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Transfer of Paralytic Shellfish Toxins *via* Marine Food Chains: A Simulated Experiment[1]

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Objective To study the transfer of paralytic shellfish toxins (PST) using four simulated marine food chains: dinoflagellate *Alexandrium tamarense*→Artemia *Artemia salina*→Mysid shrimp *Neomysis awatschensis*; *A. tamarense*→*N. awatschensis*; *A. tamarense*→*A. salina*→Perch *Lateolabrax japonicus*; and *A. tamarense*→*L. japonicus*. **Methods** The ingestion of *A. tamarense*, a producer of PST, by *L. japonicus*, *N. awatschensis,* and *A. salina* was first confirmed by microscopic observation of *A. tamarense* cells in the intestine samples of the three different organisms, and by the analysis of *Chl.a* levels in the samples. Toxin accumulation in *L. japonicus* and *N. awatschensis* directly from the feeding on *A. tamarense* or indirectly through the vector of *A. salina* was then studied. The toxicity of samples was measured using the AOAC mouse bioassay method, and the toxin content and profile of *A. tamarense* were analyzed by the HPLC method. **Results** Both *A. salina* and *N. awatschensis* could ingest *A. tamarense* cells. However, the ingestion capability of *A. salina* exceeded that of *N. awatschensis.* After the exposure to the culture of *A. tamarense* (2 000 cells·mL^{-1}) for 70 minutes, the content of *Chl.a* in *A. salina* and *N. awatschensis* reached 0.87 and 0.024 μg·mg^{-1}, respectively. Besides, *A. tamarense* cells existed in the intestines of *L. japonicus*, *N. awatschensis* and *A. salina* by microscopic observation. Therefore, the three organisms could ingest *A. tamarense* cells directly. *A. salina* could accumulate high content of PST, and the toxicity of *A. salina* in samples collected on days 1, 4, and 5 of the experiment was 2.18, 2.6, and 2.1 MU·g^{-1}, respectively. All extracts from the samples could lead to death of tested mice within 7 minutes, and the toxin content in artemia sample collected on the 1st day was estimated to be 1.65×10^{-5} μg STX equal/individual. Toxin accumulation in *L. japonicus* and *N. awatschensis* directly from the feeding on *A. tamarense* or indirectly from the vector of *A. salina* was also studied. The mice injected with extracts from *L. japonicus* and *N. awatschensis* samples that accumulated PST either directly or indirectly showed PST intoxication symptoms, indicating that low levels of PST existed in these samples. **Conclusion** Paralytic shellfish toxins can be transferred to *L. japonicus*, *N. awatschensis,* and *A. salina* from *A. tamarense* directly or indirectly *via* the food chains.

Key words: Paralytic shellfish poisoning toxins; *A. tamarense*; *L. japonicus*; *N. awatschensis*; *A. salina*; Marine food chains

INTRODUCTION

Paralytic shellfish toxin (PST) is one of the most common and deadly phycotoxins in the sea, produced mainly by the dinoflagellates-including 11 *Alexandrium* species, *Gymnodinium catenatum* and *Pyrodinium babamense* var. *compressum*[1-3]. Previous studies showed that these toxic algae could lead to mass mortalities of marine organisms[4-8]. Besides, PST could be accumulated by a part of marine organisms, such as plankton[9-10], crustacean[11] and shellfish[12-13], due to their feeble swimming abilities. Toxins accumulated in these organisms could be further transferred via marine food web to organisms at higher trophic levels, such as fish, birds and mammals in the sea[1,4,10-11,14-15]. Furthermore, human illness caused by the consumption of PST has been reported all over the world[16-17].

Recently, the harmful algal blooms (HABs) caused by dinoflagellate *Prorocentrum donghaiense* and *Alexandrium* spp. occurred in East China Sea at a high frequency, and the *Alexandrium* species can produce a high level of PSP toxins[18]. Since Zhoushan fishery, the most important fishery of China, is just located in this area, it is important to know whether and how the organisms living in this area accumulate PSP toxins when HABs occur. It is also important to know whether the fishes or shrimps coming to the HAB area later accumulate PSP toxins indirectly by ingesting toxic zooplankton after the HAB event.

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Three typical organisms were chosen in our study to examine the transfer of PST *via* food chains. Perch *Lateolabrax japonicus* is an important commercial fish at northern part of China. Mysid shrimp *Neomysis awatschensis* belonging to genus *Mysidopsis*, is widely distributed along the coast of China, and is used as the standard test organism in toxicity bioassay[19]. Artemia *Artemia salina*, a common diet for the larva of marine organism, can be taken as a representative species of zooplankton. Four simulating food chains: dinoflagellate→artemia→mysid shrimp, dinoflagellate→mysid shrimp, dinoflagellate→ artemia→perch, and dinoflagellate→perch were set up in the experiment. This paper reports the results of the simulated experiment on PST transfer *via* the food chains.

MATERIALS AND MATHODS

Maintenance of Algae and Test Organisms

A strain of *A. tamarense* (ATHK) isolated from South China Sea was cultured in a 5 L bottle with 4 L of f/2 medium at 20℃ under a light intensity of 52 $\mu Em^{-2}s^{-1}$ (14:10 h L:D cycle). *Isochrysis* sp. was cultured in a 20 L bottle with f/2 medium at 20℃ under natural light intensity. Both algae at exponential phase (*A. tamarense*: 0.75×10^4 cells·mL^{-1}; *Isochrysis* sp.: 2.8×10^6 cells·mL^{-1}) were used for experiments.

Juvenile fishes of perch *L. japonicus* (about 5 cm long) were purchased from a culture-farm in Qingdao, China, and acclimated to experimental conditions for two weeks in a 100 L aquarium with flow through water supply prior to experiment. Mysid shrimps *N. avatschensis* were collected from the west coast of Jiaozhou Bay, Qingdao, and maintained in a 100 L aquarium with flow through water supply. Healthy and active mysid juveniles of 5±1 d were collected and used in the experiment. Perch and mysid cultures were continuously supplied with sand-filtered seawater pumped from Taipingjiao (a site out of Jiaozhou Bay without pollution history). Eggs of artemia *A. salina* (hatching rate is about 80% under 25℃) were purchased from American Salt Creek Inc. The 48 h old larvae of artemia(mean wet-weight was 0.054±0.001 mg) were used for experiments.

Mice (ICR strain) purchased from Qingdao Institute for Drug Control were used for bioassay of PSP toxicity, as described in AOAC[20].

Feeding Experiments

To test the ability of *N. awatschensis* and *A. salina* to ingest of *A. tamarense*, the animals were starved for 24 hours prior to the experiment. Diluted culture of *A. tamarense* collected at the exponential phase (2000 cells·mL^{-1}) with fresh seawater was used in the experiment, *Isochrysis* sp. (40 000 cells·mL^{-1}) was used as control. For the experiment of *N. awatschensis*, 200 individuals were put into 800 mL algae medium in a 1 L beaker. For the experiment of *A. salina*, 500 individuals were put into 400 mL algae medium in a 500 mL beaker. Each group had four replicates. One hundred individuals of *A. salina* and 30 individuals of *N. awatschensis* were collected on a GF/C membrane after 0, 30, 50, 70 minutes, respectively. To determine the *Chl.a* level, a certain number of cells of *A. tamarense* and *Isochrysis* sp. were also collected to the GF/C membrane to analyze the relationship between *Chl.a* level and cell number.

All samples were ground and extracted with 10 mL 90% acetone, and stored at -20℃ in dark for 24 hours. The *Chl.a* level was determined with a spectrophotometer using the methods described in the Criterion of PRC (GB12763.6-91): Marine Investigation Criterion for marine biological investigation.

Except for the analysis of *Chl.a*, the intestine samples were also observed for *A. tamarense* cells to confirm the ingestion of *A. tamarense* cells by the different organisms. The ingestion of *A. tamarense* cells by *L. japonicus* was tested only by this method due to the difficulty in analysis of *Chl.a* in *L. japonicus*.

PST Transfer via Food Chains

All accumulation experiments were carried out in 10 L glass tanks, with 8 L fresh seawater or diluted culture of *A. tamarense*. Seawater or culture of *A. tamarense* was replaced every day. Continuous aeration was given during the experiment. The temperature was 20℃-22℃.

In experiment of *A. salina*, about 10^4 individuals were put into each tank containing diluted culture of *A. tamarense*, and 20 mL *Isochrysis* sp. (5×10^5 cells·mL^{-1}) were added as food everyday. The experiment lasted for 5 days. Live animals on the 2nd and 5th days, and the excreta on the 4th day were collected for toxicity assay. Artemia used in the toxin transfer experiment were exposed to the diluted culture of *A. tamarense* to accumulate PST for 24 hours. These toxic artemia were then used as food of *N. awatschensis* and *L. japonicus* in the following experiments.

In experiment of *N. awatschensis*, 100 individuals were put into each tank. For simulated food chain *A. tamarense*→*N. awatschensis*, mysid shrimps were exposed to culture of *A. tamarense*, and non-toxic artemia (about 3×10^4 individuals, wet

weight 1.5 g) were added as food twice daily. The experiment lasted for 6 days. For simulated food chain *A. tamarense→A. salina→N. awatschensis*, mysid shrimps were put into fresh seawater, and fed with toxic artemia twice daily. The amount of toxic artemia added was the same as that described above. The experiment lasted for 8 days.

In experiment of *L. japonicus*, 20 individuals were put into each tank. For simulated food chain *A. tamarense→L. japonicus*, perch was exposed to diluted culture of *A. tamarense*, and non-toxic artemia (about 4×10^4 individuals, wet weight 2 g) were added as food twice daily. The experiment lasted for 6 days. For simulated food chain *A. tamarense→A. salina→L. japonicus*, perch was put into fresh seawater, and fed with toxic artemia. The amount of toxic artemia added was the same as that described above. The experiment lasted for 8 days. Samples were collected after the experiment and stored at -20℃.

Samples of perch, mysid and artemia that did not touch *A. tamarense* were taken as control and collected into test tube, and *A. tamarense*cells used in the experiment (7 500 cells·mL^{-1}, about 1 085 mL) were collected by filtration with a GF/C membrane to analyze the toxin content and profile. All samples were stored at -20℃. The toxicity of the animal and *A. tamarense* samples was assayed by the AOAC method, and the *A. tamarense* sample was also analyzed with high-performance liquid chromatography (HPLC).

HPLC Analysis

The PSP toxin profile and content of *A. tamarense* were analyzed with HPLC[21]. Sample was extracted with 0.1 mmol/L acetic acid. For C toxin analysis, the sample was hydrolyzed by 0.1 mmol/L hydrochloric acid and reanalized[21]. The excitation and emission wave length used for detection of PSP toxins were 330 nm and 390 nm, respectively. Temperature set for the post column derivatization was 80℃. All solvents used were HPLC grade and 1-heptanesulfonic acid was purchased from Sigma. The other chemicals were of analytical grade. Water used for HPLC was prepared by the Millipore Ultra Pure Water System (Millipore, Milford, USA). Toxin standard, including GTX1, GTX2, GTX3, and GTX4 were purchased from the National Research Council, Canada, Marine Analytical Chemistry Standards Program, Halifax NS, Canada.

Mouse Bioassay

Samples collected during the experiment were extracted with 0.1 mmol/L acetic acid and 0.1 mmol/L hydrochloric acid (HCl). Toxicity of the samples was then assayed with mice (ICR strain), according to the protocol of AOAC (Association of Official Methods of Analytical Chemists)[20,22]. The symptoms of mice after the injection (intraperitoneally [i.p]) were observed, and the lethal time was recorded. PSP toxin contents of *A. salina* samples were estimated according to the results of HPLC and mouse bioassay.

RESULTS

Feeding Experiments

The *Chl.a* level of *A. tamarense* and *Isochrysis* sp. increased with the number of algae cells. The *Chl.a* content in a single *A. tamarense* cells was about 200 times as high as in a single *Isochrysis* sp. cell, which was similar with the cell bulk ratio (Fig. 1) between the two different algae. The results of the feeding experiment showed that both *N. awatschensis* and *A. salina* could ingest *A. tamarense* and *Isochrysis* sp. cells directly, but *A. salina* was more inclined to ingest algae than *N. awatschensis*. After 70 minutes, the *Chl.a* level of *A. tamarense* in *A. salina* and *N. awatschensis* was 0.87 and 0.024 μg·mg^{-1}, respectively. The Chl.a level of *Isochrysis* sp. in *A. salina* and *N. awatschensis* was 0.024 and 0.004 μg·mg^{-1}, respectively (Fig. 2). *A. tamarense* cells were observed in the intestines of *L. japonicus*, *N. awatschensis* and *A. salina* after these organisms were exposed to 0.2×10^4 cells·mL^{-1} *A. tamarense* for 24 hours (Fig. 3). However, only a few cells of *A. tamarense* could be observed in the intestine of *L. japonicus*, compared to the congregated cells of *A. tamarense* in the intestine of *A. salina*, suggesting that *A. salina* could accumulate PST more easily from *A. tamarense* than *L. japonicus*.

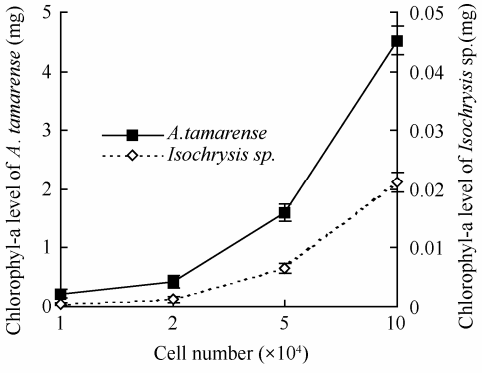

FIG.1. The relation between chlorophyll-a level and cell number of *A. tamarense* and *Isochrysis* sp.

Profile and Content of PSP Produced by A.

tamarense (ATHK)

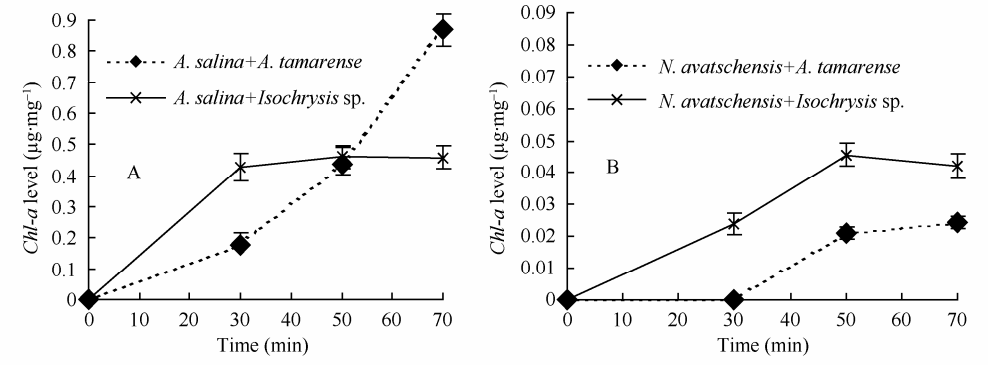

FIG. 2. The *chlorophyl-a* level in *A. salina* (A) and *N. awatschensis* (B) during intaking process.

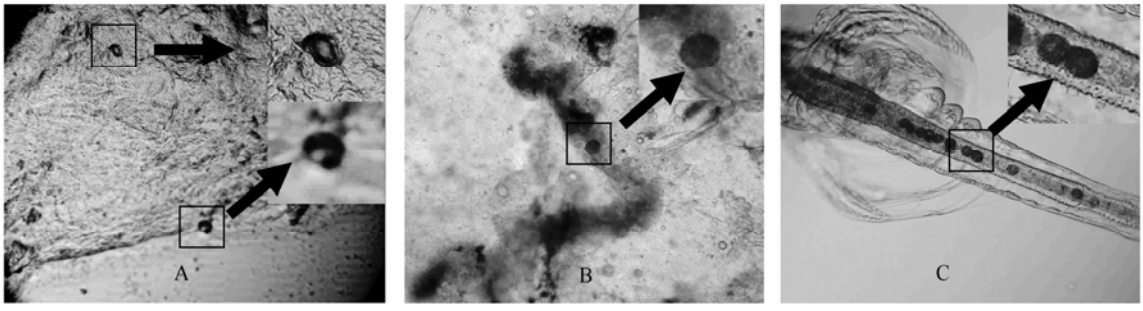

FIG. 3. The *A. tamarense* cells in the intestines of *L. japonicus* (A), *N. awatschensis* (B) and *A. salina* (C).

The results showed that PST produced by *A. tamarense* was mainly composed of C, B1 (GTX5), GTX1/4, and GTX2/3. The proportion of GTX toxins was the highest, accounting for 54.84% of the total toxins. The total PSP content of this strain was 18.5 fmol STX Equal/cell (Fig. 4 and Table 1).

Toxicity of Algae, Artemia, Mysid, and Perch Samples

TABLE 1

Toxin Composition and Content of PST Produced by *A. tamarense* (ATHK) at Stationary Phase

Toxins	C1	C2	B1	GTX 4	GTX1	GTX3	GTX2
Quantity (pg/cell)	2.30	5.82	018	4.84	3.10	0.18	0.12
Quantity (fmol/cell)	0.468	1.224	0.048	1.176	0.754	0.046	0.030
Percent (%)	12.49	32.67	1.03	31.39	20.13	1.02	0.55
Toxicity (pg STX Equal/cell)	—	0.188	0.010	3.16	2.04	0.10	0.04
Total	5.538 pg STX Equal/cell or 18.5 fmol STX equal/cell						

Note. C, B1: N-sulfocarbamoy1 toxins; GTX1,2,3,4: Gonyautoxin1,2,3,4.

From Table 2, it could be seen that *A. tamarense* sample had strong toxicity to the mice, with a toxicity of 1.83 $MU \cdot mL^{-1}$. Both live *A. salina* and the facets contained a high content of PST. The toxicity was 2.18 and 2.1 $MU \cdot g^{-1}$ for the live *A. salina* samples collected on the 1st and 5th day after the exposure to *A. tamarense*. The facet sample collected on the 4th day had a toxicity of 2.6 $MU \cdot g^{-1}$. The tested mice died within 7 minutes and showed typical symptoms caused by PST, such as gasp, convulsions, leap, staggering during the experiment. Samples of *N. awatschensis* and *L. japonicus* collected from the direct or indirect accumulation experiments also showed typical symptoms of PSP intoxication in tested mice, though no death of tested mice was observed, probably due to the low PST content in these samples suggesting that *A. salina* could directly accumulate PST from *A. tamarense*, and *N. awatschensis* while *L. japonicus* could accumulate PST directly from *A. tamarense* or indirectly from *A. salina* as a vector.

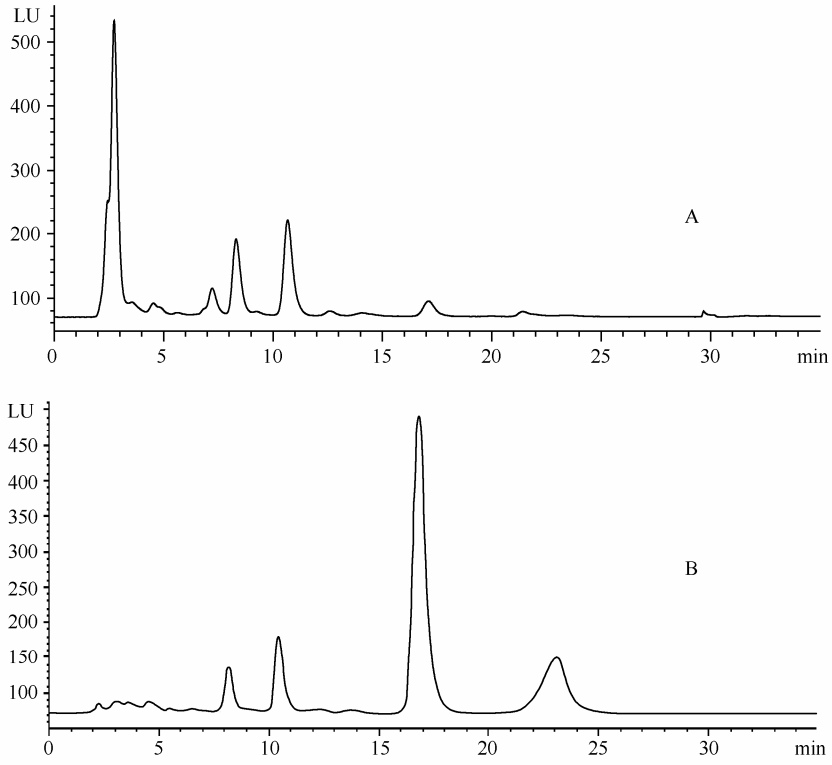

FIG. 4. Chromatograms of toxins extracted from *A. tamarense* (ATHK) before (A) and after (B) hydrolysis.

TABLE 2

The Toxin Level of *A. tamarense*, *A. salina*, *N. awatschensis,* and *L. japonicus* Samples Analyzed Using Mouse Bioassay

Samples			Symptoms of Mouse	Effects/Toxicity
Control	0.1 mol·L^{-1} Hydrochloric Acid (HCl)		Painful and Uneasy, But Recovered Soon	-
	0.1mol·L^{-1} Acetie Acid		Painful and Uneasy, But Recovered Soon	-
	A. salina	Extracted by HCl	Painful and Uneasy, But Recovered Soon	-
		Extracted by Acetie Acid	Painful and Uneasy, But Recovered Soon	-
	N. avatschensis	Extracted by HCl	Painful and Uneasy, But Recovered Soon	-
		Extracted by Acetie Acid	Painful and Uneasy, But Recovered Soon	-
	L. japonicus	Extracted by HCl	Painful and Uneasy, But Recovered Soon	-
		Extracted by Acetie Acid	Painful and Uneasy, But Recovered Soon	-
1 mL *A. tamarense* (9×10^4 Cells)			Gasp, Convulsions, Leap, Respiratory Failure, and Died at 5′25″	++++ (1.83 MU·mL^{-1})
A. salina (0.1 mol·L^{-1} Acetie Acid)	Lasted for 1 Day		Gasp, Convulsions, Leap, Respiratory Failure, and Died at 6′48″	++++ (2.18 MU·g^{-1})
	Lasted for 5 Days		Gasp, Convulsions, Leap, Respiratory Failure, and Died at 6′28″	++++ (2.1 MU·g^{-1})
	Facets Collected on the 4th Day		Gasp, Convulsions, Leap, Respiratory Failure, and Died at 5′51″	++++ (2.6 MU·g^{-1})
N. avatschensis (0.1 mol·L^{-1} Acetie Acid)	Accumulated Directly (*A. tamarense*→*N. avatschensis*)		Irregular Breathing, Convulsions, Staggering, Leap, Did Not Die	+
	Accumulated Indirectly (*A. tamarense*→*A. salina*→*N. avatschensis*)		Irregular Breathing, Convulsions, Staggering, Leap, Did Not Die	+
L. japonicus (0.1 mol·L^{-1} Acetie Acid)	Accumulated Directly (*A. tamarense*→*L. japonicus*)		Irregular Breathing, Convulsions, Staggering, Leap, Did Not Die	+
	Accumulated Indirectly (*A. tamarense*→*A. salina*→*L .japonicus*)		Irregular Breathing, Convulsions, Staggering, Leap, Did Not Die	+

Note. ++++ means strong toxicity; + means weak toxicity; - means negative control.

Estimation of PSP Level in A. salina

Based on the results of mouse assay and HPLC analysis, the content of PSP produced by *A. tamarense* was 5.538 pg STX equal·cells^{-1}, or 2.0×10^{-5} MU·cells^{-1}. The toxicity of *A. salina* exposed to *A. tamarense* for 1 day was 2.2 MU·g^{-1}, which was about 6.092×10^{5} pg STX equal·g^{-1}. The average wet-weight per individual of *A. salina* was 0.054 mg, and the toxicity was about 3.29 pg STX equal/ individual.

DISCUSSION

Growth and toxin production of dinoflagellate are greatly influenced by the environmental factors such as light, salinity, temperature and nutriment[23-27], as well as algale growth rate and nutrition metabolism[28]. The biosynthesis, transform, decomposition and excretion of phycotoxins have a relationship with algae physiology, which is strongly affected by environmental factors. In this paper, C1, C2, GTX1, GTX2, GTX3, GTX4, and GTX5 were detected in *A. tamarense* with the total toxicity of 18.5 fmol STX Equal/cell. The toxin content and profile of *A. tamarense* were slightly different from the previously reported result[29] which might be due to the sample collection time. Martins *et al.*[30] found that saxitoxin production is lost in *A. lusitanicum* during routine culture maintenance.

We found that both *N. awatschensis* and *L. japonicus* could accumulate PST by direct ingestion of *A. tamarense* cells, despite the carnivorous characteristics of these organisms, suggesting that this process is not the major way for nutrient uptake of these organisms. The algal cells found in their intestines may be due to the mistaken intake or other reasons, such as water swallow. It has been reported that *Calanus finmarchicus*, a planktonic copepod, could feed upon the non-toxic diatom *Thalassiosira weiffflogii* and avoid toxic dinoflagellate *A. excavatum* when presented with a mixture of both algae[9], suggesting that it may have some *A. excavatum* cells in its body and that the copepod could ingest the toxic dinoflagellate, either in a wrong way or during exploratory bouts of feeding. Toxin analysis results of *C. finmarchicus* samples showed a similar toxin profile to that of the toxic dinoflagellate, suggesting that the copepod could accumulate toxins from *A. excavatum*[9]. White *et al.* [31] also has demonstrated that first-feeding larvae of red sea bream, *Pagrus major*, could ingest *A. excavata* directly. However most studies showed that crustaceans[11] and fishes[1,15] could accumulate PSP indirectly by ingesting toxic zooplankton or other organisms. The PSP mainly occurs in the liver, gut, gill, *etc.*[1,15]. Since PSP does not appear to accumulate in muscle of fish, and humans who consume only the muscle are unlikely to become intoxicated, whereas those who eat the viscera are more easily to become sick[32-34].

Zhoushan fishery is one of the most important fish industries in China. The HAB caused by *Prorocentrum donghaiense* and toxic *Alexandrium* species not only do harm to the different marine organisms through the toxic effects, but also pose a potent threat to human-beings through the toxin transfer process *via* marine food chains. Marine zooplankton, crustaceans or fishes could accumulate PST directly from toxic algae or by ingesting toxic zooplankton after the HAB event. Therefore, to protect the environment and human health, great attention should be paid to HAB and marine algal toxins from the environmental and epidemiological view.

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