeff et al., 1991).

Stock cultures of *A. minutum* maintained in GSe modified medium (Blackburn et al., 2001) in 60 mL tissue culture flasks were used to establish experimental cultures in 600 mL tissue culture flasks with initial densities of $\sim 4 \times 10^3$ cells mL^{-1}. High biomass batch cultures were set up from the experimental cultures in 2 L Pyrex bottles, with initial densities of $\sim 6.5 \times 10^3$ cells mL^{-1}. All cultures were maintained at 21 °C ± 1 °C, with cool white fluorescent illumination (90 μmol photon m^{-2} s^{-1}) and a 12:12 light:dark cycle. The high biomass cultures were aerated (air/CO_2 100:1, v/v). When these cultures reached $\sim 4 \times 10^5$ cells mL^{-1} they were used to establish 2 × 15 L high biomass cultures in 20 L carboys (initial densities $\sim 2 \times 10^4$ cells mL^{-1}) which were maintained as outlined above. Algal cells were harvested from the carboys (in stationary phase ~164,000 ± 5000 cells mL^{-1}) by centrifugation then incorporated into abalone feed pellets as described in Section 2.3.

PST production during the growth of the culture was confirmed by taking three separate 250 mL samples from the high biomass culture at stationary phase and centrifuging them for 5 min at 3000 × gravity. The supernatant from each sample was decanted and discarded and the cellular pellet was frozen to −20 °C to stabilise the cells. The pellets were then thawed and re-extracted using 5 mL of 0.01 M acetic acid, then sonicated for 5 min to release the toxin from the cells. The extracts were then re-frozen to −20 °C and toxin analysis was undertaken (Section 2.5).

2.2. Abalone stock

Commercial farm-reared greenlip abalone (*H. laevigata*) (approximately 80 mm shell length) were used throughout the study. Assessment of the animals' physical condition, including total weight and stimulated reflex action of the mantle, was checked on receipt from the farm before they were translocated into a 5000 L polyethylene aquarium at a density of one abalone per 100 L seawater. The aquarium was maintained with flow-through seawater (~37.5 psu; 100 L min^{-1} flow), which was piped in from 1.45 km offshore, and underwent several purification steps (screen filtration, settlement, sand filtration and then held in holding tanks) before being introduced into the system. The stock abalone were maintained under ambient environmental conditions with aeration sufficient to ensure oxygen saturation, and at a temperature of 16.5 ± 2.6 °C. Prior to experimental work being undertaken, the abalone were held in the 5000 L stock aquarium for a minimum two week acclimatisation phase following relocation from the farm, to identify animals that showed signs of disease or abnormal behaviour. During this period, the animals were fed 0.5 g per animal of 2 mm Eyre Peninsula Aquafeeds (Cummins, South Australia) commercial abalone pellet diet every second day. The abalone were accustomed to this diet on receipt at the experimental facility. Daily maintenance of the abalone in the stock aquarium included 50% water changes, siphoning of waste (left over feed and faeces), nitrate (NO_3) measurements, monitoring of temperature and checking the delivery of dissolved oxygen to the aquarium. Abalone health was also assessed by performing shell-lift checks to establish a strength response of the animal. A strong unwavering suction was interpreted as a healthy response.

2.3. Toxic feed production

The most likely mode of abalone intoxication is the direct uptake of toxic dinoflagellate cells or cysts that have become entrapped in the mucosal sheath surrounding the macroalgae on which abalone graze. In order to mimic this process experimentally, feed pellets were manufactured containing toxic dinoflagellate cells.

2.3.1. Incorporation of A. minutum into abalone feed pellets

Two batches of homogenous feed containing different concentrations of PSTs were produced by substituting the water component of the feed with *A. minutum* cell concentrates. To prepare the toxic pelleted feed batches, cell counts on the high biomass culture of *A. minutum* were undertaken using a Sedgwick rafter chamber (Seamer, 2001) to determine when the algae was in stationary phase. Two batches (3.75 L and 10.0 L) of stationary phase culture were centrifuged (in 50 mL aliquots) for 5 min at 3000 × gravity. The resultant pellets for each batch were then resuspended together in a total of 300 mL of cell culture supernatant. The two cell concentrates were then added to a feed mash and binding agent (Eyre Peninsula Aquafeeds Pty Ltd commercial mash and binding agent, Cummins, South Australia) in place of the water component. The low PST dose feed contained 8×10^5 cells g^{-1} feed, and the high dose contained 2×10^6 cells g^{-1} feed. To ensure that the *A. minutum* cells were equally distributed throughout the feed, a commercial grade Hobart™ Planetary A200C 20 L mixer was used to consistently knead the mixture while the cell concentrate was gradually added via a sterile syringe. The mixtures were then extruded twice through a Fimar™ commercial grade pasta machine to ensure that the end products were densely compacted. The toxic feed batches were then dried overnight at 40 °C and broken up to produce a palatable pellet. Dry weight measurements were conducted by subsampling 3 × 1 g portions of each of the feed batches. The samples were then incubated at 105 °C overnight and reweighed to determine the loss in mass.

To maintain freshness and toxin stability, the feed was vacuum sealed then stored at −20 °C. The level of PSTs present in the feed was determined for 3 × 0.5 g sub-samples of each batch at the time of production. In addition, 3 × 0.5 g sub-samples of the low dose feed was analysed following the 50 day archival period (to determine any degradation over time or due to freezing). As described in Homan et al. (2010), palatability trials were undertaken on the low and high dose feeds to determine which feed was preferential for the abalone and the low dose feed was selected for the toxin accumulation trial (Homan et al., 2010).

2.4. Toxin accumulation trial

Abalone were selected in a non-systematic random manner from the stock aquarium for inclusion in the following toxin accumulation trial described below.

2.4.1. Toxin uptake and tissue distribution study

Ten *H. laevigata*, weighing an average of approximately 87 g (total weight including shell), were transferred from the 5000 L stock aquarium to individual aquaria (300 × 300 × 500 mm), each containing 12–15 L of static aerated seawater. Following an initial six day acclimatisation period (three feeding cycles) during which feed was withheld, the animals were each fed 0.5 g of the low dose manufactured feed, which contained approximately 410,000 *A. minutum* cells, every second day over a period of 50 days (26 feeding cycles). No food was provided on alternate days. During each feeding cycle, the uneaten portions were removed from the aquaria 24 h after provision of food, dehydrated and weighed to determine the proportion of feed that was consumed throughout the 50 day experiment. To determine the dry weight of leftover feed, the abalone faeces were first removed from the aquaria and discarded. The uneaten food was then collected in a fine mesh net, transferred to ceramic pots, dried overnight at 105 °C and weighed. The amount of food consumed by individual abalone was calculated as follows:

[dry weight of food presented to abalone (0.5 g)] – [5% loss to system] – [dry weight of left over food] = food consumed by abalone.

A 5% loss of food to the system was included in the calculations to account for particles that were unable to be recovered and weighed due to dissipation of the food into fine particles in the water column. This proportion of loss was demonstrated in separate abalone nutritional studies (Dr David Stone, SARDI Aquatic Sciences, personal communication).

At the completion of the study, the surviving nine abalone were euthanased by sedating them in a slurry of ice water before severing their foot from their shell. Six out of the nine animals were scrubbed prior to analysis (mimicking the commercial canning preparation) to remove the pigment on the epipodial fringe and to determine if this reduced toxin levels. All nine animals were then dissected into foot tissue (muscle, mouth without oesophagus and epipodial fringe) and viscera (stomach, gut, heart, gonad, gills and mantle). All tissues were frozen to −20 °C and analysed via HPLC analysis (as described in Section 2.5) to determine the quantity of PSTs present.

2.4.2. Control animals

Three groups of control animals were analysed throughout the experiments ('pre-experimental', 'post-experimental' and 'farm-direct'). The pre and post-experimental controls ($n = 7$ each) were harvested from the stock aquarium prior to and following the 50 day toxin uptake and distribution study (Day 0 and Day 50 respectively) (Section 2.4.1). The farm-direct control animals ($n = 3$) were requested directly from the farm of origin and were not introduced into the stock aquarium prior to analysis. These animals were received from the farm 36 days after the experimental abalone were received. All control animals were dissected into foot and viscera portions, but were not scrubbed prior to analysis.

2.5. Toxin quantification and confirmation

2.5.1. Sample preparation

Extraction, oxidation and quantitation of abalone tissue sample homogenates was done according to AOAC Official Method 2005.06 Paralytic Shellfish Poisoning Toxins in Shellfish (Lawrence et al., 2005). As discussed previously, several foot samples were scrubbed prior to analysis to remove the pigment. Algal extracts (Section 2.1) in 0.01 M acetic acid were defrosted and centrifuged at 3000 × gravity for 10 min. The supernatant was then cleaned up over an SPE C18 cartridge.

Feed samples (Section 2.3.1) were extracted by boiling 0.5 g of the feed with 3 mL of 1% acetic acid for 5 min, followed by cooling in ice and centrifugation at 3000 × gravity for 10 min. The supernatant was decanted then the remaining pellet was extracted twice more and the extracts were combined. The volume was made up to 10 mL with water and mixed. Further processing followed the published protocol (Lawrence et al., 2005).

No matrix modifier was used for the periodate oxidations. Spiking experiments on abalone foot and viscera samples showed the oxidation yields for these samples were similar to those obtained for the standards (close to 100%) so no recovery factors were applied. Since the sample contained very little GTX-2,3, the third oxidation peak of GTX-1,4 was used for quantitation because it showed better calibration and stability as well as a lower limit of detection than the second oxidation peak used in the published method (Lawrence et al., 2005). The second peak was used for confirmation.

2.5.2. High Pressure liquid chromatography (HPLC) analysis

HPLC analysis was performed on a Waters Acquity UPLC system (Waters, Milford, MA) coupled to a Waters Acquity FLR detector. Separation was achieved with a Waters Acquity C18 BEH 1.7 μm 2.1 × 50 mm column at 30 °C, eluted at 0.2 mL min^{-1}. Mobile phases were 0.1 M ammonium formate (A) and 0.1 M ammonium formate in 5% acetonitrile (B), both adjusted to pH 6 (for feed samples a mobile phase at pH 4.2 was used). The gradient consisted of 100% A for 0.5 min, a linear gradient to 80% B over 3.5 min, then returning to initial conditions over 0.1 min and held for 1.9 min. The fluorescence detector had excitation set to 340 nm and emission to 395 nm.

2.5.3. Analytical standards

Analytical standards of PSTs, including NEO, STX, GTX-1,4 and GTX-2,3, were obtained from the National Research Council Canada. Each toxin in the abalone, feed and *A. minutum* samples was quantitatively determined by direct comparison with the analytical standards. For the determination of GTX-1,4 in feed samples the pH of the mobile phases was changed to 4.2 to separate interfering peaks from the third oxidation peak of GTX-1,4. The detection limit for GTX-1,4 and STX were 1.0 and 0.20 μg

100 g^{-1} in the foot tissue and 4.0 and 2.0 μg 100 g^{-1} respectively for the visceral portions.

2.5.4. Liquid chromatography mass spectrometry (LC-MS/MS) analysis

LC-MS/MS analysis was undertaken to confirm the identity of STX detected by HPLC in abalone foot tissue. The homogenates of four archived abalone foot samples, previously found to contain STX by HPLC, were combined to give a 33 g mixed foot sample. This was first extracted with 30 mL of 1% acetic acid, then boiled for 5 min. The sample was then extracted again with 30 mL 1% acetic acid and the two extracts combined. The combined extracts were then purified over a 20 g Strata-X polymeric sorbent cartridge to remove any lipophilic compounds. The purified extract was then evaporated at 30 °C under vacuum until approximately 6 g was left. At that point a substantial amount of salt became visible. To desalt the sample 44 mL of ethanol was added and mixed well. 20 μL of acetic acid was also added to stabilize the PSP toxins. The sample was left overnight at 4 °C and centrifuged at 3000 × gravity for 10 min. The supernatant was decanted and evaporated at 30 °C under vacuum until the volume was reduced to 2 mL. To confirm the presence of STX the sample was then analyzed by HPLC using a post-column oxidation method (Rourke et al., 2008) and also by LC-MS.

LC-MS/MS was performed on a Waters Acquity UPLC system (Waters, Milford, MA) coupled to a Waters–Micromass Quattro Premier XE triple quadrupole mass spectrometer (Manchester, UK) with electrospray source. Separation was achieved with a Primesep 500 5 μm 2.1 × 150 mm column (SIELC, Illinois, USA) at 21 °C, eluted at 0.4 mL min^{-1} with linear gradients of 50 mM ammonium formate in 50% acetonitrile (A) and 50 mM formic acid in 50% acetonitrile (B). The gradient consisted of 0–15% B over 3 min, increasing to 35% B at 4 min, increasing to 45% B at 6 min and held for 1 min before returning to initial conditions. The electrospray ionization source was operated in positive-ion mode at 100 °C, desolvation temperature 400 °C, capillary 3 kV, cone 45 V, nitrogen gas desolvation 900 L h^{-1} (350 °C) and cone gas 50 L h^{-1}. The collision energy was set at 20 eV for NEO and 25 eV for STX with 3 mbar argon. Parent ions of m/z 316 for NEO and m/z 300 for STX were fragmented and fragment ions were scanned from m/z 50–320.

To confirm PST identity, a daughter ion scan of both STX and NEO was performed on a concentrated abalone foot extract, and certified standards of both compounds and the spectra were compared.

2.6. Statistical analysis

All data analyses were performed using the R software version 2.10.1 (R Core Development Team, 2009). Summary measures are presented in the form of mean ± standard deviation, calculated from the untransformed analytical results.

The five treatment groups: farm controls, pre-experimental controls, post-experimental controls, exposed not scrubbed and exposed scrubbed, were tested for significant differences in the mean $\log_{10}$ STX concentrations using an Analysis of Variance (ANOVA) – the analytical results were transformed to the $\log_{10}$ scale to satisfy the assumptions for the statistical analysis. The assumption of equal variance was assessed using Bartlett's test of homogeneity of variance (Bartlett, 1937). The fit of the ANOVA model was assessed using standard diagnostic plots. Unless otherwise stated statistical analyses have been undertaken with all data, including outliers.

Comparisons between the pairs of treatments were made using Tukey's Honest Significant Differences (TukeyHSD) including adjustments for unequal sample sizes in the treatment groups (Yandell, 1997).

3. Results

3.1. Animal husbandry

The *H. laevigata* used in these studies were received from the farm of origin in good health. Throughout the entire experimental period (acclimatisation through to accumulation study) they were observed consuming food and there were no mortalities of control animals. In the accumulation study, dry weight measurements of leftover feed demonstrated that the abalone were consuming toxic feed on every feeding occasion. Shell-lift measurements and non-elevated nitrate levels indicated that the abalone were in good health throughout the exposure period, however there was one mortality of an experimental animal on Day 18 of the study (see Section 3.4.1).

3.2. Control animals

PSTs were not detected in the viscera of any control abalone throughout these experiments. The foot tissues of the pre-experimental control abalone (that had been subjected to 15 days acclimatisation prior to the start of the uptake and distribution study) contained minute, but detectable, levels of STX (0.77 ± 0.43 μg STX-equivalent 100 g^{-1}). The mean and standard deviation are inflated due to the presence of an outlier in this group (Fig. 1). The corresponding animal was re-tested without change in analytical result and no explanation for this abnormally large value could be found. Excluding this outlier, STX levels of 0.61 ± 0.13 μg STX-equivalent 100 g^{-1} were detected in the pre-experimental controls.

Following the detection of baseline levels of STX in the 'pre-experimental control' animals, additional animals were requested directly from the farm of origin ('farm-direct controls') to determine if the toxin was introduced into the abalone in the stock aquarium, or if in fact baseline levels might have existed prior to the experiment. Testing of farm-direct abalone controls showed very low, but detectable levels of STX in the edible portion (0.57 ± 0.08 μg STX-equivalent 100 g^{-1}) (Fig. 1). There was no significant difference in mean $\log_{10}$ STX content between the pre-experimental controls and the farm-direct controls ($P = 0.94$). This indicated that the baseline levels of STX were likely to have been present upon receipt of the animals from the farm and suggested that the toxin was not introduced from the experimental holding system.

In the post-experimental control abalone, which were fed with uncontaminated food for the duration of the

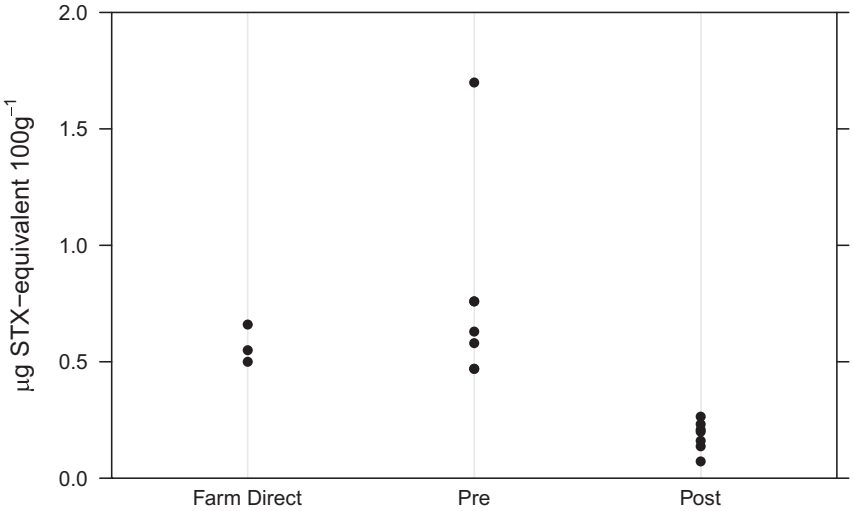

Fig. 1. Levels of STX detected in abalone foot samples: Farm-Direct = direct from the farm of origin 36 days post receipt of experimental animals; Pre = at the start of the experiment after 15 days maintenance in a toxin-free stock aquarium; and Post = at the end of the 50-day experiment (after a total of 65 days maintenance in a toxin-free stock aquarium). Limit of detection of STX in abalone foot tissue = 0.20 µg 100 g^{-1}.

experiment, an average STX level of 0.21 ± 0.03 µg STX-equivalent 100 g^{-1} was detected in the foot tissue (Fig. 1). This was significantly less than the STX levels in the foot tissue of both the pre-experimental controls ($P = 0.00001$) and the farm-direct controls ($P = 0.0018$). Comparison of the levels of STX in the foot tissues of the post-experimental controls and the pre-experimental controls indicated a reduction of approximately ~73% over the 50 days of the experiment.

3.3. Toxin composition profiles

3.3.1. Algae and feed

The *A. minutum* culture predominantly contained GTX-1,4 (Fig. 2, Panel A). Levels of STX and GTX-2,3 below the level of quantitation were also detected (Fig. 3, Panel D). Using HPLC analysis, sub-samples of the *A. minutum* culture sampled at stationary growth phase (~164,000 ± 5000 cells mL^{-1}) contained approximately 0.09 ± 0.007 pg GTX-1,4 $cell^{-1}$. GTX-1,4 was also the predominant toxin detected in the low dose manufactured feed used in the accumulation study (Fig. 2, Panels E & F). Three sub-samples of the low dose feed analysed immediately after production were found to contain 12 ± 3.0 µg STX-equivalent 100 g^{-1}. Three additional samples were re-analysed following a 50 day period of storage at −20 °C and contained 14 ± 0 µg STX-equivalent 100 g^{-1} of toxin. Results from analysis of samples of blank feed ('PST free') confirmed the absence of PSTs.

3.3.2. H. laevigata tissues

GTX-1,4 and GTX-2,3 were not detected in any *H. laevigata* tissues throughout this study (Fig. 2, Panel C). In the

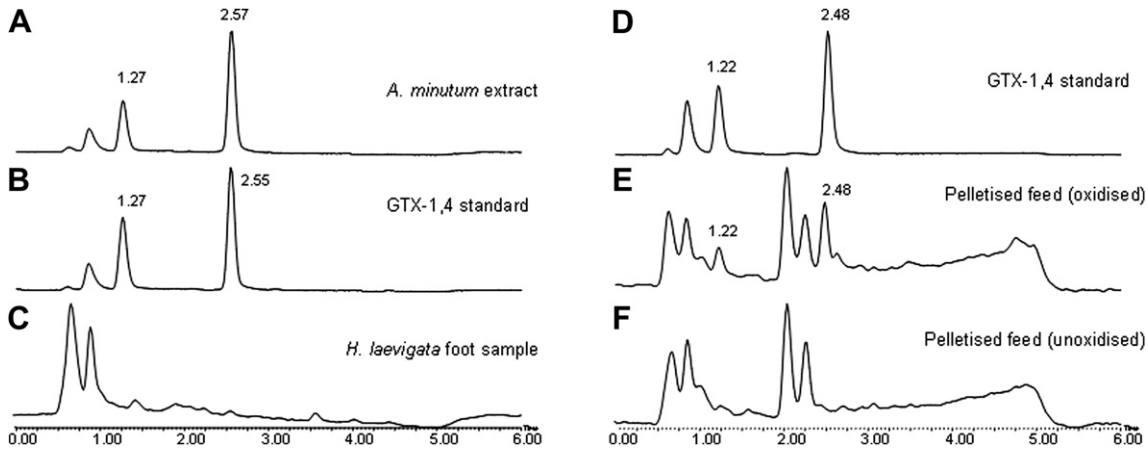

Fig. 2. HPLC (utilising fluorescence detection) chromatograms (generated using a mobile phase at pH 6) of periodate oxidised GTX-1,4 detected in an extract of *A. minutum* culture (Panel A), a certified reference standard (Panel B), and an extract of an *H. laevigata* foot tissue (Panel C). Panels D and E show HPLC chromatograms (generated using a mobile phase at pH 4.2) of periodate oxidised GTX-1,4 detected in a certified reference standard and in an extract of the toxic feed pellets, respectively. While Panel F shows the HPLC chromatogram of a non-oxidised toxic feed pellet sample.

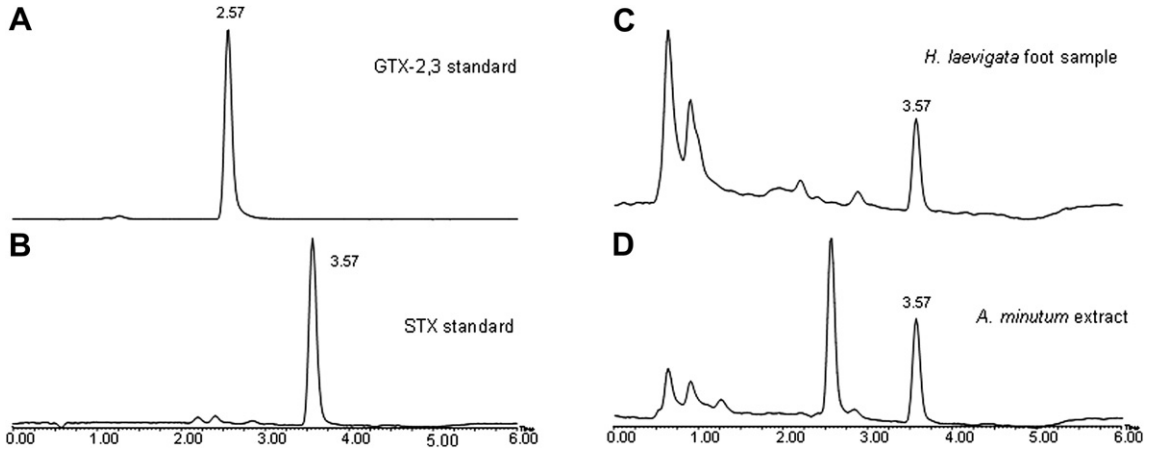

Fig. 3. HPLC (utilising fluorescence detection) chromatograms (generated using a mobile phase at pH 6) of peroxide oxidised GTX-2,3 (Panel A) and STX (Panel B) in certified reference standards, STX in an extract of an *H. laevigata* foot sample (Panel C) and GTX-2,3 and STX in an extract of *A. minutum* culture sub-sample (Panel D).

samples of *H. laevigata* analysed by HPLC in this study, only the foot tissue contained quantifiable levels of STX (Fig. 3, Panel C). Trace quantities of NEO were also detected in these samples (data not shown). The viscera of some animals contained trace amounts of STX, however this was unable to be accurately defined as the levels detected were below the limit of quantification for the method (detection limit for viscera = 4.0 and 2.0 μg 100 g^{-1} respectively for GTX-1,4 and STX) (data not shown).

3.4. Toxin accumulation trials

3.4.1. Uptake and distribution study

The dry weight measurements taken during the uptake and distribution study demonstrated that each of the ten animals were consuming, on average, 0.33 ± 0.09 g of the toxic food at each feeding occasion (Table 1).

Nine of the ten abalone exposed to toxic feed during the uptake and distribution study survived the full 50 day exposure treatment. One abalone died on day 18. The cause of this mortality is unknown and daily observations had detected no signs of changed behaviour or disease. All control abalone survived through the duration of the uptake and distribution study.

Table 1
Average (mean) weight of toxic feed containing mainly GTX-1,4 (12 μg STX-equivalent 100 g^{-1}) consumed by individual abalone (*H. laevigata*) on each feeding occasion (26 feeding days) during a 50 day period.

Abalone ID	Mean weight consumed per feeding occasion (mean g ± SD)
1	0.42 ± 0.10
2	0.33 ± 0.13
3	0.11 ± 0.54
4	0.32 ± 0.15
5	0.41 ± 0.18
6[a]	0.31 ± 0.30
7	0.40 ± 0.45
8	0.33 ± 0.23
9	0.35 ± 0.23
10	0.30 ± 0.19

[a] Animal 6 died on exposure day 18.

Following the exposure period, the abalone were dissected into foot tissue and viscera. On average, the foot tissue weighed 19.02 ± 1.89 g and the viscera weighed 13.94 ± 3.44 g. Trace levels of PSTs were detected in some of the viscera samples taken from the abalone exposed to the toxic feed for 50 days, but quantifiable levels were detected in the foot tissue of these animals. The pre-experimental control animals contained significantly less STX (0.77 ± 0.43 μg STX-equivalent 100 g^{-1}) than the animals exposed to the toxic feed for 50 days that did not undergo commercial scrubbing ($P = 0.048$)[1]. Abalone that had the epipodial fringe surrounding the foot tissue scrubbed (as would occur in commercial processing) contained 0.48 ± 0.20 μg STX-equivalent 100 g^{-1}; significantly less than those that were not scrubbed, which contained 1.6 ± 0.27 μg STX-equivalent 100 g^{-1} ($P = 0.0015$) (Fig. 4).

3.4.2. Confirmation of PSTs in uptake abalone by LC-MS/MS

The presence of STX (Fig. 5, Panels A & B) and NEO (Fig. 5, Panels C & D) in a mixed abalone foot tissue sample was confirmed by LC-MS/MS and daughter ion scans. 400 ng of STX was found in the mixed foot tissue sample. Retention times and fragmentation patterns of both compounds in the concentrated extract were identical to certified standards and conclusively confirmed the presence of these toxins in the abalone foot tissue.

4. Discussion

4.1. Uptake of PSTs by H. laevigata

H. laevigata can accumulate very low levels of PSTs after ingesting feed pellets that contain toxic *Alexandrium minutum* cells. Following uptake of the toxic feed pellets,

[1] Note that this marginal *P*-value is substantially inflated due to the presence of one extreme outlier in the pre-experimental treatment group. Excluding this outlier from the analysis results in a *P*-value of 0.0055.

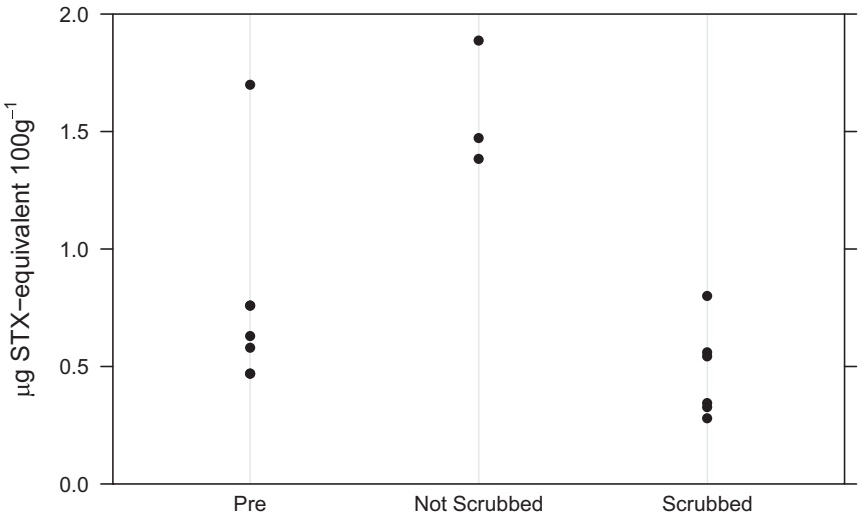

Fig. 4. Levels of STX detected in abalone foot samples: Pre = after two weeks maintenance in a toxin-free stock aquarium; Not Scrubbed = following 50 days of feeding abalone 'low dose' toxic feed pellets; and Scrubbed = following 50 days of feeding abalone 'low dose' toxic feed pellets plus removal of pigment by scrubbing prior to toxin analysis. Limit of detection of STX in abalone foot tissue = 0.20 μg 100 g^{-1}.

quantifiable levels of STX and trace levels of NEO were found in the foot tissue of the abalone, however only trace levels of STX (non quantifiable) were identified in the viscera. The unexpected low trace levels of STX in the viscera of animals used in this study may be, in part, due to the low overall level of uptake of toxins by the animals and a higher limit of detection for STX in the viscera compared with the foot tissue. Consistent with the results of this study, Bravo et al. (1996) and Nagashima et al. (1995) also found significantly higher levels of PSTs present in abalone foot tissue (220 μg STX-equivalent 100 g^{-1} and 10600 Mouse Units 100 g^{-1} respectively) than the viscera (104 μg STX-equivalent 100 g^{-1} and 1560 MU 100 g^{-1} respectively).

While final levels of PSTs in foot tissue were extremely low, each abalone ingested ~1.02 μg STX-equivalents (on average) over the course of the 50 day uptake period, and 16.5% of the ingested toxin was retained in the foot tissue at the end of the exposure period (as calculated on a molar basis). It is important to acknowledge that in a natural environment abalone graze on macroalgae in the benthic zone whereas *A. minutum* cells are generally suspended in the water column and migrate to modulate light and nutrient availability. Therefore, despite the retention of PSTs in the abalone foot tissue when fed artificially contaminated pellets, it is unlikely that abalone are able to consume large numbers of toxic *A. minutum* cells due to the occupation of different zones in the marine environment.

4.2. Toxin composition

Different analogues of PSTs were detected in the algae and feed samples (predominantly GTX-1,4) than those that were in the abalone tissue (predominantly STX). This suggests that there is significant metabolism or interconversion of these toxins in *H. laevigata*. Since these animals were maintained in a closed laboratory system, the only source of toxin was the feed containing GTX-1,4. Similarly, *H. midae* from South Africa were found to contain only STX (Nagashima et al., 1995) indicating biotransformation of PSTs occurs in several species of abalone. This is likely to be due to physico-chemical reactions that are catalysed by enzymes and bacteria contained within the gut of the shellfish (Dell'Aversano et al., 2008; Jaime et al., 2007). As shown in Section 3.3.1, the GTX-1,4 content of the feed samples did not decrease after 50 days storage at −20 °C. This demonstrates that there was no degradation or loss of the toxin in the feed samples or interconversion of GTX-1,4 into other PST analogues during storage.

4.3. Toxin reduction via scrubbing process

While the experimental abalone foot tissue accumulated levels of STX significantly higher than those found in control animals, additional results showed that the removal of the pigment on the epipodial fringe by scrubbing reduces the STX levels in the foot tissue of *H. laevigata* by ~70%. This result is consistent with the findings of a detailed anatomical study undertaken on *Haliotis tuberculata* by Bravo et al. (1999) in which the epithelium of the abalone foot was shown to contain significantly higher levels of toxins in comparison with the gut and the foot tissue. It was estimated that the epithelium alone contributed ~64% of the total toxicity of the abalone and that removal of both the foot tissue epithelium and gut tissue would decrease the toxicity of the abalone by around 75%. Similar work undertaken in South Africa on *Haliotis midae* also described a decrease in toxin levels due to scrubbing (Nagashima et al., 1995; Pitcher et al., 2001). Collectively, these studies on three different *Haliotis* species indicate that there is probably no major species variation in localisation of PSTs in the tissues. Removal of the pigment is already a common undertaking for commercial product destined for canning (due to the market preference for this type of presentation)

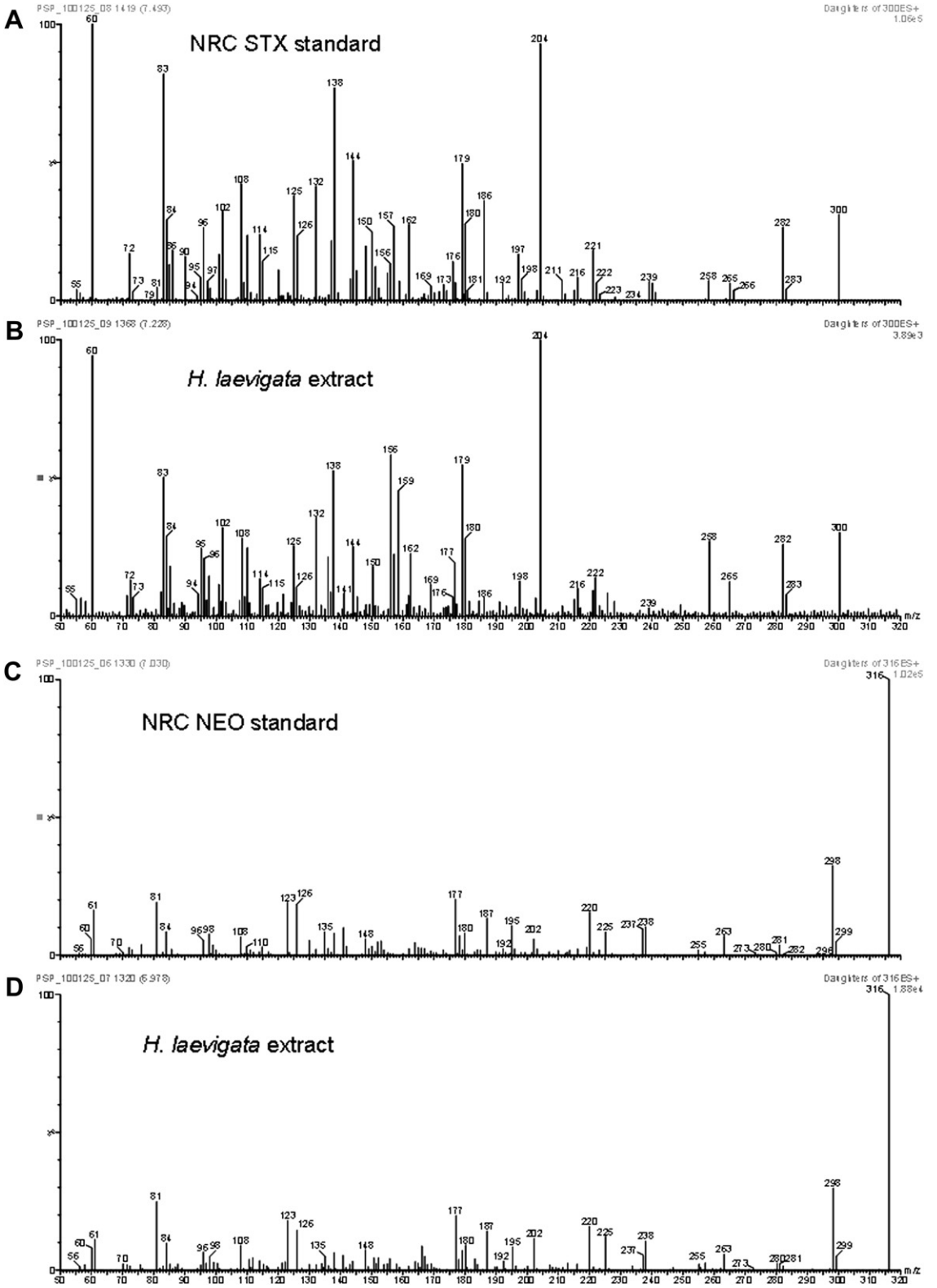

Fig. 5. LC-MS/MS ion spectra of: an STX analytical standard (A) produced by a daughter ion scan of *m*/*z* 300 (STX) (B) and an *H. laevigata* foot extract. Followed by an ion spectra of: a NEO analytical standard (C) produced by a daughter ion scan of *m*/*z* 316 (NEO) (D) and an *H. laevigata* foot extract.

and as such provides an effective risk reduction step, however, the effect the actual canning step has on the toxin profile and levels in abalone is unknown.

4.4. Toxin reduction via extended depuration

The testing of pre-experimental and post-experimental controls, combined with the finding of low levels of STX in the foot tissues of the pre-experimental controls, provided the opportunity to observe the depuration of STX in the control animals over the 50 days of the experiment. *H. laevigata* maintained for 50 days in constantly replenished fresh seawater displayed a ~73% decrease in the levels of STX in foot tissues compared with pre-experimental control animals. Consistent with these findings, Etheridge et al. (2004) found that toxin levels in *H. midae* decreased at a rate of 6.27 μg STX-equivalent 100 g^{-1} tissue d^{-1} over a two week period when fed an artificial feed diet. Other studies on depuration of PSTs from abalone fed their normal macroalgal diet, have shown that there was no depuration over an extended timeframe (Bravo et al., 1996; Pitcher et al., 2001; Etheridge et al., 2004). The observation that toxins depurated by a significant amount in *H. laevigata* raises a potential mitigation strategy for the abalone industry to reduce the risk of biotoxin contamination should a toxic event arise. Further research is needed however, to determine: (a) how quickly the toxins depurate in the initial days and weeks following contamination; and (b) whether toxins depurate to the same level when the abalone feed on macroalgae rather than commercially manufactured diets.

5. Conclusions

A. minutum is a prolific bloom species in nature, commonly reaching densities of 10^2–10^3 cells mL^{-1}, but reported to reach as high as 10^5 cells mL^{-1} in dense blooms (Hallegraeff et al., 1991). The abalone in this study were fed food pellets containing ~4 × 10^5 *A. minutum* cells every second day for 50 days. Despite this relatively high exposure, the maximum levels in the foot tissue were ~50 times lower than the maximum permissible limit (80 μg STX 100 g^{-1} tissue) for PSTs in molluscan shellfish. In contrast to this, *H. laevigata, H. midae* (South Africa) and *H. tuberculata* (Spain) have all been reported to contain STX levels well in excess of the regulatory limit (Martinez et al., 1993; Nagashima et al., 1995; Pitcher et al., 2001). The mode by which these species of abalone accumulated such high levels of these toxins still remains unclear. The results from this study suggest the possibility of a non-dietary mechanism of uptake and this should be clarified.

The low level of uptake in this study, when abalone were exposed to relatively high numbers of *A. minutum* cells over a prolonged period, may indicate a low risk of PSP poisoning to humans from the consumption of *H. laevigata* that has been exposed to a bloom of potentially toxic *A. minutum* in Australia. This finding, in conjunction with the fact that there have been no internationally recorded human illnesses relating to marine biotoxin contamination of abalone supports the recent decision made by the European Commission to exempt Gastropods from being harvested from classified production areas that have routine biotoxin monitoring programs in place.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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