Transfer of Paralytic Shellfish Toxins via Marine Food Chains: A Simulated Experiment

ZHI-JUN TAN*,†, TIAN YAN*,§, REN-CHENG YU*, and MING-JIANG ZHOU*

*Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, Shandong, China; †Graduate School, Chinese Academy of Sciences, Beijing 100039, China

Objective To study the transfer of paralytic shellfish toxins (PST) using four simulated marine food chains: dinoflagellate Alexandrium tamarense→Artemia Artemia salina→Mysid 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. Toxic 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 1.65×105 µg STX equal/individual. Toxin accumulation in A. salina from the tamarense directly or indirectly via the food chains. 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[4-9]. 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 Zoushan 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 PST toxins indirectly by ingesting toxic zooplankton after the HAB event.

REFERENCES

1The work was supported by National Basic Research Project No. 2001 CB409700, NNSFC KZCX2-YW-208.
2Correspondence should be addressed to Tian YAN. Fax/Tel: 86-532-82898589. Fax: 86-532-82893088. E-mail: tianyan@ms.qdio.ac.cn

Biographical note of the first author: Zhi-Jun TAN, male, born in 1978, graduate student of IOCAS and GSCAS, majoring in marine ecotoxicology and HAB. E-mail: zhijuntandy@hotmail.com

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Three typical organisms were chosen in our study to examine the transfer of PST via food chains. Perch \textit{Lateolabrax japonicus} is an important commercial fish at northern part of China. Mysid shrimp \textit{Neomysis awatschensis} belonging to genus \textit{Mysisidopsis}, is widely distributed along the coast of China, and is used as the standard test organism in toxicity bioassay\cite{19}. Artemia \textit{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.

\section*{MATERIALS AND METHODS}

\subsection*{Maintenance of Algae and Test Organisms}

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

Juvenile fishes of perch \textit{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 \textit{N. awatschensis} 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 \textit{A. salina} (hatching rate is about 80% under 25°C) 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\cite{20}.

\subsection*{Feeding Experiments}

To test the ability of \textit{N. awatschensis} and \textit{A. salina} to ingest of \textit{A. tamarense}, the animals were starved for 24 hours prior to the experiment. Diluted culture of \textit{A. tamarense} collected at the exponential phase (2000 \text{cells·mL}^{-1}) with fresh seawater was used in the experiment, \textit{Isochrysis} sp. (40 000 \text{cells·mL}^{-1}) was used as control. For the experiment of \textit{N. awatschensis}, 200 individuals were put into 800 mL algae medium in a 1 L beaker. For the experiment of \textit{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 \textit{A. salina} and 30 individuals of \textit{N. awatschensis} were collected on a GF/C membrane to analyze the relationship between \textit{Chl.a} level and cell number.

All samples were ground and extracted with 10 mL 90% acetone, and stored at -20°C in dark for 24 hours. The \textit{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 \textit{Chl.a}, the intestine samples were also observed for \textit{A. tamarense} cells to confirm the ingestion of \textit{A. tamarense} cells by the different organisms. The ingestion of \textit{A. tamarense} cells by \textit{L. japonicus} was tested only by this method due to the difficulty in analysis of \textit{Chl.a} in \textit{L. japonicus}.

\subsection*{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 \textit{A. tamarense}. Seawater or culture of \textit{A. tamarense} was replaced every day. Continuous aeration was given during the experiment. The temperature was 20°C-22°C.

In experiment of \textit{A. salina}, about 10^4 individuals were put into each tank containing diluted culture of \textit{A. tamarense}, and 20 mL \textit{Isochrysis} sp. (5×10^5 \text{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 \textit{A. tamarense} to accumulate PST for 24 hours. These toxic artemia were then used as food of \textit{N. awatschensis} and \textit{L. japonicus} in the following experiments.

In experiment of \textit{N. awatschensis}, 100 individuals were put into each tank. For simulated food chain \textit{A. tamarense}→\textit{N. awatschensis}, mysid shrimps were exposed to culture of \textit{A. tamarense}, and non-toxic artemia (about 3×10^4 individuals, wet
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 \(A. \text{salina}\) samples were estimated according to the results of HPLC and mouse bioassay.

### RESULTS

**Feeding Experiments**

The Chl-a level of \(A. \text{tamarense}\) and Isochrysis sp. increased with the number of algae cells. The Chl-a content in a single \(A. \text{tamarense}\) 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 \(N. \text{awatschensis}\) and \(A. \text{salina}\) could ingest \(A. \text{tamarense}\) and Isochrysis sp. cells directly, but \(A. \text{salina}\) was more inclined to ingest algae than \(N. \text{awatschensis}\). After 70 minutes, the Chl-a level of \(A. \text{tamarense}\) in \(A. \text{salina}\) and \(N. \text{awatschensis}\) was 0.87 and 0.024 \(\mu\)g·mg\(^{-1}\), respectively. The Chl-a level of Isochrysis sp. in \(A. \text{salina}\) and \(N. \text{awatschensis}\) was 0.024 and 0.004 \(\mu\)g·mg\(^{-1}\), respectively (Fig. 2). \(A. \text{tamarense}\) cells were observed in the intestines of \(L. \text{japonicus}\), \(N. \text{awatschensis}\) and \(A. \text{salina}\) after these organisms were exposed to 0.2×10\(^5\) cells·mL\(^{-1}\) \(A. \text{tamarense}\) for 24 hours (Fig. 3). However, only a few cells of \(A. \text{tamarense}\) could be observed in the intestine of \(L. \text{japonicus}\), compared to the congregated cells of \(A. \text{tamarense}\) in the intestine of \(A. \text{salina}\), suggesting that \(A. \text{salina}\) could accumulate PST more easily from \(A. \text{tamarense}\) than \(L. \text{japonicus}\).

**HPLC Analysis**

The PSP toxin profile and content of \(A. \text{tamarense}\) 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 reanalyzed\(^{21}\). 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°C. 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
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, Mysis, and Perch Samples

| 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 | 0.18 | 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-sulfocarbamoyl 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·mL⁻¹. Both live *A. salina* and the facets contained a high content of PST. The toxicity was 2.18 and 2.1 MU·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·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.
TABLE 2

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

<table>
<thead>
<tr>
<th>Samples</th>
<th>Symptoms of Mouse</th>
<th>Effects/Toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 0.1 mol·L⁻¹ Hydrochloric Acid (HCl)</td>
<td>Painful and Uneasy, But Recovered Soon</td>
<td>-</td>
</tr>
<tr>
<td>0.1 mol·L⁻¹ Acetic Acid</td>
<td>Painful and Uneasy, But Recovered Soon</td>
<td>-</td>
</tr>
<tr>
<td><em>A. salina</em></td>
<td>Painful and Uneasy, But Recovered Soon</td>
<td>-</td>
</tr>
<tr>
<td>Extracted by HCl</td>
<td>Painful and Uneasy, But Recovered Soon</td>
<td>-</td>
</tr>
<tr>
<td><em>N. awatschensis</em></td>
<td>Painful and Uneasy, But Recovered Soon</td>
<td>-</td>
</tr>
<tr>
<td>Extracted by HCl</td>
<td>Painful and Uneasy, But Recovered Soon</td>
<td>-</td>
</tr>
<tr>
<td><em>L. japonicus</em></td>
<td>Painful and Uneasy, But Recovered Soon</td>
<td>-</td>
</tr>
<tr>
<td>Extracted by Acetic Acid</td>
<td>Painful and Uneasy, But Recovered Soon</td>
<td>-</td>
</tr>
</tbody>
</table>

1 mL *A. tamarense* (9×10⁴ Cells)

<table>
<thead>
<tr>
<th>Samples</th>
<th>Symptoms of Mouse</th>
<th>Effects/Toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. salina (0.1 mol·L⁻¹ Acetic Acid)</td>
<td>Gasp, Convulsions, Leap, Respiratory Failure, and Died at 5′25″ (1.83 MU·mL⁻¹)</td>
<td>++++</td>
</tr>
<tr>
<td>Lasted for 1 Day</td>
<td>Gasp, Convulsions, Leap, Respiratory Failure, and Died at 6′48″ (2.18 MU·g⁻¹)</td>
<td>+++++</td>
</tr>
<tr>
<td>Lasted for 5 Days</td>
<td>Gasp, Convulsions, Leap, Respiratory Failure, and Died at 6′28″ (2.1 MU·g⁻¹)</td>
<td>++++</td>
</tr>
<tr>
<td>Facets Collected on the 4th Day</td>
<td>Gasp, Convulsions, Leap, Respiratory Failure, and Died at 5′51″ (2.6 MU·g⁻¹)</td>
<td>+++++</td>
</tr>
</tbody>
</table>

*N. awatschensis* (0.1 mol·L⁻¹ Acetic Acid)

Accumulated Directly

(A. tamarense→*N. awatschensis*)

Irregular Breathing, Convulsions, Staggering, Leap, Did Not Die

Accumulated Indirectly

(A. tamarense→*A. salina*→*N. awatschensis*)

Irregular Breathing, Convulsions, Staggering, Leap, Did Not Die

*L. japonicus* (0.1 mol·L⁻¹ Acetic 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 \(2.0 \times 10^5\) 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\(\times 10^5\) 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 nutrient\(^{[23-27]}\), as well as algal 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\(^{[30]}\), 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 tox profile to that of the toxic dinoflagellate, suggesting that the copepod could accumulate toxins from A. excavatum\(^{[31]}\). 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, gall, 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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