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Original Article

Tetrodotoxin and paralytic shellfish poisons in gastropod species from Vietnam analyzed by high-performance liquid chromatography and liquid chromatography–tandem mass spectrometry



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ABSTRACT

Among marine toxins, tetrodotoxin (TTX) and paralytic shellfish poisons (PSPs) are known as notorious neurotoxins that induce serious food poisoning incidents in the Southeast Asia region. The aim of this study was to investigate whether TTX and PSP toxins are important issues of seafood safety. Paralytic toxicity was observed in mice exposed to 34 specimens from five species of gastropods using a PSP bioassay. Five species of gastropods, *Natica vitellus*, *Natica tumidus*, *Oliva hirasei*, *Oliva lignaria*, and *Oliva annulata*, were collected from the coastal seawaters in Nha Trang City, Vietnam, between August 2007 and October 2007. The average lethal potency of gastropod specimens was 90 ± 40 (mean \pm standard deviation) mouse units (MU) for *N. vitellus*, 64 ± 19 MU for *N. tumidus*, 42 ± 28 MU for *O. hirasei*, 51 ± 17 MU for *O. lignaria*, and 39 ± 18 MU for *O. annulata*. All toxic extracts from the sample species were clarified using a C18 Sep-Pak solid-phase extraction column and a microcentrifuge filter prior to analysis. High-performance liquid chromatography coupled with fluorescence detection indicated that the toxins of the olive shell (*O. hirasei*, *O. lignaria*, and *O. annulata*) were mainly composed of saxitoxin (STX) (73–82%), gonyautoxin (GTX) 2, 3 (12–22%), and minor levels of TTX (5–6%). The toxins of *N. vitellus* and *N. tumidus* were mainly composed of STX (76–81%) and GTX 1, 4 (19–24%). Furthermore, liquid chromatography–tandem mass spectrometry analysis was used to verify the identity of the PSPs and TTX. Our evidence shows that these gastropods have novel toxin profiles.

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1. Introduction

Among marine toxins, tetrodotoxin (TTX) and paralytic shellfish poisons (PSPs) have frequently been found along the coasts of Southeast Asia, including China [1], Vietnam [2,3], Thailand [4,5], Cambodia [6,7], Malaysia [8], and the Philippines [9,10]. TTX was originally discovered and isolated from puffer fish in 1964 [11]. Later, it was found in many other organisms from terrestrial [12] and marine habitats [13–15]. The molecular formula of TTX is C₁₁H₁₇O₈N₃ (molecular weight = 319 Da), which has more than 10 analogs, and TTX owns the highest toxicity among them [11,14]. The molecule consists of a positively charged guanidinium group and a pyrimidine ring that helps stabilize the TTX–sodium channel binding complex at the aqueous interface (Fig. 1A).

In the past decades, PSPs have been responsible for the most severe seafood poisoning in humans worldwide [1,15–20]. The source of PSPs in Vietnam was thought to be the toxic dinoflagellates, including *Alexandrium minutum* and *Alexandrium affine* [3,21,22]. The PSP toxins from a group of closely related tetrahydropurine compounds can be categorized into up four subgroups: carbamate, N-sulfocarbamoyl, decarbamoyl, and deoxydecarbamoyl components, including saxitoxin (STX), neosaxitoxin (neo-STX), several gonyautoxins (GTXs), and their variants (Fig. 1B), which act by blocking the

flux of sodium ions through voltage-gated sodium channels on excitable cell membranes. GTXs, especially GTX 1,4 epimers and GTX 2,3 epimers, are most abundant in mollusk extract samples, being around 75–85% of the total toxin content in most of the samples analyzed, indicating that they account for the high toxicity of the shellfish in China, Chile, and South America. Different GTXs may have different toxicities and may be converted to/from each other through various mechanisms [3,7,23].

In Vietnam, the first evidence of TTX association with food poisoning was the intoxication of six humans after ingesting puffer fish [2]. PSP and TTX compounds have been linked to seafood poisoning involving gastropods of the genera *Nassariidae*, *Naticidae*, and *Olividae* in China, Taiwan, Japan, Hong Kong, Malaysia, and Australia [17,20,24–27].

We undertook further examination of the lethal potency of gastropods consumed in Vietnam because of food safety concerns. To determine the toxicity of various compounds, a mouse bioassay was employed, followed by high-performance liquid chromatography with fluorescence detection (HPLC–FLD) and liquid chromatography–tandem mass spectrometry (LC–MS/MS) [28–30]. This study showed for the first time that PSPs and TTX can be found in gastropods from Vietnam. As identified by HPLC–FLD and LC–MS/MS, the examined gastropods exhibit novel toxin profiles.

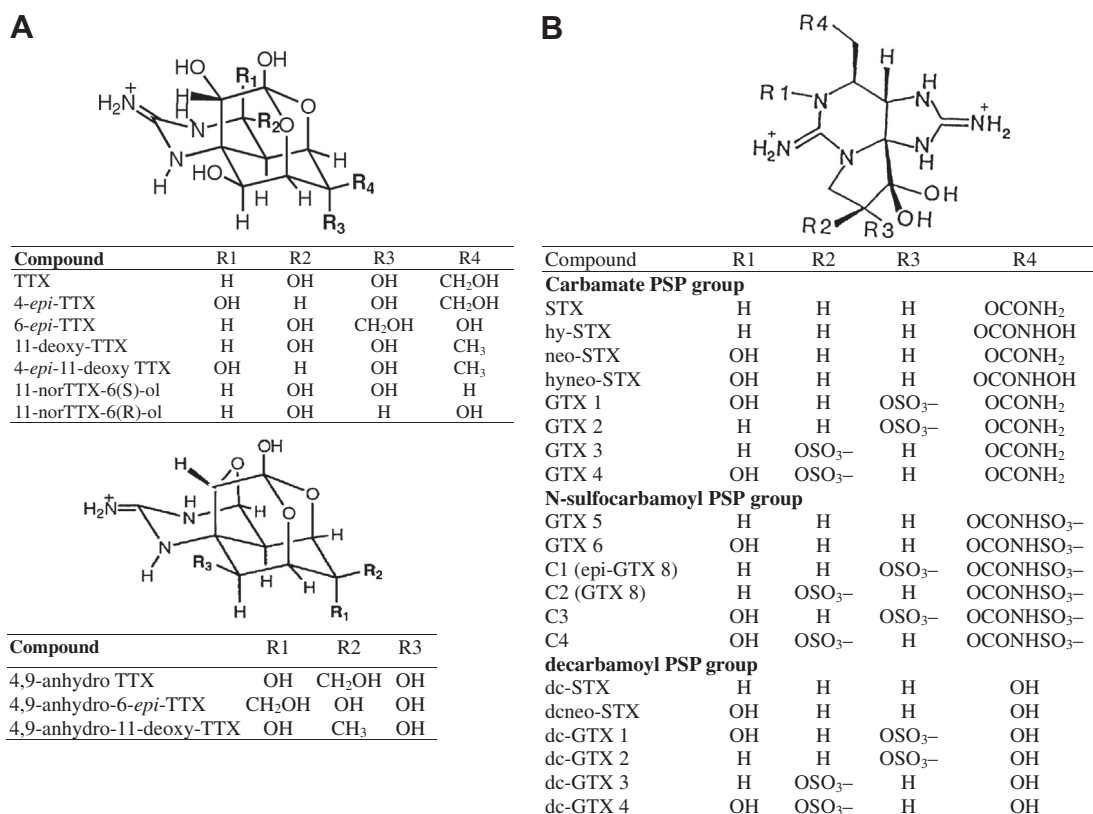


Fig. 1 – Structures of (A) TTX and (B) PSP components. GTX = gonyautoxin; PSP = paralytic shellfish poison; STX = saxitoxin; TTX = tetrodotoxin.

2. Methods

2.1. Materials

Five species of gastropods, *Natica vitellus* (8 specimens), *Natica tumidus* (6 specimens), *Oliva hirasei* (6 specimens), *Oliva lignaria* (8 specimens), and *Oliva annulata* (6 specimens), were collected from the coastal seawaters in Nha Trang City, Vietnam (Fig. 2), between August 2007 and October 2007. The frozen gastropods were delivered to the laboratory of the National Taiwan Ocean University, and dissected into edible portions of muscle tissue and digestive glands to determine the anatomical distribution of toxins. The Institute of Cancer Research (ICR) mouse strain was purchased from the National Taiwan University Hospital (Taipei, Taiwan). Healthy mice weighing between 18 g and 20 g were used. Authentic TTX and anhydrotetrodotoxin were obtained from Wako Pure Chemical Industries (Tokyo, Japan). Authentic PSP compounds, including GTX 1–4, were isolated and purified from the purple clam *Soletellina diphos* found in Taiwan [28]. In addition, authentic STX and neo-STX were obtained from the crab *Zosimus aeneus* found in Japan [31]. These authentic PSP toxins were verified and purified according to the methods of Sullivan and Iwaoka [32]. The calibration curve for the toxins was confirmed by NRC CRM-

STXdiAc, CRM-GTX 1, 4-C, and CRM-GTX 2, 3-C agents (National Research Council Canada, Nova Scotia, Canada). The toxins were freeze dried separately as stock solutions (1000 MU/mL) in several small black tubes and stored at -70°C . Only one stock toxin at a time was dissolved in 1 mL 30 mM acetic acid and kept at -20°C until use.

2.2. Toxin extraction and toxicity assay

Toxic muscles and digestive gland were homogenized for 5 minutes with three volumes of 1% acetic acid in methanol and centrifuged (20,000g, 20 minutes). The operation was repeated twice again. Supernatants were combined, concentrated under reduced pressure at 45°C , and examined for toxicity using a mouse assay for TTX. Lethal potency was expressed in mouse units (MU), where 1 MU = 0.178 μg TTX, defined here as the amount of toxin required to kill 20 g of ICR strain male mice in 30 minutes after an intraperitoneal injection [28]. A series of test solutions were prepared by diluting the unknown toxin extract with 0.1% acetic acid.

In the same way, the supernatants were tested for toxicity using a mouse bioassay for levels of PSPs. The lethal potency was also expressed in MU. One MU is equal to 0.18 μg of STXs. The MU was defined as the amount of toxin required to kill a 20 g ICR strain male mouse 15 minutes after an intraperitoneal injection [33].

2.3. Toxin purification from gastropods

The remaining toxic extracts from each species were mixed, purified partially using a cartridge column, and then ultra-filtered as described previously [29,30]. Briefly, the extract was passed through a cartridge column (C18 Sep-Pack cartridges; Millipore, Waters, MA, USA) that was regenerated previously with 10 mL methanol and equilibrated with 10 mL water. The toxin was absorbed on the column and then eluted with 10 mL methanol. The eluate was then freeze dried, dissolved in 2 mL 0.5% acetic acid, and filtered through a 3000 molecular weight cutoff Ultrafree microcentrifuge filter (Micron YM-3; Millipore). The filtrate was then dried down, reconstituted into a 1 mL volume, and used for subsequent HPLC–FLD [29,34].

2.4. HPLC–FLD analysis for TTX and PSPs

All the chemicals and solvents used were of HPLC or analytical grade. Reversed-phase HPLC (L-2100; Hitachi Ltd, Tokyo, Japan) was performed over a reversed-phase column (Merck Lichromsper 100 RP-18, 4 mm ID \times 20 cm; E. Merck, Darmstadt, Germany) with a fluorescence detector (F-1000; Hitachi Ltd). The mobile phase for TTX analysis consisted of sodium 1-heptanesulfonate (2 mM) in methanol (1%)-potassium phosphate buffer (0.05 M, pH 7.0). The TTX was detected by mixing the eluate with 3 N NaOH at a ratio of 1:1, followed by heating at 99°C for 0.4 minutes, and then monitored by the fluorescence emission at 505 nm and excitation at 381 nm [35]. For PSP analysis, the mobile phase contained sodium 1-heptanesulfonate (2 mM) as an ion-pairing reagent. For GTX detection, this mobile phase also contained 10 mM ammonium phosphate (pH 7.1). For STX detection, the mobile phase consisted of sodium 1-heptanesulfonate (2 mM) in 30 mM



Fig. 2 – Map of Vietnam showing the collection site (arrow). **Note.** From <http://www.alex4ever.de/vietnam/evkarte.htm>, by A. Birner, 2013, Copyright 2013, Alex Birner. Reprinted with permission.

Table 1 – Lethal potency in specimens of gastropods from Vietnam.

Specimens	Body weight (g)	Body length (cm)	Digestive gland weight Toxicity (g) (MU/g)	Muscle weight Toxicity (g) (MU/g)	Total toxicity ^a (MU/specimen)		
<i>Natica vitellus</i>							
1	16.3	5.2	2.3	16	3.7	ND ^b	37
2	18.0	5.3	2.9	14	3.9	ND	41
3	18.4	5.4	2.8	22	3.5	ND	62
4	19.7	6.3	3.8	33	4.2	ND	125
5	20.1	6.4	4.5	28	5.6	ND	126
6	21.2	6.0	5.1	26	4.5	ND	133
7	21.3	6.3	4.6	18	5.2	ND	83
8	22.5	6.5	4.2	27	6.2	ND	113
Mean ± SD	19.7 ± 2.0	5.9 ± 0.5	3.8 ± 1.0	23.0 ± 6.6	4.6 ± 1.0	0	90 ± 40
<i>Natica tumidus</i>							
1	4.6	2.5	2.3	25	1.0	ND	58
2	4.8	2.8	2.5	30	1.2	ND	75
3	5.0	3.1	2.8	32	1.3	ND	90
4	5.1	3.6	2.6	29	1.4	ND	75
5	5.3	3.3	2.6	15	1.3	ND	39
6	5.5	3.5	2.7	17	1.7	ND	46
Mean ± SD	5.2 ± 0.3	3.1 ± 0.4	2.8 ± 0.5	24.7 ± 7.1	1.3 ± 0.2	0	64 ± 19
<i>Oliva hirasei</i>							
1	16.0	4.5	1.2	ND	2.5	10	25
2	16.2	4.5	1.3	ND	2.7	23	62
3	17.2	4.8	1.7	ND	3.5	4	14
4	19.0	5.1	2.4	ND	3.2	7	22
5	19.3	5.1	2.8	ND	3.4	12	41
6	20.2	5.2	2.7	ND	3.2	27	86
Mean ± SD	18.0 ± 1.8	4.9 ± 0.3	2.0 ± 0.7	0	3.1 ± 0.4	13.8 ± 9.2	42 ± 28
<i>Oliva lignaria</i>							
1	15.7	4.2	1.4	ND	2.3	23	53
2	15.8	4.6	1.4	ND	2.5	18	45
3	15.8	4.6	1.4	ND	2.8	16	45
4	16.1	4.7	1.1	ND	2.9	10	29
5	16.2	4.6	1.2	ND	2.8	26	73
6	16.3	4.3	1.3	ND	3.0	23	69
7	16.3	4.7	1.2	ND	2.2	13	29
8	16.5	4.3	1.2	ND	3.2	21	67
Mean ± SD	16.1 ± 0.3	4.5 ± 0.2	1.3 ± 0.1	0	2.7 ± 0.3	18.7 ± 5.5	51 ± 17
<i>Oliva annulata</i>							
1	16.1	4.8	1.2	ND	2.7	17	46
2	18.1	5.1	1.2	ND	3	12	36
3	18.2	5.0	1.5	ND	2.8	8	22
4	19.2	5.0	1.3	ND	2.1	12	25
5	20.2	5.5	1.4	ND	3.8	19	72
6	20.4	5.1	1.4	ND	2.2	15	33
Mean ± SD	18.7 ± 1.6	5.2 ± 0.4	1.3 ± 0.1	0	2.8 ± 0.6	13.8 ± 3.9	39 ± 18

ND = not detected; SD = standard deviation.

^a Total toxicity was calculated from weight and toxicity of digestive gland and muscle in gastropods.

^b ND implies less than 4 MU/g and is assumed to be zero for calculation.

phosphoric acid (pH 7.1) containing 5% acetonitrile. The mobile phase was pumped at a flow rate of 0.6 mL/min. In all cases, the eluate from the column was oxidized continuously with periodic acid (7 mM) in 50 mM potassium phosphate buffer (pH 9.0) while passing through Teflon tubing. The eluate was then heated at 90°C for 0.5 minutes and mixed with an equal volume of acidifying reagent (0.5 M acetic acid) prior to entering a fluorescence detector. The intensity of the fluorescence was measured at 330 nm with an excitation wavelength at 390 nm [36]. The PSP and TTX levels in gastropod extracts were determined by comparing the peak areas of each toxin component with a standard.

2.5. LC–MS/MS analysis of PSPs and TTX

The tested solutions were separated on a liquid chromatographer HP 1100 (Agilent Technologies, Waldbronn, Germany) consisting of a quaternary pump for the mobile phase and a Cosmosil Hilic 4.6 × 150 mm² column (Waters, MA, USA).

Mobile phase A consisted of 0.1% formic acid in water, whereas mobile phase B consisted of methanol. The mobile phase gradient started with 10% B for 5 minutes and then linearly increased to 90% B within 15 minutes. The mobile phase was kept isocratically at 90% B for 10 minutes and then re-equilibrated for another 10 minutes. The total analysis time

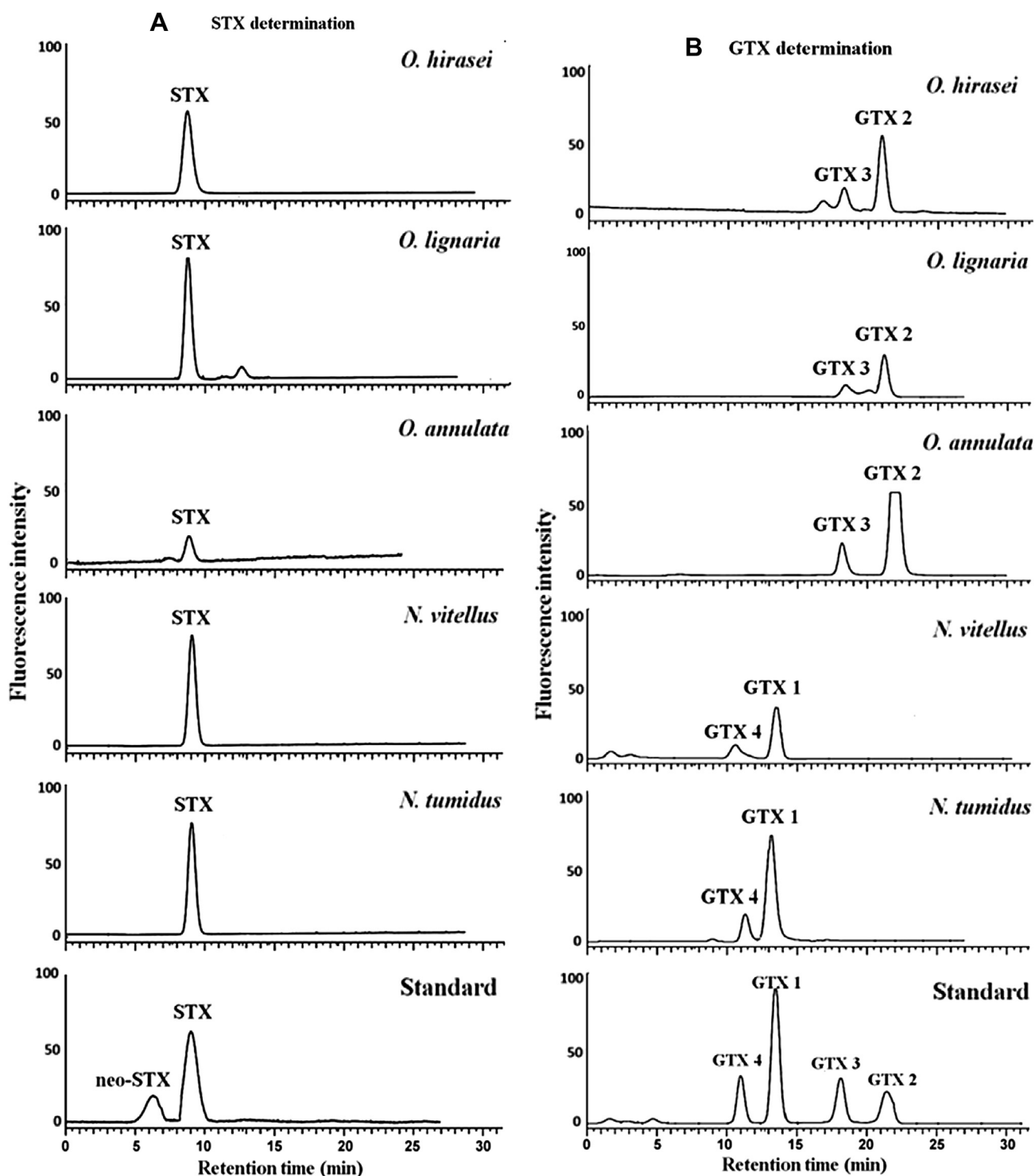


Fig. 3 – (A) STX, (B) GTX and (C) TTX determination of HPLC chromatography of toxin extract from five species of gastropods (*Natica vitellus*, *Natica tumidus*, *Oliva hirasei*, *Oliva lignaria*, and *Oliva annulata*), and authentic STX, GTXs, and TTX. GTX = gonyautoxin; HPLC = high-performance liquid chromatography; STX = saxitoxin; TTX = tetrodotoxin.

was 15 minutes, and the flow rate was 700 $\mu\text{L}/\text{min}$. All LC–MS/MS experiments were recorded on a 4000Q TRAP mass spectrometer (ABI-Sciex, Toronto, Canada) equipped with an electrospray ion source with the data system in positive-ion mode. The optimum ion source parameters for MS/MS were as follows: curtain gas = 10 psi; ion spray voltage = 5500 V;

temperature = 550°C; ion source gas 1 = 50 psi; and ion source gas 2 = 50 psi. The collision gas was set to a medium mode and the interface heater to an on mode. Tuning parameters were optimized to obtain the best signal to noise ratio for PSPs and TTX. The mass spectrometer was operated in MS/MS mode using a multiple reaction monitor to detect the transition of

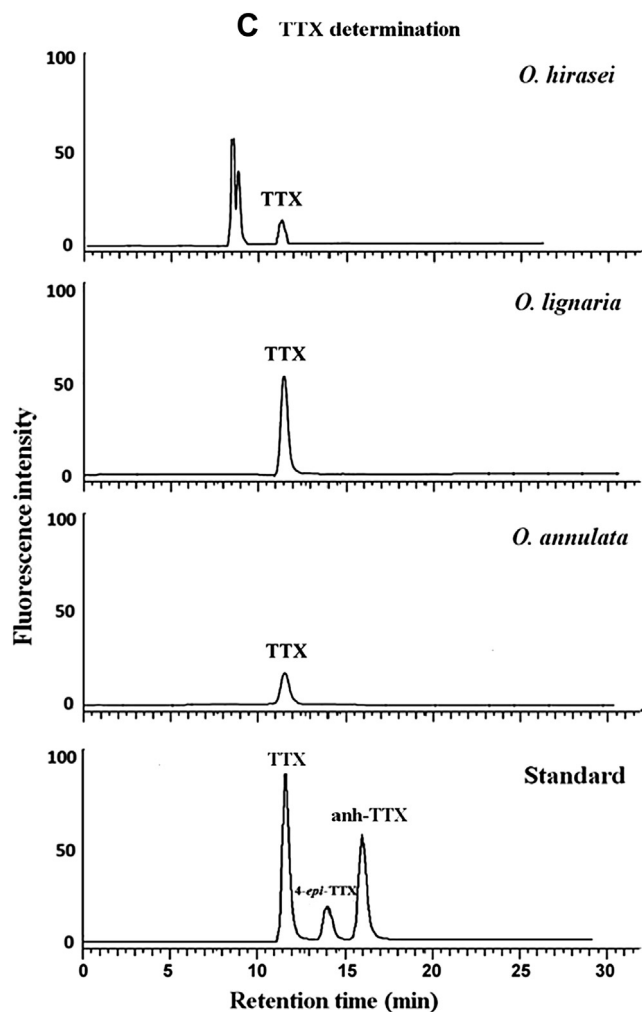


Fig. 3 – (continued).

specific precursor ions to product ions for each sample. For TTX analysis, the collision full scan (Q1) spectra were collected in the mass range m/z 100–330. The mass spectral Q1 and Q3 transitions, monitored for TTX, were m/z 320/302 and m/z 320/162, respectively [37,38]. For PSP analysis, the Q1 spectra were collected in the mass range m/z 100–600. The mass spectral Q1/Q3 transitions were monitored for STX (m/z 300/282), GTX 1 (m/z 412/332 and m/z 396/314), GTX 2 (m/z 396/298), GTX 3 (m/z 396/298 and m/z 396/316), and GTX 4 (m/z 412/314 and m/z 412/394) [23,39].

3. Results

3.1. Toxicity assay

Results of the toxicity assay for five species of toxic gastropods, namely *N. vitellus*, *N. tumidus*, *O. hirasei*, *O. lignaria*, and *O. annulata*, are summarized in Table 1. All the specimens analyzed were toxic. The mean value of lethal potency was 90 ± 40 MU/specimen (mean \pm standard deviation) in *N. vitellus*, 64 ± 19 MU/specimen in *N. tumidus*, 42 ± 28 MU/

specimen in *O. hirasei*, 51 ± 17 MU/specimen in *O. lignaria*, and 39 ± 18 MU/specimen in *O. annulata*. Toxicity of olive shells (*O. hirasei*, *O. lignaria*, and *O. annulata*) was localized mainly in the muscle, rather than in the digestive gland (<4 MU/g). However, the Naticidae gastropods *N. vitellus* and *N. tumidus* contained the majority of toxins in the digestive gland, and not in the muscle.

3.2. Matrix

Solid-phase extraction was optimized to be robust as the matrix did not affect the accuracy of the method significantly, as evidenced by analyzing tissue. The extent of the matrix effect may vary with the source of a given biological matrix. In this case, the use of a C18 cartridge as a preliminary clean-up step is essential to remove material from samples. Therefore, spiked samples were prepared by dissolving 25 ng/mL, 50 ng/mL, and 100 ng/mL of TTX or STX in the normal matrix. Recoveries of TTX and STX spiked in three amounts (25 ng/mL, 50 ng/mL, 100 ng/mL) were in the range of 94.7–96.3% and 93.3–97.2%, respectively. The average recoveries of TTX and STX were 95.8% and 96.2%, respectively.

3.3. HPLC–FLD analysis for toxin profile and contents

HPLC–FLD provided a rapid and quantitative means to differentiate various toxins, including PSPs and TTX. In the HPLC–FLD analysis for PSPs, carbamoyl-*N*-methyl derivatives of STX and GTX 1–4 were detected in five gastropod species. The tissue extracts of the five gastropods produced a main peak with a retention time of 9 minutes. This retention time was consistent with that of the STX standard (Fig. 3A). *O. hirasei*, *O. lignaria*, and *O. annulata* toxins consisted of two peaks that had the same retention times (18 minutes and 21.5 minutes) as those of GTX 2, 3 (Fig. 3B). The main toxin of gastropod specimen was confirmed by HPLC–FLD analysis. The detection limit of TTX and STX was $1 \mu\text{g/mL}$, and the calibration curve was linear in the range of 1–500 $\mu\text{g/mL}$. The retention time of one peak almost coincided with that of TTX (11.5 minutes). Toxins of three gastropod species (*O. hirasei*, *O. lignaria*, and *O. annulata*) revealed one peak with the same retention time as that of authentic TTX (Fig. 3C). Relative quantities of TTX and PSPs were estimated using chromatographic peak heights to calculate the molar ratio. In these three gastropod species, STX accounted for 73–82 mole% of toxins. Trace amounts of GTX 2, 3 (18 mole%) and TTX (5 mole%) were also detected (Fig. 4A–C).

HPLC–FLD analysis of the toxic gastropods revealed that *N. vitellus* and *N. tumidus* contained two peaks with the same retention times (11.2 minutes and 13.5 minutes) as those of GTX 1, 4 (Fig. 3B). Neither *N. vitellus* nor *N. tumidus* contained TTX, but STX accounted for 76–81 mole% of toxins. A lower amount of GTX 1,4 (19–24 mole%) was detected in *N. vitellus* and *N. tumidus* (Fig. 4D and E).

3.4. LC–MS/MS analysis for PSPs and TTX

Since the spectra for PSP toxins have been reported [23], Q1 mass spectra and Q3 product ion spectra of all STX analogues available to us were examined. For the LC–MS/MS analysis of

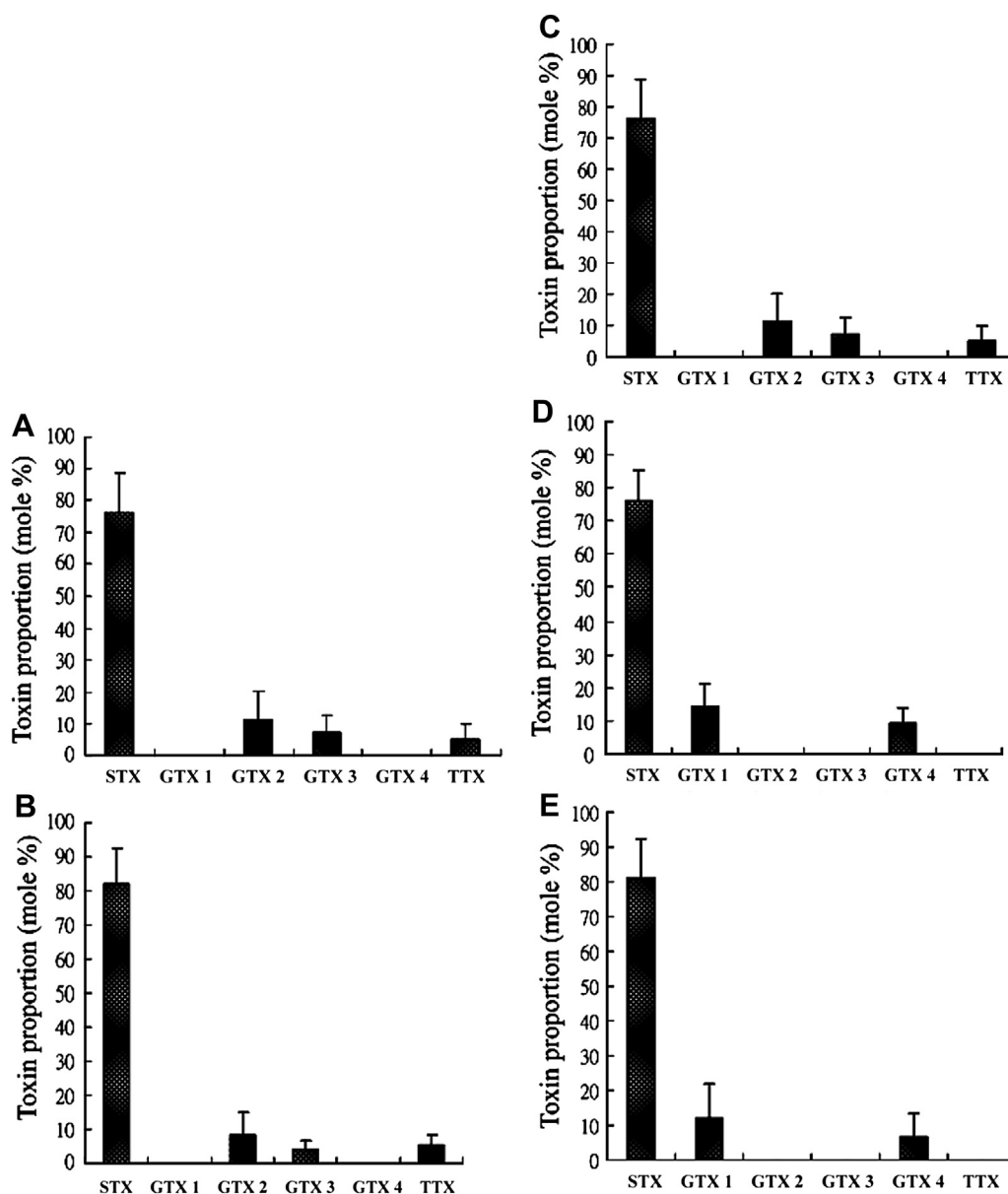


Fig. 4 – Comparison of the toxin component (mole %) profile of five gastropods: (A) *Oliva hirasei*, (B) *Oliva lignaria*, (C) *Oliva annulata*, (D) *Natica vitellus*, and (E) *Natica tumidus*. The proportion of each toxin component was expressed as mole ratio (%). Each column represents mean \pm standard deviation. GTX = gonyautoxin; STX = saxitoxin.

PSPs, we determined molecular masses of STX (300 Da), GTX 1 (412 Da), GTX 2 (396 Da), GTX 3 (396 Da), and GTX 4 (412 Da; Table 2). The presence of PSPs in the five species of gastropods was also confirmed by LC–MS/MS analysis. We found selective ions, corresponding to the ions of STX (m/z 300 \rightarrow m/z 282; Fig. 5), GTX 1 (m/z 412 \rightarrow m/z 332 and m/z 412 \rightarrow m/z 314) and GTX 4 (m/z 412 \rightarrow m/z 314 and m/z 412 \rightarrow m/z 332; Fig. 6A and B), and GTX 2 (m/z 396 \rightarrow m/z 298) and GTX 3 (m/z 396 \rightarrow m/z 298 and m/z 396 \rightarrow m/z 316; Fig. 6C–E) fragmentation in *O. hirasei*, *O. lignaria*, and *O. annulata*. Furthermore, the ions of STX fragmentation were found in *N. vitellus* and *N. tumidus*.

For the LC–MS/MS analysis of TTX, we found a molecular mass of 320 Da, assignable to TTX + H ($C_{11}H_{17}N_3O_3 = 320$). Multiple reaction monitoring was performed at unit resolution using mass transition ion pairs m/z 320 \rightarrow m/z 302 and m/z

320 \rightarrow m/z 162 for TTX. Gastropod samples containing TTX were applied to the LC–MS/MS in the electrospray ion mode detecting at m/z 320–162 for TTX. The presence of TTX in *O. hirasei*, *O. lignaria*, and *O. annulata* was confirmed by LC–MS/MS analysis (Fig. 7). Specific selective ions m/z 162 Da, 302 Da, and 320 Da corresponded to the ions from TTX fragmentation [37,38].

4. Discussion

Our results showed that five species of Vietnamese gastropods, *N. vitellus*, *N. tumidus*, *O. hirasei*, *O. lignaria*, and *O. annulata*, were toxic. The highest lethal potencies of the specimens were 133 MU for the *N. vitellus* and 90 MU for the *N. tumidus*. In the

Table 2 – Parent fragment ion combinations used for multiple reaction monitoring.^a

Compound	Q1 spectra		Q3 product ion spectra			Decustering potential (V)	Collision energy (V)
	[M + H] ⁺	Fragment ions (m/z)	Precursor ion	Production ion	Lost part		
TTX	320	302	320	302	–H ₂ O	88.57	34.75
STX	300	282	300	222	–H ₂ O–NH ₃ –CO ₂	46.01	12.87
				204	–2H ₂ O–NH ₃ –NHCO		
				179	–H ₂ O–NH ₃ –CO ₂		
				282	–HNCNH		
GTX 1	412	332	412	332	–H ₂ O	50.42	13.24
				314	–SO ₃ –H ₂ O		
				298	–SO ₃ –H ₂ O		
GTX 2	396	316	396	298	–SO ₃ –H ₂ O	46.01	13.68
				316	–SO ₃		
GTX 3	396	298	396	298	–SO ₃ –H ₂ O	46.01	15.53
				316	–SO ₃		
				378	–SO ₃ –2 H ₂ O–NH ₃		
GTX 4	412	394	412	314	–NHCO	50.42	15.41
				332	–SO ₃ –H ₂ O		
				332	–SO ₃		
				253	–SO ₃ –H ₂ O–NH ₃ –CO ₂		

ESI = electrospray ion; GTX = gonyautoxin; STX = saxitoxin; TTX = tetrodotoxin.

^a ESI, positive ion mode.

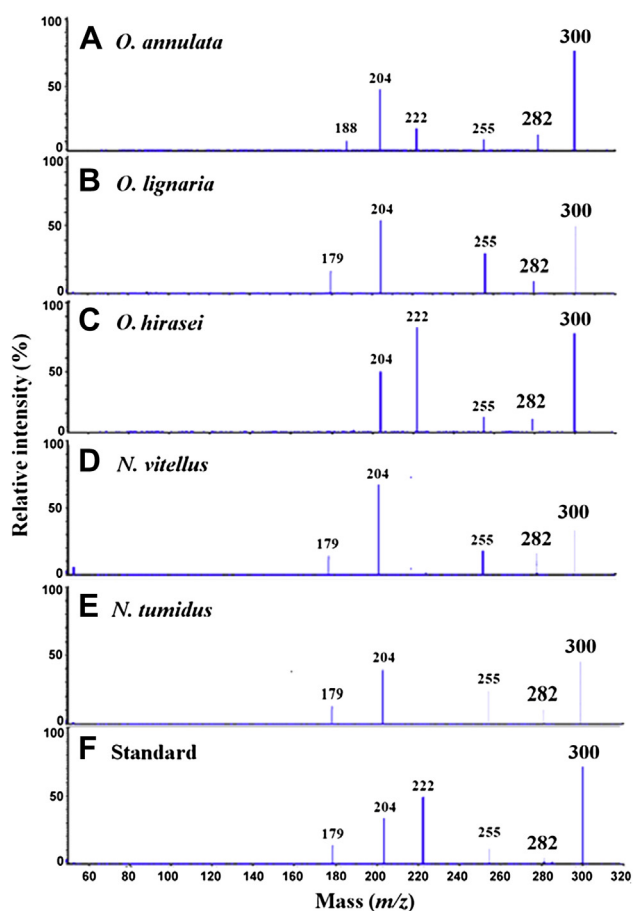


Fig. 5 – Fragmentation ion profile in an LC–MS/MS system of five gastropod species: (A) *Oliva annulata*, (B) *Oliva lignaria*, (C) *Oliva hirasei*, (D) *Natica vitellus*, and (E) *Natica tumidus*, and (F) authentic STX. LC–MS/MS = liquid chromatography–tandem mass spectrometry; STX = saxitoxin.

N. vitellus and *N. tumidus*, 76–81 mole% of STX and 19–24 mole % of GTX 1, 4 were detected in the digestive gland. However, the toxicity of *O. hirasei*, *O. lignaria*, and *O. annulata* was localized mainly in the muscle, rather than in the digestive gland. The moderate lethal potency of the specimens was 86 MU, 73 MU, and 72 MU in *O. hirasei*, *O. lignaria*, and *O. annulata*, respectively. Based on HPLC–FLD analysis, the toxins from these species were identified as STX (73–82 mole%), GTX 2,3 (12–22 mole%), and TTX (5–6 mole %). In summary, the five toxic gastropods from Vietnam contained a high amount of STX toxins and lower levels of GTXs and TTX.

In Australia, some gastropods, including *Tectus fenestratus*, *Tectus niloticus*, *Tectus pyramis*, *Tectus hanleyanus*, and *Turbo argyrostomus*, contain only PSPs [40]. However, several gastropods in Taiwan, including *Nassarius papillosus* [20], *Nassarius clathrata* [41], and *Nassarius lineata* [42], were found to contain TTX only, except in the springtime when they were found to contain TTX along with a small amount of PSPs.

Oliva miniacea, *Oliva mustelina*, and *Oliva nirasei* were reported to contain only TTX in the muscle; they may secrete TTX as a defensive agent when they encounter predators or as a paralyzing agent when they attack a prey [17]. This may explain why certain gastropod species contain higher levels of toxins in the muscle, whereas other species contain higher levels in the digestive gland. Furthermore, another study indicated that a TTX-binding material of high molecular weight was found in the muscle of five toxic gastropods (*Polinices didamy*, *N. lineata*, *O. miniacea*, *O. mustelina*, and *O. hirasei*) [26]. In recent years, accumulation of TTX in the skin of the wild Cambodian freshwater puffer (*Tetraodon turgidus*) has been demonstrated [7]. Newts were noted to have glands in their skins that secrete TTX. It has also been reported that TTX-containing animals may absorb and accumulate TTX and related substances produced by bacteria [43–45].

Among the five species of gastropods we examined, STX was noticed to be the major component. STX is one of the

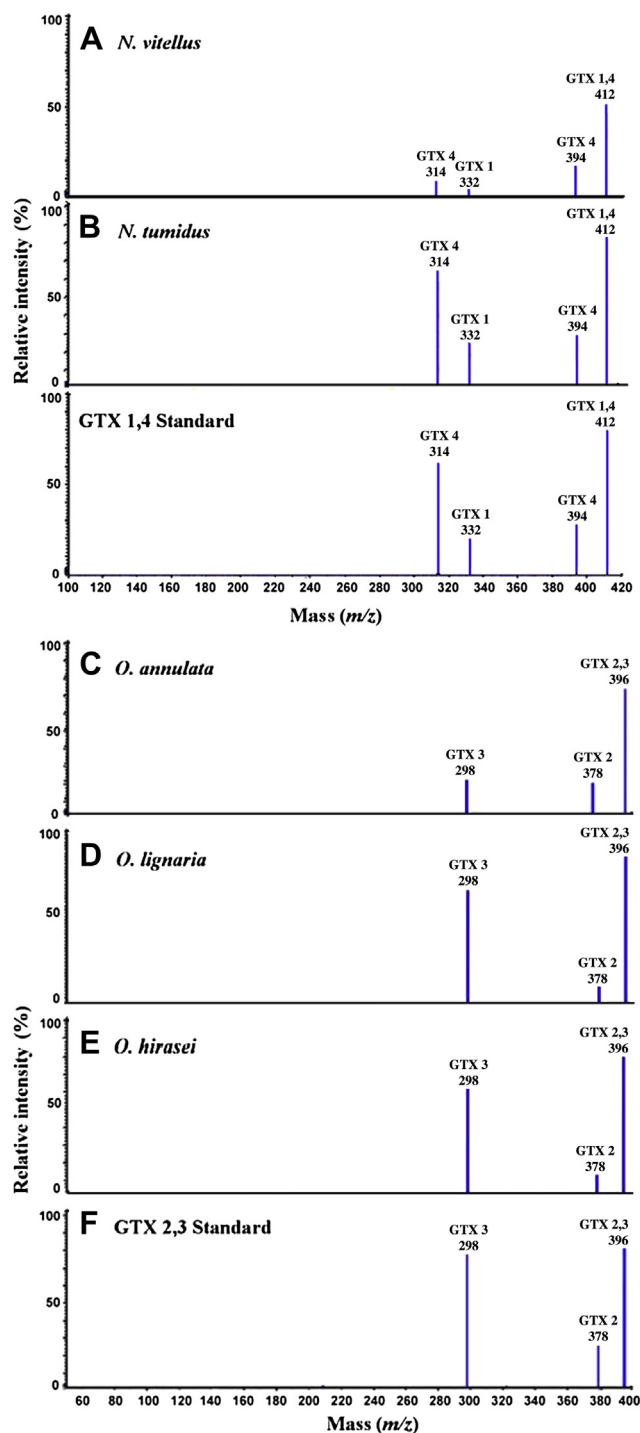


Fig. 6 – Fragmentation ion profile in an LC–MS/MS system of five gastropods: (A) *Natica vitellus*, (B) *Natica tumidus*, (C) *Oliva annulata*, (D) *Oliva lignaria*, and (E) *Oliva hirasei*, and (F) authentic GTX 2,3. GTX = gonyautoxin; LC–MS/MS = liquid chromatography–tandem mass spectrometry.

most potent neurotoxins and is just as lethal in mice as TTX. The intoxicated dose for a man ranges from 1000 MU to 5000 MU (equivalent to 200–1000 μg of STX), and the lethal dose for a man is 3000–30,000 MU (equivalent to 600–6000 μg

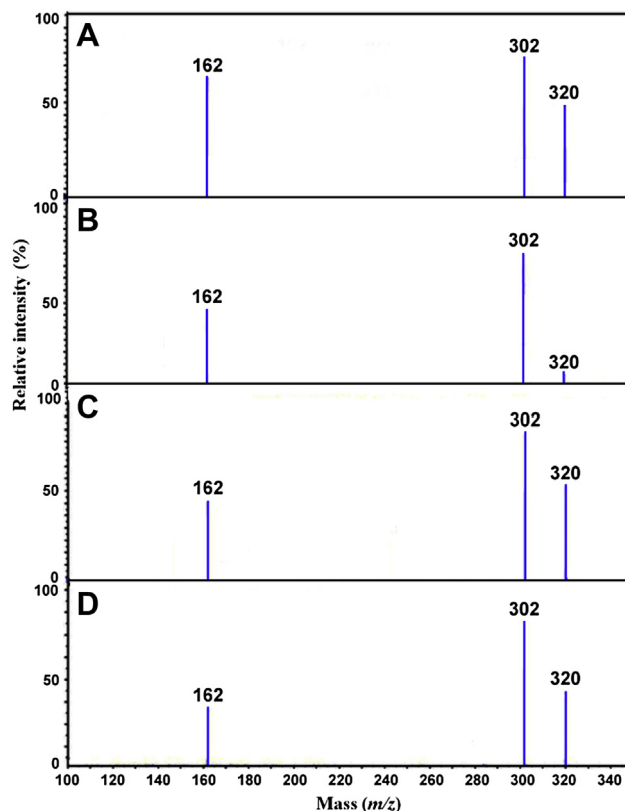


Fig. 7 – Fragmentation ion profile in an LC–MS/MS system of three gastropods: (A) *Oliva annulata*, (B) *Oliva lignaria*, and (C) *Oliva hirasei*, and (D) authentic TTX. LC–MS/MS = liquid chromatography–tandem mass spectrometry; TTX = tetrodotoxin.

of STX). In most countries, the regulatory limit for safe consumption of shellfish is set at 80 μg STX/100 g tissue [46,47].

As reported previously, results demonstrated that PSP is derived possibly from the food chain, starting from PSP-producing cyanobacteria [47]. The edible shellfish may become toxic as a result of accumulated PSPs from toxic dinoflagellates through the filter-feeding system of the red tide [13]. Other researchers noted that the occurrence of a potentially toxic *A. minutum* species in coastal waters of Vietnam is well documented [21,22]. The toxin profile of *A. minutum* in Vietnam was characterized by the presence of GTX 1–4, neo-STX, and dc-STX [3].

Taken together, we suggest that the mechanism of PSP toxicity in the gastropods begins with the PSPs produced by dinoflagellates. Later, these toxins accumulate in plankton feeders and may be converted into related compounds. Next, the gastropods search out plankton feeders as food and, consequently, accumulate those toxic compounds. Our results indicate that PSPs and TTX are distributed in several gastropods in the coastal seawaters of Vietnam and that these gastropods show different toxin profiles.

In conclusion, five gastropod species were collected from Vietnam and found to be toxic using a PSP bioassay. According to HPLC–FLD and LC–MS/MS analyses, toxins of olive shell (*O. hirasei*, *O. lignaria*, and *O. annulata*) consisted of STX (73–82%), GTX 2,3 (12–22%), and TTX (5–6%), and those in *N. vitellus* and

N. tumidus were STX (76–81%) and GTX 1,4 (19–24%). These data show unique toxin profiles for individual gastropod species.

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