Harmful Algae 8 (2009) 369–376

Contents lists available at ScienceDirect

Harmful Algae

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

Uptake of paralytic shellfish poisoning and spirolide toxins by paddle crabs (*Ovalipes catharus*) via a bivalve vector

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ARTICLE INFO

Article history:
Received 3 April 2008
Received in revised form 9 August 2008
Accepted 11 August 2008

Keywords:
Alexandrium
Paddle crab
Paralytic shellfish poisoning (PSP) toxins
Ovalipes catharus
Spirolides
Food web

ABSTRACT

The uptake of paralytic shellfish poisoning (PSP) toxins and spirolides by the paddle crab (*Ovalipes catharus*) was investigated in two laboratory feeding trials using Greenshell™ mussels (*Perna canaliculus*), which had been fed toxic strains of either *Alexandrium catenella* or *A. ostenfeldii*, as a vector. Toxin uptake by crabs occurred in both feeding trials and was limited to the visceral tissue; no toxins were detected in the body meat or the gills. The first trial utilized a strain of *A. catenella* that had high total PSP toxin content, 442.3 ± 91.6 fmol/cell, that was dominated by low toxicity N-sulfocarbamoyl toxins resulting in a low cellular toxicity, 5.5 ± 1.6 pg STXequiv./cell. In this trial, toxin accumulation in the crabs was highly variable and ranged from 3.8 to 221.5 μg STXequiv./100 g, with 3/4 of the crabs exceeding the regulatory limit of 80 μg STXequiv./100 g. Eight days after feeding on toxic mussels the crabs still retained high levels of toxin suggesting that depuration rates in this species may be slow. In the second feeding trial, the *A. ostenfeldii* strain fed to mussels produced low levels of both PSP toxins (52.0 ± 19.5 fmol/cell; 1.4 ± 0.3 pg STXequiv./cell) and spirolides (1.8 pg/cell) and, as a result, the concentration transferred to crabs via the mussels was very low-PSP toxins ranged from 2.5 to 6.8 μg STXequiv./100 g and spirolides from 6 to 7 μg/kg. The results of our study demonstrate that paddle crabs are capable of acquiring both PSP toxins and spirolides and suggest that this may occur in the wild during a toxic shellfish event. It also highlights the need to remove the viscera before consumption.

1. Introduction

The uptake of algal toxins by filter-feeding shellfish is widely recognized as a risk to public health, however there are also a variety of other higher-order carnivores that can accumulate potentially hazardous levels of toxins. Among these alternate vectors are scavenging crustaceans, which are gaining attention as organisms that can transfer toxins to seafood consumers (Shumway, 1995). A variety of harmful algal bloom toxins, including paralytic shellfish poisoning (PSP) toxins, diarrhetic shellfish toxins, palytoxins and domoic acid, have been identified in the edible tissue of crabs and lobsters from around the world (Table 1; also see review by Shumway, 1995), and have even resulted in human fatalities on rare occasions (Alcala et al., 1988; Llewellyn et al., 2002). The primary route of exposure to lobsters and crabs is through the consumption of bivalve molluscs and, though toxins have been detected in several tissue types (e.g. gills, guts, gonads, body meat), the hepatopancreas is the organ that typically contains the highest concentrations and is most frequently contaminated (Shumway, 1995; Torgersen et al., 2005; Jiang et al., 2006; Sephton et al., 2007). The hepatopancreas, known as crab "butter" or "mustard," is not commonly consumed, however it is considered a delicacy by some (e.g. in Japan; Oikawa et al., 2002) and the presence of high toxin concentrations in this organ warrants concern.

Throughout New Zealand the paddle crab (*Ovalipes catharus*) is a commercially harvested crustacean marketed both locally and in Japan. The paddle crab is particularly vulnerable to consuming algal toxins through its diet because, while it is an opportunistic predator, it primarily preys upon molluscs (Wear and Haddon, 1987). Evidence that these crabs concentrate algal toxins in nature has yet to be reported, though Rhodes et al. (2007) determined through laboratory experiments that paddle crabs do accumulate palytoxins in the digestive and reproductive organs when fed a diet of toxic mussels. Those findings, along with the general ability of crabs to uptake algal toxins, raises the concern that paddle crabs may also acquire other algal toxins commonly found in New Zealand coastal waters. Currently, the New Zealand Food Safety Authority requires that crabs harvested in an area where biotoxins are present in shellfish must be gutted prior to cooking and/or export (Busby, 1999).

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doi:10.1016/j.hal.2008.08.002

Table 1
Representative algal toxins detected in select crustaceans and maximum reported concentrations

Crustacean species	Toxin type	Maximum reported concentration[a]	Tissue type	Reference
Crabs				
Actaeodes tomentosus	PSP toxins	4.2 MU/g	Whole crab	Ho et al. (2006)
Camposcia retusa	PSP toxins	3.0 MU/g	Whole crab	Ho et al. (2006)
Cancer magister	Domoic acid	90 μg/g	Viscera	Wekell et al. (1994)
Cancer pagurus	Okadaic acid	600 μg OA equiv./kg	Hepatopancreas	Torgersen et al. (2005)
Demania reynaudii	Palytoxin	800 MU[b]	Leg meat	Alcala et al. (1988)
Lophozozymus pictor	Palytoxin	100 mg/kg	Viscera	Yasumoto et al. (1986)
Telmessus acutidens	PSP toxins	80.0 MU/g	Viscera	Oikawa et al. (2002)
Xanthias lividus	PSP toxins	4.5 MU/g	Whole crab	Ho et al. (2006)
Zosimus aeneus	PSP toxins	461.5 ± 92.7 μg STXequiv./100 g	Whole crab	Llewellyn et al. (2002)
Lobster				
Homarus americanus	PSP toxins	447.26 ± 88.18 μg STXequiv./100 g	Hepatopancreas	Sephton et al. (2007)

[a] Units are given as in original publication.
[b] Total amount of toxin present in two crab legs.

In this study we examine the uptake of two unique groups of phycotoxins, PSP and spirolide toxins, by paddle crabs using a bivalve vector, the Greenshell™ mussel (*Perna canaliculus*). Both PSP and spirolide toxins have been detected in New Zealand shellfish, and in this region they are produced by particular species within the dinoflagellate genus *Alexandrium* (Rhodes et al., 2001; MacKenzie et al., 2004). PSP toxins, the more widely studied of the two groups of toxins, consist of a suite of toxins derived from a potent neurotoxin, saxitoxin (STX), and are produced by several dinoflagellate genera throughout the world. PSP toxins have been detected in New Zealand isolates of *Alexandrium minutum* (MacKenzie and Berkett, 1997), *A. catenella*, *A. tamarense* and *A. ostenfeldii*, though the only species that has been associated with significant shellfish poisoning events is *A. catenella* (MacKenzie et al., 2004). Because the various STX analogues differ in specific toxicity (low toxicity N-sulfocarbamoyl toxins, moderately toxic decarbamoyl toxins and the highly toxic carbamate toxins), the overall toxicity of a given *Alexandrium* strain or contaminated shellfish sample will be dependent on the relative proportions of these analogues. More recently discovered and unrelated to PSP toxins, spirolides are produced only by *A. ostenfeldii* and are comprised of macrocyclicimine compounds that cause "fast-acting" neurotoxic symptoms, including rapid death, in mice (Hu et al., 1995; Cembella et al., 2000; Richard et al., 2001). While PSP toxins have been widely recognized as a threat to human health for several decades, the actual risk that spirolides pose to seafood consumers is an area of active research.

In the experiments presented here we used known toxin-producing strains of *A. catenella* and *A. ostenfeldii* to contaminate Greenshell mussels that were then fed to paddle crabs in two individual feeding trials. The first trial examines the uptake and retention of PSP toxins produced by *A. catenella*, and the second examines the uptake of PSP toxins and spirolides simultaneously produced by *A. ostenfeldii*.

2. Materials and methods

2.1. Toxin uptake experiments

2.1.1. A. catenella and A. ostenfeldii batch cultures

Two toxic *Alexandrium* isolates maintained within the Cawthron Culture Collection of Micro-algae, *A. catenella* (CAWD45) and *A. ostenfeldii* (CAWD135), were used as the source of PSP toxins and/or spirolide toxins in two separate feeding trials carried out at the Cawthron Institute in Nelson, New Zealand. In these experiments paddle crabs were fed toxic mussels to examine the uptake of (1) PSP toxins produced by *A. catenella* and (2) spirolide and PSP toxins produced by *A. ostenfeldii*. In preparation for feeding to Greenshell mussels, several batch cultures (ca. 12 l) of each strain were grown in aerated 100% GP medium (Loeblich and Smith, 1968) at 18 °C under 90–100 μmol m^{-2} s^{-1} photon flux (12:12 h light:dark). On the day cultures were to be fed to Greenshell mussels, 50 ml subsamples were collected from stationary phase cultures and either preserved in Lugol's for cell counts or pelleted by centrifugation at 3000 × *g* and stored at −20 °C for toxin analysis by high performance liquid chromatography (HPLC) or liquid chromatography–mass spectrometry (LC–MS). Cell densities were determined by counting settled 0.1 ml aliquots of preserved culture in triplicate.

2.1.2. Feeding of A. catenella and A. ostenfeldii to Greenshell mussels

Approximately 200 live Greenshell mussels were purchased at a local seafood retailer (Guyton Fisheries Ltd., Nelson, New Zealand) and acclimated at 18 °C for a minimum of 24 h prior to feeding. Before the mussels were used, three replicates of unexposed mussels (eight whole individuals per sample) were submitted to the Biotoxin Lab at Cawthron for a full toxin screen by LC–MS (McNabb et al., 2005) in order to document any background toxin concentrations. Mussel samples were negative for all toxins, with the exception of one mussel sample that contained trace levels of domoic acid and *iso*-domoic acid C. No spirolide or PSP toxins were detected. Approximately 30 non-toxic mussels were set aside for feeding to control crabs. The remaining mussels were placed on mesh racks in aerated cylindrical glass tanks (12–14 specimens per cylinder) containing 4 l of 0.3 μm, UV-filtered seawater. *A. catenella* or *A. ostenfeldii* culture was added to the cylinders incrementally over 8 h, left over night and the process repeated for 2 or 3 days. At the end of the exposure period mussels were frozen whole at −20 °C. A subset of each group of exposed mussels was set aside for toxin analysis and the rest were fed to paddle crabs in the following feeding trials.

2.1.3. Feeding of toxic Greenshell mussels to paddle crabs

Male and female paddle crabs from the Marlborough Sounds were collected by commercial fishermen on two different days (June 15 and July 3, 2007) and promptly transferred to the laboratories at Cawthron where they were placed, three individuals per container, in filtered seawater and fed a diet of non-toxic Greenshell mussels. As with mussels, a composite sample of whole unexposed crabs from each collection date (three crabs from June 15 and 4 crabs from July 3) were determined to be free of toxins after a full biotoxin screen (LC–MS; McNabb et al., 2005). In preparation for the experiments, crabs were acclimated for a minimum of 5 days in 0.3 μm, UV-filtered seawater at 18 °C

(exchanged at least once daily) and each crab fed one non-toxic mussel every 1–2 days. As required by the New Zealand Animal Welfare Act of 1999, Animal Welfare Ethics approval was obtained through the Nelson Marlborough Institute of Technology's Animal Ethics Committee before commencing with the feeding trials.

Preliminary feeding observations revealed that paddle crabs readily fed on thawed Greenshell mussel tissue, but would not always consume the whole mussel. Therefore, in the following feeding trials each crab was fed 1/2 of a thawed mussel (ca. 6 g) per feeding to ensure consumption of the visceral mass, the bivalve tissue with the greatest body burden shortly after toxification (Bricelj and Shumway, 1998). The two feeding trials were carried out sequentially.

In feeding trial #1, 14 crabs were arranged in the following experimental set up to examine the uptake and retention of PSP toxins produced by *A. catenella*:

- One control treatment (two crabs) fed non-toxic mussel for 5 days.
- Two uptake treatments (U1 and U2; three crabs each) fed toxic mussel for 5 days.
- Two retention treatments (R1 and R2; three crabs each) fed toxic mussel for 4 days, then fed non-toxic mussel periodically until harvested on days 9 and 12.

Starting on day 3 of the experiment a daily sample of crab fecal pellets from the two "retention" treatments was collected. After feeding each day, fecal pellets were siphoned from the tank periodically throughout the day and the following morning prior to daily cleaning and feeding (ca. 24 h between sampling). Each day's sample was centrifuged at $3000 \times g$, the supernatant poured off and frozen at −20 °C until extraction. At the end of the experiment crabs were anesthetized by covering with a salted crushed ice slurry for at least 20 min. Once crabs showed no movement or response to touch they were sacrificed by crushing the cerebral ganglion and then dissected into three tissue types, the gills, viscera (digestive system, hepatopancreas and gonads) and body meat. For each treatment the gills or body meat of individual crabs were pooled with like tissue resulting in one sample each per treatment. Because toxin was expected to accumulate primarily in the visceral tissue, the viscera for each crab was collected and analyzed separately. After dissection, these tissues were frozen at −20 °C pending toxin analyses.

In feeding trial #2, 12 male crabs were fed mussels daily for 4 days to examine the uptake of spirolides and PSP toxins produced by *A. ostenfeldii*. In this experiment there were three replicate treatments (three crabs each) fed toxic mussels and one control (three crabs) fed non-toxic mussels. At the end of the experiment, crabs were anesthetized and dissected as described above. The viscera, body meat and gills for all three crabs in each treatment were pooled to give one sample per tissue type per treatment.

In addition to the feeding trials, one crab was submerged in 10 l of aerated *A. catenella* culture (5.9×10^6 cells l^{-1}) for 24 h to determine the potential for PSP toxin contamination in crab gills and viscera after exposure to a "bloom."

2.2. Toxin extraction and analysis

2.2.1. PSP toxin extraction and analysis by HPLC

PSP toxins were extracted from cell pellets of *A. catenella* and *A. ostenfeldii* (see Section 2.1.1) in 0.5 ml of 0.5 M acetic acid. After the addition of extraction solvent, samples were sonicated on ice for 30 s and then centrifuged at $3000 \times g$ for 15 min. The supernatant was passed through a 0.45 μm minisart RC4 syringe filter (Sartorius, Auckland, New Zealand), collected in a HPLC vial and stored at −20 °C until analysis.

Crab feces samples from feeding trial #1 were thawed and the wet weight recorded prior to extraction in 1.0 ml of 0.5 M acetic acid. Samples were sonicated, centrifuged, filtered and stored at −20 °C, as in the *Alexandrium* culture samples.

PSP toxins were extracted from thoroughly homogenized mussel and crab tissue following the AOAC (2000) protocol used for analyzing shellfish. Briefly, equal volumes of extraction solvent (0.1N HCl) to sample weight (8.5–10.0 g) were well mixed, pH adjusted to 3 ± 0.5 and boiled for 5 min. If necessary, samples were pH adjusted again then centrifuged for 15 min at $3000 \times g$. The resulting supernatant was passed through a Sep-Pak C18 cartridge column, spun at $4500 \times g$ for 15 min and filtered through a 0.45 μm minisart RC4 syringe filter. Samples that would not pass through the syringe filter were placed in a Millipore Ultrafree-MC (10,000 NMWL) centrifugal filter unit and centrifuged at $20{,}000 \times g$ until sufficient volume for HPLC analysis had been collected. All extracts were stored in sealed HPLC vials and frozen at −20 °C until analysis (maximum 3 weeks). Note that this AOAC extraction method results in a partial conversion of low toxicity N-sulfocarbamoyl toxins into their more potent carbamate counterparts, though it is still useful in evaluating samples by HPLC (Luckas et al., 2003).

Using certified reference materials obtained from the National Research Council of Canada (Institute for Marine Biosciences, Halifax, Nova Scotia, Canada) we were able to determine the concentration of the following PSP toxins: carbamoyl toxins [gonyautoxins 1–4 (GTX 1–4), neosaxitoxin (neoSTX) and STX], decarbamoyl toxins (dcGTX 2–3, dcSTX) and N-sulfocarbamoyl toxins (GTX 5). National Research Council of Canada reference materials were used for indicative calculation of C1–4 analogues. Individual PSP toxins were resolved by HPLC with post-column derivatization based on the method described by Oshima (1995). Specifically, sample extracts were analyzed on a Shimatsu HPLC system consisting of a SIL-10A auto injector, LC-10AT mobile phase pump (1.0 ml/min flow rate) and a RF-10A fluorescence detector set at excitation and emission wavelengths of 330 and 390 nm, respectively. The post-column oxidation system was comprised of a LC-10AD double-head reaction pump for the oxidizing reagent (7 mM periodic acid in 50 mM potassium phosphate buffer, pH 7.8) and acidifying reagent (0.5 mM acetic acid), both delivered at 0.4 ml min^{-1}, and a CRB-6A reaction oven set at 85 °C containing 10 m of 0.5 mm i.d. Teflon tubing.

Each sample was analyzed in two independent runs; the first run used a gradient elution to resolve GTX 1–5, dcGTX 2–3, dcSTX, neoSTX and STX with a stop time of 60 min and the second used an isocratic gradient to resolve C1–4 with a stop time of 30 min. For GTX/STX determination, samples were passed through a 4.0 mm × 3.0 mm C18 guard column and a reverse phase 250 mm × 4.6 mm Synergi 4u Polar RP-80A column (Phenomenex, North Shore City, New Zealand) maintained in a 40 °C oven. Toxins were separated by a gradient elution using four mobile phases, Milli Q water, HPLC grade acetonitrile, 50 mM ammonium phosphate (pH 7.1) and 50 mM sodium heptanesulphonic acid. The ratio of the mobile phases delivered at a total flow rate of 1.0 ml/min changed in the following stages: (1) 0–12 min at 67% Milli Q, 11% 50 mM ammonium phosphate and 22% 50 mM sodium heptanesulphonic acid, (2) 13–33 min at 30% Milli Q, 10% acetonitrile, 38% 50 mM ammonium phosphate and 22% 50 mM sodium heptanesulphonic acid, (3) 34–60 min at the initial ratios listed in stage 1. For determination of C-toxins, samples were similarly passed through a C18 guard column and a reverse phase 250 mm × 4.6 mm Synergi 4u Hydro RP-80A column (Phenomenex, North Shore City, New Zealand) and then separated by isocratic elution using 2 mM tetrabutyl ammonium phosphate (pH 5.8). Prior to use, all mobile

phases were filtered through a 0.45 μm hydrophilic polypropelene membrane filter (Pall Corp., Auckland, New Zealand). Injection volumes for samples and standards, which were run after every 5th or 6th sample, were 25 μl for GTX/STX runs and 10 μl for C-toxins with the exception of fecal pellet samples. Fecal pellet extracts were only analyzed for GTX/STX analogues and injection volumes were 10 μl.

The concentration of toxin in the sample extract was determined using the conventional method for an external standard as detailed in Oshima (1995). The toxin concentration of each individual analogue was converted to fmol/cell for *Alexandrium* cultures and to nmol/g for tissue samples and then summed to give the total toxin content of the sample. The biological toxicity relative to the most potent analogue, STX, or the "specific toxicities," were calculated using the conversion factors in Oshima (1995) and reported as pg STXequivalents (STXequiv.)/cell for *Alexandrium* cultures and as μg STXequiv./100 g for mussel and crab tissue. These values were similarly summed and presented as toxicity.

2.2.2. PSP toxin determination by Jellett Rapid PSP test kit

A selection of crab viscera, body meat and fecal pellet samples were screened for presence/absence of PSP toxins using the Jellett Rapid PSP test (JRPT; Jellett Rapid Testing Ltd., Nova Scotia, Canada). Test kits were confirmed valid using a saxitoxin standard prepared in 0.5 M acetic acid. Sample extracts as prepared above were diluted in the manufacturer's buffer solution and applied to the test cartridge as directed. After 35 min, the results were recorded as negative or positive based on the strength of the "test" line relative to the "control" line and the JRPT cartridges photographed.

2.2.3. Spirolide toxin analysis

All samples extracted and analyzed for spirolides were contracted to the Biotoxin Lab, Cawthron Institute. Samples of *A. ostenfeldii*, Greenshell mussels, crab viscera, crab body meat and crab gills from feeding trial #2 were tested by LC–MS for the full suite of known spirolide toxins, including A, B, C, D, 13-desMethyl-C (13-desMe-C), 13-desMethyl-D (13-desMe-D), 13, 19-dides-Methyl-C, E, F, G and 20-methyl-G using the method described in McNabb et al. (2005). Certified standard, spirolide 13-desMe-C (National Research Council of Canada, Institute for Marine Biosciences, Halifax, Nova Scotia, Canada), was used as an external standard.

3. Results

3.1. Feeding trial #1—uptake and retention of PSP toxins produced by *A. catenella*

3.1.1. HPLC results

All six batch cultures of *A. catenella* used in this experiment produced PSP toxins. The toxin profile results were similar to earlier measurements of *A. catenella* isolates from New Zealand (MacKenzie et al., 2004) and were dominated by low toxicity N-sulfocarbamoyl toxins (98.01 mol%). Small amounts of carbamate toxins (1.96 mol%) and trace levels of decarbamoyl toxins (0.03 mol%) were also present in these cultures. Overall, the average cellular toxin content was quite high, 442.3 ± 91.6 fmol/cell, but the relative toxicity 5.5 ± 1.6 pg STXequiv./cell was low due to the overwhelming dominance of N-sulfocarbamoyl toxins.

On average, each mussel for feeding trial #1 was exposed to a total of 4.4×10^6 cells of *A. catenella* over 3 days. While, PSP toxins were produced consistently across the six batch cultures fed to mussels, there was a degree of variability in the relative proportions of toxins produced. This variation is reflected in the total toxin content and the specific toxicity of the cultures (Fig. 1A) and contributed to the difference in toxin content and toxicity observed between batches 1 and 2 of mussels (Fig. 1B).

HPLC analysis of the crab samples showed that PSP toxins were only present in the viscera and in fecal pellets. No toxins were detected in either the body meat or gills of the crabs. The total concentration of PSP toxins in crab viscera varied considerably, ranging from 5.1 to 110.0 nmol/g in the "uptake" crabs harvested on the day of the last feeding (U1 and U2) and from 0.2 to 49.0 nmol/g in the "retention" crabs harvested 4 and 7 days after the last feeding (R1 and R2). There was no statistical difference between treatments for either the total toxin content or toxicity (one-way ANOVA), suggesting that significant amounts of toxins were still retained at 8 days post-exposure. Two behaviors were noted during feeding observations that could result in the wide range of values detected: (1) in some instances the dominant crab within a treatment would take mussel from the other crabs or (2) an individual would reject the mussel leaving it for the other crabs to eat.

The relative proportions of carbamoyl, decarbamoyl and N-sulfocarbamoyl toxins present in mussel and crabs were similar to *A. catenella* (Table 2), despite the partial hydrolysis of N-sulfocarbamoyl toxins that likely occurred during the extraction process.

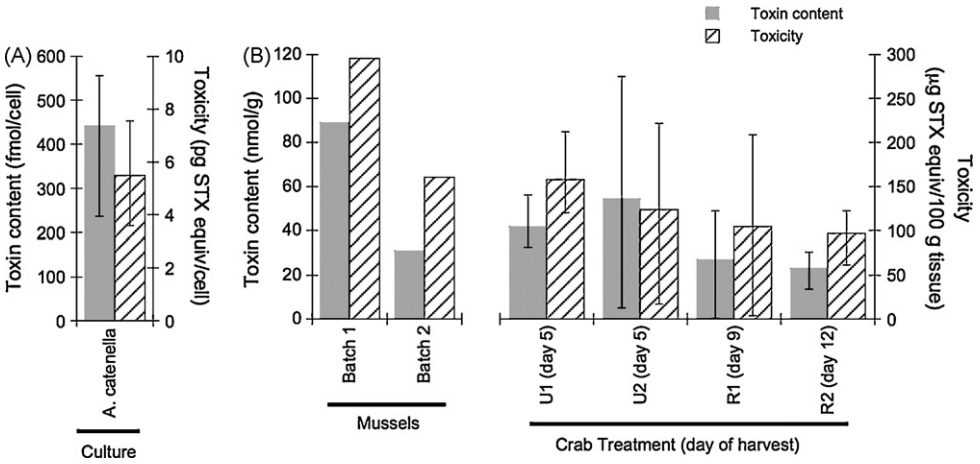

Fig. 1. PSP toxin results from feeding trial #1. (A) Mean PSP toxin content and toxicity of *A. catenella* ($n = 9$). (B) Average toxin content and toxicity in Greenshell mussels after feeding on *A. catenella* and in paddle crab viscera after feeding on toxic mussels (U = uptake and R = retention treatments, all $n = 3$). Error bars indicate the range of values observed.

Table 2
Mole percent distribution of PSP toxins in *A. catenella*, Greenshell mussels and paddle crabs from feeding trial #1 (values are mol% ± standard deviation)

Sample type (sample size)	N-sulfocarbamoyl	Decarbamoyl	Carbamoyl
Culture			
A. catenella ($n = 9$)	98.01 ± 0.5	0.03 ± 0.02	1.96 ± 0.52
Mussels			
Batch 1	94.68	0.05	5.27
Batch 2	86.04	0.17	13.79
Crabs			
Uptake 1 ($n = 3$)	95.01 ± 3.06	0.17 ± 0.31	4.81 ± 3.86
Uptake 2 ($n = 3$)	98.16 ± 0.26	0.00 ± 0.01	1.84 ± 0.26
Retention 1 ($n = 2$)[a]	95.03 ± 1.90	0.03 ± 0.03	4.93 ± 1.93
Retention 2 ($n = 3$)	96.81 ± 3.44	0.00 ± 0.00	3.19 ± 3.44

[a] Excluded values from crab who rejected mussel, only trace levels of GTX 1 and GTX 5 were detected.

Because the concentrations of N-sulfocarbamoyl toxins were very high in comparison to the other toxins, partial conversion of these toxins into their more toxic carbamate counterparts had only a small impact on the mol% distribution of toxins. However, carbamate toxins are significantly more toxic so this conversion will affect the relative toxicity of samples that undergo boiling in weak acid as part of the extraction method, a standard procedure used by regulatory officials in New Zealand and elsewhere around the world to monitor the safety of seafood (AOAC, 2000). Using this method, the average toxicity in crab viscera exceeded the regulatory limit (80 μg STXequiv./100 g of tissue) in all four treatments.

PSP toxins were also detected in fecal pellets collected from the two "retention" treatments (Fig. 2). The peak in toxin content occurred on the final day of feeding (day 4) then steadily decreased until the crabs were harvested. Unfortunately we were only able to measure a subset of PSP toxins (GTX 1–5, dcGTX 2–3, dcSTX, neoSTX and STX) in fecal pellets, therefore the toxin content reported in Fig. 2 does not include C-toxins.

The single crab that was submerged in *A. catenella* culture for 24 h had no measurable toxin in the guts or body meat and only traces of GTX 2 and GTX 3 present in the gills. It is unlikely that significant amounts of toxin would accumulate in the gills, or other tissues, in the event of a dense bloom of *A. catenella*.

3.1.2. Jellett Rapid PSP test (JRPT) results

The qualitative results of the nine crab samples analyzed by JRPT support those results obtained by HPLC. These JRPT were performed as independent tests to confirm the toxicity or lack thereof in each sample type (body meat, guts and fecal pellets). Samples that tested negative by JRPT included five body meat samples, one control gut sample and one control fecal pellet sample. In three individual gut samples (U1 treatment crabs) the "test" line was completely absent suggesting they were strongly positive. One fecal pellet sample was tested and determined to be positive (faint "test" line present).

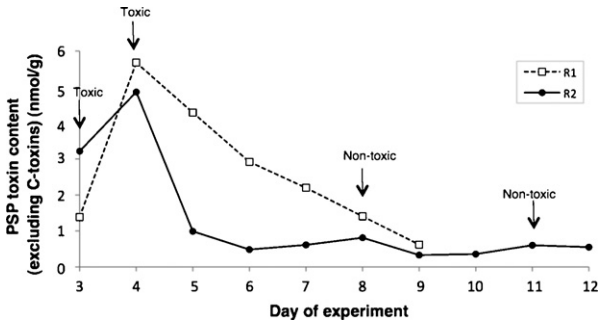

Fig. 2. PSP toxin content (excluding C-toxins) in crab fecal pellets collected over time from two "retention" treatments in feeding trial #1. Arrows indicate days on which crabs were fed mussels and whether they contained PSP toxins.

3.2. Feeding trial #2—uptake of PSP and spirolide toxins produced by *A. ostenfeldii*

The batch cultures of *A. ostenfeldii*, strain CAWD 135, that were used in feeding trial #2 produced both PSP and spirolide toxins. Similar to the *A. catenella* strain used, the PSP toxin profiles of the *A. ostenfeldii* cultures were dominated by the presence of N-sulfocarbamoyl toxins (90.48 mol%), with lesser amounts of decarbamoyl (1.68 mol%) and carbamoyl (7.84 mol%) toxins. The average toxin content, 52.0 ± 19.5 fmol/cell, and specific toxicity, 1.4 ± 0.3 pg STXequiv./cell, was much lower however (Fig. 3A). In addition to PSP toxins, this strain of *A. ostenfeldii* also produced 3 of the known spirolide toxins, 13-desMe-C (97.58 mol%), 13-desMe-D (1.89 mol%) and D (0.53 mol%). The total cellular spirolide toxin content was 1.8 pg/cell (Fig. 3C).

Greenshell mussels were fed *A. ostenfeldii* for 2 days, exposing them to an average total of 3.7×10^6 cells/mussel. During this period mussels accumulated relatively low levels of PSP toxins (4.3 nmol/g) compared to the previous feeding trial, and the specific toxicity was well below the regulatory limit (Fig. 3B). Given an average cellular toxin content of 52.0 fmol/cell, and if we assume an average mussel weight of 12 g, we can estimate that approximately 9.9×10^5 cells of *A. ostenfeldii* were ingested. Using this value, we calculated the expected concentration of spirolides in mussel tissue to be 148 μg/kg (based on the total cellular spirolide content of 1.8 pg/cell). The actual concentration of spirolides measured was reasonably close to this value, at 131 μg/kg, and was comprised of 13-desMe-C (110 μg/kg), 13-desMe-D (3 μg/kg) and D (18 μg/kg). It has been suggested that 32 μg/kg of mussel meat containing a mixture of spirolides with predominantly 13-desMe-C is a sufficient amount of toxin to result in the death of mice (Aasen et al., 2005). The concentration present in the mussels after feeding on moderately toxic *A. ostenfeldii* could therefore be considered potentially harmful. While under consideration, there currently are no safety limits set for spirolide toxins in New Zealand (P. McNabb, personal communication).

Paddle crabs that were fed the mussels containing both PSP and spirolide toxins showed very little uptake of either toxin group (Fig. 3B and D). As in feeding trial #1, no toxins were detected in the body meat or the gills. Trace levels of PSP toxins were present in the viscera (0.1–0.2 nmol/g) and were predominantly carbamate toxins. This suggests that any N-sulfocarbamoyl toxins, which made up 94% of the PSP toxins in the mussel tissue, were completely hydrolyzed during the extraction process. Despite the high spirolide content in mussels, the concentrations in crab viscera (6–7 μg/kg) were very low and consisted of only 13-desMe-C.

4. Discussion

The results of these two feeding trials demonstrate that paddle crabs are capable of acquiring significant concentrations of PSP toxins, and at least low levels of spirolides, in visceral tissues if they feed on toxic mussels. In the first trial, in which the greatest uptake of PSP toxins occurred, individual feeding behaviors contributed to a high degree of variability in total toxin content and toxicity between individual crabs. Nonetheless, toxicities above the regulatory limit of 80 ug STXequiv./100 g were measured in 9 of the 12 crabs exposed to PSP toxins and the crab "butter" from these specimens would therefore be deemed

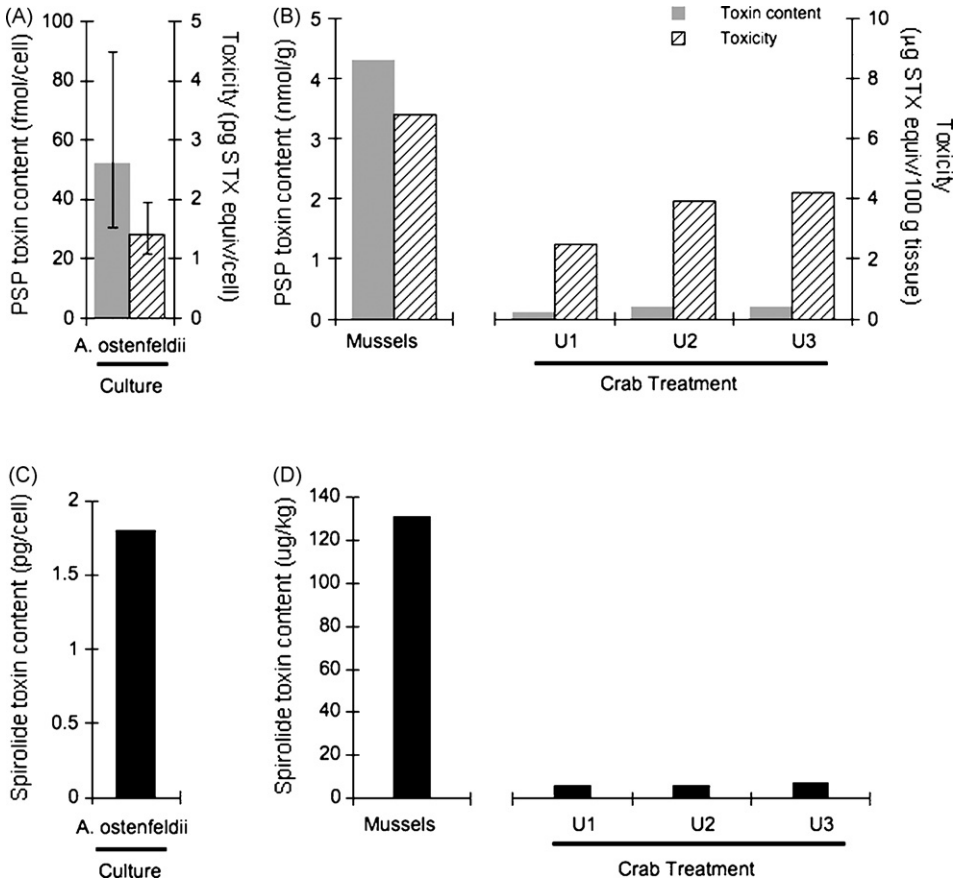

Fig. 3. PSP and spirolide toxin results from feeding trial #2. (A) Mean PSP toxin content and toxicity of *A. ostenfeldii* ($n = 7$); error bars indicate the range of values observed. (B) Average PSP toxin content and toxicity in Greenshell mussels after feeding on *A. ostenfeldii* and in paddle crab viscera after feeding on toxic mussels. (C) Total spirolide toxin content in *A. ostenfeldii*. (D) Total spirolide toxin content in Greenshell mussels and crab viscera.

unsafe for human consumption. The overall toxin uptake in the second feeding trail was very low relative to the first, and neither PSP toxins nor spirolides reached concentrations that would result in illness. We also did not detect either group of toxins in the most commonly consumed part of the crab, the body meat. The distribution of toxins in crab tissues that we observed is consistent with that of other scavenging crustaceans in which the highest concentrations are found in the hepatopancreas (a substantial proportion of the visceral mass) and only rarely detected in body meat (Shumway, 1995; Oikawa et al., 2002, 2007; Jiang et al., 2006; Sephton et al., 2007).

With respect to PSP toxins, the highest toxicity achieved in crabs in these feeding trials, 221 μg STXequiv./100 g viscera, occurred after only 5 days of feeding on moderately toxic shellfish. In a similar laboratory study, Oikawa et al. (2005) observed a comparable average toxicity of 12.8 ± 3.8 MU/g [256 ± 76 μg STXe-STXequiv./100 g; given 1 MU = 0.2 μg STXequiv. (Wekell et al., 2004)] in the hepatopancreas of the edible shore crab, *Telmessus acutidens*, after 20 consecutive days of feeding on toxic mussels. Further studies by Oikawa et al. (2002, 2004, 2007) have shown that PSP toxins are commonly detected in *T. acutidens* in the field and can also reach very high concentrations (up to 85.3 MU/g). The fact that these two species achieve similar toxicities in the lab indicates that paddle crabs may also rapidly acquire high levels of PSP toxins in nature during a toxic bloom, at least in the viscera.

The retention of toxins after exposure to algal toxins is of significant concern for regulatory purposes. In feeding trial #1 the "retention" treatment crabs that were fed toxic mussels for 4 days still contained high concentrations of PSP toxins 5 and 8 days after exposure (Fig. 1B). Despite being fed one less day than the "uptake" crabs, there was no significant difference in the total toxin content or the toxicity measured in those crabs vs. the "retention" crabs. While this was likely due to the high variability among individuals, the elevated mean concentrations indicate that PSP toxins are not rapidly depurated. Oikawa et al. (2005) examined the depuration of PSP toxins in *T. acutidens* under fed and non-fed conditions over 20 days and found that in both situations a significant reduction to ca. 50% of the initial toxicity occurred after only 5 days of depuration. However, after that initial loss, they did not observe further significant reduction until 20 days post-exposure, suggesting that depuration rates slowed. Additional evidence that toxins are retained in this crab for a significant period of time are given in a separate field study where the toxicity of *T. acutidens* hepatopancreas remained above regulatory levels even after levels in shellfish had declined to safe concentrations (Oikawa et al., 2007). Furthermore, two studies on the depuration rates of PSP toxins from lobster by Desbiens and Cembella (1997) and Haya et al. (1994) demonstrated that it could take several weeks to several months for toxin elimination in the hepatopancreas to occur, given initial concentrations of 452 μg STXequiv./100 g and 1554 μg STXequiv./100 g, respectively. In this study we were unable to document any significant toxin loss from the viscera after 8 days, but we did observe the excretion of some toxin in fecal pellets immediately after toxic mussel feedings ceased (Fig. 2). However, the rate of toxin loss quickly decreased over time suggesting that this is not a primary route of detoxification. Though we are limited in the conclusions we can make about PSP toxin retention in paddle crabs, the results indicate that the toxins

are depurated slowly from this species, as in *T. acutidens* and lobster.

It is clear from our experiments that the degree of intoxication in crabs was related to the amount of toxin produced by the cells. Both *A. catenella* and *A. ostenfeldii* had low cellular PSP toxicities, 5.5 ± 1.6 and 1.4 ± 0.3 pg STXequiv./cell, respectively, however the total toxin content in *A. catenella* was on average 8.5 times greater than that in *A. ostenfeldii*. The lower cell densities of *A. ostenfeldii* in the batch cultures combined with this discrepancy in the cellular toxin quota was clearly reflected in the mussels that fed on these cells; *A. catenella* fed mussels had much higher PSP toxin concentrations than those fed *A. ostenfeldii* and crabs acquired toxins from the shellfish accordingly. In New Zealand coastal waters, *A. ostenfeldii* is more widely distributed than *A. catenella*, but is typically present at very low densities and has not yet been associated with a toxic shellfish event (MacKenzie et al., 2004). Interestingly, some local strains of *A. ostenfeldii* are highly toxic (217 pg STXequiv./cell; MacKenzie et al., 1996) indicating that this species does have the potential to contaminate shellfish under favorable conditions. In contrast, *A. catenella* is known to cause PSP events in Northland where maximum toxicities have reached 1007 ug STXequiv./100 g in Greenshell mussels (MacKenzie et al., 2004), three times the maximum toxicity we achieved in this study and clearly enough toxin to be transferred to higher predators. Given the occurrence of these two species, it is more likely that blooms of *A. catenella* would result in an event in which paddle crabs may become toxic.

5. Conclusion

The results presented here provide the first evidence that paddle crabs are capable of rapidly acquiring PSP toxins when feeding on toxic shellfish. Though high concentrations of spirolides were not transferred to the crabs in these experiments, our findings indicate that they are susceptible to the uptake of these toxins as well. The presence of these highly toxic compounds suggests that crabs harvested in the vicinity of a shellfish toxicity event related to *Alexandrium* spp. should be examined for PSP toxins—a standard practice in many coastal regions with seafood monitoring programs, including Japan, Canada and the US (Watson-Wright et al., 1991; Lawrence et al., 1994; Marien, 1996; Oikawa et al., 2007). The primary concern, therefore, are recreational fishers and markets that target the whole crab, or crab "butter," especially export regions such as Japan, where consuming boiled hepatopancreas is preferred (Oikawa et al., 2002). These results corroborate current recommendations of the New Zealand Food Safety Authority and the Ministry of Helath to eviscerate crabs prior to consumption and/or export.

Acknowledgements

The authors would like to express our gratitude to J. Adamson, K. Smith, T. Dodgshun and K. Heasman, the Cawthron Biotoxin lab (especially P. Holland, P. McNabb, R. van Ginkel and A. Selwood), and the Royal Society of New Zealand for their support. This project was carried out as part of the National Science Foundation East Asia and Pacific Summer Institute (EAPSI) for U.S. graduate students and received financial support from the "Seafood Safety" programme, funded by the NZ Foundation for Research, Science and Technology (Contract No. CAWX0703).[SS]

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