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Detection of marine biotoxin in plankton net samples from the Bulgarian coast of Black Sea

Z. V. Peteva[1*], B. Krock[2], T. Max[2], M. D. Stancheva[1], S. K. Georgieva[1]

[1]*Medical University - Varna, Department of Chemistry, Tsar Osvoboditel Blvd., 84, 9000 Varna, Bulgaria*
[2]*Alfred-Wegener-Institut Helmholtz-Zentrum für Polar- und Meeresforschung, Chemische Ökologie, am Handelshafen 12, 27570 Bremerhaven, Germany*

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Some diatoms and dinoflagellates can produce marine toxins, which can accumulate in, e.g. filter-feeding bivalves, posing a potent treat to seafood consumers. In this study, concentrated net plankton samples were collected from mussel cultivation regions (Kavarna bay) and zones for wild catch (Varna bay) in two periods - winter to fall 2018 and spring 2019. A method using liquid chromatography-tandem mass spectrometry (LC-MS/MS) was employed to analyze domoic acid (DA), okadaic acid, dinophysistoxins, yessotoxin, pectenotoxin-2 (PTX2), gymnodimine A (GYM), 13-desmethyl spirolide C (SPX1), and goniodomin A (GDA). Paralytic shellfish toxins (PSTs) were investigated by high performance liquid chromatography with post-column derivatization and fluorescence detection. Results indicated the presence of DA, PTX2, SPX1 and GDA reaching maximum levels of 1.4 $ng.NH^{-1}.m^{-1}$ DA, 115.5 $ng.NH^{-1}.m^{-1}$ PTX2, 0.2 $ng.NH^{-1}.m^{-1}$ SPX1 and 8.6 $ng.NH^{-1}.m^{-1}$ GDA. No PSTs were detected in the investigated samples. The maximum toxin load of the samples was due to the presence of PTX2. Detection of DA, PTX2, SPX1 and GDA in the samples points to the possible toxigenic nature of phytoplankton species along the Bulgarian coast. These data may be used to evaluate the probability of potential risks to local aquaculture and seafood from wild catch.

Keywords: Black Sea, domoic acid, PTX2, SPX1, goniodomin A, LC-MS/MS, HPLC-FLD

INTRODUCTION

Natural toxins are harmful organic compounds that have a biogenic origin. Some of them are also bio-contaminants – they are produced by microorganisms and may accumulate in food and food products. Among them are mycotoxins, bacterial toxins and marine biotoxins. Mycotoxins are a chemically diverse group of compounds that are secondary metabolites of fungi. They have a strong capacity to produce acute toxicity in animals and humans. Bacterial toxins are proteins produced by a large variety of bacterial pathogens. They can act, e.g. as primary virulence factors. Marine biotoxins are produced mostly by certain freshwater and marine microalgae. They find their way to humans through the food chain causing severe illness when exceeding certain levels.

Scientific interest on the occurrence of natural toxins in Bulgaria is not persistent. Just in 1996, the first survey on the natural occurrence of *Fusarium* mycotoxins in Bulgarian wheat was published [1], although the breeding of these crops has long tradition in Bulgaria [2]. More standing but still not contemporary is the investigation on the possible role of mycotoxins for the development of endemic nephropathy by the rural population [3-5]. Lately, an approach based on the immunosensor quantification of aflatoxins was proposed [6].

More recent are studies on genetics [7, 8] and occurrence [9-11] of toxin-producing bacteria. Even more, Stratev *et al.* (2016) [12] proved a risk to human health through consumption of fish and fish products due to the presence of *A. hydrophila* strains that form different virulence factors.

In 2017 Stoyneva-Gärtner *et al*. [13] published a summary of results on the assessment of cyanoprokaryote blooms and of cyanotoxins (freshwater phycotoxins) in Bulgaria in a 15-years period (2000-2015), incl. drinking-water reservoirs, recreational lakes and sites of nature conservation importance, indicating a particular scientific activity in this field. Furthermore, a hygienic assessment of some water reservoirs based on determination of cyanotoxins was also made [14].

Marine biotoxins were investigated in seafood within the national monitoring program [15-17] and by our scientific team by means of human exposure estimation [18-20]. Due to the bioaccumulation processes, chemical levels in marine biota (e. g. mussels) are by orders of magnitude larger than in

* To whom all correspondence should be sent:
E-mail: zlatina.peteva@mu-varna.bg

water. Even more, some marine toxins undergo chemical changes through the food chain transfer [21, 22]. Additionally, the evidence of potentially toxigenic microalgae on the Bulgarian coast [23-25] and other parts of the Black Sea [26] is increasing.

Therefore, to better understand the origin of marine toxins, a research down to the lowest trophic levels is required. The aim of this study is to determine the phycotoxin levels in plankton net samples from the Bulgarian coast.

EXPERIMENTAL

Sampling and extraction procedure

Sampling area covered Kavarna and Varna bays, situated on the north Bulgarian coast, and the two periods - winter to fall 2018 and spring 2019. Plankton samples (N = 37) were collected by horizontal net tow hauls with a 20-μm mesh and an aperture of 40-cm diameter. Net haul concentrates were adjusted to a defined volume of 500-1000 mL (depending on the net tow volume) using 20 μm filtered seawater. The samples were separated into two aliquots. Each aliquot was filtered on 0.45 μm Whatman® nylon membrane filters. Filters were then washed with 200-1000 μL of 100% methanol for domoic acid and lipophilic toxins and with 200-1000 μL of 0.03M acetic acid for paralytic toxins.

The methanolic and acetic acid suspensions were then sonicated (40 Hz, 15 min) and centrifuged at 4000 × g for 10 min at 10 °C. The supernatants were filtered through syringe filters (0.45 μm pore size, ⌀ 25 mm, Minisart, Sartorius, Germany). Filtrates (150-1000 μL) were transferred into chromatographic vials and kept at -20 °C until further analysis.

Chromatographic analysis

The hydrophilic paralytic toxins were determined by HPLC-FLD with post-column derivatization according to Krock *et al.* (2007) [27] on a LC1100 series liquid chromatograph (Agilent, Waldbronn, Germany) coupled to a PCX 2500 post-column derivatization system (Pickering Laboratories, Mountain View, CA, USA) and dual monochromator fluorescence detector (G1321A) as described in detail in our previous study (Peteva *et al.* 2019). Briefly, the mobile phase contained two eluents: A – 6 mM octanesulfonic acid, 6 mM heptanesulfonic acid and 40 mM ammonium phosphate and B - 13 mM octanesulfonic acid and 50 mM phosphoric acid maintained by isocratic elution program (flow rate was 1 mL min^{-1}). As stationary phase 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 was used.

Post-column derivatization constituted of oxidation of the eluate with 10 mM periodic acid dissolved in 555 mM ammonium hydroxide followed by an acidification with 0.75 M nitric acid. Detection was performed on following wavelengths - λex 333 nm; λem 395 nm. Calculated limits of detection (LOD) are reported in Table 1. The hydrophilic domoic acid (DA) and lipophilic toxins – gymnodimine A (GYM), 13-desmethyl spirolide C (SPX1), okadaic acid (OA), dinophysistoxins-1 and -2 (DTX1,2), pectenotoxin-2 (PTX2), goniodomin A (GDA), yessotoxin (YTX) and azaspiracid-1 (AZA1) were analyzed according to Krock *et al.* (2008) [28] on an LC-MS/MS system. It consists of Agilent model 1100 LC coupled to an API-Sciex 4000 QTrap triple-quadrupole mass spectrometer (Sciex, Darmstadt, Germany) equipped with a Turbo Spray interface. Measurements were carried out in positive-ion mode by selected reaction monitoring (SRM) experiments. Toxins were separated by reverse-phase chromatography on an analytical column (50 × 2 mm) packed with 3 mm Hypersil BDS 120 Å (Phenomenex, Aschaffenburg, Germany). Gradient elution was performed with two eluents, where eluent A was water and B was acetonitrile/water (95:5v/v), both containing 2.0 mM ammonium formate and 50 mM formic acid. Calculated limits of detection are presented on Table 1.

The quality control was performed by regular analysis of procedural blanks and certified reference material (National Research Council, Canada). Quantification of detected toxins was done by integration of the areas of the chromatographic peaks.

Calculations

Toxin contents are expressed as nanograms per net tow and meter (ng. NT^{-1} m^{-1}).

RESULTS AND DISCUSSION

With the aim to determine the qualitative and quantitative composition of marine biotoxins in plankton net samples a variety of toxins were analyzed, where DA, PTX2 (Table 2), SPX1 and GDA were detected.

Domoic acid is a hydrophilic marine biotoxin that represents a pyrrolidine carboxylic acid and belongs to a group of amino acids called kainoids [29]. DA was detected in the samples from winter-spring 2018. The highest concentration of 1.4 $ng.NH^{-1}.m^{-1}$ was registered in a sample from April 2018. This value is much lower than the concentrations detected

by Almandoz *et al.* (2017) [30] – 97 – 5041 ng.NT^{-1} in Argentinian Sea during a summer expedition. Still, it should be considered that this difference could be due to variation in salinities of both Argentinian and Black Sea.

Domoic acid was determined in plankton net samples from Marmara Sea [31], as well as in Tyrrhenian [32] and North Sea [33], which was always associated with the presence of a natural population of the toxic diatom *Pseudo-nitzschia*. As also on the Bulgarian coast there is an evidence of the same genus [23-25], the presence of this toxin in plankton samples was expected. Spirolides and gymnodimines are cyclic imines. Structure elucidation of SPX1 showed that it contains a 6-5-5-polyether ring system in addition to a heptacyclic imine ring [34, 35]. Production of SPX1 is linked to *A. ostenfeldii* [36, 37]. This species is also recorded in the Black Sea [38, 39]. Often along with SPX1 also paralytic toxins and gymnodimines are present in the plankton samples [40-42]. There are also studies reporting that in geographical isolates either GYMs or SPX1 [43] are detected. Our investigation showed that SPX1 was registered in the samples from summer-fall 2018. Highest value (0.245 ng.NH^{-1}.m^{-1}) was determined in a sample from July 2018.

Table 1. Limits of detection (LOD) of analyzed marine biotoxins

HPLC- FD method		LC-MS/MS method	
Paralytic toxins analyzed	LOD, ng.NH^{-1}.m^{-1}	Marine toxins analyzed	LOD, ng.NH^{-1}.m^{-1}
C1/2	2.1	DA	1.1
GTX 4	20.1	GYMA	0.2
GTX 1	26.0	SPX1	0.1
dc-GTX2	0.8	OA	1.5
dc-GTX 3	0.9	DTX2	1.1
GTX 2	1.0	DTX1	20.3
B1	5.5	PTX2	1.0
GTX 3	1.3	GDA	4.6
Neo STX	10.3	YTX	3.8
dc-STX	1.5	AZA1	0.3

Table 2. Levels of detected phycotoxins in mussel samples

Period studied	Detected toxins – positive concentration range, ng.NT^{-1}.m^{-1}		
	DA	*PTX2*	*SPX1*
Spring-Fall 2018	0.5-1.4	1.3-115.5	0.054-0.245
Spring 2019	nd	69.8-109.1	nd

It is lower than the SPX1 levels (0.5 - 143.6 ng.NT^{-1}) detected in the northern Patagonian shelf in the Argentinian Sea by Guinder *et al.* (2018) [36].

The samples were also analyzed for paralytic toxins by HPLC-FLD, but all were negative. The absence of paralytic toxins is in agreement with our previous study on plankton samples from the same area in 2017 [18], where no paralytic toxins were detected in the plankton samples as well.

Gymnodimines are the smallest molecules of the group of cyclic imines. The chemical structures of GYM A, 12-methyl GYM A [44], GYM B, GYM C, GYM D [45], 16-desmethyl GYM D and GYM E [46] have been structurally elucidated by now. All GYM analogues have a six-membered imino ring. Their macrocycle contains 16 carbon units and one ether bridge [47].

The investigation on GYMs showed that some samples from spring-summer 2018 were near but below the LOD.

In two samples from summer-fall 2018 a putative gymnodimine A-like compound was detected. The difference in mass of this compound (*m/z* 540/522, RT 3.21 min) to GYM A (*m/z* 508/490, RT 3.02 min) is 32 Da which could be a variant with two additional water molecules. However, abundances were too low for confirmatory analysis. Calculated concentrations of this putative gymnodimine A-like compound are 1.1 and 3.0 ng.NH^{-1}.m^{-1}.

Gymnodimines are not often reported in field samples. GYM-A and its analogues are mostly detected in isolates and cultures of the species from, e.g. the Netherlands [46], the Baltic Sea [45], etc. Detected concentrations in the Bulgarian samples were much lower than the reported by Kremp *et al.*, (2019) [48] for Limfjord (up to 590 ng.NT^{-1}) and the North Sea (up to 100 ng. NT^{-1}). Goniodomin-A

(GDA) is a linear polyether macrolide. GDA was detected in two samples from summer 2018 with values of 8.6 and 5.5 ng.NH^{-1}.m^{-1}. GDA is known to be produced by species with a wide abundance like *A. pseudogonyaulax* [49, 50] and the two rare species *A. monilatum* and *A. hiranoi* [51, 52]. These species were also registered in the Black Sea [53-55] and even on the Bulgarian coast [56]. Nevertheless, to our knowledge SPX1 and GDA have never been detected in plankton samples from the Black Sea.

Pectenotoxins are also linear polyether compounds. Their common structural features include a spiroketal group, three oxolanes, a bicyclic ketal and a six-membered cyclic hemiketal [57]. The most dominant lipophilic toxin was PTX2 (Table 2), which was detected in 67 % of the samples from April-July 2018, as well as in single samples from August and September 2018 and from March and April 2019 (Fig. 1). In 2018 the highest value (115.5 ng.NH^{-1}.m^{-1}) was detected in a sample from July. This value is close to the highest value registered in spring 2019 (109.1 ng.NH^{-1}.m^{-1}). In July 2018 the PTX2 concentrations were higher compared to the other investigated season when the toxins were detected in single samples.

The PTX2 concentration ranges of 2018 and 2019 are comparable with PTX2 levels reported by Guinder *et al.* [36] (2018) in Argentinian Sea.

Okadaic acid (OA), dinophysistoxins (DTXs) (also known as diarrhetic shellfish toxins) and pectenotoxins are toxic polyether compounds produced by planktonic species of the genus *Dinophysis* and benthic species of *Prorocentrum* [58,59]. These toxins very often cooccur [60-62], but also only PTX2 containing isolates are reported [63]. Hereby, the toxin profile of plankton net samples also includes only the PTX2, which could be due to a geographical specification of the producing species. Neither OA, DTXs nor YTX and AZA1 were detected in the samples.

Investigation on the toxin load of the samples showed that only eight samples, all from 2018, contained more than one toxin (Fig. 2).

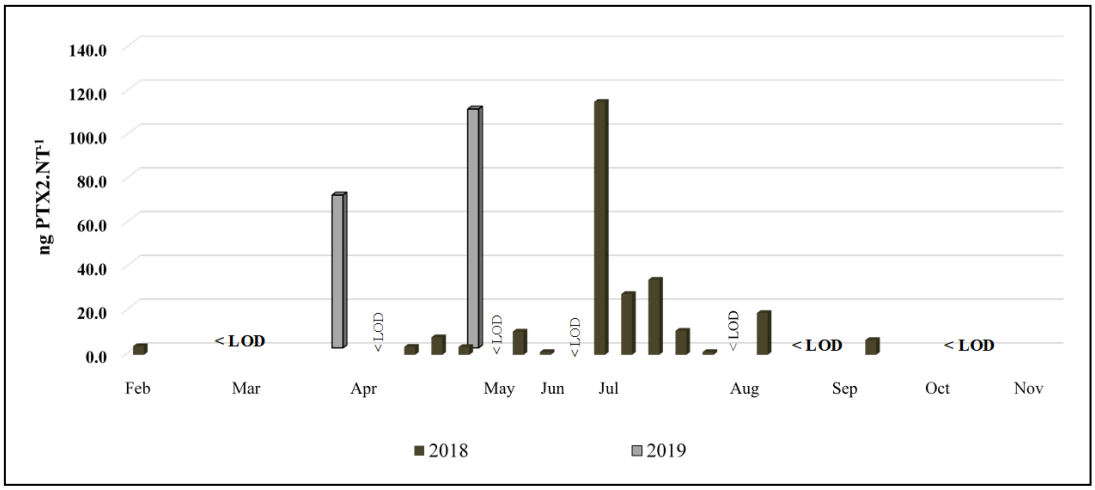

Figure 1. Distribution of PTX2 in plankton samples

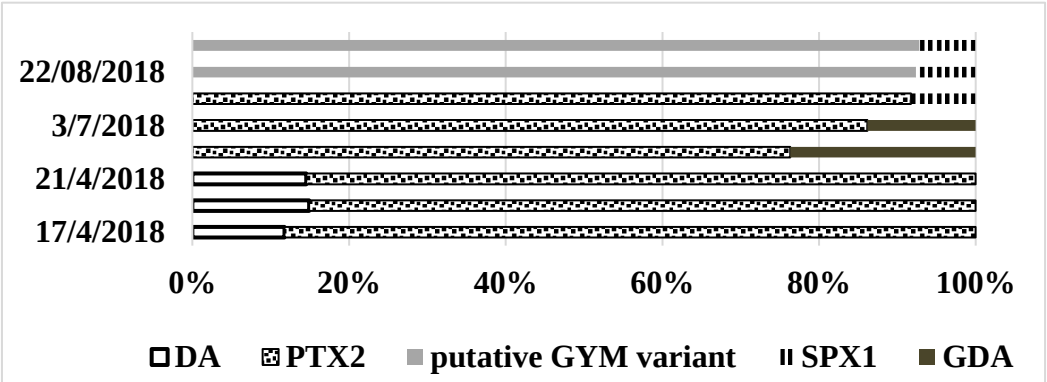

Figure 2. Toxin load of multitoxin plankton samples

The major contribution in the toxin content of the samples is due to the presence of PTX2 (6 samples) and in two samples to the availability of the "putative GYM variant". However, two pairs of samples are

collected on the same time; no seasonal variation could be concluded. Still, as domoic acid is present in only three multitoxin samples and in one more sample and no paralytic toxins were detected, it is reasonable to conclude that the toxin profile of plankton net samples is based on the presence of lipophilic toxins.

CONCLUSION

During a sampling campaign in 2018 and 2019 the marine algal toxins – PTX2, SPX1, DA and GDA were detected in phytoplankton net samples from North Bulgarian coast. The maximum toxin load was due to the presence of PTX2. This is the first report for the SPX1 and GDA in plankton samples from Bulgarian Black Sea coast.

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