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Multi-year assessment of paralytic shellfish toxins in hard clam species along the coastline of Jiangsu Province, China

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Abstract

Paralytic shellfish toxins (PSTs) are notorious neurotoxins that threaten public health and food safety worldwide. Although PST monitoring programs have recently been established throughout China, the profiles and variation of PSTs in important commercial clams (e.g., *Mactra veneriformis*, *Ruditapes philippinarum*, and *Meretrix meretrix*) along the Jiangsu Province coastline remain largely unexplored. In this study, a validated hydrophilic interaction liquid chromatography–tandem mass spectrometry (HILIC-MS/MS) method was used to examine PST profiles and levels in 540 clam samples from natural production areas along Jiangsu Province coastline during 2014–2016. Although the PST levels (≤6.38 μg saxitotoxin equivalents (eq)/kg) were consistently below European Union regulatory limits (≤800 μg saxitotoxin eq/kg) during this time period, saxitotoxin, decarbamoylsaxitotoxin, and gonyautoxins 1 and 4 were detected, and nearly 40% of the samples were saxitotoxin-positive. The PST levels also varied significantly by seasons, with peak values observed in May during 2014–2016. This is the first systematic report of PSTs in clams from Jiangsu Province, and additional research and protective measures are needed to ensure the safety of clams harvested in this area.

Key words: paralytic shellfish toxin, HILIC-MS/MS, clam, seasonal variation, Jiangsu Province

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1 Introduction

The hard clams *Mactra veneriformis* (surf clam), *Ruditapes philippinarum* (steamer clam), and *Meretrix meretrix* (Asiatic hard clam) are commercially important species that are harvested in coastal areas in China, and the coastal tidal flat of Jiangsu Province is a major culturing area for these clams, owing to its naturally favorable conditions (Fig. 1). Indeed, in 2013, more than 600 kt of hard clams were harvested from Jiangsu Province, much of which was exported to Japan, Korea, Europe, and other regions (Ni, 2013; Shen et al., 2012; Liao, 2012; Chen and Yao, 2005; Li et al., 2014; Qin and Shang, 2008; Liu et al., 2015). However, due to rapid industrial development and the deterioration of coastal water bodies, assessing the safety of seafood products from this region has become increasingly more important (Jiang et al., 2015).

One of the main health concerns is paralytic shellfish poisoning. Paralytic shellfish poisoning is a common seafood toxicity problem that occurs worldwide (Etheridge, 2010). The illness typically results from the consumption of bivalves that are contaminated with paralytic shellfish toxins (PSTs), which include a range of alkaloids that are based on the 3, 4, 6-trialkyltetrahydropurine skeleton (Fig. 2) (Bricelj and Shumway, 1998). These compounds are potent neurotoxins that block voltage-gated sodium channels in excitable cells, thereby suppressing ion permeation and causing a variety of symptoms, including tingling, numbness, headaches, weakness, and difficult breathing in humans, which can subsequently result in death (Hall and Strichartz, 1990). At least 30 PSTs have been identified to date, the better-known of which include the carbamate toxins, e.g., saxitoxin (STX), neosaxitoxin (NEO), and the gonyautoxins (GTX1 to GTX4); the decarbamoyl toxins, e.g., decarbamoylsaxitoxin (dcSTX), decarbamoylneosaxitoxin (dcNEO), and the decarbamoylgonyautoxins (dcGTX1 to dcGTX4); and the *N*-sulfocarbamoyl toxins, e.g., B1 (GTX5), and C1 to C4; and the compounds vary in toxicity, owing to their different affinities to sodium channels, with the carbamate toxins being the most toxic and the *N*-sulfocarbamoyl derivatives being the least toxic (Etheridge, 2010; Bricelj and Shumway, 1998; Hall and Strichartz, 1990).

Bivalve filter feeders, such as clams, cockles, and mussels, generally accumulate these toxins after feeding on blooms of PST-producing microalgae, especially marine dinoflagellates of the genera *Alexandrium* and *Gymnodinium* (Asakawa et al., 2015). Notably, the distribution and frequency of PST-producing microalgae red tide have increased over the past few years, and specific strains, including *Gymnodinium catenatum* (*G. catenatum*) and *Alexandrium tamarense* (*A. tamarense*), have been reported to occur dominantly in the red tides of the Jiangsu coastal water (Table 1) and other China sea areas (Li et al., 2012;

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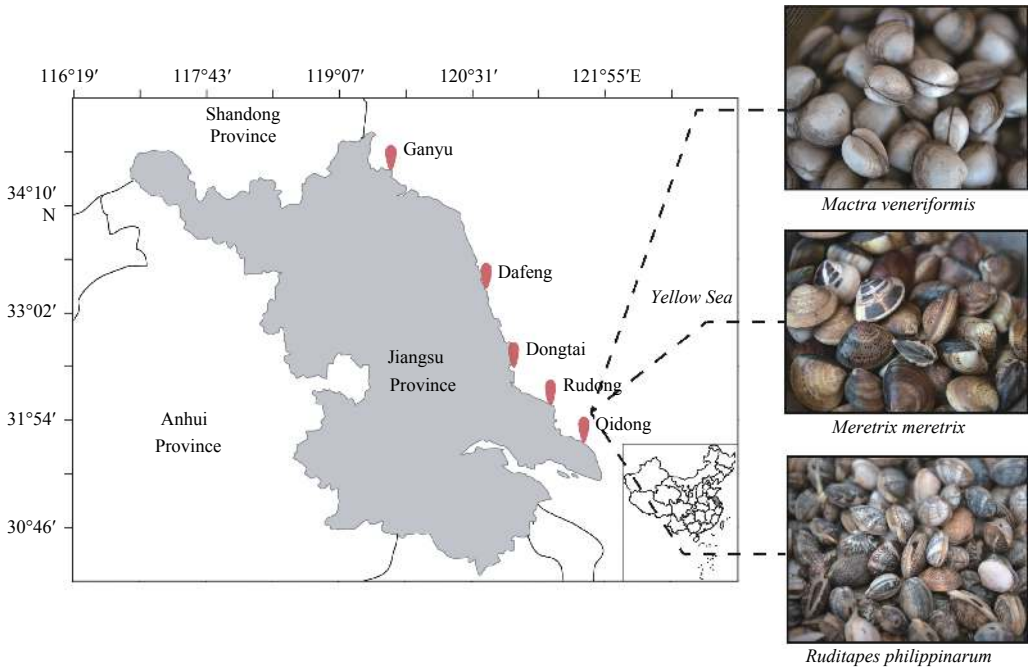
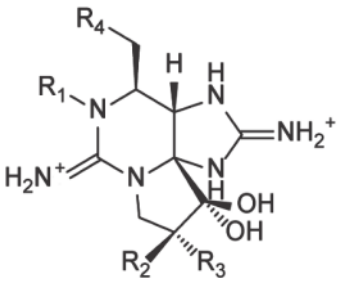


Fig. 1. The sampling locations along Jiangsu coastline and representative clam samples collected from Qidong County, Jiangsu Province.



Toxins	R_1	R_2	R_3	R_4	Toxicity factor
C1	H	H	$-OSO_3^-$	—O, $NHSO_3^-$	NA
C2	H	$-OSO_3^-$	H		0.1
STX	H	H	H		1
GTX2	H	H	$-OSO_3^-$		0.4
GTX3	H	$-OSO_3^-$	H	—O, NH_2	0.6
NEO	OH	H	H		1
GTX1	OH	H	$-OSO_3^-$		1
GTX4	OH	$-OSO_3^-$	H		0.7
GTX5	H	H	H	—O, $NHSO_3^-$	0.1
dcSTX	H	H	H		1
dcGTX2	H	H	$-OSO_3^-$		0.2
dcGTX3	H	$-OSO_3^-$	H	-OH	0.4
dcNEO	OH	H	H		0.4

Fig. 2. Chemical structure of PSTs and relative potency of STX and analogues calculated by EFSA.

Lin et al., 2012; Wang et al., 2011, 2004, 2008; Zhou et al., 1999; Wang and Hsieh, 2005; Yu and Luo, 2016). As a result of ingesting these microalgae and accumulating PSTs, various bivalve species have become serious hazards to public health in China. In fact, since 1967, shellfish poisoning incidents have occurred frequently along the country's coastline, and most of these events have been attributed to PSTs or similar toxins, based on mouse bioassays or the symptoms of the victims (Yu and Luo, 2016).

Table 1. PST-producing microalgae outbreaks along Jiangsu coastline during 2005–2013[1)]

Year	No. of outbreaks	Outbreak area /km^2	Dominant microalgae species
2005	4	1 275	*G. catenatum*
2006	1	600	
2010	2	220	

Note: [1)] Data obtained from *Jiangsu Marine Environmental Quality Bulletin* published by Ocean and Fishery Bureau of Jiangsu Province in 2013.

Accordingly, effective monitoring programs for PSTs in shellfish are important both from a public health perspective and for protecting the region's aquaculture industry. Currently, the mouse bioassay method is used for the routine monitoring of shellfish for PSTs. However, this method suffers from low sensitivity (LOD approximately 40 μg STX eq/(100 g) shellfish), poor re-

producibility, and high variability, in addition to ethical arguments, owing to the assay's use of live animals (Ben-Gigirey et al., 2012; Association of Official Analytical Chemists, 2000). Reversed phase column coupled with post-column oxidation and fluorescence detection (LC-ox-FLD) has also been used as a high-sensitivity technique for detecting PSTs; however, the method typically involves a complex set-up and protocol, along with daily maintenance of the equipment (Turner et al., 2011). More recently, the use of hydrophilic interaction liquid chromatography-tandem mass spectrometry (HILIC-MS/MS) is recommended by many researchers, which is more rapid, specific, and sensitive in the quantification analysis of PSTs, and the method has subsequently been used to determine the PSTs contents of a variety of sample matrices, including microalgae and commercial shellfishes (Turner et al., 2015; Zhuo et al., 2013; Boundy et al., 2015; Jansson and Åstot, 2015; Dell'Aversano et al., 2005; Costa et al., 2009; Humpage et al., 2010; Watanabe et al., 2013).

Because the profiles and monthly variation of PSTs in clam samples cultivated along the coastline of Jiangsu Province have yet to be reported (although the red tides bloom have been frequently reported in last decades) and because the use of HILIC-MS/MS for detecting multiple PSTs in clam matrices cultivated in Jiangsu has not been reported, the main goals of the present study were (1) to establish a routine HILIC-MS/MS-based PST monitoring program for clams harvested in Jiangsu Province; (2) to determine the profiles and seasonal variation of PSTs in clam species cultivated along the Jiangsu coastline (*M. veneriformis*, *R. philippinarum* and *M. meretrix*); and (3) to generate information that will help farmers and consumers to ensure the safety of the local clam commodities. This is the first time that multiple PSTs have been analyzed on a monthly basis from multiple production areas in Jiangsu Province.

2 Materials and methods

2.1 *Chemicals and materials*

STX (66.3 μmol/L); dcNEO (28.9 μmol/L); C1 (113.4 μmol/L); C2(33.9 μmol/L); dcGTX2 (116 μmol/L), dcGTX3 (26.1 μmol/L), dcSTX (65.0 μmol/L), GTX1 (60.4 μmol/L), GTX4 (19.7 μmol/L), GTX2 (114.2 μmol/L), GTX3 (43.4 μmol/L), NEO (65.6 μmol/L), and GTX5 (55.7 μmol/L) were all purchased from the National Research Council, Institute for Marine Biosciences (NRC-CNRC; Halifax, NS, Canada).

MS-grade acetonitrile was obtained from Merck (Darmstadt, Germany); HPLC-grade formic acid was purchased from Tedia (Fairfield, CA, USA); ultra-pure water was generated using a Milli-Q system (Millipore, Billerica, MA, USA), and F/2 culture medium was purchased from the Guangyu Biological Technology Co., Ltd (Shanghai, China). All other chemicals and reagents were of analytical grade, and Supelco ENVI-Carb cartridge (250 mg/(3 mL)) were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2 *Field sampling*

From January to December during the year 2014–2016, our laboratory collected three commercially important clam species (*M. veneriformis*, *R. philippinarum* and *M. meretrix*) from the intertidal zone of Jiangsu, China, on a monthly basis. The specific collecting sites (Fig. 1) included Qidong (Lvsi Harbor, 32°01′N, 121°40′E, Area 1), Rudong (Changsha County, 32°25′N, 121°18′E, Area 2), Dongtai (Tiaozini, 32°45′N, 120°54′E, Area 3), Dafeng (Dongsha County, 33°16′N, 120°50′E, Area 4), and Ganyu County (34°29′N, 121°49′E, Area 5); and control samples (n=5, per species) that had not been exposed to PSTs were kindly provided by Xihe Wan from the Institute of Oceanology and Marine Fisheries (Jiangsu Province, China).

The exterior surfaces of all of the clam samples were thoroughly cleaned with fresh water, in order to remove sand and other foreign material, and then the tissues were removed from the shells, chopped and homogenized with a mixer, and stored at –20°C until analyzed. In addition, the identities of all the collected samples were authenticated by an expert, and all specimens were deposited at the College of Pharmacy, Nanjing University of Chinese Medicine, Nanjing, China.

2.3 *Standard preparation and sample spiking*

Stock solutions of STX (493.6 μg/L), dcNEO (498.8 μg/L), C1 (449.2 μg/L); C2 (134.3 μg/L), dcSTX (428.0 μg/L), NEO (413.4 μg/L), GTX1 (497.0 μg/L), GTX4 (162.0 μg/L), GTX2 (903 μg/L), GTX3 (343.2 μg/L), dcGTX2 (817.4 μg/L), dcGTX3 (184 μg/L), and GTX5 (422.6 μg/L) were prepared using 1% (v/v) aqueous acetic acid, and these stock solutions were further diluted with 1% aqueous acetic acid to different concentrations, which were used as working standards for calibration curves and method validations. All the solutions were stored at 4°C.

Standard samples were prepared for calibration by spiking control *M. veneriformis*, *R. philippinarum* and *M. meretrix* tissues with working standards: 1.86–59.64 μg/kg for GTX1, 2.43–38.88 μg/kg for GTX4, 1.55–49.60 μg/kg for NEO, 1.86–59.86 μg/kg for dcNEO, 1.68–53.90 μg/kg for C1, 2.01–32.23 μg/kg for C2, 1.53–49.04 μg/kg for dcGTX2, 1.38–44.16 μg/kg for dcGTX3, 1.69–54.18 μg/kg for GTX2, 2.57–41.18 μg/kg for GTX3, 1.58–50.72 μg/kg for GTX5, 0.80–51.36 μg/kg for dcSTX, and 0.93–59.24 μg/kg for STX. Quality control (QC) samples were independently prepared in the same way, using 3.72, 14.88 and 59.64 μg/kg GTX1; 2.44, 9.72 and 38.88 μg/kg GTX4; 3.1, 12.4 and 49.6 μg/kg NEO; 3.06, 12.26, and 49.04 μg/kg dcGTX2; 3.72, 29.84 and 59.64 μg/kg dcGTX3; 3.72, 29.76 and 59.86 μg/kg dcNEO; 3.36, 26.88 and 53.90 μg/kg C1; 2.01, 16.12 and 32.23 μg/kg C2; 3.4, 27.08 and 54.18 μg/kg GTX2; 2.56, 20.6 and 41.18 μg/kg GTX3; 3.16, 25.36, and 50.72 μg/kg GTX5; 1.6, 12.84, and 51.36 μg/kg dcSTX; and 1.86, 14.8, and 59.24 μg/kg STX. Both the standard and quality control samples were then subjected to the same extraction and analysis procedures as the clam samples (described below).

2.4 *Sample extractions and SPE cleanup*

The extraction of PSTs from the clam samples was performed according to the AOAC 2005.06 double extraction acid procedure (Lawrence et al., 2005). Briefly, (5±0.1) g of each clam homogenate was independently vortexed (Thermo Scientific, Waltham, MA, USA) with 5 mL of 1% (v/v) aqueous acetic acid for 90 s, incubated in a boiling water bath for 5 min, cooled to room temperature, and then vortexed for another 90 s. Afterward, the samples were centrifuged at 4 500 r/min for 10 min, and the supernatants were transferred to graduated polypropylene tubes. The remaining homogenate was extracted again using the same conditions, after which the supernatants were combined and diluted to 15 mL using 1% (v/v) aqueous acetic acid.

The acetic acid PST extracts were prepared for UFLC-MS/MS analysis, using an automated SPE cleanup process with a SUPELCO Visiprep SPE Vacuum Manifold liquid handler (Bellefonte, PA, USA). Briefly, 1.5 mL of each of the acetic acid extracts was transferred to a polypropylene tube and 5 μL of NH_4OH added, then 500 μL of sample extracts independently loaded onto an Supelco ENVI-Carb cartridge (250 mg/(3 mL), Sigma–Aldrich, St. Louis, MO, USA), which had been previously conditioned us-

ing 3 mL of acetonitrile/water/acetic acid (20:80:1, v/v/v) and equilibrated using 3 mL of water/NH_4OH (1 000:1, v/v), then washed with 1 mL of water at 3 mL/min. After each step, an air push was used to ensure the complete elution of the reagents. Next, the sample extracts were eluted and collected by adding 2 mL of acetonitrile/water/acetic acid (20:80:1, v/v/v) at 3 mL/min. The resulting eluent was vortexed, and centrifuged at 12 000 r/min for 5 min before UFLC-MS/MS injection.

2.5 *Ultra-fast liquid chromatography (UFLC) -MS/MS method*

Chromatographic analysis was performed using a Prominence UFLC system (Shimadzu, Kyoto, Japan). Separation was achieved using an Agilent Zorbax Rx-SIL column (100 mm×4.6 mm; i.d. 5 μm) with a guard cartridge at 30°C, and the mobile phase was composed of acetonitrile (A) and 0.1% formic acid aqueous solution (B), with an elution gradient as follows: 0–12 min, 85%–78% of A; 12–15 min, 78%–50% of A; and 15–20 min 50% of A. The flow rate was set to 0.6 mL/min, and an injection volume of 5 μL was selected. The full chromatographic cycle time is 25 min, all the toxins were eluted within 20 min, and during the rest time, the column was cleaned, readjusted to the initial conditions, and equilibrated.

Triple-quadrupole linear ion trap mass spectrometers (5500 Q-Trap; Applied Biosystems, Foster City, CA, USA) that were equipped with a TurboIonSpray source were tested in positive ionization mode. Instrument control, data acquisition, and processing were performed using the associated Analyst 1.5.2 software. MS/MS data acquisition was performed in the MRM mode. In order to obtain the maximum sensitivity for detecting PSTs, the ion source temperature was set to 550°C, and the ion source voltages were set to 5.5 kV. The ion source gasses (1 and 2) were set at 55 arbitrary units and the curtain gas was set at 35 arbitrary units. The analyte-specific parameters are shown in Table S1.

2.6 *Method verification*

The methods were verificated according to Document SANCO 10684/2009 (EU Reference Laboratories for Residues of Pesticides, 2009), and the verification assessed the method's selectivity, linearity, LOQ, matrix effect, precision, accuracy, and extraction recovery. The specific UFLC-MS/MS verification procedures are described in the Supplementary materials.

2.7 *Sample quantification and toxicity equivalency factors*

The collected clam samples were prepared and analyzed as described above for PST monitoring, and the recommended European Food Safety Authority toxicity equivalency factors (Fig. 2) were used to calculate total toxicity (μg STX eq/kg) (Alonso et al., 2016; European Food Safety Authority, 2009).

2.8 *Effect of temperature on cell densities of G. catenatum*

Isolate of *G. catenatum* was kindly provided by Xihe Wan, Institute of Oceanology and Marine Fisheries, which obtained from the coastal water of Qidong County, Jiangsu Province in 2014. Temperature effects on cell densities were determined in the ALM algae incubator (Jiangnan Instrumental Inc., China) at nine temperatures (including 4, 8, 12, 16, 18, 20, 22, 24 and 28°C) between 4 and 28°C (the coastal water temperature range of Jiangsu Province). Light was supplied by four cool white fluorescent lamps set on a 12 h:12 h light-dark cycle, with an irradiance reported previously (Band-Schmidt et al., 2014). Microalgae cells in the logarithmic phase were inoculated by triplicate in 150 mL flasks filled with 20 mL of F/2 culture medium and 80 mL of artificial seawater (prepared according to Doblin et al., 1999), then cultured in nine different temperatures. After the cultivation for 7 d, cell densities of *G. catenatum* cultivated under different temperatures were measured with Countstar Automated Cell Counter (Inno-Alliance Biotech Inc., USA) under the manufacturer's instructions.

2.9 *Statistical analysis*

Analysis of variance (ANOVA) was used to compare the positive rates of PSTs in clam samples between different collecting areas, years and species using the Student-Newman-Keuls (S-N-K) procedure in Statistical Package for the Social Sciences 22.0 (SPSS, Inc., Chicago, IL, USA).

3 Results and discussion

3.1 *Optimization of UFLC-MS/MS*

Previous studies reported that HILIC columns were capable of separating PST mixtures (Turner et al., 2015; Zhuo et al., 2013; Boundy et al., 2015; Jansson and Åstot, 2015; Dell'Aversano et al., 2005). Therefore, we compared the efficacy of several typical HILIC columns, which included the Xbridge BEH amide (Waters, Santry, Ireland), Xbridge BEH HILIC (Waters), ZORBAX Rx-SIL (Agilent Technologies, Santa Clara, CA, USA), and TSKgel amide 80 (Tosoh Bioscience, Tokyo, Japan) columns. Of these, the ZORBAX Rx-SIL column separated epimeric pairs (i.e., GTX1 & GTX4, GTX2 & GTX3, dcGTX2 & dcGTX3, and C1 & C2) most effectively in current UFLC system. In addition, an acetonitrile-water mobile phase system was selected since acetonitrile provided narrower peaks, higher responses, and lower column pressure than methanol; and as reported previously, the addition of 0.1% formic acid to the aqueous portion significantly improved the ionization efficiency and MS sensitivity for STX groups (The MRM chromatograms of PST standards were all shown in Fig. 3).

Mass spectrometry indicated that all of the analytes exhibited a sufficient MS response of $[M+H]^+$, and the optimization of daughter ions and their declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP) was performed using the compound optimization tool in Analyst 1.5.2 (Table S1).

3.2 *Optimization of sample cleanup*

Previous studies reported that salts in shellfish matrices were determined to be the main cause for suppressing electrospray ionization for LC-MS/MS analysis, and the graphitized carbon solid phase extraction (GCB-SPE) was able to overcome this issue by reducing the salt interference from the matrices without significant PST losses (Boundy et al., 2015). Therefore, GCB-SPE cartridge (Superclean ENVI-Carb 250 mg/(3 mL) SPE cartridge (Sigma-Aldrich, St. Louis, MO)) was trialed for clam matrices cleanup in this study.

As recorded by Boundy et al. (2015), the acetic acid solution used in extraction procedure was found to decrease PST retention in GCB-SPE. Therefore, a dilute ammonium hydroxide solution was used to neutralize this effect. With 1, 2.5, 5, 10 μL additions of ammonium hydroxide solution to the crude clam extract, improvements to the recovery of STX and dcSTX (the least well retained PST analogues) were observed (10%–16% increase with the 0 μL control). No significant differences in MS response of STX was observed between 5 and 10 μL, therefore, 5 μL of NH_4OH was added to sample extracts before loaded onto the GCB-SPE cartridges.

GCB-SPE Elution conditions were optimized using 500 μL of elution solvent which increased in acetonitrile by 2% until all the PSTs had eluted. It was found that GTXs were the last toxins

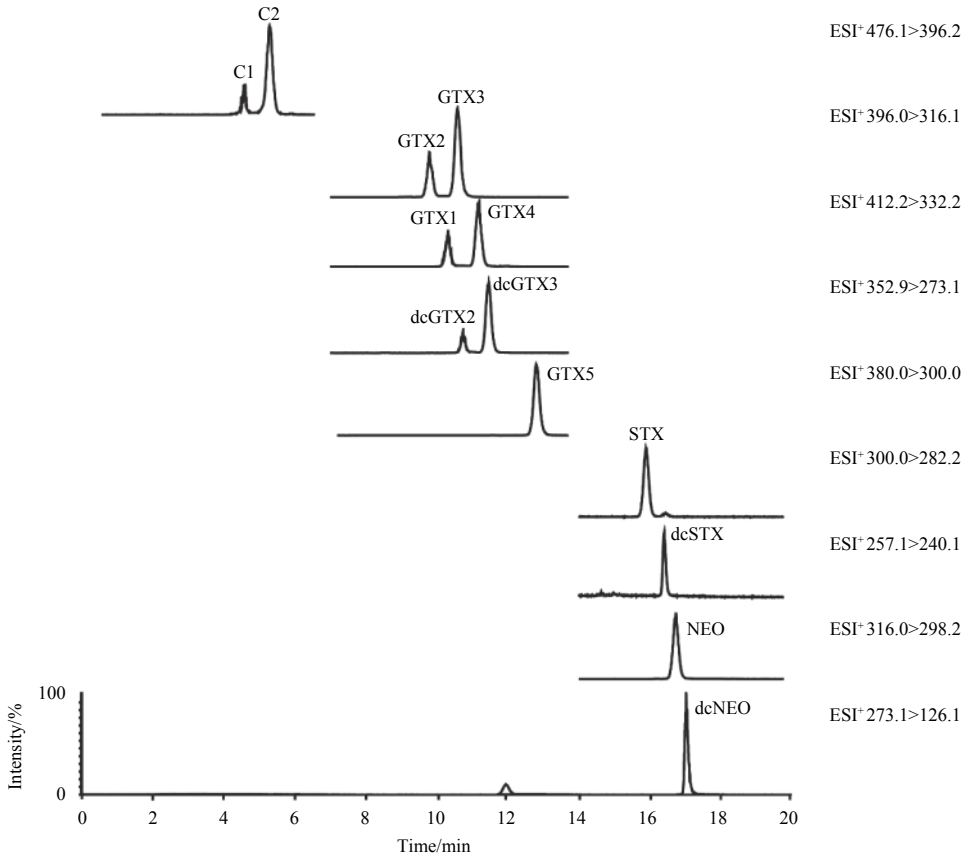


Fig. 3. MRM chromatograms of PST standards.

eluted off the cartridge with 16:84 (v/v) acetonitrile/water. Therefore, elution solution was attempted with acetonitrile/water (18:82, v/v). A total of 2 mL was required to elute all the PST analogues from the carbon. It was also found that addition of the acetic acid can significantly increase the peak response for the STXs group. For this reason, acetic acid was included in the elution solvent in this study.

3.3 *Method verification*

Using the UFLC and sample cleanup methods described above, the resolution of epimeric pairs was acceptable (Fig. 3 and Fig. S1). Furthermore, no significant interferences were observed for the retention times of any of the transitions, and the target compounds were only detected in the spiked clam samples at their specific retention times, which indicated the method's high specificity (Fig. S1). In addition, calibration curve slope ratios (matrix matched to pure solvent), which were calculated to assess signal enhancement or suppression (Granby et al., 2004), indicated that matrix effects occurred when the slope ratio values of the matrix curve of three clam tissue matrices were between 0.81–1.18 (Table S2). Therefore, either ionization suppression or signal enhancement occurred; however, the effect was not significant.

The calibration curves of PSTs all exhibited good regression linearity, with determination coefficients (R^2) that ranged from 0.993 4–0.999 9, and the calibration ranges adequately covered the variation in analyte level in each sample. In addition, the limit of quantification (LOQ) corresponded to the lowest fortification level analyzed from the different matrices, and the LOQs of all PSTs were ⩽3.5 μg/kg. In general, these limits are considered acceptable for analyzing PSTs in real clam samples.

Precision studies, which were performed to ensure both intra- and inter-day repeatability and reproducibility (Table S3), indicated that the relative standard deviation (RSD) of 13 PSTs was between 0.7% and 6.6% for the intra-day studies and between 1.0% and 7.7% for inter-day studies, which were all within the acceptable range.

The accuracy of the method was also verified by measuring the recovery of PSTs from spiked blank samples of the three different matrices (Table S4), and the overall recovery rate of the 13 PSTs was 75.1%–93.6% for the clam samples, with RSD values of <17.5%. According to the EU guideline (recovery of 70%–110% and RSD⩽20%) (EU Reference Laboratories for Residues of Pesticides, 2009), the proposed method was considered accurate, with a satisfactory recovery rate for clam matrices. Therefore, the method was validated in all three clam species, demonstrated to exhibit both excellent accuracy and reproducibility, and deemed suitable for use in the routine monitoring of PSTs in clam samples from Jiangsu Province.

3.4 *PSTs in clams from Jiangsu Province*

To fully understand the distribution and variation of PSTs in bivalves cultivated in Jiangsu Province, specimens of the three main cultivated species (*M. veneriformis*, *R. philippinarum*, and *M. meretrix*) were collected from five cultivating bases along the coastline of Jiangsu Province, during 2014–2016, respectively, and analyzed as described above. Of the 13 PSTs investigated, only STX, dcSTX, GTX1 and GTX4 were detected (Fig. S2, Tables S5–7), and among these four, STX was detected most frequently (Fig. 4a). In fact, during the monitoring period, over 40% of the clams ana-

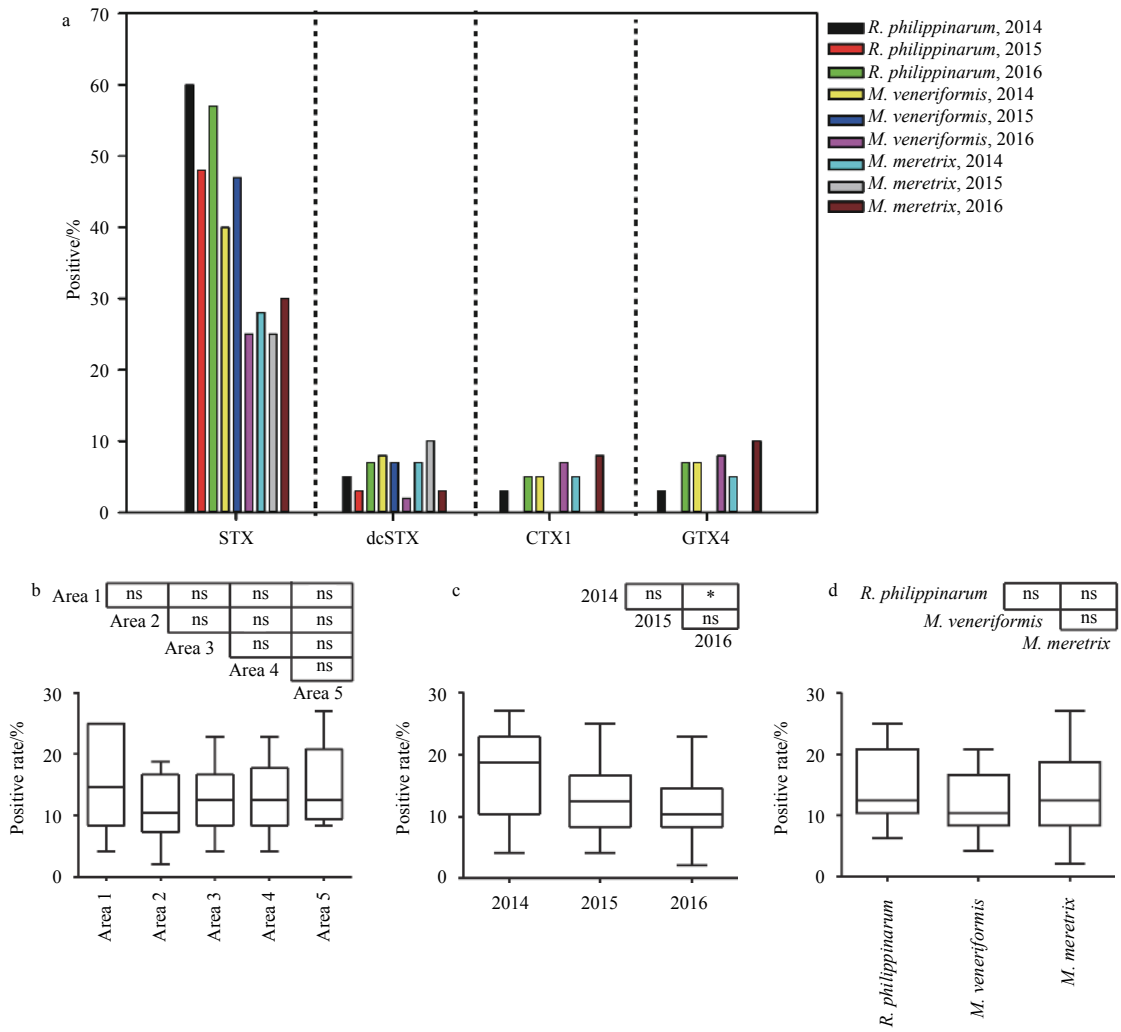


Fig. 4. PST profiles of the clam samples collected from Jiangsu coastline during 2014–2016. a. Occurrence of PST in tested clam samples during 2014–2016; comparison of the PST positive rate between different breeding areas (N=108, b), years (N=180, c) and clam species (N=180, d) in all tested clam samples, the inserted tables indicate significant differences between them. ns indicates non-significant and *p<0.05.

lyzed were contaminated with STX, followed by dcSTX, for which ~8% of the specimens were positive. Both GTX1 and GTX4 were observed, as well; however, the frequency of their detection (~4%) was much lower than those of STX and dcSTX.

STX and dcSTX are produced by the red tide microalgae such as *G. catenatum* and *A. tamarense*. According to the data provided by Professor WAN Xihe, Ocean and Fishery Bureau of Jiangsu Province (Table 2), the dominant PST-producing microalgae observed in the coastal water of Jiangsu Province during 2014–2016 were the *G. catenatum* strains, which suggest that the PSTs we detected in the clam samples were mainly originated from this microalga.

The effects of breeding area, year, and species on the variation of PST profiles in the clam specimens were also investigated. The rate of positive PST detection was not significantly affected by either locations or species (Figs 4b and d); however, it did vary by sampling years, with PSTs detected in up to 16.5% of the specimens in 2014 but only in 10.9% of the specimens in 2016 (p<0.05; Fig. 4c). According to Table 2, the average cell number of red tide microalgae varied significantly between years occurred in Jiangsu coastal water in 2014 was larger than that in 2016, which may be the reason for the high detection rate of PST in clam samples collected in 2014.

The UFLC-MS/MS quantification data indicated that the PST levels of all the clam samples during 2014–2016 (≤6.38 μg STX eq/kg) were much lower than the EU regulatory limits (800 μg STX eq/kg) (European Food Safety Authority, 2009), which due to no large-scale red tide bloom during the monitoring period.

Table 2. Characteristic of the red tide microalgae in the coastal water of Qidong, Jiangsu Province[1)]

Year	Cell density/cell·L^{-1}	Dominant microalgae species	Maximumdensity month
2014	2.91×10^3–9.57×10^5	*Skeletonema costatum*, *Encampia zoodiacus*, *Chaetoceros lorenzianus*, *G. catenatum*	May
2015	9.50×10^2–9.18×10^5	*Skeletonema costatum*, *Encampia zoodiacus*, *G. catenatum*, *Heterosigma akashiwa*	May
2016	6.33×10^2–5.55×10^5	*Skeletonema costatum*, *Encampia zoodiacus*, *G. catenatum*	May

Note: [1)] Data have been kindly provided by the Ocean and Fishery Bureau of Jiangsu Province.

Moreover, we were surprised discovered that the PST concentrations varied significantly by seasons (Fig. 5a). For all the sampling locations, the maximum PST concentrations were all observed in May during 2014–2016, whereas the minimum levels were observed during the Winter (January, February and December). Overall, the PST concentrations were highest during the spring, followed by autumn, summer and winter, respectively.

In our opinion, the observed trends in PST concentration can likely be attributed to seasonal variation in the temperature of the coastal waters. As shown in Fig. 5b, the surface temperatures of the waters we measured from collecting areas varied from 5.2 to 27.8°C in 2014, from 4.3 to 27°C in 2015, and from 6.2 to 27.1°C in 2016. The water temperature changes (from 4 to 27°C), as we discovered, have affected the growth of *G. catenatum* significantly. The maximum cell densities, as shown in Fig. 5c, were observed at the water temperature of 18°C (up to 4 900 cell/mL). According to Fig. 5b, the water temperatures occurred in May (17–18.2°C) of Jiangsu coastline during 2014–2016 were the most suitable temperatures for the growth of *G. catenatum*, which subsequently resulted in high levels of PSTs detected in the clam samples. On the other hand, when the water temperatures <8°C or >24°C, which unfavorable for the growth of *G. catenatum*, resulted in the low cell densities (320–1 540 cell/mL) and correspondingly low content of PSTs detected in the clam samples collected in the winter (seawater temperature between 4.3–8.5°C in December, January, February, and March) and summer months (seawater temperature between 23.3–27.8°C in July, August and September).

Moreover, as depicted in Figs 6a–c, the PST profiles of the three clam species were dominated by STX (mean of 81% for *R. philippinarum*, 93% for *M. veneriformis*, and 72% for *M. meretrix*). Its dicarbamoyl derivative dcSTX (mean 9% for *R. philippinarum*, 7% for *M. Veneriformis*, and 6% for *M. meretrix*), and GTX1&4 were detected at much lower levels (mean 9% for *R. philippinarum* and 22% for *M. meretrix*, respectively). In addition, the total PST concentrations of the winter and summer samples were almost exclusively composed of STX. In contrast, during the spring and autumn seasons, when the toxin concentrations were relatively higher, the proportion of STX dropped significantly, to a mean of 62.0%, whereas the relatively concentrations of dcSTX, GTX1 and GTX4 significantly increased. To fully understand this phenomenon, the toxin profile variations in *G. catenatum* strains collected from different seasons in Jiangsu coastal water should be studied in the near future.

4 Conclusions

In conclusion, the results of the present study illustrate that (1) the combination of weak cationic exchange SPE and HILIC-UFLC/MS is suitable for quantifying PSTs in commercial clam species cultivated in Jiangsu Province, owing to the method's

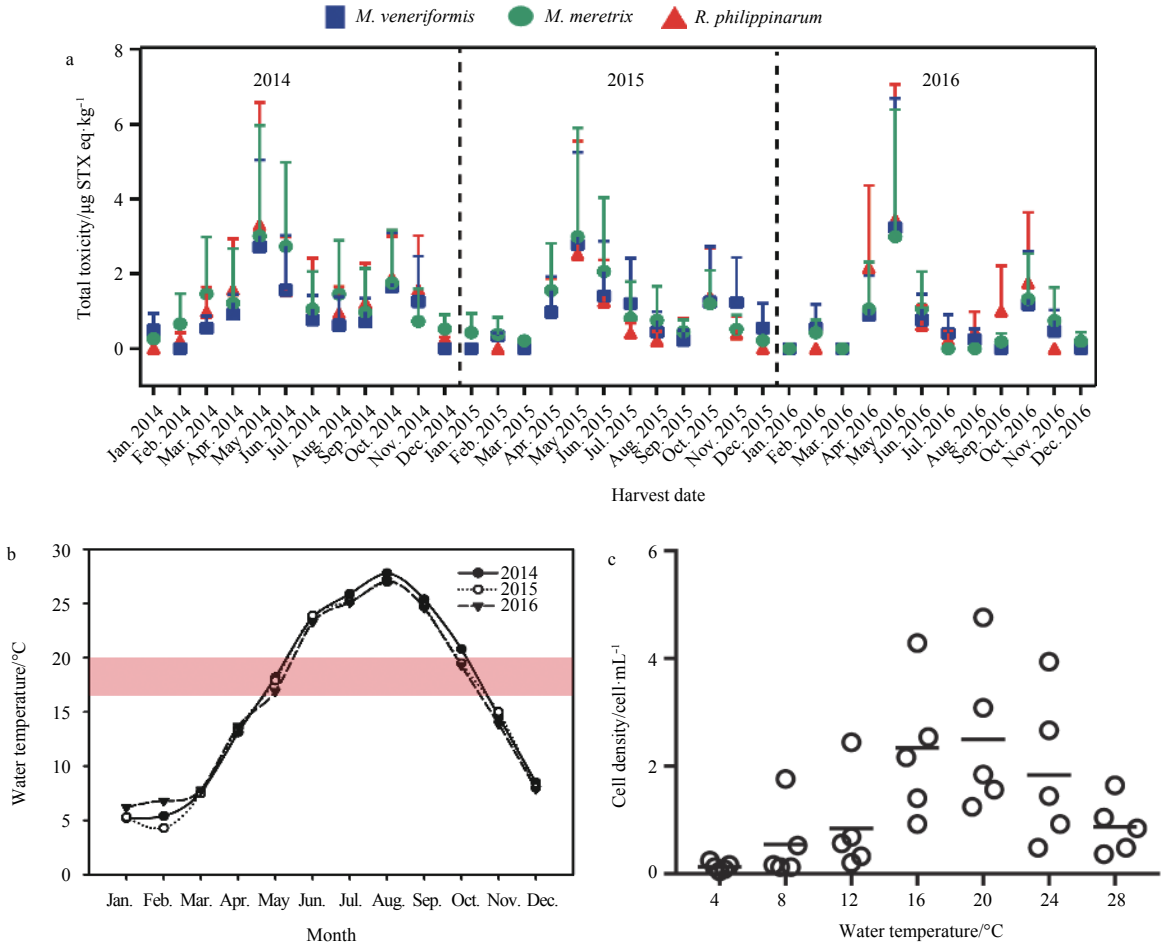


Fig. 5. Monthly variations in concentration (μg STX eq/kg) of the four PST detected in clam samples cultivated in Jiangsu (a); monthly seawater temperature variations along Jiangsu coastline (b) and cell densities of *G. catenatum* strains cultivated at different water temperatures (c).

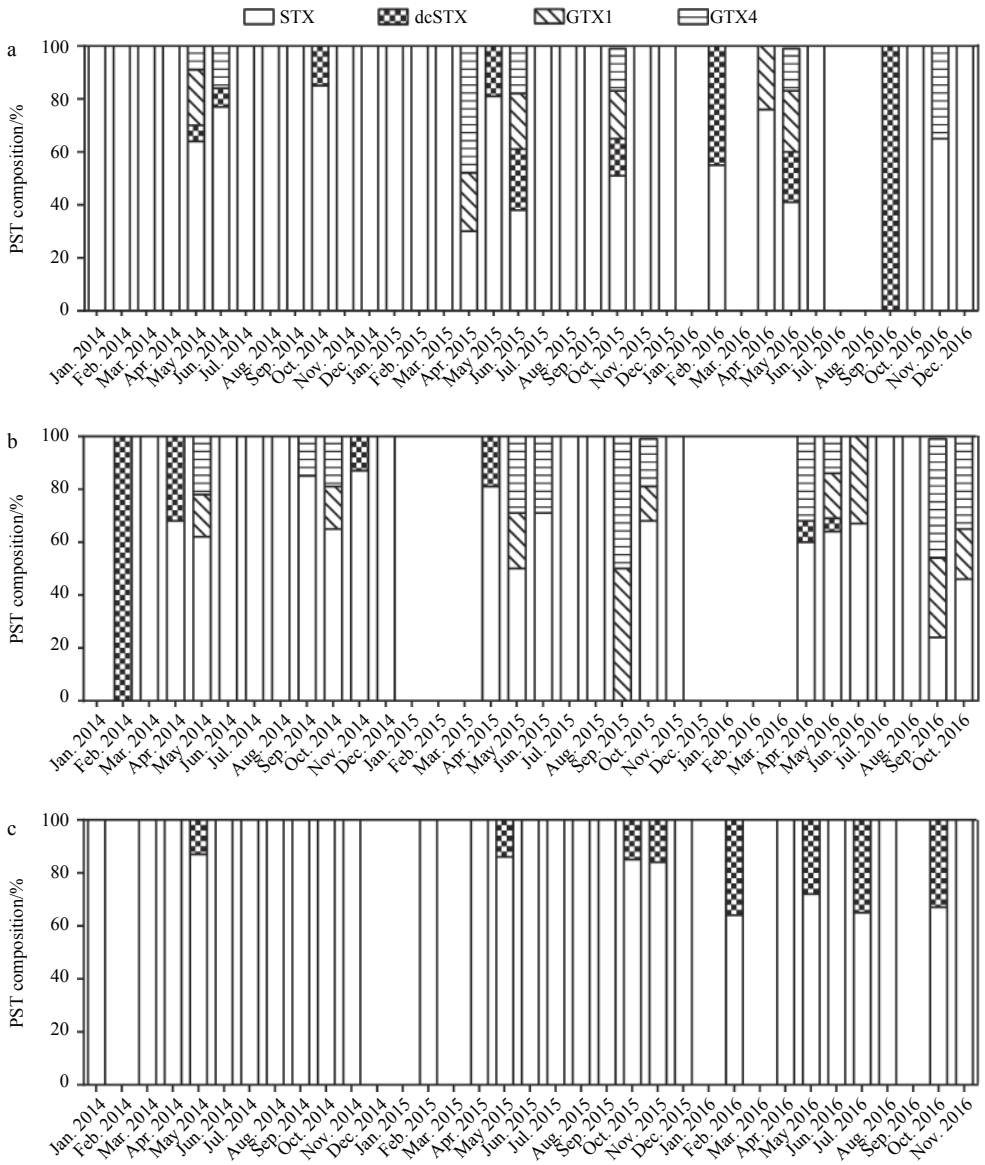


Fig. 6. Monthly variations in relative abundance (%) of the four PST detected in clam samples cultivated in Jiangsu during 2014–2016. *R. philippinarum* (a), *M. Veneriformis* (b), and *M. Meretrix* (c).

precision, sensitivity, repeatability, recovery, and suitability for routine monitoring; (2) four PSTs (STX, dcSTX, GTX1, and GTX4) occur in clams cultivated along the coastline of Jiangsu Province during 2014–2016, and ~40% of the clam specimens were STX-positive, because no large-scale of PST-producing microalgae outbreak has been occurred during this period, the toxin levels in clam samples were consistently far below the EU regulatory limits; and (3) the concentration of detected PSTs varied significantly by seasons, with peak values observed in May, when the water temperatures were favorable for growth of the dominant PST-producing microalgae *G. catenatum*'s in Jiangsu coastal water. Therefore, the PST monitoring method validated in the present study can be used to evaluate the potential risk or safety of bivalves for local consumers. The occurrence and monthly variation data presented may also be used to guide local aquaculture farmers and consumers to choose appropriate seasons for harvesting and consumption. Further studies should focus on improving our current understanding of the toxin profiles of PST-producing algae under different seasons, as well as of the transportation and bio-transformation of PSTs in different clam species.

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Supplementary information:

Fig. S1. MRM chromatograms of a blank clam extract (*R. philippinarum*) before (A) and after (B) spiked with PST standards.

Fig. S2. MRM chromatograms of tested clam sample extracts collect from Jiangsu coastline.

Table S1. MS/MS conditions used for the MRM acquisition windows for the detection of PSTs.

Table S2. Regression equation, correlation coefficients (R^2), limit of quantification (LOQ), and matrix effect of the investigated PSTs.

Table S3. Precision test of ten investigated PSTs.

Table S4. Recovery test of the ten investigated PSTs.

Table S5. Concentrations (μg/kg) of the four PSTs detected in *R. philippinarum* cultivated along Jiangsu coastline.

Table S6. Concentrations (μg/kg) of the four PSTs detected in *M. veneriformis* cultivated along Jiangsu coastline.

Table S7. Concentrations (μg/kg) of the four PSTs detected in *M. meretrix* cultivated along Jiangsu coastline.

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