Accumulation and Self-Cleaning of PSP Toxins in Green Mussel, *Perna viridis*, Fed on Various Cell Density of *Alexandrium minutum*

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ABSTRACT

Paralytic shellfish poisoning (PSP) is a serious threat to marine ecosystems and human health, caused by toxins from harmful algal blooms. This study investigates the accumulation and self-cleaning processes of PSP toxins in the green mussel, *Perna viridis*, fed various cell densities of *Alexandrium minutum*, a major PSP toxin producer. The first part assessed the clearance rate of the green mussels over 24 h, feeding them *A. minutum* at concentrations of 100, 500, 1,000, and 3,000 cells·mL^{-1}. Results showed that clearance rates increased with higher cell density. The total PSP toxins accumulated in the mussels were proportional to the total toxin found in *A. minutum*. The second part of the study examined the accumulation of PSP in mussels fed *A. minutum* for two consecutive weeks at a cell density of 500 cells·mL^{-1}, followed by two weeks of self-cleaning while being fed the non-toxic alga *Isochrysis* sp. The results showed that the PSP toxin content increased during feeding on *A. minutum*, surpassing the regulatory limit (0.8 µgSTXeq·g^{-1}) after 10 days. PSP toxin levels peaked at 2.16±0.21 µgSTXeq·g^{-1} after 14 days, approximately 2.7 times the regulatory limit. During the self-cleaning period, toxin levels gradually declined to safe concentrations within one day, with no detectable toxins at the end of the experiment. The elimination of GTX1,4 toxin (highly toxic derivatives) was particularly rapid during the self-cleaning phase. These findings suggest that mussels could serve as an effective indicator species for early warning of PSP outbreaks.

Keywords: *Alexandrium minutum*, Paralytic shellfish poisoning, *Perna viridis*, PSP toxins

INTRODUCTION

Paralytic shellfish poisoning (PSP) toxins are potent neurotoxins that block voltage-gated sodium-ion channels (Kao, 1996). These toxins are produced by several species of dinoflagellates, including those from the genus *Alexandrium, Gymnodinium catenatum*, and *Pyrodinium bahamense var. compressum* (Kodama, 2010; Anderson *et al*., 2012). PSP toxins consist of 58 closely related compounds based on a tetrahydropurine skeleton (Burrell *et al*., 2012). Structural differences among these toxins result in variations in their toxicity levels. Shellfish contamination with PSP toxins, particularly during toxic events, can lead to significant economic losses in the marine aquaculture industry (Qiu *et al*., 2018). Bivalves that feed on toxic dinoflagellates accumulate PSP toxins, which can then be transferred through the food chain (Kodama and Sato, 2008). Human consumption of contaminated bivalves can lead to paralytic shellfish poisoning syndrome (McFarren *et al*., 1961).

In 2001, an *A. minutum* bloom in Malaysia resulted in one case with seven fatalities (Lim *et al*., 2004). This species has been frequently found in several bivalve culture areas in Malaysia and the Philippines (Bajarias *et al*., 2006; Lim *et al*., 2012;

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Lau *et al*., 2017). In the Gulf of Thailand, *A. minutum* has been reported since 1991 (Songroop, 1998) although at very low cell densities. This species produces PSP toxins, with GTX 1,4, a highly toxic component, being the main toxin profile (Piumsomboon *et al*., 2001; Phumphoung *et al*., 2019). However, no PSP incidents from *A. minutum* have been reported in the region.

Green mussels (*Perna viridis*) are commonly found along the coasts of Southeast Asia and are an important economic resource in Thailand, with an annual production of approximately 52,067.29 metric tonnes valued at about 587.12 million baht in 2021 (Department of Fisheries, 2022). In the inner Gulf of Thailand, green mussel is known to occur near four major rivers: the Bang Pakong River, Chao Phraya River, Mae Klong River, and Tha Chin River (Prakoon *et al*., 2010).

The first PSP outbreak in the Gulf of Thailand occurred in May 1983 at the Pranburi River mouth, resulting in at least 63 patients and one fatality after consuming local green mussels (Suvapepun, 1984). No further cases have been reported since. However, low cell densities of toxic dinoflagellates such as *A. minutum* and *A. tamiyavanichii* have been detected in phytoplankton samples collected from coastal areas of the Gulf of Thailand (Piumsomboon *et al*., 2001; Fu *et al*., 2021), a major mussel farming area. In response, Thailand Department of Fisheries monitors shellfish toxicity, closing affected areas when PSP levels exceed the regulatory limit of 0.80 µg STXeq·g^{-1}.

Given the widespread distribution of *P. viridis* along the Thai coast, particularly in the Gulf of Thailand, these mussels face a risk of toxin contamination. As such, they are suitable for monitoring short-term and long-term changes in PSP toxins content and profiles. This study aims to investigate the accumulation and self-cleaning (depuration) of PSP toxins in green mussels fed *A. minutum*, a confirmed PSP toxin producer.

MATERIALS AND METHODS

Biological materials and algal culture methods

The toxic dinoflagellate, *A. minutum* (CU-MPL-Am1 strain) was isolated from the Chao Phraya River mouth and maintained in T1 medium at a salinity of 28 psu and 25 °C, under a 12:12 h light-dark cycle. Cells of *A. minutum* in a late-exponential growth phase (12–18 days) were harvested for feeding experiments. The total toxicity of *A. minutum* cells used in this study was previously reported (Phumphoung *et al*., 2019), ranging from 3.75 to 4.46 pgSTXeq·cell^{-1}, with GTX1,4 (95.86± 2.84%), GTX2,3 (3.69±2.78%), dcGTX2,3 (0.25± 0.17%), and dcNEO (0.21±0.12%), with no detection of NEO, C1, C2, or STX. *Isochrysis* sp., used as a nontoxic live feed for control purposes, was maintained under the same conditions.

A total of 950 market-sized mussels (*P. viridis*) with an average shell length of 7.74± 0.81 cm were collected from the Sriracha Fisheries Research Station, Kasetsart University, and transported to the Angsila Research Station at Chulalongkorn University in November 2018. Byssus threads were gently removed, and mussels were acclimatized for 24 h in fiberglass tanks containing 250 L of 28 psu filtered water (using a 1 µm pore size cartridge filter) with continuous aeration. The mussels were not fed during the acclimatization period. Prior to experimentation, mussels were randomly sampled, and their toxicity was analyzed to confirm the absence of PSP toxins.

Clearance rates experiments

The first part of this study aimed to assess PSP toxin levels in *A. minutum* and the clearance rate of 150 green mussels exposed to *A. minutum* at varying cell densities (100, 500, 1,000, and 3,000 cells·mL^{-1}) in triplicate aquariums (20 cm length × 20 cm width × 10 cm height) containing 1,500 mL of filtered seawater. Ten mussels were placed into each aquarium and fed *A. minutum* four times daily at 12:00 p.m., 6:00 p.m., 12:00 a.m., and 6:00 a.m. Every 6 h, the entire volume of seawater was replaced to remove feces and replenish with seawater

containing *A. minutum*. Clearance rates were determined using the formula described by Coughlan (1969) and Jørgensen (1996) as follows: $C = (V/Nt) \times \ln(C_0/C_t)$, where V is the water volume (l), N is the number of mussels, t is time of the grazing rate experiments, and C_0 and C_t are the initial and final cell concentrations (in cells·mL^{-1}), respectively. Toxin accumulation and profiles in mussels were analyzed after the feeding period. The whole-body tissue samples were weighed and immediately frozen at -20 °C before HPLC analysis. Control mussels were fed the non-toxic alga *Isochrysis* sp., at 100,000 cells·mL^{-1}.

Feeding experiments

The second part of the study aimed to examine the accumulation and self-cleaning of PSP toxins in 800 green mussels. Continuous aeration was provided in experimental tanks containing 100 L of filtered seawater. Mussels were divided into two groups: (1) mussels fed *A. minutum* at 500 cells·mL^{-1} (based on the results from the first part of this study) every 12 h for 14 days, followed by *Isochrysis* sp. at 100,000 cells·mL^{-1} for 14 days, and (2) a control group fed *Isochrysis* sp. at 100,000 cells·mL^{-1} for 28 days. Water management was conducted as in part 1. Ten mussels were collected at various intervals (0, 0.5, 1, 2, 3, 4, 5, 10, 14, 14.5, 15, 16, 17, 18, 19, 24, and 28 days) for toxin analysis. Whole tissues were immediately frozen at -20 °C prior to toxin analysis.

Chemical analysis by HPLC-FLD

PSP toxins were extracted and analyzed using the HPLC-FLD pre-chromatographic oxidation method, based on the AOAC International 20th Edition 2016 with minor modifications (Fish Inspection and Quality Control Division, Department of Fisheries, 2016). The toxin analysis was conducted at the Fish Inspection and Quality Control Division, Department of Fisheries in Bangkok. Briefly, mussel tissues (whole edible parts from 10 individuals in each group) were homogenized with 1% acetic acid and heated in a boiling water bath for 5 min. The homogenate was then centrifuged at 3,000 rpm for 15 min, and the supernatant was collected and passed through a C18 SPE cartridge for purification. The eluate was ultrafiltered, and analyzed using HPLC-FLD. All toxins were identified and quantified against an external calibration curve containing gonyautoxins 1 and 4 (GTX1,4), neosaxitoxin (NeoSTX), decarbamoyl neosaxitoxin (dcNeoSTX), saxitoxin (STX), decarbamoyl saxitoxin (dcSTX), gonyautoxins 2 and 3 (GTX 2,3), gonyautoxins 5 (GTX 5), decarbamoyl gonyautoxins 2 and 3 (dcGTX 2,3), and N-sulfocarbamoyl-11-hydrosulfate toxins (C 1,2) (National Research Council, Canada).

Statistical analysis

Statistical analyses were conducted using SPSS software (IBM Corp., 2017). One-way ANOVA was employed to assess differences in clearance rates and toxin accumulation. Duncan's Multiple Range Test was utilized to analyze differences between treatments at a significance level of $p<0.05$.

Ethic statement

The experimental protocol for this study was approved by the Animal Care and Use Committee of the Faculty of Science, Chulalongkorn University (Protocol Review No. 1823005).

RESULTS AND DISCUSSION

Effect of Alexandrium minutum densities on clearance rates

The mean clearance rates of the mussels during the feeding experiments indicated an increase as the *A. minutum* cell density increased. As shown in Figure 1, the clearance rate for the low cell density of 100 cells·mL^{-1} was 115.13±0.00 mL·ind^{-1}·h^{-1}, while for the cell densities of 500 and 1,000 cells·mL^{-1}, the rates were 155.32±0.10 and 172.68±0.02 mL·ind^{-1}·h^{-1}, respectively. The clearance rate for the high cell density of 3,000 cells·mL^{-1} was 199.85± 0.16 mL·ind^{-1}·h^{-1}. Throughout the feeding experiment, all mussels fed with *A. minutum* and *Isochrysis* sp. in each experimental set survived.

The clearance percentages of the mussels gradually declined as *A. minutum* cell density increased (Table 1). However, no statistical differences were observed for densities between 100–1,000 cells·mL^{-1}. The lowest feeding percentage, significantly different ($p<0.05$), was observed at the high cell density of 3,000 cells·mL^{-1}. Although *A. minutum* cells were seldom observed in the exchange seawater (less than 2% of supplied cells), this indicating that the cells supplied were almost completely filtered by the mussels. The results of our study suggest that the clearance rate of green mussels was not affected, and the mussels were able to ingest toxins as food sources. The feeding behavior exhibited a similar pattern, consistent with findings for other bivalves fed on PSP producers such as the Pacific oyster (*Crassostrea gigas*) exposed to *A. minutum* (Haberkorn *et al*., 2011) and the scallop (*Chlamys nobilis*) and clam (*Ruditapes philippinarum*) exposed to *Alexandrium tamarense* (Li *et al*., 2001).

In Table 2, the amount of toxin retained in mussels at the end of each feeding experiment was directly proportional to the total cellular toxin content in *A. minutum*. The toxin content in the mussel ranged from 0.10±0.01 to 2.93±0.30 µg STXeq·ind^{-1}, occasionally exceeding the regulatory limit for human consumption (0.8 µgSTXeq·g^{-1}). The maximum toxin content was observed in the mussel fed on *A. minutum* at a high cell density of 3,000 cells·mL^{-1}, while the lowest toxin content was found at a low cell density of 100 cells·mL^{-1}. These results suggested that green mussels fed on *A. minutum* could accumulate toxins rapidly and reach the regulatory level within 24 h of exposure.

However, the percentages of PSP toxins accumulated (Table 2) indicated that there was no significant difference in the percentages of PSP toxin accumulation at the end of each feeding experiment (ranging from 37.14±3.53% to 42.14±5.58%). Two possible explanations exist for the fate of the ingested toxin. First, PSP toxins may be lost through gut evacuation of undigested cells. Several studies have shown that not all ingested cells are digested in mussels, with intact dinoflagellates often passing through the digestive system and being ejected in

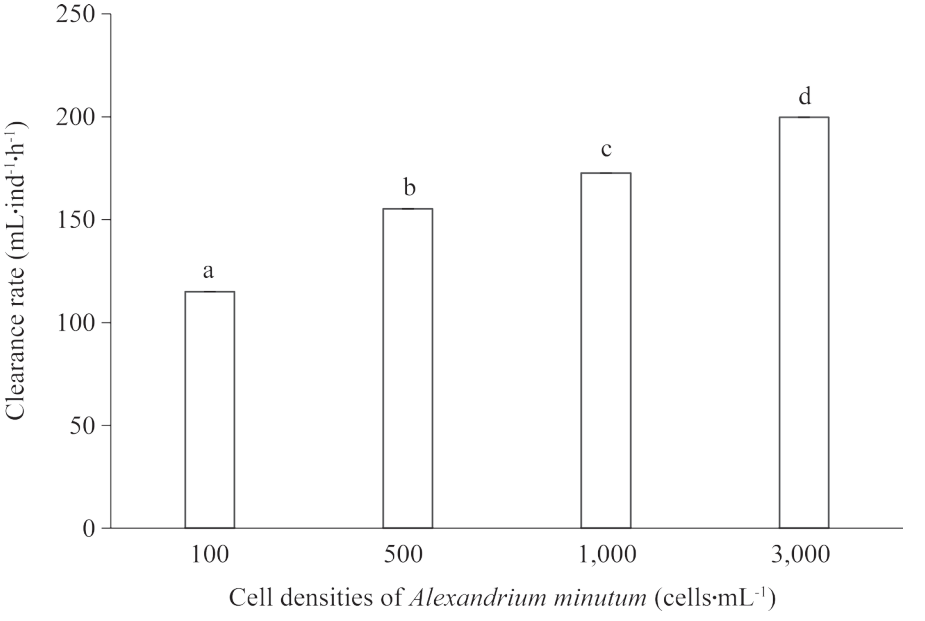

Figure 1. Clearance rates of green mussels fed four different cell densities of *Alexandrium minutum*. Bars represent the mean, with error bars showing the standard deviation (SD), ranging from 0 to 0.16. Bars with different lowercase letters are significantly different ($p<0.05$).

Table 1. Initial cell densities of *Alexandrium minutum*, total cells provided, total cells ingested, and clearance percentage of the mussels in each experimental set.

Initial cell density (cell·mL^{-1})	Total cells provided (cell·ind^{-1}±SD)	Total cells ingested (cell·ind^{-1}±SD)	Clearance percentage (±SD)
100	6.0×10^{4}±0.00	6.0×10^{4}±0.00	100±0.00^{b}
500	3.0×10^{5}±0.00	2.9×10^{5}±772.90	99.69±0.25^{b}
1,000	6.0×10^{5}±0.00	5.9×10^{5}±565.24	99.94±0.09^{b}
3,000	1.8×10^{6}±0.00	1.7×10^{6}±10,994	98.80±0.61^{a}

Note: Means±SD within each column, superscripted with different lowercase letters are significantly different ($p<0.05$).

Table 2. Initial cell density, total toxin supplied, total toxin in whole mussel tissue, and percentages of PSP toxins accumulated by the mussels.

Initial cell density (cell·mL^{-1})	Total toxin supplied (µgSTXeq·ind^{-1}±SD)	Total toxin in whole mussel tissue (µgSTXeq·ind^{-1}±SD)	Percentages of PSP toxins accumulated (±SD)
100	0.26±0.00^{a}	0.10±0.01^{a}	42.14±5.58^{a}
500	1.33±0.00^{b}	0.52±0.11^{b}	40.16±8.17^{a}
1,000	2.67±0.00^{c}	1.06±0.03^{c}	40.75±1.13^{a}
3,000	7.93±0.04^{d}	2.93±0.30^{d}	37.14±3.53^{a}

Note: Means±SD within each column, superscripted with different lowercase letters are significantly different ($p<0.05$).

the feces (Guéguen *et al*., 2008; Hégaret *et al*., 2008). Second, PSP toxins may be released in dissolved form, as they are relatively soluble in water (Wang *et al*., 2011). Sekiguchi *et al*. (2001) found that the amount of PSP toxins in the rearing water was equivalent to the amount lost from mussels in feeding experiments. Additionally, Asakawa *et al*. (2006) observed an increasing trend in dissolved PSP toxins in the rearing water over time during feeding experiments of oysters (*C. gigas*), fed with the toxic dinoflagellate *A. tamarense*

Accumulation and self-cleaning of PSP toxins in green mussel

The results showed that the toxin content in green mussel tissue during the exposure period to *A. minutum* increased proportionally with the amounts of cellular PSP toxin content in the toxic cells (Figure 2). After 10 days of exposure, the toxin content became 2.7 times higher than the regulatory limit of 0.80 µgSTXeq·g^{-1}, reaching a maximum level of 2.16±0.21 µgSTXeq·g^{-1} on day 14. To investigate the self-cleaning of the toxins in mussels, the above experiment was extended. On day 15 of the experiment, non-toxic algae was provided for the mussels in the tank for another 14 consecutive days. The changes in the increasing PSP content over the 14–day period showed a sharp decrease in toxin content, falling below the regulatory limit (red line in Figure 2) within one day (Day 15), followed by a relatively constant level during the next 13 days. This finding suggests that mussels have a highly efficient self-cleaning process to eliminate toxins, reducing them to below the regulatory limit of 0.40±0.02 µgSTXeq·g^{-1} within one day and reaching non-detectable levels after 9 days of self-cleaning (Day 23). Throughout the feeding experiment, the total toxin concentration in the mussels from the control group, which were fed with *Isochrysis* sp., remained non-detectable.

These results are consistent with findings from other studies, indicating that mussel groups are tolerant to PSP toxins and can temporarily retain high levels of these toxins in their tissues (Bricelj

et al., 1990). Kwong *et al*. (2006) reported similar results in controlled laboratory studies, where the green mussels accumulated approximately 1.5 µgSTXeq·g^{-1} after being fed *A. fundyense* at 100 cells·mL^{-1} for 7 days. This phenomenon has been widely reported in other bivalve species in natural environments. For example, during a massive red tide of *A. catenella* in the southern Argentina region, the toxicity level of *Mytilus edulis* reached 1,204.4 µgSTXeq·g^{-1} (Carreto and Benavides, 1993). Similarly, Montojo *et al*. (2012) reported that the green mussel accumulated up to 226 µgSTXeq·g^{-1} of PSP toxins in their tissues after being fed *P. bahamense* at cell density of 57 cell·mL^{-1}. Furthermore, the level at which bivalves can accumulate PSP toxins varies among species of bivalves and toxic dinoflagellates. Studies have indicated that mussels exhibit a strong tolerance to PSP toxins and have a high efficiency in toxin accumulation (Wu *et al*., 2022).

The percentage of PSP toxin accumulation in mussels increased on first two days, reaching a maximum of 47.67±4.53% (Day 2), and then gradually decreased to 25.97±2.49% on the last day of exposure (Day 14). When the toxic dinoflagellates were replaced with non-toxic algae, the mussels lost 50% of the PSP toxin accumulation from the last day of exposure, reducing it to 11.68±0.93% (Day 14.5). The percentage of PSP toxin accumulation continued to decrease on the subsequent days. This finding is consistent with those observed in other bivalves, such as 35% in the mussel *Mytilus galloprovincialis* fed with *A. tamarense* (Ichimi *et al*., 2001), 32% in the short-necked clam (*Tapes japonica*) fed with *Alexandrium catenella* (Samsur *et al*., 2006), and about 50% in the green mussel fed with *Alexandrium cohorticula* (Wisessang *et al*., 1991). Another interesting result of this study was that the percentage of PSP toxin accumulation decreased from about 47.67% at the beginning to about 25.97% at the end of exposure. Ichimi *et al*. (2001) suggested that this change might be due to a decreased rate of toxin uptake in bivalves when exposed to a high density of toxic dinoflagellates, making it difficult for the bivalves to absorb all the toxins that were available. It is also noteworthy that PSP toxins in mussel tissue could be released into the rearing water due to their water-soluble nature (Suzuki *et al*., 2003; Samsur *et al*., 2006).

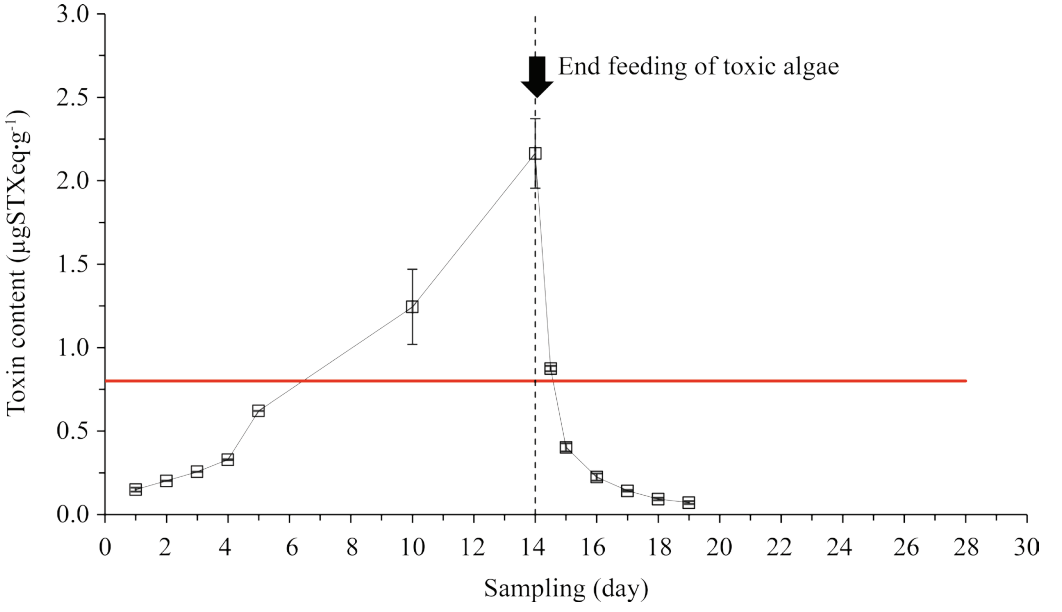

Figure 2. Changes of PSP toxins content in the mussels during accumulation and self-cleaning periods. Red line represents the regulatory limit. Error bars represent ±SD (n = 3).

It has been well documented that bivalves of the genus *Mytilus* and *Perna* can rapidly eliminate PSP toxins (Bricelj and Shumway, 1998). Recent research on green mussels has shown that they can eliminate 60% of total PSP toxins within 24 h during the self-cleaning period (Kusnoputranto *et al*., 2014). Moreover, Choi *et al*. (2003) and Mok *et al*. (2012) suggested that self-cleaning of PSP toxins in mussels is characterized by rapid elimination within the first day, followed by slower elimination thereafter. This is in accordance with Velásquez and Navarro (2014), who observed that the mussel (*Mytilus chilensis*) can accumulate and eliminate toxins in a shorter period of time. Studies have indicated that rapid toxin elimination occurs in the digestive gland during gut evacuation of unassimilated toxins, while slower elimination occurs in the tissue due to the loss of toxins that had been assimilated (Liu *et al*., 2020).

The compositions of PSP toxins are depicted in Figure 3. GTX1,4, GTX2,3, dcNEO, and dcGTX2,3 were found in both mussels and *A. minutum*, while NEO, C1, C2, and STX were detected only in mussels. GTX1,4, the major toxin in *A. minutum*, accounted for 96.10% of the total toxin on a µgSTXeq·g^{-1} basis. However, its composition decreased in mussels fed on *A. minutum* for 14 days, ranging from 76.40% to 94.47% of the total toxin. During self-cleaning, the composition of GTX1,4 further decreased, ranging from 69.56% to 73.19% on the first day and gradually decreasing until undetectable on Day 23. Similarly, the composition of GTX2,3 differed from that of *A. minutum*. In mussels fed on *A. minutum* for 14 days, GTX2,3 accounted for 5.04% to 19.53% of the total toxins while during self-cleaning, it ranged from 21.16% to 49.31%. In contrast, dcNEO toxins increased over the experimental period ($p<0.05$), reaching a maximum composition of 33.2% on the last detectable date (Day 19). NEO was only detected in mussels fed on *A. minutum* from Day 3 to 14, while small amounts of STX and C1, C2 were observed only in mussels fed on non-toxic algae from Day 16 to 19, respectively, but were not observed in *A. minutum* cells.

It is worth noting that in this feeding experiment, almost all of the PSP toxin composition was contributed by the toxic dinoflagellate GTX1,4, followed by GTX2,3, dcGTX2,3, and dcNEO, while NEO, C1, C2, and STX were not detected. Interestingly, when the PSP toxins were transferred from *A. minutum* to mussels, the proportion of

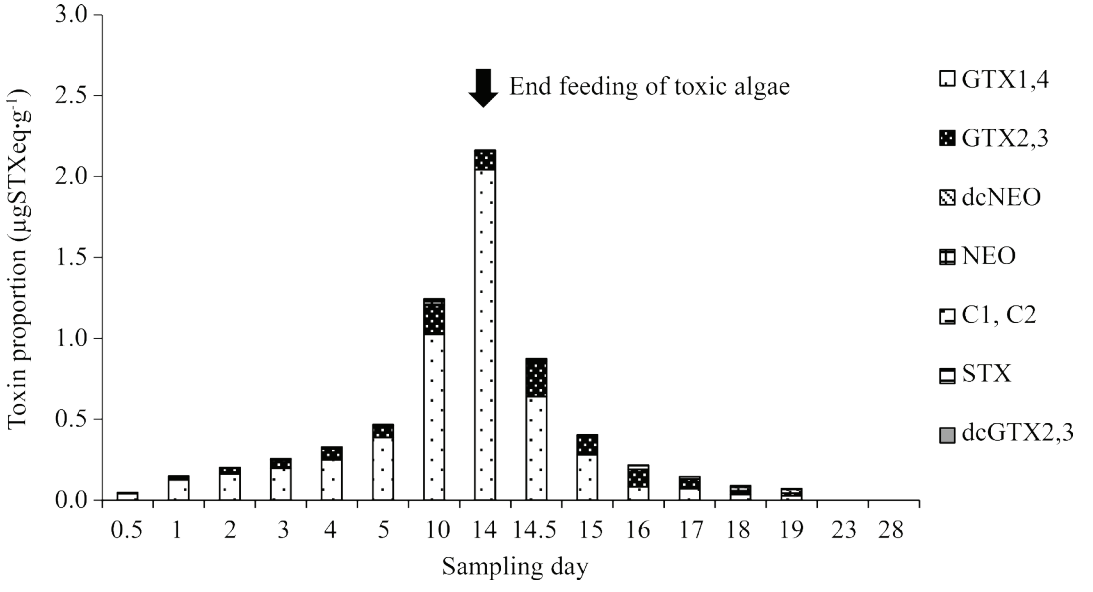

Figure 3. Changes in the percentages of PSP toxin profile in mussels during accumulation and self-cleaning periods.

GTX2,3 became higher than that in *A. minutum*, and NEO was detected during the intoxication period. These findings suggest that reductive conversions of GTX1,4 occurred in mussels. In fact, several studies have shown that the transformation of GTX1,4 to GTX2,3 might occur due to chemical or enzymatic transformations and/or selective retention of individual toxins in bivalves (Bricelj *et al*., 1990; Asakawa *et al*., 2006; Wiese *et al*., 2010). Additionally, the appearance of NEO in mussels may have originated from the reductive cleavage of the O-sulfate group on the R2 or R3 side chain of GTX1,4 in tissues (Shimizu and Yoshioka, 1981).

These results align with the findings of Chen and Chou (2001), who reported a longer half-life for the GTX2,3 toxin compared to the GTX1,4 toxin, and Shimizu (1978), who studied the stability of saxitoxin derivatives and found that the GTX2,3 toxin was more stable than the GTX1,4 toxin. This suggests that the elimination time for GTX1,4 toxin from mussel tissue is shorter than that for GTX2,3 toxin. Importantly, these findings carry significant implications for health and risk assessment. High-potency toxins, such as GTX 1,4, can be effectively eliminated from mussel tissue much more quickly than low-potency toxins, such as GTX2,3. Consequently, this leads to a reduction in the total PSP toxins of STXeq in mussels during both the accumulation and self-cleaning periods.

CONCLUSIONS

The first part of this study revealed that green mussels rapidly accumulated PSP toxins within 24 h of exposure to *A. minutum*. The clearance rate of mussels increased with higher cell densities of *A. minutum*, indicating their high capacity to filter toxic phytoplankton. Moreover, the total PSP toxins accumulated in mussels were directly proportional to the toxin supplied by *A. minutum*, with the highest levels observed at high cell densities.

In the second part of the study, continued feeding on *A. minutum* led to a linear increase in PSP toxins in mussels, exceeding regulatory limits after 10 days and reaching maximum levels by the end of the experiment. Due to their insensitivity to PSP toxins, green mussels effectively accumulated toxins to regulatory levels. However, they demonstrated the capacity for self-cleaning, rapidly eliminating toxins within the first few days. Notably, the more potent toxin, GTX1,4, was efficiently eliminated, leading to a significant decrease after exposure.

These findings highlight the potential of mussels as indicator species for early warning of PSP outbreaks. They also underscore the importance of monitoring and preventive measures to safeguard consumer health, such as the temporary closure of shellfish culture areas when PSP levels exceed safety criteria.

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