



(This is a sample cover image for this issue. The actual cover is not yet available at this time.)

This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

<http://www.elsevier.com/copyright>



Contents lists available at SciVerse ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

First report of Paralytic Shellfish Poisoning (PSP) in mussels (*Mytilus galloprovincialis*) from eastern Adriatic Sea (Croatia)

Ivana Ujević^a, Romana Roje^{b,*}, Živana Ninčević-Gladan^a, Ivona Marasović^a

^a Institute of Oceanography and Fisheries, Šetalište I. Meštrovića 63, 21000 Split, P.O. Box 500, Croatia

^b Erasmus Mundus Master of Science in Marine Biodiversity and Conservation, Dvorska 9, 21000 Split, Croatia

ARTICLE INFO

Article history:

Received 3 March 2011

Received in revised form

14 October 2011

Accepted 22 October 2011

Keywords:

PSP

Shellfish toxicity

Saxitoxin

Mussels

HPLC-FLD

Adriatic Sea

ABSTRACT

The chromatographic HPLC-FLD method was introduced for the first time to identify and quantitatively determine individual Paralytic Shellfish Poisoning toxins accumulated in aquacultured shellfish from Croatian coastal waters. Populations of Mediterranean mussels (*Mytilus galloprovincialis*) were contaminated with PSP toxins throughout January to April 2009 leading to the positive test results by Mouse Bioassay (MBA). Until 2009 there was no evidence of PSP toxins in the examined samples. For the first time an instrumental method revealed the PSP toxin profile of samples taken along the eastern Adriatic coast and identified saxitoxin (STX) as the main representative of this toxin group that may cause paralysis and death in consumers of contaminated shellfish. This phenomenon may have serious health and economic consequences. Following these potential consequences, marine biotoxins (PSP, ASP and DSP) are continuously assessed in bivalves from 25 breeding and harvesting areas along the Croatian Adriatic coast. Positive MBA results were confirmed by instrumental method in two out of three recorded samples. Saxitoxin was the dominant PSP toxin extracted from contaminated mussels within the range of 53.17–1298.17 $\mu\text{g g}^{-1}$, that contributed more than 70% to the total shellfish toxicity, followed by gonyautoxins 2 and 3 (GTX 2,3) which contributed 27% and decarbamoylsaxitoxin (dcSTX) that accounted for less than 2%, considering all stations.

© 2011 Elsevier Ltd. All rights reserved.

1. Introduction

1.1. Croatian shellfish production

Shellfish farming is widespread in Croatia along its entire coastline with particularly long tradition on the far south of the Croatian coast (Pelješac Peninsula and Mali Ston Bay; Fig. 1). Mali Ston Bay is the most important shellfish farming area in Croatia with more than one hundred years long tradition. Croatian shellfish industry, with its production of 3000–4000 tons of mussels and 1 million oysters per year is small when compared to the other countries, but it has a high potential for aquaculture expansion within its 33 200 km² of territorial waters, 5835 km of coastline and 1246 islands (Anonymous, 2008).

There are 25 sampling sites from shellfish breeding and harvesting areas along the Croatian coast of the Adriatic Sea included in continuous monitoring (in accordance with EU Directives) of shellfish and seawater quality that has begun in July 2000. These

sites include: 9 sites near Istria Peninsula, 4 near Zadar, 4 near Šibenik, 1 near Split and 7 sites in Mali Ston Bay, Pelješac Peninsula and Mljet Island (Fig. 1).

1.2. The occurrence of phytotoxic species and shellfish toxicity in the Adriatic Sea

Alexandrium species have been observed in the Adriatic since 1976 (Boni, 1983). Shellfish toxicity outbreaks in the Adriatic Sea were documented in the northwestern part of the Adriatic Sea in 1989 (Boni et al., 1992), however PSP toxicity was first recorded during an *Alexandrium minutum* bloom in the coastal waters of the northern Adriatic (Emilia Romagna) in the spring of 1994 (Honsell et al., 1996). Orhanović, Ninčević, Marasović, and Pavela-Vrančić (1996) reported massive fish kills by intensive red tide bloom of *Lingulodinium polyedrum* accompanied by *A. minutum* in Kaštela Bay (central Adriatic) in the summer of 1994, but the presence of PSP was not specifically stated in the report. Although *L. polyedrum* is considered to produce yessotoxins (Paz, Riobó, Fernández, Fraga, & Franco, 2004) and there is no strong relationship with the PSP toxicity, Bruno, Gucci, Pierdominici, Ioppolo, and Volterra (1990) reported the presence of saxitoxin (STX) in water samples taken

* Corresponding author. Tel.: +385 21 467 906; fax: +385 21 358 650.
E-mail address: romana.roje@ugent.be (R. Roje).



Fig. 1. Geographic locations of regular sampling sites (marked with green circles) along the Croatian coast with designated locations (stations S1, 2 and 3, Istria peninsula and Mali Ston Bay are marked with red arrows) where PSP toxins were established during the 2000–2009 period of investigation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

during a bloom of *L. polyedrum* in the Adriatic Sea. During 1995 and 1996, Marasović, Ninčević, Pavela-Vrančić, and Orhanović (1998) speculated that PSP toxins were detected in shellfish, but not at the level considered hazardous to human health based on the MBA test. There are more reports of PSP toxic dinoflagellate occurrence than of PSP shellfish contamination in the Mediterranean waters (Ciminiello, Fattorusso, Fiorino, & Montresor, 2000; Giacobbe et al., 2004; Honsell et al., 1996; Lilly, Kulis, Gentien, & Anderson, 2002; Montresor, John, Beran, & Medlin, 2004; Vila, Garcés, Masó, & Camp, 2001). Marasović et al. (2007) stated that PSP toxicity has never been recorded along the eastern Adriatic coast since the Croatian 'National monitoring programme of shellfish farms' began in 2000, while ASP toxicity was recorded in a very few samples with concentrations below the limits set by the European Commission and there were extremely high DSP shellfish toxicity events recorded in 2005 in breeding areas along the western Istrian coast in the northern Adriatic (Ninević-Gladan et al., 2008; Ujević et al., 2010).

Therefore, in this study we introduced HPLC-FLD (Lawrence, Niedzwiedek, & Menard, 2005) method for qualitative and quantitative identification of PSP toxin types in Croatian shellfish, as an effort to substitute MBA and to implement it in continuous monitoring. We also established the first PSP toxin types list for Croatian coastal waters and tried to find the relationship between the occurrences of PSP shellfish toxicity and the presence of causative phytoplankton species, based on available data.

2. Materials and methods

From the year 2000 through 2009 sampling frequency per site was once a month from November until May and twice a month from May through October. Istria Peninsula had a weekly sampling frequency from April through November in 2007 and 2008. Starting in 2009, samples were acquired fortnightly from January through March and weekly from April through December, with the exception of sampling sites at Istria Peninsula which had a weekly sampling throughout the entire year. Mediterranean mussel (*Mytilus galloprovincialis*) is the dominant cultured species in this area, while European flat oyster (*Ostrea edulis*), Mediterranean scallop (*Pecten jacobaeus*) and proteus scallop (*Flexopecten proteus*) are present as wild populations at three stations on the West coast of Istria. For this survey all the 3408 shellfish samples have been taken into consideration for PSP toxins testing by MBA. Among all, only 12 mussel samples from S1 location in the northern Adriatic Sea (Fig. 1) showed the PSP toxins presence by MBA, and we subsequently analyzed 11 samples (for one sample there was not enough tissue left after used for DSP analysis). Except these samples, there were 19 shellfish samples that were suspected (death of 1 mouse within 60 min or death time interval in two or three mice between 1 and 24 h, by MBA) for PSP presence during this monitoring period. Among these, 5 were missing, therefore HPLC-FLD analysis was performed on 14 of them.

2.1. Mouse bioassay

Preliminary analysis of PSP toxins was done by MBA reference method (959.08; AOAC, 1990). 100 g of homogenized shellfish tissue was boiled for 5 min in 100 mL of 0.1 M HCl, pH adjusted to 3 and centrifuged for 10 min at 3000 rpm. One millilitre of the acidic extract was inoculated into the peritoneum of the mouse (strain ICR (Swiss), weight limits 18–20 g). The method is based on the dose of PSP toxins (equivalent amount of saxitoxin) that provokes death in mice within 1 h after intraperitoneal injection of the shellfish extract (Hall, 1991). Three parallel tests were done and the reactions of the mice were observed for 1 h after the treatment or until death.

2.2. HPLC toxin analysis

Shellfish samples were extracted with 1% acetic acid. Extracts were cleaned up using solid-phase extraction (SPE) C18 cartridges. For neosaxitoxin (NeoSTX), gonyautoxins 1 and 4 (GTX 1,4) and decarbamoylneosaxitoxin (dcNeoSTX) determination, extracts were further purified utilising SPE-COOH ion exchange cleanup. For HPLC detection, peroxide and periodate oxidative conversions of PSP toxins (in sample extracts and standard solutions) to fluorescent derivatives were necessary. The applied method was as close as possible to that of Lawrence's original method, and performed according to the scheme depicted in Fig. 2.

The PSP extracts were analyzed based on the HPLC pre-chromatographic oxidation method with fluorescence detection (Lawrence et al., 2005), using Varian ProSTAR 230 HPLC analytical system coupled with ProStar 363 fluorescence detector (excitation wavelength set to 340 nm and emission to 390 nm) and ProStar 410 autosampler. Separation of toxin oxidation products was carried on reversed-phase C18 column (Restek, Pinnacle II 250 × 4.6 mm, 5 µm particle size) protected by a guard cartridge Pinnacle II C18, 20 × 4.0 mm (Varian, SAD). Column temperature was kept at 30 °C and run time was set to 15.00 min. PSP toxin profile was identified by comparing chromatograms of standard solutions: saxitoxin (STX-e), decarbamoylsaxitoxin (dcSTX), neosaxitoxin (NeoSTX-c), decarbamoylneosaxitoxin (dcNeoSTX-b), gonyautoxins 1 and 4 (GTX 1,4-c), gonyautoxins 2 and 3 (GTX 2,3-c), decarbamoylgonyautoxins 2 and 3 (dcGTX 2,3-b), gonyautoxin 5 (GTX 5-b) and N-sulfocarbamoylgonyautoxin 1 and 2 (C 1,2); NRC, Halifax, Canada. All reagents used were of HPLC or analytical grade obtained

from Sigma–Aldrich and all solutions were prepared in deionized water. All water used was distilled and deionized and referred to as DI water in the text. Mobile phases consisted of (A) 0.1 M ammonium formate and (B) 0.1 M ammonium formate in 5% acetonitrile (both pH = 6.0). Programmed solvent elution was done with a mixture of mobile phase A and mobile phase B with flow rate set at 1.5 mL/min.

To show linearity, a calibration curves (five points) in the range of 0.16–0.96 µg ml⁻¹ STX, dcSTX, NeoSTX and GTX 1,4; 0.016–0.096 µg ml⁻¹ C 1,2 and GTX 5; 0.08–0.48 µg ml⁻¹ dcNeoSTX; 0.096–0.576 µg ml⁻¹ GTX 2,3; 0.064–0.384 µg ml⁻¹ dcGTX 2,3 were obtained ($r > 0.99$). HPLC was calibrated by injections of 25 or 50 µL of all individual toxin standard solutions of three mixtures with different combination of toxin standards and one mixture with all the PSP analytical standards available.

The four mixtures were prepared as follows:

Mix I.

NeoSTX, dcNeoSTX and GTX 1,4 (periodate oxidation)

Mix II.

STX, dcSTX, GTX 2,3, dcGTX 2,3 and GTX 5 (peroxide oxidation, Fig. 3)

Mix III.

C 1,2 (periodate and peroxide oxidation)

Mix IV.

STX, dcSTX, NeoSTX, dcNeoSTX, GTX 1,4, GTX 2,3, dcGTX 2,3, GTX 5, C 1,2 (periodate oxidation, Fig. 4)

Retention times (chromatographic peaks) obtained after periodate and peroxide oxidations of PSP standard solutions are shown in Figs. 3 and 4. A few of the PSP toxins (GTX 1,4, GTX 2,3, C 1,2 and dcGTX 2,3) consist of an isomeric toxin pairs that are detected together during the HPLC analysis, consequently these toxins are quantified together by calculating the highest toxicity factor of the two coeluted compounds. As a result, the proportion of each toxin in the pair remains unknown.

Chromatographic separation was used to identify individual toxins which were quantitatively determined by direct comparison with calibration solutions of analytical standards. Limit of detection

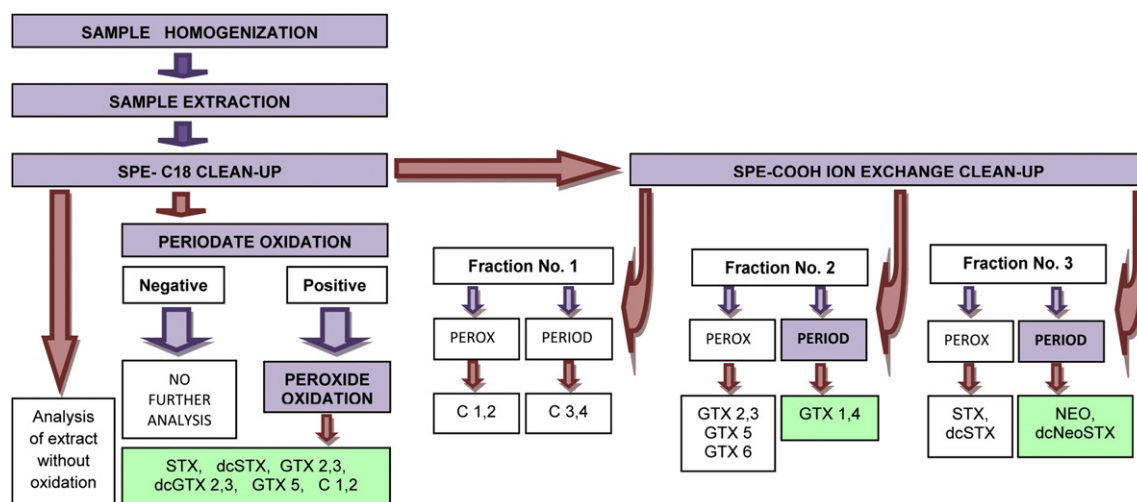


Fig. 2. Pre-column PSP toxins derivatization scheme (SPE, solid-phase extraction; green boxes indicate oxidation products needed for quantitation). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

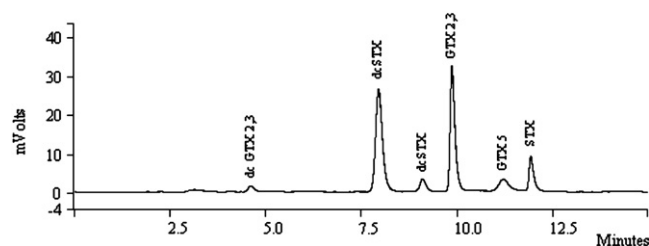


Fig. 3. Chromatogram obtained by HPLC-FLD with pre-column peroxide oxidation of the Mixture II containing STX, dcSTX, GTX 2,3, dcGTX 2,3 and GTX 5.

(LOD) was determined based on a generally accepted 3:1 signal-to-noise ratio. It was performed by comparing the measured signals from samples with known low toxin concentrations to those of the blank samples and establishing the minimum concentration at which the toxin can be reliably detected. The limit of detection was found to be $25.3 \mu\text{g kg}^{-1}$ for STX, $20.5 \mu\text{g kg}^{-1}$ for GTX 2,3 and $7.2 \mu\text{g kg}^{-1}$ for dcSTX. Toxicity Equivalence Factors (TEFs) are required to calculate the total $\mu\text{g STX dihydrochloride equivalents } 100 \text{ g}^{-1}$ sample. Specific toxicity factors of PSP toxins by Oshima (1995) were used for calculations. Integrated seawater samples (from surface to bottom) for phytoplankton analysis were taken by PVC sampling tubes and subsequently fixed with glutaraldehyde solution (final concentration of 0.5%). Phytoplankton species were identified and counted under the inverted microscope (Olympus IX50) according to Utermöhl method (Utermöhl, 1958).

3. Results

Station S1 was represented by STX, GTX 2,3 and dcSTX, with the first two being present in mussel samples from January through April 2009 (Fig. 5). STX showed three outbreaks, in February ($1298.17 \mu\text{g kg}^{-1}$) and March ($929.38 \mu\text{g kg}^{-1}$ and $732.01 \mu\text{g kg}^{-1}$) with the highest concentrations recorded during the whole study period, resulting in total toxicities of $823.84 \mu\text{g STX eq. kg}^{-1}$, $1314.82 \mu\text{g STX eq. kg}^{-1}$ and $1550.49 \mu\text{g STX eq. kg}^{-1}$ per sample (Fig. 6). As STX and GTX 2,3 exhibited high concentrations at this station and taking into account that their toxicity factors are the highest, three of these results reached the regulatory limit. Range of dcSTX went from its lowest value found in this study $7.06 \mu\text{g kg}^{-1}$ to $84.14 \mu\text{g kg}^{-1}$.

In autumn 2008, mussel samples both from the northern (S2) and the southern (S3) Adriatic Sea stations showed only dcSTX presence in concentrations of $64.96 \mu\text{g kg}^{-1}$ and $8.68 \mu\text{g kg}^{-1}$, respectively. However, these have to be considered with caution as these were the old samples and during MBA test mice survived approximately 22 h. Decarbamoyl group, with its intermediate toxicity and low concentrations results in low total toxicity of these samples ($33.32 \mu\text{g STX eq. kg}^{-1}$ and $4.44 \mu\text{g STX eq. kg}^{-1}$; Fig. 7).

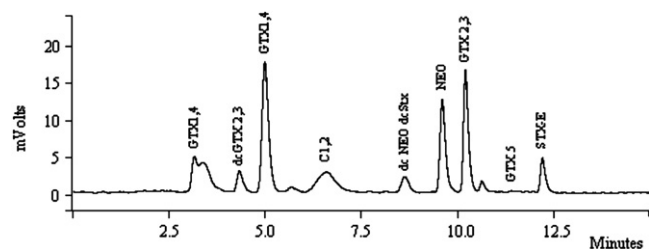


Fig. 4. Chromatogram obtained by HPLC-FLD with pre-column periodate oxidation of the Mixture IV containing STX, dcSTX, NeoSTX, dcNeoSTX, GTX 1,4, GTX 2,3, dcGTX 2,3, GTX 5 and C 1,2.

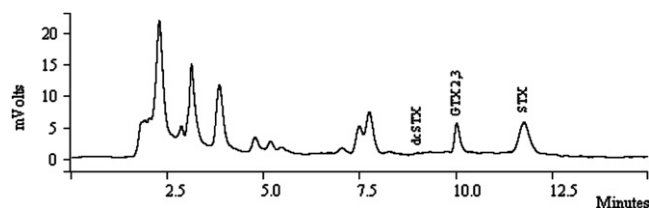


Fig. 5. Chromatogram obtained by HPLC-FLD with pre-column periodate oxidation of the mussel (*M. galloprovincialis*) C-18 extract sampled at station S1 (16th March 2009), containing STX and GTX 2,3.

In addition, we analyzed 12 shellfish samples that were suspected to contain PSP toxins established by MBA. These suspicious samples did not contain PSP toxins according to the HPLC-FLD method.

Positive PSP Mouse Bioassay results compared with those obtained by the HPLC for mussel samples from breeding area at station S1 in the northern Adriatic Sea are shown in Fig. 8. Four samples exceeded the regulatory limit of $800 \mu\text{g STX eq. kg}^{-1}$. Among these, only sample from 9th March showed the same resulting values by both methods, while in other two samples HPLC showed higher values than MBA. It is important to notice that according to HPLC two results exceeded the regulatory limit, while that was the case for only one of them according to the MBA method. Conversely, sample from 4th February exhibited $1903.37 \mu\text{g STX eq. kg}^{-1}$ by MBA, while HPLC recorded only $400.02 \mu\text{g STX eq. kg}^{-1}$ for the same sample. Short mouse survival time interval when testing the sample may be the reason for calculating the high content of STX eq. by biological method, as it would require dilution of acidic extract for testing of these samples. It was not possible to compare S2 and S3 stations since MBA showed no response (one mouse died within 24 h).

4. Discussion

4.1. PSP toxins in Croatian mussels

Some of the advantages of HPLC method would be that it is suitable for identification and quantitative determination of toxins, while its high selectivity makes it suitable for monitoring as preventive measure; moreover, HPLC-FLD is mentioned in Commission regulation EC No 2074/2005 (Anonymous, 2005) as an alternative to the Mouse Bioassay. In contrast, not all toxins have available reference materials (standards) and structurally unrelated and unknown toxins cannot be detected. During 2000–2009 period shellfish samples were analyzed using Mouse Bioassay. Among 3408

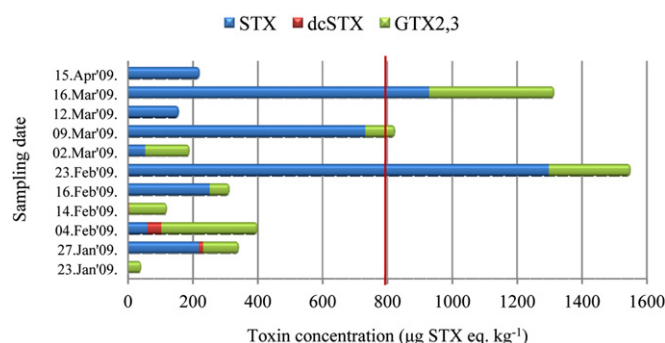


Fig. 6. Total PSP toxicity ($\mu\text{g STX eq. kg}^{-1}$) from mussel (*M. galloprovincialis*) samples at station S1 (regulatory limit of $800 \mu\text{g STX eq. kg}^{-1}$, marked as red vertical line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

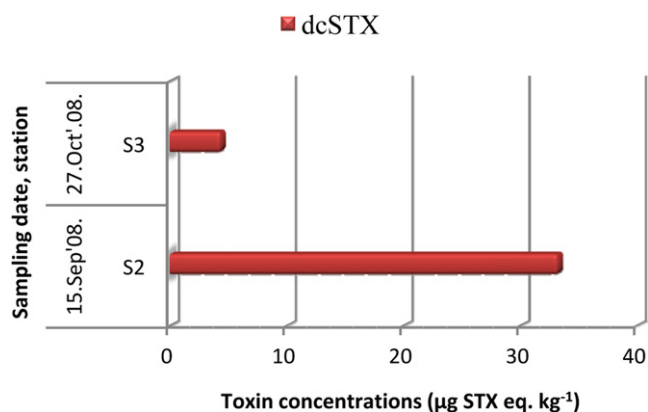


Fig. 7. Total PSP toxicity ($\mu\text{g STX eq. kg}^{-1}$) from mussels (*M. galloprovincialis*) at stations S2 and S3.

analyzed samples, 12 showed the presence of PSP by MBA and subsequent HPLC-FLD analysis was conducted on 11 of them. Both methods revealed 3 mussel samples that exceeded the regulatory limit of $800 \mu\text{g STX eq. kg}^{-1}$. High performance liquid chromatography analysis of SPE-C18 cleaned mussel extracts after periodate oxidation revealed qualitative composition of PSP toxins, while the same analysis of peroxide oxidised extracts was used for quantitation of STX, GTX 2,3, GTX 5, C 1,2, dcSTX and dcGTX 2,3. Although there was no evidence of GTX 1,4, NeoSTX and dcNeoSTX presence in periodate extracts after SPE-C18 cleanup, further analyses of SPE-COOH cleaned F2 and F3 periodate fractions (according the scheme on Fig. 2) were performed to prove their absence in PSP toxin profile for the eastern Adriatic Sea mussels. During our study, the PSP contamination mostly affected mussels sampled from the North Adriatic Sea (stations S1 and S2) with one incident in the southern Adriatic area of Mali Ston Bay (station S3). As shown in Fig. 6, STX, dcSTX and GTX 2,3 were the toxins determined to be present in Croatian PSP toxin profile. A typical contaminated mussel sample during this period of investigation is depicted in Fig. 5. Toxin profiles of six samples demonstrated $>70\%$ dominance of STX, while four samples revealed $>70\%$ of GTX 2,3 predominance. Decarbamoylsaxitoxin was the least represented in the PSP toxin profile of S1, ranging from 3 to 13% in three mussel samples at the beginning of 2009, while samples from stations S2 and S3 reached 100% in 2008, however in low concentrations (Table 1). Decarbamoylsaxitoxin coincided with the greater proportions of GTX 2,3 in the sample

Table 1

Percentage of each PSP toxin type contribution to total concentration ($\mu\text{g STX eq. kg}^{-1}$) from mussel (*M. galloprovincialis*) samples at stations S1, 2 and 3.

Station	Sampling date	% STX	% dcSTX	% GTX 2,3
S1	15.Apr '09.	100	0	0
	16.Mar '09.	60.68	0	39.32
	12.Mar '09.	100	0	0
	9.Mar '09.	83.61	0	16.39
	2.Mar '09.	19.97	0	80.03
	23.Feb '09.	76.71	0	23.30
	16.Feb '09.	72.66	0	27.34
	14.Feb '09.	0	3.72	96.28
	4.Feb '09.	9.87	13.83	76.30
	27.Jan '09.	52.45	6.28	41.27
S2	23.Jan '09.	0	0	100
	15.Sep '08	0	100	0
S3	27.Oct '08.	0	100	0

profiles that exhibited low total toxicities (Table 1, Fig. 6). Accumulation of higher proportion of carbamate toxins (GTX) relative to the other group of PSP toxins (C toxins) was observed in the mussel tissue also by Kwong, Wang, Lam, and Yu (2006). These authors recorded carbamate toxins accounted for 33.4% in *Alexandrium* species, while the same toxins accounted for 53.0% on average in the mussel samples. Such difference could result in differential retention or biotransformation of PSP toxins.

4.2. PSP profile from Croatian mussels compared with other reported profiles

Toxins GTX 1,4, dcGTX 2,3, GTX 5, NeoSTX, dcNeoSTX and C1,2 were not recorded in shellfish tissue, but there is also a possibility of undetected presence of C 3,4 and GTX 6 toxins, since their standard solutions were not commercially available at the time of conducting this study. The overall toxicity obtained by HPLC-FLD analysis ranged from 41.31 to $1550.49 \mu\text{g STX eq. kg}^{-1}$, while STX and GTX 2,3 were the two dominating toxins in terms of concentration and toxicity contribution considering all sampled mussels. This is in partial agreement with the study of Ciminiello et al. (1995) which revealed the presence of GTX 2,3 in *M. galloprovincialis* from the northwest Adriatic Sea at low toxicity levels. Similarly, Álvarez et al. (2009) in June 2006 documented two episodes of shellfish PSP toxicity in Chile ranging from 270 to $340 \mu\text{g STX eq. kg}^{-1}$, with mostly STX and GTX 2,3 toxins present. GTX 1,4, NeoSTX and STX played the same role in shellfish samples from the Norwegian coastline (Sayfritz, Aasen, & Aune, 2008). In comparison, Abouabdellah et al. (2008) found the dominance of GTX 2,3 in mussels contaminated with *A. minutum* from southern Atlantic coasts of Morocco, while in shellfish from northern Pacific coast of Japan GTX 1,4 and GTX 2,3 were the two dominant toxins, and C 1,2, STX and dcSTX were much less represented (Kaga, Sekiguchi, Sato, & Kodama, 2003).

4.3. MBA and HPLC-FLD methods comparison

The obtained results reveal that MBA gives higher values of toxicity than HPLC (average result values were $626.39 \mu\text{g STX eq. kg}^{-1}$ and $403.12 \mu\text{g STX eq. kg}^{-1}$, respectively), as expected by previous studies (Lawrence, Menard, & Cleroux, 1995). Maximum overall toxicities were recorded in mussels from station S1 in February with $1903.37 \mu\text{g STX eq. kg}^{-1}$ and $1550.48 \mu\text{g STX eq. kg}^{-1}$ by MBA and HPLC, respectively. Different extraction procedures may result in different toxin products. Extraction with heated hydrochloric acid (AOAC, 1990) converts the N-sulfocarbamoyl toxins into more toxic carbamoyl type, hence increasing the overall

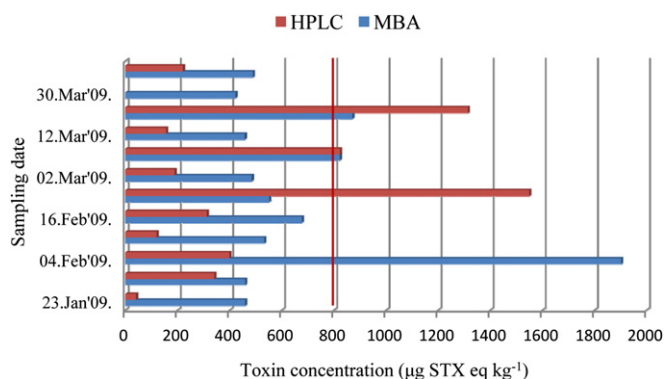


Fig. 8. Saxitoxin equivalents per 1 kg of shellfish sample tissue ($\mu\text{g STX eq. kg}^{-1}$) determined by HPLC-FLD (red bars) and MBA (blue bars) for the samples from S1 station. The regulatory action level is indicated by red vertical line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

toxicity that could be the explanation for higher MBA results (Botelho, Gomes, Rodrigues, & Vale, 2008; Hall, 1982). It has to be considered that MBA presents high variability, as small discrepancies between real and recorded survival time intervals when testing the sample may be the reason for calculating different content of STX eq. Also, samples involved in HPLC analysis were extracted with a mild acetic acid that is considered to maintain the original toxin composition. The transformation of toxins can alter their overall toxicity, as reported by Kwong et al. (2006) that C 2 toxin can be converted into GTX 3 under acidic conditions, increasing the toxicity by six-fold. For the majority of shellfish samples PSP contamination occurs during January to April period, which is in agreement with periodicity found for the Mediterranean coast (Taleb, Vale, Jaime, & Blaghen, 2001). Mons, van Egmond, and Speijers (1998) suggest that dinoflagellates develop at relatively high temperatures and abundant sunlight which, in Europe, results by cases of intoxications mainly between May and November.

4.4. Possible causative phytoplankton species

Phytoplankton community surveys of the S1 station area recorded dinoflagellate *A. minutum* present at the end of March 2009. Since *A. minutum* had been the only PSP causative species recorded in this area, we believe that it has to be the responsible one for the toxicity, but due to the sampling method used it was not detected. Namely, PVC sampling tubes were employed during this study, which are known to collect less water than phytoplankton nets that are proven better tools for toxic species sampling (Ninević-Gladan et al., 2008; Sidari, Cok, Cabrini, Tubaro, & Honsell, 1995). As *A. minutum* periodically occurs along the West Istrian coast and Mali Ston Bay it was consequently found in water samples from 29th September (80 cells L⁻¹) and 20th October (1.1 cells m⁻²) 2008. These findings are in agreement with those of Honsell et al. (1996) where the authors identified *A. minutum* as a saxitoxin producer along the coasts of Emilia Romagna at northwest Adriatic stations in May 1994, when MBA results confirmed by HPLC analysis revealed GTX 2 and GTX 3 present in mussel samples. Chou, Chen, and Chen (2004) have found GTX 1,4 and GTX 2,3 as the only representatives of the saxitoxins that occur in *A. minutum*. Conversely, according to this study STX is dominantly present in Croatian PSP toxin profile. That fact creates an implication of the same strain of phytoplankton organism in different, as well as in the same region, exhibiting different PSP profile and makes the comparison considerably difficult, similar to conclusion of Ichimi, Suzuki, and Ito (2002). Research by Frangópoulos, Spyarakos, and Guisande (2011) provides the first report of *Noctiluca scintillans* grazing rates on *Alexandrium* cells. These authors support the hypothesis that *Noctiluca* may cause grazing pressure on the growth of PSP causative species and therefore reach a role of a regulator of phytoplankton PSP toxins production. In the study of Asakawa, Miyazawa, Takayama, and Noguchi (1995), GTX 4 and C 2 were identified as major contributors to *Alexandrium tamarensis* toxin profile and Suzuki, Ichimi, Oshima, and Kamiyama (2003) found GTX 1,4 and C 1,2 to be the predominant toxins in the same dinoflagellate, mussel and seawater samples. Bearing that in mind and adding the fact that there were no detections of C 1,2 and GTX 1,4 in our currently analyzed tissue samples, it is almost logical that we obtained results of *A. tamarensis* absence from our seawater samples. Bower, Hart, Matthews, and Howden (1981) faced the similar situation when found the presence of saxitoxins in shellfish and crabs, but without detection of the causative organism. In 1992, mussels from the Atlantic coast contained saxitoxins with no toxin producing algae detected in the water (CRL, 1995). Álvarez et al. (2009)

reported an outbreak of *Alexandrium* species in May 2006, in Chile, where the authors have not detected saxitoxins in wild and cultured dinoflagellate; as at the same time, shellfish samples revealed the presence of GTX 2,3, dcGTX 2 and C 2. The same authors, in June 2006, documented two episodes of shellfish PSP toxicity with STX and GTX 2,3 toxins present. Possible explanation could be that of FAO (2004) suggesting the possibility of non-toxic algal species to acquire ability of toxin production when exposed to atypical nutrient regimes. This unlocks an option of so far considered non-toxic dinoflagellate to cause the PSP toxicity in this part of the Adriatic waters, or that certain non-monitored toxin producing algae may be responsible for present shellfish contamination. This resembles the findings of Bruno et al. (1990) when saxitoxins presence was confirmed in the Adriatic seawater samples taken during a *L. polyedrum* bloom. At the same location, formerly considered non-toxic, cyst forming *Alexandrium andersoni* was found to express the presence of mostly STX and NeoSTX (Ciminiello et al., 2000). The latter goes well with the assumption of various organisms producing the PSP toxins, such as supposed non-toxic dinoflagellates, some marine bacteria or may be other. Another possible origin of PSP toxicity recorded in winter 2009 in the northern Adriatic Sea might be the cysts of toxic dinoflagellates, which according to Schwinghamer, Hawryluk, Powell, and MacKenzie (1994) can be found incorporated into the sediment. Mussels located over depositional basins in embayments with shallow sills and orientation along the strong winds had persistently high toxin levels. There is also a hazard of introducing toxic cysts or vegetative cells of alien organisms through the ships' ballast waters (McMinn, Hallegraeff, Thomson, Jenkinson, & Heijns, 1997). If we consider higher food supply during the winter months when enhanced mixing and resuspension of organic matter above the benthic sediments occur, then the recorded toxicity during December to April period is reasonable.

5. Conclusions

This study reveals the PSP toxins presence in aquacultured mussels collected along the eastern Adriatic coast in concentrations below and above the maximum permissible level of 800 µg STX eq. kg⁻¹ by the Croatian and European legislation. Therefore it represents the first report of individual PSP toxin types and ascertains the PSP toxin profile for Croatian coastal waters. Toxicity was detected in samples collected during winter 2009, mainly in February and March. STX was determined as the prevalent toxin and the main contributor (more than 70%) to the total shellfish toxicity. It was followed by GTX 2,3 which contributed 27%, and the lowest contribution was that of dcSTX which accounted for less than 2% of toxicity considering all stations. To protect both public health and aquaculture it is very important to determine qualitative and quantitative composition of all PSP toxin types that accumulate in cultured bivalves and cause shellfish toxicity. According to these findings, it would be very difficult to make possible predictions about its occurrence since the PSP toxicity was found to be present in the investigated area, but without detection of the expected causing phytoplankton organisms.

Acknowledgements

Erasmus Mundus Master of Science in Marine Biodiversity and Conservation scholarship and the Croatian Ministry of Science, Education and Sports of the Republic of Croatia (through the grant 001-0010501-0848) supported this study.

References

- Abouabdellah, R., Taleb, H., Bennouna, A., Erler, K., Chafik, A., & Moukrim, A. (2008). Paralytic shellfish poisoning toxin profile of mussels *Perna perna* from southern Atlantic coasts of Morocco. *Toxicon*, 51(5), 780–786.
- Álvarez, G., Uribe, E., Vidal, A., Ávalos, P., González, F., Mariño, C., et al. (2009). Paralytic shellfish toxins in *Argopecten purpuratus* and *Semimytilus algosus* from northern Chile. *Aquatic Living Resource*, 22, 341–347.
- Anonymous. (2005). Commission Regulation (EC) No. 2074/2005. European Union Commission. *Official Journal of the European Union*, L 338, 27.
- Anonymous. (2008). Ministarstvo kulture, Državni zavod za zaštitu prirode, Republika Hrvatska. *Izješće o stanju prirode i zaštite prirode u Republici Hrvatskoj za razdoblje 2000–2007*. Zagreb.
- AOAC. (1990). Paralytic shellfish poison. Biological method. Final action. In K. Hellrich (Ed.), *Official methods of analysis* (15th ed.). (pp. 881–882) Arlington, Virginia, USA: Association of Official Analytical Chemists, Sec 959.08.
- Asakawa, M., Miyazawa, K., Takayama, H., & Noguchi, T. (1995). Dinoflagellate *Alexandrium tamarense* as the source of paralytic shellfish poison (PSP) contained in bivalves from Hiroshima Bay, Hiroshima Prefecture, Japan. *Toxicon*, 33(5), 691–697.
- Boni, L. (1983). Red tides off the coast of Emilia Romagna (north-western Adriatic Sea) from 1975 to 1982. *Inflore Botan Italian*, 15, 8–24.
- Boni, L., Mancini, L., Milandri, A., Poletti, R., Pompei, M., & Viviani, R. (1992). First cases of diarrhetic shellfish poisoning in the northern Adriatic Sea. In R. A. Vollenweider, R. Marchetti, & R. Viviani (Eds.), *Marine coastal eutrophication* (pp. 419–426). Elsevier.
- Botelho, M. J., Gomes, S. S., Rodrigues, S. M., & Vale, P. (2008). The study of cryptic PSP toxicity depending upon the extraction procedure. In In Ø. Moestrup (Ed.), *Proceedings of the 12th international conference on harmful algae* (pp. 338–340). Copenhagen: International Society for the Study of Harmful Algae and Intergovernmental Oceanographic Commission of UNESCO.
- Bower, D. J., Hart, R. J., Matthews, P. A., & Howden, M. E. (1981). Nonprotein neurotoxins. *Clinical Toxicology*, 18, 813–863.
- Bruno, M., Gucci, P. M. B., Pierdominici, E., Ioppolo, A., & Volterra, L. (1990). Presence of saxitoxin in toxic extracts from *Gonyaulax polyedra*. *Toxicon*, 28, 1113–1116.
- Chou, H. N., Chen, Y. M., & Chen, C. Y. (2004). Variety of PSP toxins in four culture strains of *Alexandrium minutum* collected from southern Taiwan. *Toxicon*, 43(3), 337–340.
- Ciminiello, P., Fattorusso, E., Magno, M., Oshima, Y., Poletti, R., Vivian, R., et al. (1995). Determination of PSP toxins in mussels from the Adriatic Sea. *Marine Pollution Bulletin*, 30(11), 733–735.
- Ciminiello, P., Fattorusso, E., Fiorino, M., & Montresor, M. (2000). Saxitoxin and neosaxitoxin as toxic principles of *Alexandrium andersoni* (Dinophyceae) from the Gulf of Naples, Italy. *Toxicon*, 38, 1871–1877.
- CRL. (1995). *State of the art NRLs on marine biotoxins*. CRL Rep 01/95. Vigo, Spain: European Community Reference Laboratory on Marine Biotoxins (CRL).
- FAO. (2004). *Marine biotoxins*. Food and Agriculture Organization of The United Nations.
- Frangópulos, M., Spyarakos, E., & Guisande, C. (2011). Ingestion and clearance rates of the red *Noctiluca scintillans* fed on the toxic dinoflagellate *Alexandrium minutum* (Halim). *Harmful Algae*, 10(3), 304–309.
- Giacobbe, M. G., Azzaro, F., Decembrini, F., Galletta, M., Gangemi, E., Raffa, F., et al. (2004). Fioritura tossica del dinoflagellato *Alexandrium minutum* in un'area costiera del Mar Ionio. *Biologia Marina Mediterranea*, 11, 703–707.
- Hall, S. (1982). Toxin and toxicity of *Protogonyaulax* from the northeast Pacific. Ph.D. Dissertation, University of Alaska.
- Hall, S. (1991). Natural toxins. In D. R. Ward, & C. Hackney (Eds.), *Microbiology of marine food products* (pp. 301–330). New York: Van Nostrand Reinhold.
- Honsell, G., Poletti, R., Pompei, M., Sidari, L., Milandri, A., Casadei, C., et al. (1996). *Alexandrium minutum* Halim and PSP contamination in the northern Adriatic Sea (Mediterranean sea). In T. Yasumoto, Y. Oshima, & Y. Fukuyo (Eds.), *Harmful and toxic algal blooms. Proceedings 7th international conference of toxic phytoplankton IOC-UNESCO, Sendai*, (pp. 77–80).
- Ichimi, K., Suzuki, T., & Ito, A. (2002). Variety of PSP toxin profiles in various culture strains of *Alexandrium tamarense* and change of toxin profile in natural *A. tamarense* population. *Journal of Experimental Marine Biology and Ecology*, 273(1), 51–60.
- Kaga, S., Sekiguchi, K., Sato, S., & Kodama, M. (2003). Toxication of bivalves and an ascidian caused by toxic dinoflagellate *Alexandrium tamarense* at Ofunato Bay, Iwate Prefecture, northern Pacific coast of Japan. *Bulletin of Iwate Prefectural Fisheries Technology Center*, 3, 63–70.
- Kwong, R. W. M., Wang, W.-X., Lam, P. K. S., & Yu, P. K. N. (2006). The uptake, distribution and elimination of paralytic shellfish toxins in mussels and fish exposed to toxic dinoflagellates. *Aquatic Toxicology*, 80, 82–91.
- Lawrence, J. F., Menard, C., & Cleroux, C. (1995). Evaluation of prechromatographic oxidation for liquid chromatographic determination of paralytic shellfish poisons in shellfish. *Journal of the Association of Official Analytical Chemists*, 78, 514–520.
- Lawrence, J. F., Niedzwizadek, B., & Menard, C. (2005). Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid chromatography with fluorescence detection: collaborative study. *Journal of the Association of Official Analytical Chemists*, 88, 1714–1732.
- Lilly, E. L., Kulis, D. M., Gentien, P., & Anderson, D. M. (2002). Paralytic shellfish poisoning toxins in France linked to a human-introduced strain of *Alexandrium catenella* from the western Pacific: evidence from DNA and toxin analysis. *Journal of Plankton Research*, 24, 443–452.
- Marasović, I., Ninčević, Ž, Pavela-Vrancić, M., & Orhanović, S. (1998). A survey of shellfish toxicity in the Central Adriatic Sea. *Journal of the Marine Biological Association of the United Kingdom*, 78, 745–754.
- Marasović, I., Ninčević-Gladan, Ž, Skejić, S., Grbec, B., Bužančić, M., & Ujević, I. (2007). Temporal distribution of *Dinophysis* spp. in relation to diarrhetic shellfish poisoning shellfish toxicity. *International Journal of Environment and Health*, 1(3), 493–506.
- McMinn, A., Hallegraef, G. M., Thomson, P., Jenkinson, A. V., & Heijnis, H. (1997). Cyst and radionucleotide evidence for the recent introduction of the toxic dinoflagellate *Gymnodinium catenatum* into Tasmanian waters. *Marine Ecology Progress Series*, 161, 165–172.
- Mons, M. N., van Egmond, H. P., & Speijers, G. J. A. (1998). Report No. 388802 005. *Paralytic shellfish poisoning; a review*, Vol. 47. National Institute of Public Health and the Environment.
- Montresor, M., John, U., Beran, A., & Medlin, L. K. (2004). *Alexandrium tamutum* sp. nov. (Dinophyceae): a new nontoxic species in the genus *Alexandrium*. *Journal of Phycology*, 40, 398–411.
- Ninčević-Gladan, Ž, Skejić, S., Bužančić, M., Marasović, I., Arapov, J., Ujević, I., et al. (2008). Seasonal variability in *Dinophysis* spp. abundances and diarrhetic shellfish poisoning outbreaks along the eastern Adriatic coast. *Botanica Marina*, 51, 449–463.
- Orhanović, S., Ninčević, Ž, Marasović, I., & Pavela-Vrancić, M. (1996). Phytoplankton toxins in the central Adriatic Sea. *Croatica Chemica Acta*, 69, 291–303.
- Oshima, Y. (1995). Postcolumn derivatization liquid chromatographic method for paralytic shellfish toxins. *Journal of the Association of Official Analytical Chemists*, 78(2), 528–532.
- Paz, B., Riobó, P., Fernández, M. L., Fraga, S., & Franco, J. M. (2004). Production and release of yessotoxins by the dinoflagellates *Protoceratium reticulatum* and *Lingulodinium polyedrum* in culture. *Toxicon*, 44, 251–258.
- Sayfritz, S. J., Aasen, J. A. B., & Aune, T. (2008). Determination of paralytic shellfish poisoning toxins in Norwegian shellfish by liquid chromatography with fluorescence and tandem mass spectrometry detection. *Toxicon*, 52, 330–340.
- Schwinghamer, P., Hawryluk, M., Powell, C., & MacKenzie, C. H. (1994). Resuspended hypnozygotes of *Alexandrium fundyense* associated with winter occurrence of PSP in inshore Newfoundland waters. *Aquaculture*, 122, 171–179.
- Sidari, L., Cok, S., Cabrini, M., Tubaro, A., & Honsell, G. (1995). Temporal distribution of toxic phytoplankton in the gulf of Trieste (Northern Adriatic Sea) in 1991 and 1992. In P. Lassus, G. Arzul, E. Erard, P. Gentien, & C. Marcaillou (Eds.), *Harmful marine algal blooms* (pp. 231–236). Technique et Documentation-Lavoisier, Intercept Ltd.
- Suzuki, T., Ichimi, K., Oshima, Y., & Kamiyama, T. (2003). Paralytic shellfish poisoning (PSP) toxin profiles and short-term detoxification kinetics in mussels *Mytilus galloprovincialis* fed with the toxic dinoflagellate *Alexandrium tamarense*. *Harmful Algae*, 2(3), 201–206.
- Taleb, H., Vale, P., Jaime, E., & Blaghen, M. (2001). Study of paralytic shellfish poisoning toxin profile in shellfish from the Mediterranean shore of Morocco. *Toxicon*, 39(12), 1855–1861.
- Ujević, I., Ninčević-Gladan, Ž, Roje, R., Skejić, S., Arapov, J., & Marasović, I. (2010). Domoic acid – a new toxin in the Croatian Adriatic shellfish toxin profile. *Molecules*, 15(10), 6835–6849.
- Utermöhl, H. (1958). Zur Vervollkommnung der quantitativen Phytoplankton Methodik. *Mitteilungen Internationale Vereinigung für Theoretische und Angewandte Limnologie*, 9, 1–38.
- Vila, M., Garcés, E., Masó, M., & Camp, J. (2001). Is the distribution of the toxic dinoflagellate *Alexandrium catenella* expanding along the NW Mediterranean coast? *Marine Ecology Progress Series*, 222, 73–83.