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Evaluation of paralytic shellfish poisoning toxin profile of mussels from Bulgarian North Black Sea coast by HPLC-FlD with post and pre-column derivatization

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Marine toxins are produced by certain toxic phytoplankton species. Harmful toxins may accumulate in the shellfish tissue, potentially impacting human health. Paralytic shellfish poisoning (PSP) is a syndrome caused by ingestion of shellfish contaminated with paralytic shellfish toxins (PST) that comprise saxitoxin and its variants (neosaxitoxin, gonyautoxins and their decarbamoyl and *N*-sulfocambamoyl analogs).

The aim of this study was to evaluate the presence of paralytic shellfish toxins (PSTs) in plankton samples and in mussels intended for human consumption.

Mussels collected in the main areas of production and recreational harvesting off the north coast of Bulgaria have been investigated for PSP toxins. Individual toxins were determined using two methods both involving fluorescence detection: ion pair-liquid chromatography with post-column derivatization (method 1) and high-performance liquid chromatographic procedure employing pre-column oxidation of the toxins (method 2). The results according method 1 demonstrated the presence of gonyautoxin 2 in 53% of the mussel samples and no toxins were detected in the plankton samples. The toxicity level - 1.6 μg STX.2HCl .kg^{-1} was far beneath the EU legislative limit of 800 μg STX.2HCl .kg^{-1} concluding in negligible risk for human health.

Due to higher limits of detection no toxins were detected via method 2. Even though, considering method 2 is recognized by European Commission as official for regulatory purposes and the relative high value of the legislative threshold, thus obtained toxin levels are enough representative to conclude if mussels are safe for consumption or not. On the other hand, the more sensitive method 1 provides important data on extremely low toxin levels which would be useful for chronic exposure estimation and for completing the knowledge about occurrence of PSTs in certain locations.

Keywords*: paralytic shellfish toxins, gonyautoxin 2, Bulgaria, mussels, plankton, LC-FLD*

INTRODUCTION

Seafood products such as shellfish are a rich source of proteins and mineral components [1, 2]. Mussels and rapana are an important source of income for the fishing community in Bulgaria [3]. However, shellfish such as mussels may contain compounds harmful to human health such as marine biotoxins.

Among them are paralytic shellfish poisoning (PSP) toxins (PSTs) that comprise saxitoxin (STX) and its variants. A dose of approximately 1 mg of saxitoxin from a single serving of contaminated shellfish is fatal to humans. PSTs are neurotoxins that specifically bind to voltage gated sodium channels and thereby block the excitation current in nerve and muscle cells, finally resulting in paralysis and other disorders in consumers of contaminated shellfish [4]. Intoxication with PSTs produces several symptoms that include tinkling, sensation or numbness around the lips, numbness of extremities, gastrointestinal problems, difficulty in breathing, and, in fatal cases, death [5]. The human PSP fatality cases vary worldwide and depend essentially on local health - care practitioner awareness of the intoxication and its treatment and the local medical system's capacity on assisting respiratory support needing victims. For example, in a series of intoxications in Europe and North America, no deaths occurred among more than 200 cases. In similar outbreaks in Southeast Asia and Latin America, where clinicians may be unexperienced with PSP, the case fatality proportion ranged between 2-14% [4]. Worldwide, PSP affects some 2000 people annually, with a 15% mortality rate [6].

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PSP toxins are produced by some cosmopolitan marine eukaryotic dinoflagellates belonging to the genera *Alexandrium* [7], *Gymnodinium* [8] and *Pyrodinium* [9]. *Alexandrium* spp., however, are the most abundant and widespread producers of PSTs [6]. Eight of the 30 known species within the genus are documented to synthesize PSTs [10]. Additionally, PST production within *Alexandrium* appears paraphyletic, and there are seemingly toxic and non-toxic strains of the same species [11]. The toxins are passed through the marine food web via vector organisms, e.g. shellfish as mussels, which accumulate the toxins by feeding on PST producing dinoflagellates without apparent harm to themselves [12].

PSP toxins are tetrahydro purine derivatives (Figure1). Classification of these toxins includes carbamoyl (STX, neosaxitoxin (NEO) and gonyautoxins (GTX1-4), *N*- sulfocarbamoyl (B1 and B2, C1-4), decarbamoyl (dcSTX, dcNEO, dcGTX1-4) derivatives and hydroxylated PSTs (M1-4).

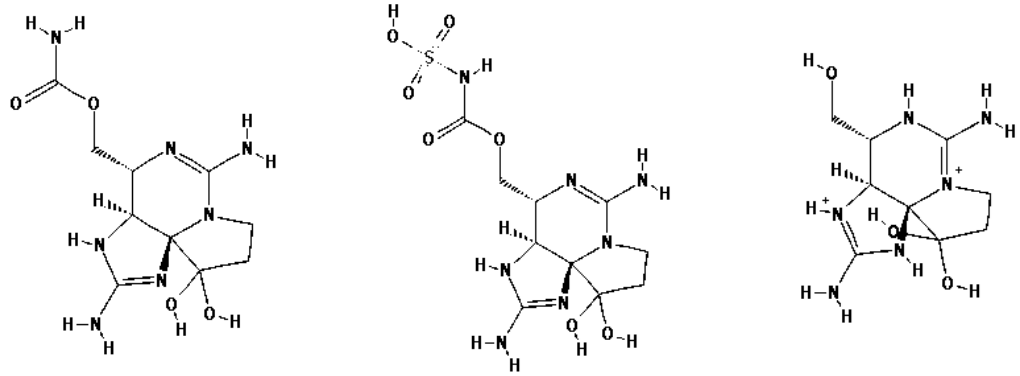


Figure 1. Chemical structures of selected representatives from the three main groups of PSTs

Within the saxitoxin group around 30 different analogues have been detected [13] but recently many more are discovered [14]. Not every analogue exhibits the same toxicity and nowadays for the most prominent analogues, toxic equivalent factors (TEF) have been established [15].

In order to be able to determine PSP toxins as non-fluorescent tetrahydro purine compounds by highly sensitive fluorescence detection (FD), PSTs have to be oxidized to fluorescent imino purine derivatives. The Official AOAC detection method is HPLC-FD with precolumn derivatization [16], the so called Lawrence method, which is widely used [17]. It is based on of prechromatographic oxidation followed by chromatographic separation of the oxidation products. However, the implementation of this method is relatively complex due to the different ionic charge states of PSTs. STX e.g. is highly polar due to the presence of two guanidino groups [18], where as C-toxins do not possess a net charge. To confirm the presence of PSTs in a sample, a detailed sample preparation procedure including solid phase extraction (SPE) separation into tree distinct fractions is required. Thereafter the fractions are treated with different oxidizing agents [19].

An alternative HPLC method for the determination of PSTs consists in chromatographic separation of underivatized PSTs followed by continuous post column derivatization and FD, the so called post-column oxidation or PCOX method. [20]. This method is very sensitive and highly specific. It can provide excellent quantitative results for known analogues and its results come in agreement with other recognized methods [20].

The aim of this study was to provide scientific information regarding the occurrence of PSTs and to assess common PSP profiles of mussel and plankton samples harvested along the north coast of Bulgaria in 2017. Total PST content was investigated by using both, the Lawrence and PCOX methods in order to assess their applicability and discuss their suitability for different applications and purposes.

EXPERIMENTAL

Sample material

Eighteen phytoplankton and 17 mussel samples were taken from 5 sites (Varna, Albena, Balchik, Kavarna and Shabla) along the north Bulgarian coast in two sampling campaigns – spring 2017 and summer – autumn 2017. Cultivated mussels were sampled from mussel farms and wild mussels from recreational harvesting sites. Locations of plankton sampling were selected in accordance of the presence of mussel aquaculture sites and wild mussel beds.

For plankton sampling a 20 μm mesh size plankton net was used. The net was hauled in different sites and depths (1 m to 5 m) depending on mussel breeding locations. In total 18 plankton samples were collected.

Wild mussels (7 samples) were harvested manually by recreational harvesters and by our co-workers. Farmed mussels (10 samples) were provided from mussel farmers directly from cultivation ropes.

Sample preparation:

The digestive glands of at least 1.5 kg specimen (~ 37.3 g) were dissected and homogenized with dispersing instrument (POLYMIX®PT 1200E, KINEMATIKA AG, Germany) for 5-10 min at maximum speed.

Extraction of Plankton:

The sample concentrate from the plankton net was collected on 20 μm mesh size sieve and washed in 50 mL centrifuge tube. The suspension was centrifuged (4000 × g, 10 min at 10 °C), the supernatant was removed and 1 mL 0.03 M acetic acid was added to the plankton pellet. For the extraction of PSTs from the plankton cells, the resulted suspension was sonicated (40 Hz, 15 min) and centrifuged again. The supernatant was filtered through syringe filters (0.45 μm pore size, ⌀ 25 mm, Minisart, Sartorius, Germany). Filtrates were transferred into chromatographic vials and kept at -20 °C until further analysis.

Extraction of digestive glands:

- for PCOX analysis:

An average 2.14 g of hepatopancreas homogenate was extracted in duplicate with 4 mL 0.2 M acetic acid with dispersing instrument for 2 min at maximum speed and centrifuged for 10-15 min at 4000 × g. The resulting supernatants were transferred to 50 mL centrifuge tubes and centrifuged again. An aliquot (1 mL) was filtered through syringe filters. Filtrates were kept in chromatographic vials at -20 °C until further analysis.

- for Lawrence method [16]:

Hepatopancreas homogenate (~ 5g) was extracted in duplicate with 3 mL 1% acetic acid. First extraction was performed at boiling water for 5 min. Subsequently a centrifugation at 4000 × g for 10 min was performed. Supernatants were combined and made up to volume of 10 mL.

Clean-up of 1 mL extract was performed with SPE C18 cartridge (SUPELCO, 500mg/3 ml) and the extract was adjusted to pH 6.5 with 1M NaOH.

Pre-column derivatization

The sample extracts were oxidized with periodate oxidant - 100 μL of sample extract were added to 100 μL of matrix modifier (PSP-free oysters were extracted and cleaned-up by the same procedure as samples) in a 1.5 mL vial. 500 μL of periodate oxidant (prepared daily by mixing 5 mL each of 0.03 M periodic acid, 0.3 M ammonium formate, and 0.3 M Na_2HPO_4, and adjusted to pH to 8.2 with 0.2 M NaOH using pH meter) were added to the vial and mixed. The solution reacted at room temperature for 1 min. Glacial acetic acid (5 μL) was added to interrupt the reaction.

HPLC- FD analysis

PCOX method (Method 1)

All mussel and plankton extracts were analyzed for PSTs by reverse-phase ion-pair liquid chromatography on a LC1100 series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) coupled to a PCX 2500 post-column derivatization system (Pickering Laboratories, Mountain View, CA, USA) and dual monochromator fluorescence detector (G1321A) following minor modifications of previously published methods [20]. Chromatographic conditions were as follows: Eluent A – 6 mM octanesulfonic acid, 6 mM heptanesulfonic acid and 40 mM ammonium phosphate; Eluent B - 13 mM octanesulfonic acid and 50 mM phosphoric acid by isocratic elution program. The flow rate was 1 mL/min and injection volume - 20 μL. The separation of analytes was performed on a 250 mm × 4.6 mm i.d., 5 μm, Luna C18 reversed-phase column (Phenomenex, Aschaffenburg, Germany) equipped with a Phenomenex SecuriGuard pre-column.

Post column derivatization

To the column eluate 10 mM periodic acid in 555 mM ammonium hydroxide was added continuously at a flow of 0.4 mL/min before the reaction coil (50 °C). After the reaction coil acidification of the eluate with 0.75 M nitric acid was performed. The toxins were detected by a dual monochromator fluorescence detector (λ_{ex} 333 nm; λ_{em} 395 nm).

Lawrence method (Method 2)

The analyses were performed on a liquid chromatograph UltiMate 3000 Thermo Fisher Scientific Dionex, equipped with quaternary pump, vacuum degasser, autosampler, injector, fluorescent detector Dionex RF 2000, chromatographic column Kinetex: C18, 150 mm × 4.6 mm, 5μm; flow rate of the mobile phase 1mL/min (Eluent A: 0.1 M ammonium formate, pH 6 with 1 M acetic acid; Eluent B: 0.1 M ammonium formate in 5% acetonitrile, pH 6 with 1 M acetic acid). Elution was achieved by a gradient program (0–5% mobile phase B/5 min, 5–70% B/4 min and 100% A for the last 6 min), injection volume was 50 μl.

The identification of PSTs for both methods was accomplished by comparison of retention time and fluorescence response at specified wavelengths to certified reference materials (CRMs) (C1/2, GTX1/4, dcGTX2/3, GTX2/3, GTX5, NEO, dc-STX, STX).

To prevent false positives caused by interference of auto-fluorescent compounds, using both methods, PSP extracts were analyzed with and without derivatization.

The limits of detection (LODs) of the nine toxins for both methods were calculated (Table 1).

Table 1. LODs of investigated PSTs via HPLC-FD with post- and pre-column derivatization (NH- net haul)

PSP toxins	HPLC FD with post column derivatization		HPLC FD with pre-column derivatization
	ng/NH/m plankton	LOD (µg STX.2HCl eq/kg) mussels	mussels
C1/C2	1.4	0.2	3
GTX 1	17.5	9.4	
GTX 4	13.5	7.2	60
dc GTX 2	0.6	0.9	13
dc GTX 3	0.6	0.9	
GTX 2	0.7	0.4	15
GTX3	0.8	0.7	
B1	3.7	0.5	3
NEO	6.9	12.1	36
dcSTX	1.0	2.2	11
STX	0.6	1.1	15

Calculations

Based on the relative potency of each STX-analogue, by means of their effect in sodium channels and in the mouse bioassay (MBA), the European Food Safety Authority (EFSA) published the toxicity equivalency factors (TEF) [15].

As the Lawrence pre-column HPLC-FD method [16] cannot distinguish between PST stereoisomers (GTX1 and GTX4; C1 and C2, dc-GTX2 and dc-GTX3, GTX2 and GTX3) by choosing the highest toxicity factor of the two co-eluted compounds. Total PSP toxicity was calculated by summing the toxicity contribution of each quantified toxin expressed in terms of µg STX .2HCl eq. kg^{-1}.

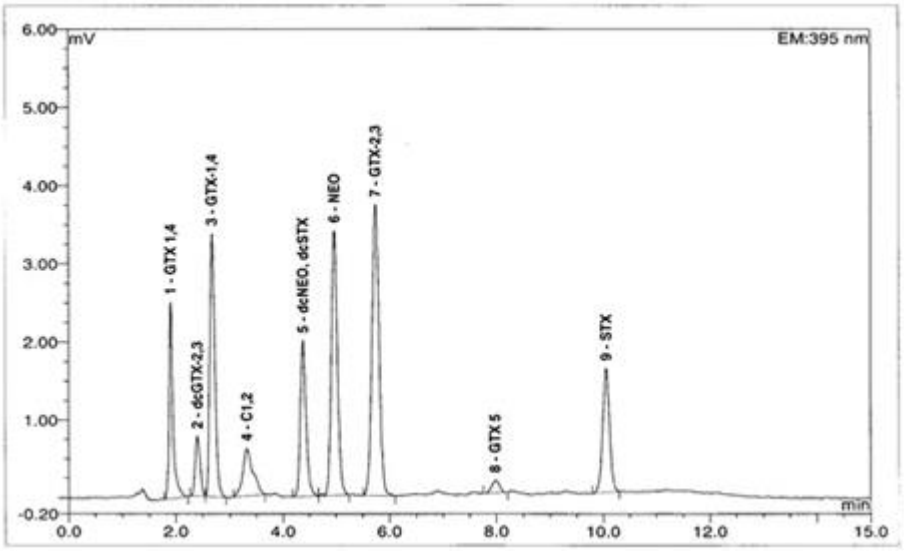
RESULTS AND DISCUSSION

In the present study, mussel and plankton samples were subjected to HPLC-FD analysis in order to detect the presence of the following PSP toxin types: dc-GTX2/3, C1/2, dc-STX, GTX2/3; B1, STX, NEO, GTX1/4 and dc-NEO.

Initially we applied the official AOAC (Lawrence) method for PSTs determination in shellfish samples. No PSTs were detected in any of the investigated samples with the precolumn derivatization method (Figure 2). The calculated LODs (Table 1) were much lower than the legislative threshold (800 µg STX.2HCl eq.kg^{-1}) which concluded that obviously there was no risk for PSP if investigated samples were consumed.

However, investigations on plankton species of the Institute of Oceanology of Bulgaria documented the presence of *Alexandrium spp*. in the studied area [21]. This lead us to conclude that there was a principal possibility for PSTs to be detected in plankton and mussels. Therefore, we performed PSTs analysis firstly of plankton samples but no PSTs were detected in any of the analyzed samples. This result came in agreement with our previous study on plankton samples from the same region where no PSTs were detected as well [22]. However, since plankton toxin data represents the situation at the time of sampling and is not valid for longer periods, we considered to reanalyze PSTs in mussels with a more sensitive PCOX method, as mussels accumulate and retain toxins for a longer period of time. The data obtained would be more informative and allow statements for longer periods.

Surprisingly, low levels of PSTs were detected by this method. Figure 3 shows the chromatogram of the sample with the highest toxicity (MU48P), analyzed by HPLC-FD with pre-column derivatization method.



Operator:User Timebase:hplc Sequence:PSP_Varna_08062018

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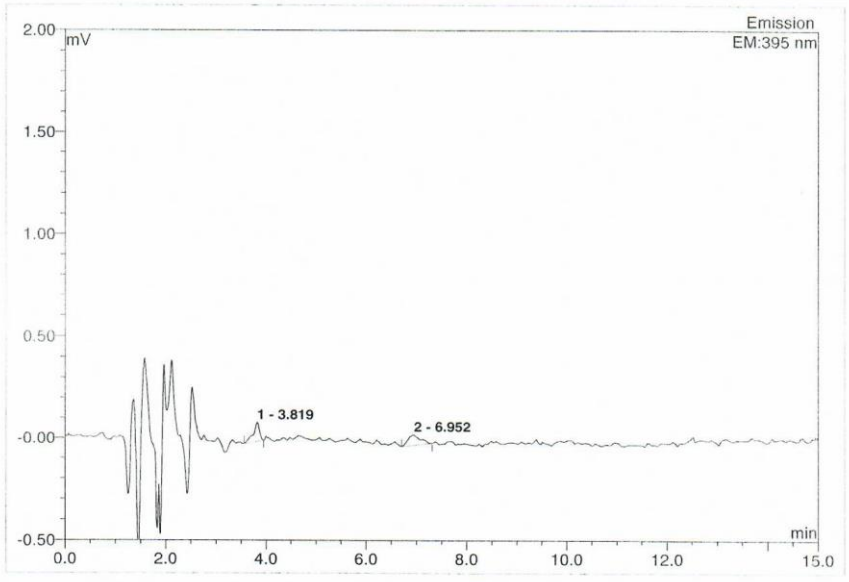
MU 48 P

Sample Name:	**samp15-MU48 P ox**	*Injection Volume:*	**50.0**
Vial Number:	**RB5**	*Channel:*	**Emission**
Sample Type:	**unknown**	*Wavelength:*	**n.a.**
Control Program:	**PSP_Kinetex_flow_1**	*Bandwidth:*	**n.a.**
Quantif. Method:	**MIX-6_PERIODAT_guard Kinetex_flow 1**	*Dilution Factor:*	**1.0000**
Recording Time:	**6/8/2018 12:39**	*Sample Weight:*	
Run Time (min):	**15.00**	*Sample Amount:*	



No.	Ret.Time min	Peak Name	Height mV	Area mV*min	Rel.Area %	Amount	Type
Total:			0.000	0.000	0.00	0.000	

Fig. 2. Chromatograms of a PSP CRMs after periodate oxidation (top) and extract of cultured mussel sample MU48P (bottom) obtained via HPLC-FD with pre-column derivatization

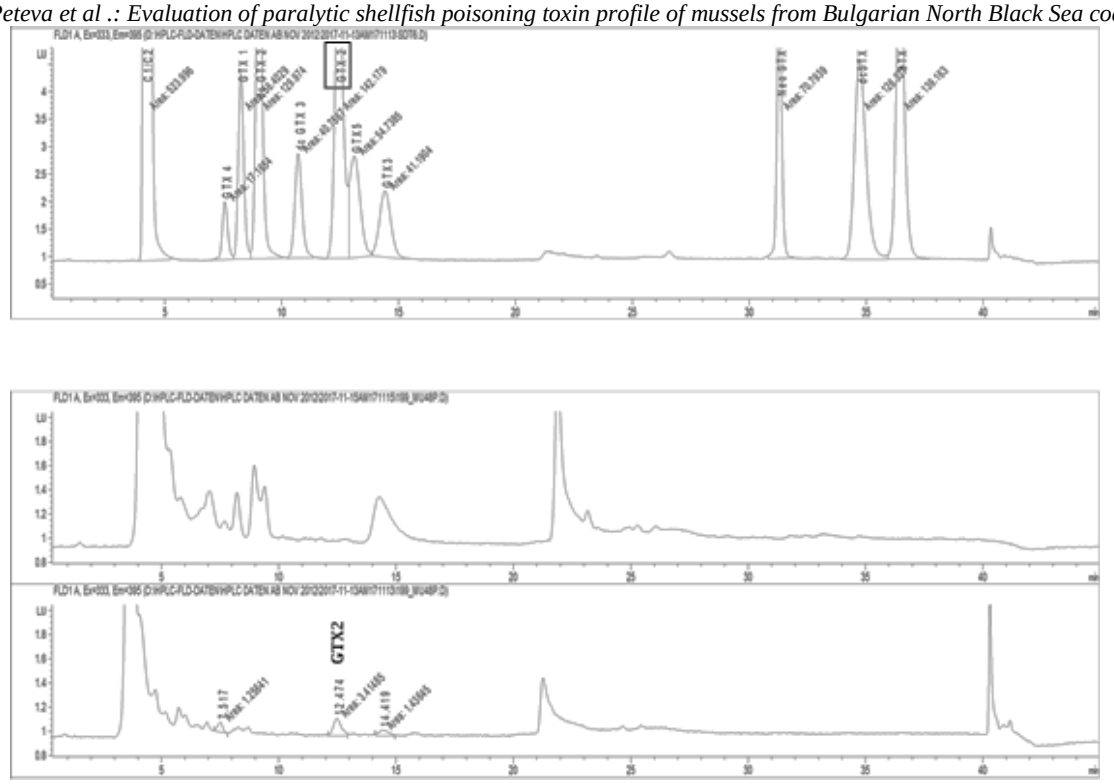


Fig. 3. HPLC-FD with post column derivatization PCOX chromatograms of a PSP standard mix containing B1, C1/2, GTX1-4, dcGTX2/3, NEO, dcSTX and STX(top) and extract of cultured mussel sample MU48P without derivatization (middle) and with derivatization (bottom).

Table 2. PSTs levels in positive samples as determined via HPLC-FD with post-column derivatization

Sample N	PSTs, µg STX.2HCl. kg^{-1} C1/2, GTX1/4, dc GTX2/3, GTX3, GTX5, NEO, dcSTX, STX	GTX 2	Total toxicity, µg STX.2HCl. kg^{-1}
MU37P farmed	nd	0.8	0.8
MU45P wild	nd	0.5	0.5
MU48P farmed	nd	1.6	1.6
MU72P farmed	nd	1.0	1.0
MU75P wild	nd	0.5	0.5
MU86P wild	nd	0.5	0.5
MU88P farmed	nd	0.7	0.7
MU87P farmed	nd	0.7	0.7
MU85P wild	nd	0.6	0.6

Table 2 summarizes the PSP toxicity of positive mussel samples, as determined by the PCOX method. As evident, only GTX2 was detected in 9 out of 17 samples investigated. Within the positive samples 5 were of cultivated mussels. This result is in contrast to our previous investigation performed at the location Kavarna, where no PSTs had been detected [22]. The highest detected level (MU48P, cultivated mussel sample) - 1.6 µg STX.2HCl .kg^{-1} was significantly lower than the EU legislative limit of 800 µg STX.2HCl .kg^{-1}. These low values confirmed the results of the measurements according to the official method and the conclusion that there was no PSP risk for mussel consumers in 2017.

As samples were collected regularly throughout the investigated period a seasonal change in PSTs contamination throughout the year 2017 (Figure 4) became evident.

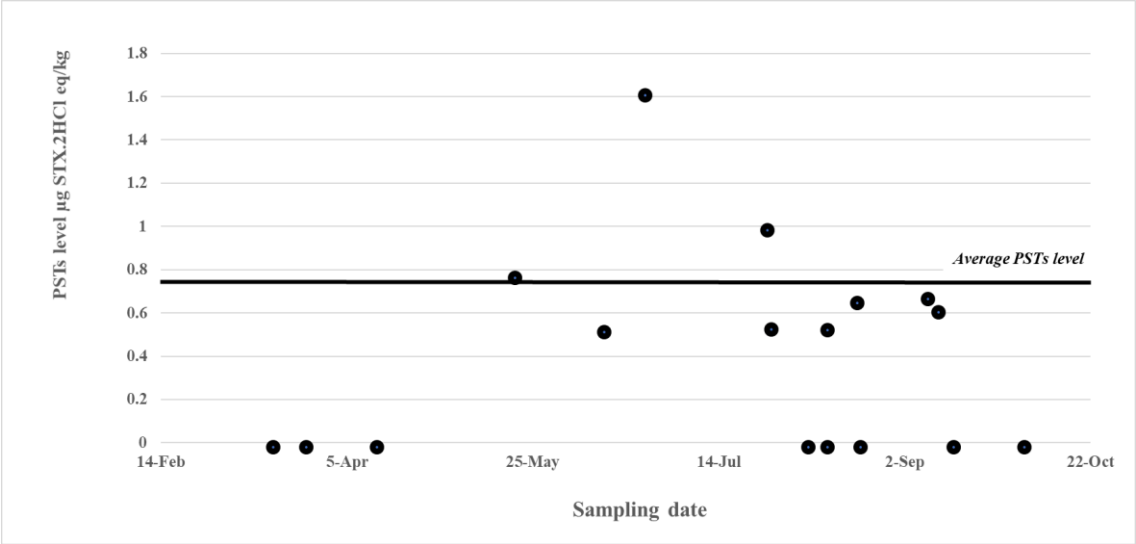


Figure 4. Seasonal changes in PSTs levels

With respect of the overall low contamination a peak in the PST level (1.6 µg STX.2HCl /kg) was observed in early summer. This increased concentration is more than two times higher than the mean PST level, which was around 0.78 µg STX.2HCl /kg. Obvious is the presence of PSTs overall harvesting seasons with exception of early spring and late autumn. However, it is still unclear whether 2017 was a typical situation or changes in environmental conditions could lead to higher *Alexandrium* densities and PSTs levels in mussels.

In similar study conducted in the same location and during the same period of time in 2015-2016 among 55 samples analyzed both STX and GTX2,3 were detected in only one sample with total toxicity of 55.1 µg STX.2HCl /kg [23], much higher than here determined.

The two methods used have their advantages and in certain cases result of both of them should be considered. HPLC-FD with pre-column derivatization is the official method recognized by the EU legislation. Thereby detected levels are low enough to consider if mussels are suitable for consumption or not. On the other hand, the method HPLC-FD with post-column derivatization is quick and lower levels of PSTs could be detected. This is important e.g. when exposure to chronic low levels of PSTs is investigated. Even more, it is still unclear whether *Alexandrium* is really responsible for PSTs in the mussels, and whether it can come in the future to higher PST loads. Therefore, research is needed to identify sources of PSTs on the Bulgarian coast, as well as whether *Alexandrium* (as a potential source of PSP) could form harmful blooms in the Black Sea.

CONCLUSIONS

This study highlights the low contamination with PSTs in mussels off the north coast of Bulgaria in 2017. Via HPLC-FD with post column derivatization were detected GTX2 levels that were lower than the LOD of the other method used – HPLC-FD with precolumn derivatization. Thus, combination of both methods will contribute in increasing awareness about PSP and by estimation of PSTs exposure.

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