

# The uptake, distribution and elimination of paralytic shellfish toxins in mussels and fish exposed to toxic dinoflagellates

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## Abstract

We exposed green-lipped mussels *Perna viridis* and black sea breams *Acanthopagrus schlegeli* to toxic dinoflagellates *Alexandrium fundyense* to evaluate the accumulation, distribution, transformation, and elimination of paralytic shellfish toxins (PSTs) in a controlled environmental condition. The mussels were fed *A. fundyense* for 7 days followed by 3 weeks of depuration, and the fish were fed toxic clams (pre-exposed to the dinoflagellates) for 5 days followed by 2 weeks of depuration. The toxin content and the compartmental distribution of PSTs were monitored throughout the experiments by high-performance liquid chromatography with post-column fluorescence derivatization (HPLC-FLD). This is the first report to assess the biokinetics of PSTs in marine fish under dietary exposure. The hepatopancreas in the mussels and the viscera in the fish accumulated most of the PSTs. Differential elimination of each toxin was observed in the mussels. The C2 toxins were eliminated rapidly in all organs; except in hepatopancreas, the more potent toxins such as GTX4, were eliminated slower during the depuration period. The relative proportions of various PSTs in the mussels changed over time, suggesting toxin-specific uptake and elimination rates, or biotransformation preferences between toxins. In the fish, the ratio of C1/C2 was 3.0 times ( $p < 0.01$ ) higher when compared to the clam tissues, indicating that conversion from C2 to C1 might have occurred when the toxin was transferred from the clams to the fish. In summary, species differences in uptake, distribution and elimination of PSTs were observed between mussels and fish, and this may influence trophic transfer of algal toxins in marine organisms.

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**Keywords:** Paralytic shellfish toxins; *Alexandrium fundyense*; *Perna viridis*; *Acanthopagrus schlegeli*

## 1. Introduction

Paralytic shellfish toxins (PSTs), among the most hazardous natural toxins, are produced by dinoflagellates such as the genus *Alexandrium* in marine environments. Paralytic shellfish poisoning (PSP) may result if people consume toxin-containing bivalves (Shumway, 1999). PSTs are water-soluble neurotoxins that act on mammalian nerve cells by blocking sodium channels, thus preventing the conductance of neuron signals leading to muscular paralysis. In severe cases, death may result due to respiratory failure (Baden and Trainer, 1993). There is no antidote available for PSP and immediate medical attention is required if the victim is suspected to be suffering from PSP.

Approximately 20 analogues of PSTs have been identified and are generally described as derivatives of saxitoxin (STX), which is the most potent PST. PSTs can be classified into three major groups: non-sulfated carbamate toxins, 11-hydroxysulfated carbamates and 21-*N*-sulfocarbamoyl toxins. On a molar basis, non-sulfated carbamate toxins, saxitoxin, and its *N*-1-hydroxy derivative neosaxitoxin (neoSTX), are the most potent toxins. The 11-hydroxysulfated carbamates, gonyautoxins (GTXs), are about half as potent as STX. The potency of 21-*N*-sulfocarbamoyl toxins (C toxins) is comparatively the lowest. PSTs are capable of transformation. The toxin conversion can be achieved by enzymatic or non-enzymatic reactions, including desulfation, oxidation, reduction and epimerization. Thus, the transformation of toxins can alter their overall toxicity. For example, C2 toxin can be converted into GTX3 under acidic conditions, which increase the toxicity by six-fold. In humans, the lethal dose ranges from 1 to 4 mgSTXeq. (Levin, 1992). Nowadays, a regulatory level of 80 µgSTXeq. 100 g<sup>-1</sup>

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of the wet weight of the shellfish tissue is implemented in many countries (Van Egmond et al., 1992).

Contamination of seafood by PSTs has posed serious problems to the fisheries industry as well as to public health (Shumway et al., 1988). There is evidence that almost all coastal waters in the temperate to tropical regions are affected to some extent by harmful algal blooms (HAB) (Hallegraeff, 2003). Apart from eutrophication, Hallegraeff and Bolch (1992) suggested that transportation of ship ballast water containing toxic algal species might be responsible for the expansion of HABs. It is well documented that PSTs can accumulate in bivalves, which act as vectors to transfer toxins to higher trophic levels. Recently, Bricej et al. (2005) found that exposure of PSTs to clams can result in a mutation that makes the clams more resistant to the toxin, resulting in a greater danger to humans. Because of their filter-feeding behavior and relative insensitivity to PSTs, bivalves are important vectors in PST transfer. Their physiology makes bivalves capable of accumulating toxins to an extremely dangerous level, and these toxins can be transferred to higher trophic levels. However, elimination rates of PSTs vary greatly among bivalve species. For example, PSTs elimination in the green-lipped mussel *Perna viridis* was relatively fast (within a few weeks, Gacutan et al., 1989), whereas removal of toxins from the butter clam is comparatively slow due to the strong binding of the siphon tissue with the highly toxic STX. Thus, considerable amounts of toxins were still observed in bivalve tissues even up to 2 years after the initial ingestion of PSTs (Beitler and Liston, 1990).

Accumulation of PSTs has also been found in other species including zooplankton, fish, crustaceans and marine mammals (Shumway, 1995; Colin and Dam, 2003; Teegarden et al., 2003; Doucette et al., 2006). Fish kills caused by PSTs have been well documented (Robineau et al., 1991a,b; Gilbert et al., 2002; Cembella et al., 2002), and during the larval stage, fish are more susceptible to PSTs (Robineau et al., 1991a). Moreover, White (1980) observed that consumption of toxin-containing zooplankton causes recurring fish kills in adult Atlantic herring. Previous studies showed that PSTs induce metabolizing enzymes in Atlantic salmon that might aid in the detoxification process (Stagg et al., 1998; Gubbins et al., 2000). In addition, there is evidence that whales, porpoises, manatees, seabirds and other wildlife can be exposed to PSTs via consumption of contaminated fish (Geraci et al., 1989; Anderson and White, 1992). Due to these circumstances, research into the bioaccumulation of PSTs in fish has become prominent. However, very little is known on the effect of PSTs on marine fish, and no study has been conducted to date on the biokinetics of PSTs via trophic transfer to fish.

In this study, we quantified the accumulation and depuration of PSTs in green-lipped mussels and black sea bream exposed to *Alexandrium fundyense* in a controlled laboratory environment. We then determined the relative anatomical distribution as well as the differential accumulation and retention of PSTs in different organs. In addition, we examined the potential bio-transformation of PSTs in each organism and through the food chain.

## 2. Materials and methods

### 2.1. Mussels, fish, and algae

Green-lipped mussels, *P. viridis* (shell length of 8 cm), and black sea bream, *Acanthopagrus schlegeli* (size of 4–5 cm), were obtained from a fish farm at Yung Shue O, Sai Kung, Hong Kong. Clams, *Ruditapes philippinarum* (shell length of 4 cm), which were used for the fish exposure experiment, were obtained from Sai Kung. After brought back to the laboratory, any epibionts on the bivalves were removed immediately. The animals were maintained in natural coastal seawater collected from Clear Water Bay, Hong Kong, at 23 °C and 30 psu, and the seawater was continuously aerated throughout the experiments. All the organisms were acclimated to laboratory conditions in a flow-through system for 5 days, during which the mussels and the fish were fed non-toxic diatoms *Thalassiosira pseudonana* (clone 3H) and commercial shrimp, respectively.

The toxic dinoflagellate *A. fundyense* (CCMP 1719) was obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton. The culture was maintained in natural seawater enriched with L1 medium (Guillard and Hargraves, 1993) at 15 °C under a 14 h light:10 h dark regime. The natural seawater was first filtered through 0.22 µm filter membranes and was sterilized at 120 °C for 20 min before the enrichment. Cells were harvested during the exponential growth phase for the exposure experiment, in which the cells were collected gently by mesh and resuspended in 0.45 µm filtered seawater prior to feeding. The non-toxic diatom *T. pseudonana* (Clone 3H) was cultured and maintained in f/2 medium (Guillard and Ryther, 1962) at 18 °C under a 14 h light:10 h dark regime.

### 2.2. Accumulation and depuration of PSTs in mussels

Twenty-five mussels were maintained in each of three tanks filled with 20 l of seawater. The mussels were initially fed a total of  $2 \times 10^6$  cells of toxic *A. fundyense*. The concentration of the dinoflagellates was continuously metered from the stock tank with a peristaltic pump to yield an approximately constant cell density of 100 cells ml<sup>-1</sup>. The number of dinoflagellate cells was counted under a microscope by fixing the cells in Lugol's iodine solution prior to feeding. A constant volume of water was also maintained by controlling the water outflow from the tanks. The mussels were exposed to toxic algae for 7 days, and then depurated for 3 weeks in a flow-through system. No pseudo-feces was observed during the experimental period. The seawater in each tank was changed daily in order to remove the feces and prevent fouling. During the depuration period, the mussels were given the non-toxic diatom *T. pseudonana*. At each sampling time point, one mussel was taken randomly from each tank ( $n=3$ ) at days 1–3, 5, 7 during exposure period and at days 1, 3, 5, 7, 11, 14 and 22 during depuration period. The mussel was dissected into gills, hepatopancreas, viscera, and foot and adductor muscle. The tissues were then stored at –80 °C until further treatment and analysis.

### 2.3. Accumulation and depuration of PSTs in fish

The clams *R. philippinarum*, which were used as the food in the feeding experiment of the fish *A. schlegeli*, were first exposed to  $2 \times 10^6$  of toxic dinoflagellates for 5 days. The clam tissues were then dissected and stored at  $-20^\circ\text{C}$  for less than 3 days for the subsequent feeding experiments. Thirty-eight fish were maintained in each of three individual tanks containing 80 l of seawater with continuous aeration. The fish were fed the toxic clams for 5 days and depurated for 2 weeks in static condition. In order for the fish to accumulate considerable amount of toxins, approximately 5% wet weight was fed to the fish each day, and two times per day. Any unconsumed clam tissues were removed after 1 h of feeding. Half of the seawater was renewed daily and any feces were removed by a siphon tube in order to keep the water quality in good condition. The toxic clams were replaced by commercial shrimp during depuration. At each sampling time point, two fish were randomly removed from each tank for composite samples ( $n = 3$ ) at days 1–5 during exposure period and at days 1–3, 5, 8, 11 and 14 during depuration period. The fish were dissected into gills, viscera and carcass and stored at  $-80^\circ\text{C}$  until further treatment and analysis.

### 2.4. Toxin extraction and quantification

Toxin extraction followed the methods described by Li et al. (2005) but with some modifications. A known amount of *A. fundyense* was first concentrated by centrifugation, followed by the addition of 0.5 ml of 0.05 M acetic acid, and sonicated in an ice bath. The extract was centrifuged at 13,000 rpm for 15 min at  $4^\circ\text{C}$ , and the supernatant was then ultra-filtered at  $5000 \times g$  for 30 min at  $4^\circ\text{C}$  by an Ultrafiltration Unit (10,000 MW, Millipore). Five microliter of the sample was injected into the high-performance liquid chromatography (HPLC) for toxin quantification.

Tissues from the mussels, fish or clams were first homogenized with a 1:1 (v/v) of 50 mM acetic acid and sonicated for 1 min. Samples were kept in an ice bath during processing. The extracts were centrifuged at 13,000 rpm for 15 min at  $4^\circ\text{C}$ . The supernatant was collected and passed through a Sep-Pak C18 cartridge column (Waters) for purification. The column was first pre-conditioned with methanol, MilliQ water and acetic acid before use. A 10–25  $\mu\text{l}$  of the samples were injected into the HPLC for toxin quantification.

The toxin profiles of the dinoflagellates, mussels, fish and clams were analyzed by HPLC with a post-column derivatization fluorescence detector (HPLC-FLD). In this study, we examined *N*-sulfocarbamoyl-11-hydroxysulfate toxins (C1 and C2), gonyautoxin I, II, III, IV (GTX 1–4), saxitoxin (STX), neosaxitoxin (neoSTX) and other decarbamoyl derivatives (dcGTX 2, dcGTX 3, dcSTX). The analytical procedures followed the method described by Oshima (1995) and Li et al. (2005). Briefly, a C8 reversed-phase column was used to separate different toxins (Inertsil C8 [film thickness, 3  $\mu\text{m}$ ; length, 150 mm; inner diameter, 4.6 mm] with Inertsil C8 [film thickness, 5  $\mu\text{m}$ ; length, 7.5 mm; inner diameter, 4.6 mm] all-guard cartridge; Alltech), aided by using three different mobile phases at a flow rate of

0.8 ml  $\text{min}^{-1}$  (three isocratic runs for each sample). The mobile phases include 1 mM tetrabutyl ammonium phosphate solution (pH 5.8) for C toxins; 2 mM sodium-1-heptanesulfonate in 10 mM ammonium phosphate solution (pH 7.1) for GTX toxins; and 2 mM sodium 1-heptanesulfonate in 30 mM ammonium phosphate (pH 7.1) for STX and neoSTX toxins. Periodic acid (7.0 mM) in 50 mM potassium phosphate and 500 mM acetic acid were used as oxidizing reagent and acidifying reagent, respectively. The mobile phases, oxidizing reagent and acidifier were freshly prepared from stock solutions each time prior to HPLC analysis, and all the reagents used were HPLC grade or analytical grades. Toxin content was quantified by comparing the fluorescent peak areas for each toxin with those of the toxin standards (National Research Council, Canada). To evaluate the combined potency of the toxins, concentrations of PSTs were expressed as STX equivalents based on mouse unit values for each toxin (Oshima, 1995): 15 for C1, 239 for C2, 1584 for GTX3, 1803 for GTX4, 2295 for neoSTX and 2483 for STX. The mouse unit was converted to STX equivalents with the conversion factor of 0.23  $\mu\text{gSTXeq. MU}^{-1}$ .

### 2.5. Data analysis

Data were checked for homogeneity and normal distribution and the percentage data were arcsine transformed for statistical analysis. One-way ANOVA was used to test for difference and Dunnett's post hoc tests were applied to identify the differences between groups. Statistical difference was accepted at  $p < 0.05$ .

## 3. Results

### 3.1. Toxin content and composition in *A. fundyense*

The toxin contents of *A. fundyense* fed to the bivalves remained moderately high ( $202.6 \text{ fmol cell}^{-1}$ ), and the toxin profiles of each batch were relatively constant throughout the whole exposure period. A mean percentage of each toxin in the algae as measured in molar ratio is given in Fig. 1a. Toxins present in the algae included: C1 (2.9%), C2 (63.7%), GTX1 (0.3%), GTX2 (7.2%), GTX3 (0.4%), GTX4 (12.0%), STX (1.4%) and neoSTX (12.1%). The most dominant toxin observed in *A. fundyense* was the C2 toxin. In addition, considerable amounts of GTX4 and neoSTX were also detected in the samples. No decarbamoyl derivatives (dcGTX 2, dcGTX 3, dcSTX) were detected.

### 3.2. Accumulation and depuration of PSTs by mussels

Similar PST analogues were found in the mussels *P. viridis* as in the toxic algae (C1 and C2, GTX 1–4, STX and neoSTX), except that the relative composition of the toxins was considerably different. In the dinoflagellates, GTX2 and GTX3 accounted for 7.2 and 0.4%, respectively, whereas in the mussel samples, GTX2 was undetectable and GTX3 was comparatively high, ranging from 4 to 8% during the whole experimental period. A trace amount of GTX1 was detected in the dinoflag-

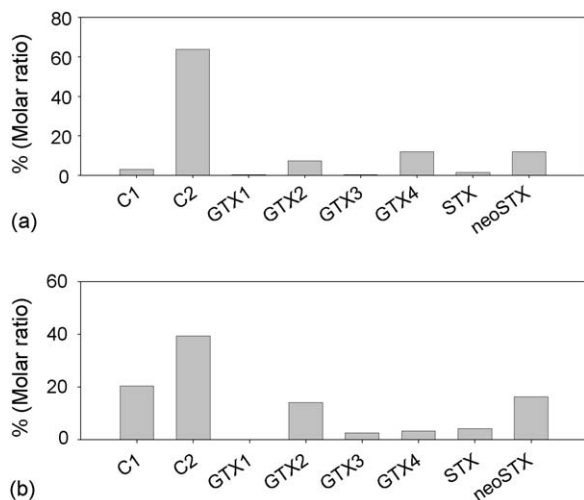


Fig. 1. Toxin composition profiles of (a) *Alexandrium fundyense* (CCMP1719) and (b) *Ruditapes philippinarum*, which were used for the fish exposure experiment.

ellates, but was below the detection limit in the mussels. A higher proportion of carbamate toxins (GTX) relative to sulfo-carbamoyl toxins (C toxins) were observed in the mussel tissue than in the algal samples. In *Alexandrium*, carbamate toxins accounted for 33.4%, while these toxins accounted for 53.0% on average in the mussel samples. In addition, the proportion of carbamate toxins increased with experimental time. No decarbamoyl derivatives were detected in the mussel samples.

The total toxin concentration in the mussels increased in parallel with the exposure time and reached a maximum level on the last day of exposure (Day 7) (Fig. 2). The total toxin concentration in all organs was significantly higher on Day 7 than Day 1 ( $p < 0.05$ ). The toxin accumulation rate in each of the compartment followed the order: hepatopancreas > viscera > gills > foot and adductor muscle. The maximum total toxin concentration accumulated in these compartments also followed the same rank order:  $1828.3 \pm 584.5$  ng/g (hepatopancreas),  $958.5 \pm 50.1$  ng/g (viscera),  $517.7 \pm 171.7$  ng/g (gills), and  $290.8 \pm 68.1$  ng/g (foot and adductor muscle). On Day 7, the total PSTs accumulated in hepatopancreas were significantly greater than in other organs ( $p < 0.01$ ). When the toxic dinoflagellates were replaced by non-toxic diatoms, the toxin content decreased during the subsequent depuration period. Though most of the toxins were accumulated

in the hepatopancreas, elimination of toxins was effective in this organ, i.e., >50% of PSTs were lost on the first day of depuration. Toxin elimination in the viscera was also comparatively effective, e.g., 45.4% of toxin was lost on the first day, followed by a gradual decrease. In contrast, toxin lost from the foot and adductor muscle was the least efficient, i.e., it took about 5 days to eliminate half of the maximum toxin content.

The toxin composition was notably different among organs. Fig. 3 shows the changes of each toxin composition in the gills, viscera, hepatopancreas, and the foot and adductor muscle. Overall, C1, C2, GTX4, GTX3, STX and neoSTX were detected in the mussel samples, and in most cases, the maximum concentration of these toxins was achieved on the last day of exposure (Day 7). Among the toxins, accumulation of C2 was the highest in all organs; nevertheless, elimination of C2 was relatively effective and was generally characterized by a rapid loss during the first few days of depuration. At Day 7, concentration of C2 in hepatopancreas was significantly higher than in gills and foot and adductor muscle ( $p < 0.05$ ), but no significant difference was found when compared with that in viscera ( $p > 0.1$ ). In addition to C2 toxin, a relatively high concentration of GTX4 was taken up by the mussels during the exposure period. However, the elimination and retention ability of GTX4 varied among the organs. In hepatopancreas, 53.4% of GTX4 was lost on the first day of depuration, and another 91.4% of GTX4 was eliminated over the subsequent period, while in the foot and adductor muscle, no GTX4 was detected after 3 days of depuration. In contrast, approximately 50% of GTX4 in the viscera was eliminated over 3 weeks of depuration. Moreover, comparatively lower concentrations of C1, GTX3, STX and neoSTX were accumulated in gills, viscera and foot and adductor muscle, whereas accumulation of these toxins were significantly higher in hepatopancreas ( $p < 0.001$ ). Interestingly, the appearance of GTX3, STX and neoSTX in the foot and adductor muscle was observed after 1, 3 and 5 days of exposure, respectively, and these toxins reached their maximum levels during the depuration period (Day 8–10).

The relative distribution of PSTs in the mussels shows that the toxin composition (in % molar ratio) temporally varied among different organs (Fig. 4). Generally, all the organs were dominated by C2 toxin during uptake period, which accounted for about 50% of the total toxins. However, the relative distribution of toxins varied among organs during the depuration period. In the gills and viscera, the proportion of GTX4 became signifi-

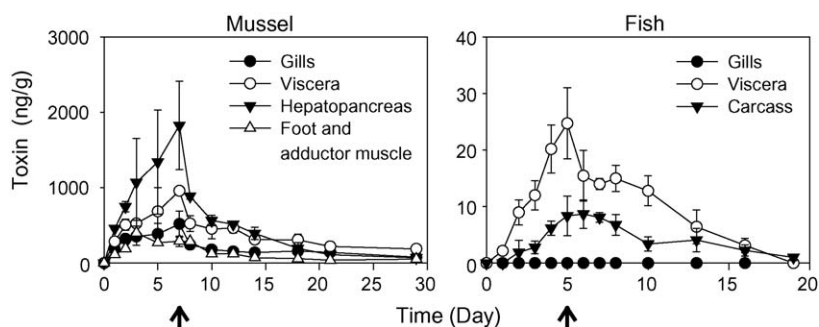


Fig. 2. Changes in total toxins of different compartments in the mussel *Perna viridis* and the fish *Acanthopagrus schlegeli* during the whole experimental period (mussel: Day 1–7 exposure, Day 8–29 depuration; fish: Day 1–5 exposure, Day 6–19 depuration). Mean  $\pm$  S.D. ( $n = 3$ ).

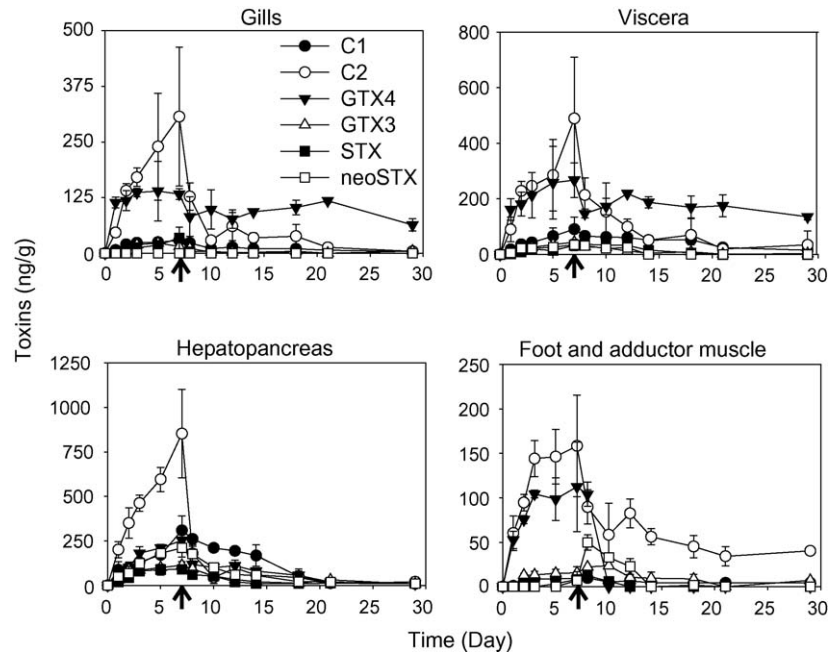


Fig. 3. Changes in toxin compositions of different compartments in the mussel *Perna viridis* during the whole experimental period (Day 1–7 exposure, Day 8–29 depuration). Mean  $\pm$  S.D. ( $n=3$ ).

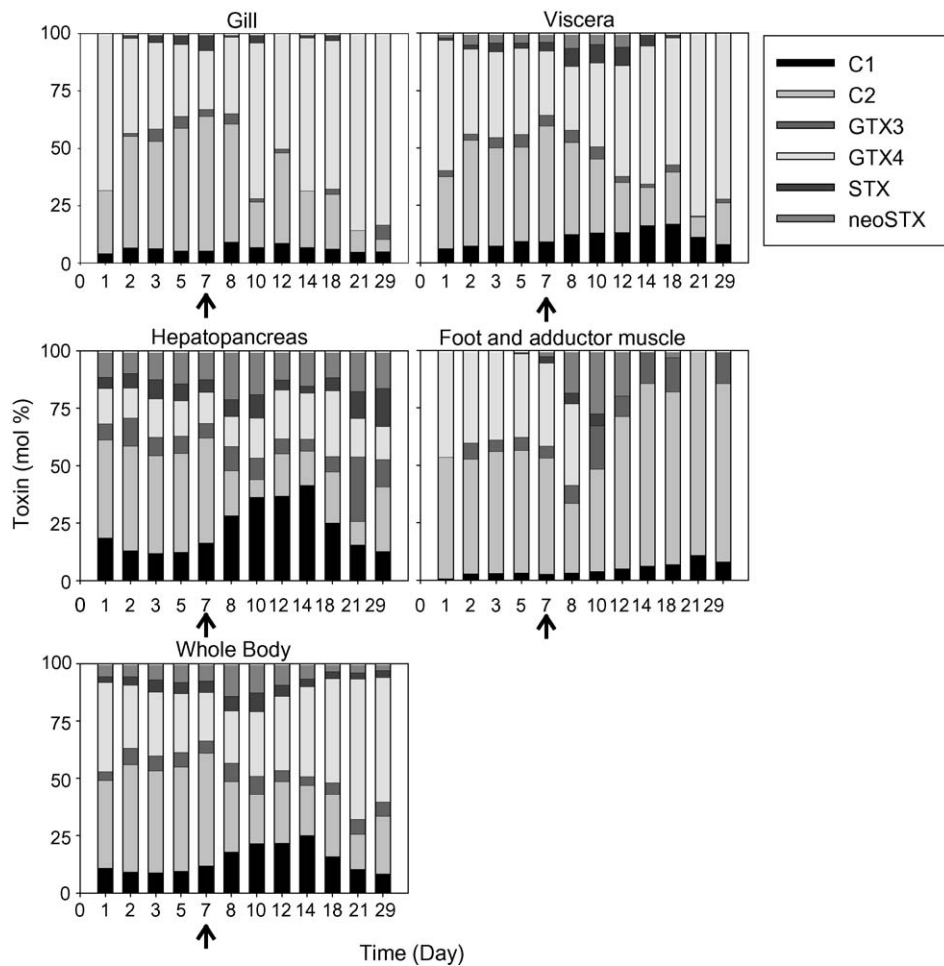


Fig. 4. Relative distribution (% in molar ratio) of PSTs in gill, viscera, hepatopancreas, foot and adductor muscle, and the whole body tissue of *Perna viridis* during the whole experimental period (Day 1–7, exposure; Day 8–29, depuration).



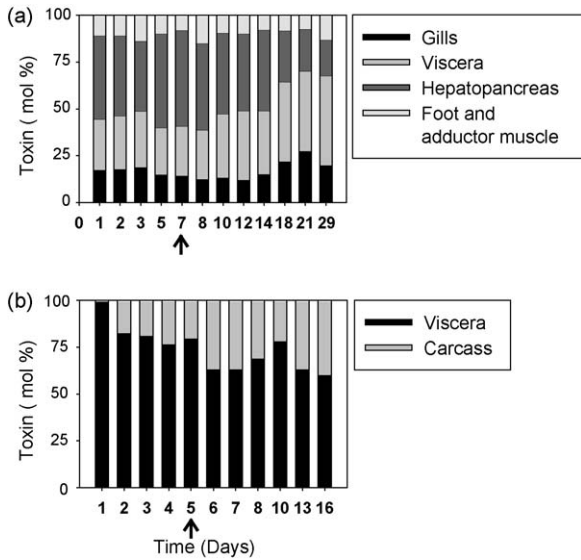


Fig. 5. Anatomical distribution (% in molar ratio) of PSTs in the mussel (a) *Perna viridis* and the fish (b) *Acanthopagrus schlegeli* during the whole experimental period (mussel: Day 1–7 exposure, Day 8–29 depuration; fish: Day 1–5 exposure, Day 6–16 depuration).

cant (>50%). In the foot and adductor muscle, the contribution of C2 increased dramatically. Contributions of other toxins (C1, GTX3 and STX) in these organs remained low throughout the whole experimental period. The toxin profile in the hepatopancreas differed significantly from other organs. During exposure, the major toxin was C2 toxin (42.4%). The ratio of C1/C2 epimer pair remained relatively steady during exposure, but C1 increased while C2 decreased during depuration. Unlike in other organs, the potent toxin neoSTX was relatively significant (14.4%) and the proportion of GTX4 remained relatively steady when compared to the gills and viscera. Moreover, the contribution of STX and GTX3 in the hepatopancreas was relatively low and did not show much variation over the course of the experiment.

The anatomical distribution of PSTs indicated that the majority of toxins were confined to the hepatopancreas, while the viscera also retained a significant amount of PSTs (Fig. 5a). On average, 39.5 and 33.5% of toxins were in the hepatopancreas and viscera, respectively. The gills contained 17.1% of toxins and only 10.3% of toxins were distributed in the foot and adductor muscle. The relative toxin distribution among dif-

ferent organs was unaltered ( $p > 0.05$ ) over the uptake period (Fig. 5a). However, the concentration of PSTs in the hepatopancreas decreased while that in the viscera, gills and the foot and adductor muscle increased during the depuration period.

Fig. 6 shows that the toxin concentration expressed as STX equivalents in the mussels increased over time during the exposure period and exceeded the mandatory limit on the second day of exposure, reaching the maximum of  $156.4 \pm 22.4 \mu\text{g STXeq. } 100 \text{ g}^{-1}$  on the last day of exposure (Day 7). When the toxic algae were replaced by non-toxic diatoms after Day 7, STXeq. decreased on the subsequent days. After 3 weeks of depuration (Day 29), the total toxin concentration decreased to  $19.7 \pm 2.0 \mu\text{g STXeq. } 100 \text{ g}^{-1}$ .

### 3.3. Accumulation and depuration of PSTs in fish

The toxin profiles of the clams *R. philippinarum* before being fed to the fish are shown in Fig. 1b. The predominant toxins detected were C1 (20.4%) and C2 (39.3%), while considerable amounts of GTX2 (14.1%) and neoSTX (16.3%) were also detected.

Fig. 7 shows the toxin composition of the fish in our experiment. Toxins were detected in the viscera and the carcass, but no toxins were detectable in the gills. The C1/C2 ratio showed a significant difference ( $p < 0.01$ ) between the fish and their prey. In the clams, the C1/C2 ratio was 1:1.9, whereas the C1/C2 ratio in the viscera of the fish was 1:0.64 on the last day of exposure (Day 5). In both organs, C1 and C2 toxins increased linearly over experimental times, and their concentrations were significantly higher on Day 5 when compared to Day 1 ( $p < 0.05$ ). In the viscera, concentrations of C1 and C2 reached a maximum of  $15.0 \pm 2.8$  and  $9.7 \pm 3.5 \text{ ng/g}$ , respectively, on Day 5. Elimination of C1 and C2 followed different patterns. C2 decreased by 57.2% during first 2 days of depuration followed by another gradual decrease, and no C2 was detectable after 16 days. In contrast, elimination of C1 was less efficient. The C1 concentration decreased by 38.6% on the first day of depuration and remained relatively steady between Day 1 and 5 of depuration, then subsequently decreased. In the carcass, the appearance of toxins was delayed until the second day of exposure. Concentrations of C1 and C2 toxins in the carcass gradually increased and reached their maximum levels of  $4.6 \pm 0.4$  and  $4.1 \pm 2.1 \text{ ng/g}$ , respectively, on the first day of depuration.

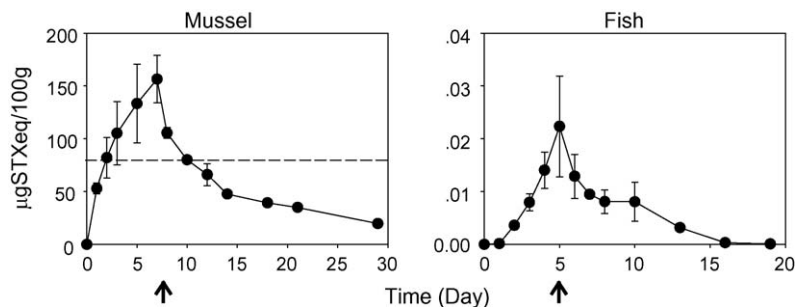


Fig. 6. Toxicity changes in the mussel *Perna viridis* and the fish *Acanthopagrus schlegeli* in the whole experimental period (mussel: Day 1–7 exposure, Day 8–29 depuration; fish: Day 1–5 exposure, Day 6–19 depuration). Dashed line represents the regulatory limit. Mean  $\pm$  S.D. ( $n = 3$ ).

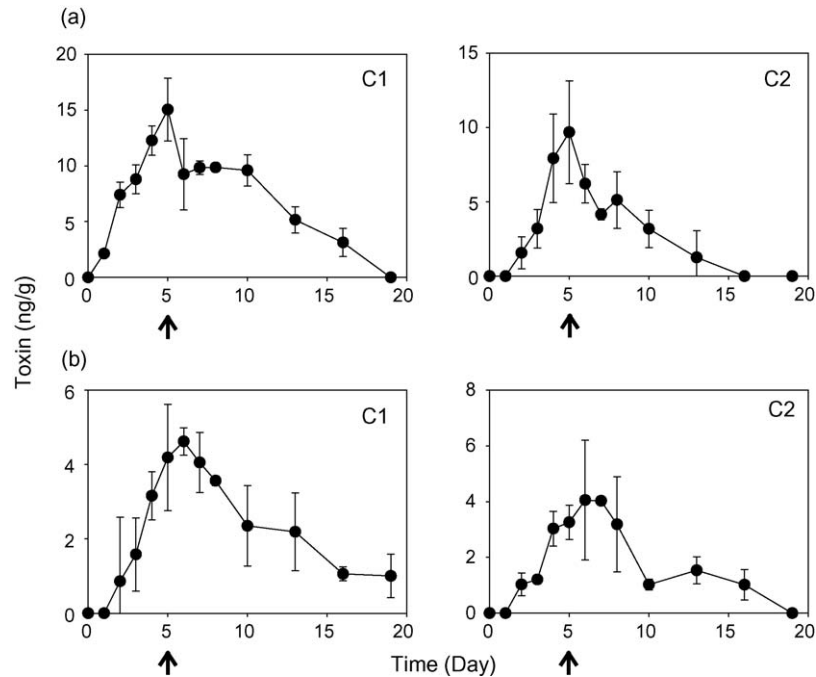


Fig. 7. Changes in concentration of C1 and C2 in (a) viscera and (b) carcass in *Acanthopagrus schlegeli* during the whole experimental period (Day 1–5, exposure; Day 6–19, depuration). Mean  $\pm$  S.D. ( $n = 3$ ).

Most toxins were confined to the viscera during the whole experimental period (Fig. 5b). However, the relative fractions accumulated in the viscera and the carcass changed between the uptake period and the depuration period. During exposure, the relative proportion of PSTs in the carcass ranged from 0 to 23.5%, while it increased to 21.6–39.9% of the total toxins during depuration. The STXeq. of the fish increased for 5 consecutive days when they were fed toxic clams, and reached the maximal toxicity of  $0.022 \pm 0.001 \mu\text{gSTXeq. } 100 \text{ g}^{-1}$  (Fig. 6). When the food was replaced by non-toxic shrimps, the STXeq. in the fish decreased subsequently; the first phase of decrease was observed within 5 days of depuration, followed by another gradual decrease.

## 4. Discussion

### 4.1. Toxin accumulation and depuration in mussels

There was a considerable difference in the PST composition between dinoflagellates *A. fundyense* and mussels *P. viridis*. An increasing proportion of carbamate toxins (GTXs) were observed in the mussels, and this proportion increased markedly over the experimental period. This phenomenon has been commonly observed in bivalves (Oshima et al., 1990; Bricelj et al., 1991; Cembella et al., 1993; Choi et al., 2003). Bivalves usually have lower proportions of *N*-sulfocarbamoyl toxins (C toxins) and higher proportions of carbamate toxins (GTX) than do the toxicogenic dinoflagellates that were ingested. Such a difference could be the result of differential retention or elimination of PSTs. In our experiments, there were no decarbamoyl derivatives detectable in either *Alexandrium* or the mussel samples, suggesting that biotransformation of PSTs into decarbamoyl

derivatives by the mussels might not have occurred. An earlier study concluded that enzymatic conversion of PSTs into decarbamoyl derivatives was not commonly found in bivalves (Bricelj and Shumway, 1998). The only transformation was observed in the viscera of a few species of clams. However, transformation of toxins into decarbamoyl gonyautoxins (dcGTX 2, dcGTX 3) was observed in *P. viridis* in a previous study (Choi et al., 2003). It is worth noting that the *Alexandrium* cells contained a high ratio of GTX2/GTX3, but we were unable to detect GTX2 in the mussel samples. Instead, there was a considerable amount of GTX3, which was presumably due to the selective accumulation of toxins by the mussels. In addition, Smith et al. (2001) suggested that bacteria isolated from bivalves have different capacities to utilize and transform PST analogues, for example, GTX 1/4 can be reductively transformed with concomitant production of GTX 2/3. Therefore, the increased GTX3 might also be the result of reduction from GTX4.

Toxin accumulation or elimination varied among the organs. In our study, the major toxin accumulated in the mussels was C2 toxin (especially in the hepatopancreas), which was due to the predominance of C2 toxin in the *Alexandrium* cells. Although the concentrations of C2 were significant, its elimination was efficient in all organs. In addition to C2 toxin, the mussels also accumulated notable amounts of GTX4, but its elimination varied among organs. In the gills and viscera, a decrease in GTX4 was relatively slow during the depuration period, suggesting that it was retained more efficiently in these organs. Elimination of GTX3, STX and neoSTX was rather effective in all of the organs. It is interesting to note that the appearance of the potent toxins STX and neoSTX was delayed in the foot and adductor muscle, which might be due to the redistribution of PSTs from other organs. Moreover, we observed that the fractions of GTX4

(12.0%) and neoSTX (12.1%) were comparable in *Alexandrium*, however, a similar percentage of accumulation of these toxins was observed only in the hepatopancreas of the mussel but not in other organs; accumulation of GTX4 was always higher than that of neoSTX in gills, viscera and the foot and adductor muscle. It suggested that different organ compartments might have different accumulation abilities depending on the type of toxin.

Bricelj and Cembella (1995) suggested that the toxin profiles in the viscera of the bivalves showed the closest resemblance to that of dinoflagellate cells, especially during the toxin exposure period. However, the composition changed markedly during the depuration period. The relative proportion of PSTs in the gills and viscera was dominated by C2 during the exposure period, whereas the contribution of GTX4 became significant during the depuration period. This was probably due to the higher rate of elimination of C2 than of GTX4. In the hepatopancreas, C2 was dominant during exposure, while an increasing percentage of C1 was observed relative to its respective C2 epimer during depuration. This might be explained by epimerization between this toxin epimer pair. In fact, a conversion from a less stable  $\beta$ -epimer (C2, GTX4, GTX3) to a more stable  $\alpha$ -epimer (C1, GTX1, GTX2) is commonly observed in bivalves (Oshima, 1995). In the present experiment, however, GTX1 and GTX2 in the mussels were always under the detection limit, possibly due to the fast removal of these toxins before the conversion, or the epimerization from GTX4 and GTX3 did not occur. On the other hand, the epimerization conversion in our experiment did not likely occur in other compartments except the hepatopancreas, suggesting that the epimerization process might mainly be confined to this organ.

Most studies have concluded that the viscera and hepatopancreas of bivalves accumulated most of the total toxins, despite the limited contribution of these organs to the total body mass of the organism (Bricelj et al., 1990; Cembella et al., 1993). Other body parts such as the foot accumulated low concentrations of toxins even though its contribution to the total body weight is substantial. In our experiment, the hepatopancreas accumulated most of the PSTs (40%), and the viscera also retained significant amounts of PSTs (34%) throughout the experimental period. In contrast, the gills contained 17% of toxins and only 10% of toxins were distributed in the foot and adductor muscle. This disproportional accumulation was in agreement with results from other bivalve studies (Cembella et al., 1993; Choi et al., 2003). Although most of the PSTs were confined to the hepatopancreas, toxins were more rapidly eliminated from this compartment/tissue. In fact, several studies have shown that the viscera had the most rapid elimination rates of PSTs relative to other tissues (Bricelj et al., 1990, 1991). Toxin elimination in the foot and adductor muscle was the slowest, despite its total toxin burden being low. On the other hand, the anatomical distribution suggested that the relative proportion of PSTs in hepatopancreas decreased, whereas the contribution in other compartments increased over time. This indicated that the relative distribution of PSTs among tissues changed over time, and the high rate of detoxification by the hepatopancreas might also be achieved by exchanging PSTs with other tissues (Bricelj and Cembella, 1995).

In the present experiment, the mussels accumulated PSTs and exceeded the regulatory level of  $80 \mu\text{gSTXeq. } 100 \text{ g}^{-1}$  within 2 days of exposure. Nevertheless, significant amounts of toxins were removed within 3 weeks, and a low level was attained thereafter. In fact, Bricelj and Shumway (1998) also suggested that green-lipped mussels could rapidly eliminate PSTs. In this study, despite the elimination of the more potent toxin GTX4 being less efficient in most organs, the predominant C2 toxin was effectively eliminated along with other potent toxins, leading to an overall decrease of STXeq. over time during depuration. However, various environmental factors and the concentration of the accumulated toxins during exposure may have a strong influence on the rate of elimination (Bricelj and Shumway, 1998).

#### 4.2. Toxin accumulation and depuration in marine fish

Previous studies found that PSTs had negative effects on fish, such as morphological abnormalities and reductions in growth and survival (Lefebvre et al., 2004). Additionally, investigations in both the field and laboratory feeding experiments revealed that dietary consumption of PSTs via algal or zooplankton vectors caused mortality in adult and larval fish (White, 1980; White et al., 1989; Gosselin et al., 1989; Robineau et al., 1991a,b), suggesting that dietary uptake of PSTs is an ecologically relevant route for exposure and a cause for acute toxicity. However, to our best knowledge, studies on the accumulation of PSTs in marine fish through dietary exposure have never been evaluated before. Due to the fact that the fish preferred clam tissues as dietary items, we used the toxin-containing clams as the primary route of toxin exposure to fish. The toxin content in the fish increased with the exposure time, highlighting that the fish accumulated PSTs from their prey.

Toxins were not detected in fish gills, possibly due to the low toxin content in the prey and the route of exposure by dietary ingestion instead of aqueous uptake. We observed only sulfo-carbamoyl toxins (C1 and C2) in the fish samples, although carbamate toxins (GTX) and saxitoxins (STX) were found in the clam tissues. Since GTX2 and neoSTX were somewhat important in the prey, we could not exclude the possibility of differential accumulation or toxin degradation in the fish gut.

We observed a significant difference in the ratio of C1/C2 between the fish and its prey. In the fish samples, the proportion of C1 ( $\alpha$ -epimer) was markedly higher than that of C2 ( $\beta$ -epimer) when compared to the clam tissues, suggesting that conversion from C2 to C1 might have occurred when the toxin was transferred from the clams to the fish. A possible mechanism of this conversion could be due to epimerization during the ingestion or assimilation process, which has been commonly observed in bivalves, where  $\beta$ -epimers (C2 toxin) can easily convert into a more stable form of  $\alpha$ -epimer (C1 toxin) (Oshima, 1995). In this case, the increased proportion of C1 reduced STXeq. following ingestion of the toxin-containing prey.

In our experiment, elimination of C2 was rather effective in both organs, whereas C1 elimination was comparatively less efficient. Apart from the possibility of a higher retention ability of C1 toxin in the tissues, toxin conversion to C1 might also account for the slower elimination rate of the C1 toxin. On the other hand,



we found that the greatest amount of PSTs was retained in the viscera, accounting for more than 74% of the total toxin on average. The viscera was the initial site for toxin assimilation and absorption, thus accumulating the greatest amount of toxins relative to the total body burden. It is also worth noting that the relative proportion of toxins in the carcass increased during the experiments, suggesting that the viscera had a comparatively faster elimination rate than did the carcass. The partitioning of toxins might have occurred via translocation of toxins from the viscera to the carcass.

The STXeq. in the fish increased for 5 consecutive days when they were fed toxic clams, and the maximum concentration was observed on the last day of exposure. Saito et al. (1985) reported that the minimum lethal dose of PSTs in marine fishes ranged from 0.75 to 4.2  $\mu\text{gSTXeq. } 100 \text{ g}^{-1}$ . White (1981) observed that the oral median lethal dose of PSTs in some marine fishes, including herring *Clupeaharengus harengus*, pollock *Pollachius virens*, flounder *Pseudopleuronectes americanus* and salmon *Salmo salar*, were from 0.40 to 0.76  $\mu\text{gSTXeq. } 100 \text{ g}^{-1}$ . However, it is difficult to ascertain the toxicity in fish in natural environments since the amount and prey types vary. A previous study showed that PSTs could be released into the water column by dinoflagellates (Hsieh et al., 2000), therefore ecological exposure could be further complicated by aqueous uptake of the dissolved toxin. In fact, Lefebvre et al. (2004, 2005) observed that dissolved STX was bioavailable to marine finfish larvae and resulted in sublethal effects and higher mortality. In this study, we demonstrated that fish accumulated PSTs through ingestion of toxic prey, and the toxin composition was modified significantly after consumption. The results indicated that the change in toxin composition alters STXeq. in fish when the toxins are transferred from their prey. However, the sublethal effects in fish under dietary accumulation of PSTs remain unclear. Since only the low potency toxins were detectable in the present study, further investigations are required to develop a more complete picture of the distribution of PSTs in fish.

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