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Comparison of seasonal and spatial phycotoxin profiles of mussels from South Bulgarian coast

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Phycotoxins (marine algal toxins) are toxic metabolites released by certain phytoplankton species. They can be responsible for seafood poisoning outbreaks because filter-feeding mollusks, such as mussels, can accumulate these toxins throughout the food chain and present a threat for consumers' health. A wide range of symptoms, from digestive to nervous, are associated to human intoxication by biotoxins, characterizing different and specific syndromes, called shellfish poisoning. The aim of this study is to compare the seasonal and spatial phycotoxin profiles of mussels (wild and farmed) harvested from South Bulgarian coast in the period 2017-2018. Analyzed were 57 samples by different analytical techniques - liquid chromatography tandem mass spectrometry (LC-MS/MS) and high-performance liquid chromatography with fluorescent detection followed by postcolumn derivatization. Domoic acid (DA), yessotoxin (YTX), pectenotoxin-2, PTX-2sa/ epi-PTX-2sa and gonyautoxin-2 (GTX2) were detected in the studied samples. Results revealed huge seasonal variations in the phycotoxin profiles of the mussels investigated. Spring 2017 profile is dominated by domoic acid present in 67% of the samples and reaching highest level of 618.9 ng. g^{-1}. In summer 2017 samples YTX is prevalent (60%) reaching a level of 8.3 ng.g^{-1}. No phycotoxins were detected in samples from fall 2017. The epimer pair PTX-2sa/ epi-PTX-2sa was with highest seasonal abundance in winter-spring 2018 – 47%. Its maximum detected level was 7.1 ng.g^{-1}. No statistically significant differences in mean phycotoxin levels of different sampling locations were determined. Generally, the herein reported marine toxins levels are comparable or even lower than in other European studies and much lower than legislative limits set in EU. Nevertheless, the huge seasonal variations in the phycotoxin profile show that for protection of consumers' health a further surveillance on marine toxins content in edible mussels is required.

Keywords: marine biotoxins**,** Black Sea, domoic acid, yessotoxin, shellfish poisoning

INTRODUCTION

The growth of aquaculture is mainly due to the increase in the human population and the general overexploitation of the fisheries [1]. Additionally, recreational harvesting has been documented to be very popular along the coast of numerous countries [2]. An important fraction of these two activities in Bulgaria is focused on mollusks and more specifically on mussels. For instance, in 2018 the commercial catch of Black Sea mussels has increased by 24% compared to 2017, becoming 13.11 tons and aquaculture production of the same species was exceeding 3000 tons [3].

Bivalves feed on the organic matter suspended in the seawater. Phytoplankton is the main component of this matter but some phytoplanktonic species can produce potent toxins (phycotoxins) which can be accumulated by mussels. Intoxications caused by consumption of contaminated mollusks are categorized according to their symptoms. The main ones are paralytic (PSP), diarrhetic (DSP) and amnesic (ASP) shellfish poisoning [4]. In Europe, the legislated groups of phycotoxins consist of six different chemical groups - paralytic toxins (saxitoxin and derivatives) (PSTs), domoic acid (DA), yessotoxins (YTXs), azaspiracids (AZAs), pectenotoxins (PTXs), and okadaic acid (OA) and its derivatives - the dinophysistoxins (DTXs). For these toxins, levels found in shellfish for human consumption must be lower than 180 μg eq STX kg^{-1}, 20 mg DA kg^{-1}, 3.75 mg eq YTX kg^{-1}, 0.16 mg eq AZA kg^{-1}, and 0.16 mg eq OA kg^{-1} (for the OA and PTX toxin group) [5, 6].

To reduce and prevent the potential risk of intoxication many studies have emphasized the importance of seasonal and spatial monitoring of phycotoxin levels in mussels intended for human consumption. Ujević *et al.* (2010) [7] found great variations in the levels of ASP causing toxin - domoic acid - in mussel samples from Adriatic Sea. Within a single winter season DA level ranged from not detected to 6.5 μg g^{-1}. The detected levels were much lower than the permissible limit and authors conclude that mussel consumption was not found to endanger human health.

In mussels purchased from Lugo (Galicia, Spain), belonging to five commercial brands of different

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origins, Otero *et al.* (2018) [8] determined that the DSP causing toxins OA and DTX-2 are the main risk in harvested mollusks. Their levels varied greatly within the investigated period 2018-2019. In the positive samples the toxicity was determined between 3.6 and 234.1 μg OA eq kg^{-1} and three samples even exceeded the legal limit of 160 μg kg^{-1}. Therefore, authors conclude that DSP toxins are the major cause for concern in local mollusks and it is necessary to monitor phycotoxins levels to check future risks derived from mussel consumption.

Given the increase in reports of fluctuations of phycotoxins levels, coupled with a high demand on Bulgarian mussels, further investigations into a potential consumer risk caused by phycotoxins is required. The aim of this study is to compare the phycotoxin profiles of mussels harvested from South Bulgarian coast in the period 2017-2018.

EXPERIMENTAL

Field samples

Cultivated mussel samples (N=41) were collected manually every two weeks and more often directly from cultivation ropes from farming areas. Wild mussel samples (N=16) were collected manually from rocks in locations used for recreational harvesting. The study area covers the south Bulgarian coast from Nessebar to Tsarevo. Samples were collected in spring, summer and fall 2017, as well as in winter and spring of 2018.

All mussel samples were drained with distilled water to discard algae and sand. Thereafter the shells were removed. The digestive glands of minimum 0.5 kg of mussels (without shells) were dissected, homogenized and used for paralytic toxins, domoic acid and lipophilic toxins analysis.

Domoic acid and lipophilic toxins analysis

Each homogenate of digestive glands (~4 g) was subjected to methanol extraction. The procedure is in detail described by Peteva *et al*. (2017) [9]. An aliquot (~ 1000 μl) of each extract was analyzed by LC-MS/MS according to Krock *et al.* (2008) [10] for presence of DA and the lipophilic toxins - OA, DTXs, YTX and PTXs. Mass spectrometric experiments were performed on a triple quadrupole mass spectrometer (model API 4000 QTrap, SCIEX, Darmstadt, Germany), equipped with a TurboSpray® interface coupled to a liquid chromatograph (model 1100, Agilent, Waldbronn, Germany). The limits of detection (LOD) for the investigated lipophilic toxins and DA (Table 1) were determined based on 3:1 signal-to-noise ratio for each series of measurements.

Table 1. Limits of detection (LOD) of lipophilic toxins and DA for samples from fall 2017 and winter-spring 2018

Analyzed phycotoxins	LOD, $ng.g^{-1}$
DA	0.250
YTX	3.178
OA	4.975
DTX1	5.800
DTX2	2.375
PTX2	2.125

Paralytic shellfish poisoning toxins (PSTs) analysis

Each homogenate of digestive gland (~2 g) was subjected to acetic acid extraction. The procedure is briefly described by Peteva *et al.* (2019) [11]. An aliquot (~ 1000 μl) of each extract was analyzed by reverse-phase ion-pair liquid chromatography coupled to a post-column derivatization system according to Krock *et al.* (2007) [12]. The limits of detection of the investigated PSTs (Table 2) were determined for each series of measurements.

Table 2. Limits of detection of the investigated PSTs

Analyzed phycotoxins	LOD, $ng.g^{-1}$	
	Spring and summer 2017 samples	Fall 2017 and winter-spring 2018 samples
C1/2	2.15	3.57
GTX 4	20.70	25.69
GTX 1	26.75	32.71
dc-GTX2	0.86	1.13
dc-GTX 3	0.90	1.16
GTX 2	1.06	1.40
GTX 5	5.67	1.84
GTX 3	1.29	6.78
Neo STX	10.55	14.71
dc-STX	1.56	2.46
STX	0.89	1.54

Calculations

As there is no reference material for PTX-2sa/ epi-PTX-2sa, their levels are given as PTX2 equivalents.

Statistical analysis

SPSS 16 was used for statistical processing of the results. Descriptive statistical analysis was applied using tabulated graphical method, mean values, distribution values, etc. Using the MS Excel 2016 descriptive statistics feature, the bar indicates the standard deviation (in absolute value) within each group. Statistical hypothesis test (t-test) was applied to establish the existence of a statistically significant difference between the mean values of toxins by type of samples and depending on the sampling location and the sampling season. Results were reported by p-values. The groups for which we proved statistically significant difference $p \leq 0.05$ are indicated with *.

RESULTS AND DISCUSSION

In this investigation, wild and cultivated mussels (N=57) were collected from eight locations on the south Bulgarian coast including important areas of mussel farming and recreational harvesting. Domoic acid and the lipophilic toxins were extracted from the digestive glands of mussels because this is the known organ where these toxins accumulate [13, 14]. Although PSTs are hydrophilic compounds [15] there is evidence that they are also concentrated in this organ [16, 17].

DA, GTX2/3, YTX, PTX2 and the epimeric pair PTX2sa/ epi-PTX2sa were detected in the samples analyzed (Table 3). OA and DTXs were not detected in the samples. Domoic acid was detected in the samples from spring 2017 and in only one sample from 2018 with a huge difference in its level. YTX was detected in spring 2017 and summer-fall 2017 samples characterized by a wide content range for both seasons. Highest level (24.559 $ng.g^{-1}$) was registered in May 2017. This level is much lower than the regulatory limit of 3.75 $mg.kg^{-1}$ [5]. PTX2 was only detected in spring 2017 samples whereas its level within the season increases up to 30 times to reach a maximum of 59.79 $ng.g^{-1}$. Comparison with legislative limit -160 μg OA $eq.kg^{-1}$ [6] showed that no risk for human health is expected. Surprisingly, at the end of the investigated period (spring 2018) PTX-2sa/ epi-PTX-2sa appeared in the studied samples. GTX2/3 was determined in a small number of samples. Few samples containing PSTs were also reported in other Bulgarian studies [18, 19].

Table 3. Levels of detected phycotoxins in mussel samples

Detected phycotoxins	Spring 2017		Summer-fall 2017		Winter-spring 2018	
	Wild mussels	Cultivated mussels	Wild mussels	Cultivated mussels	Wild mussels	Cultivated mussels
Domoic acid number of positive samples	5	9	+0	0	0	1
Domoic acid content range, $ng.g^{-1}$	247.36-576.04	108.3-618.9	<LOD	<LOD	<LOD	0.3
YTX, number of positive samples	0	9	2	7	0	0
YTX, content range, $ng.g^{-1}$	<LOD	0.009-24.559	1.596-3.926	1.606-14.806	<LOD	<LOD
PTX2, number of positive samples	3	2	0	0	0	0
PTX2, content range, $ng.g^{-1}$	1.85-59.79	0.6-1.8	<LOD	<LOD	<LOD	<LOD
PTX2sa/ epi-PTX-2sa, number of positive samples	0	0	0	0	2	5
PTX2sa/ epi-PTX2sa, content range, ng PTX2 $eq.g^{-1}$	<LOD	<LOD	<LOD	<LOD	3.0-3.3	3.1-7.1
GTX2/3, number of positive samples	1	1	0	1	0	0
GTX2/3, content range, $ng.g^{-1}$	2.63	1.79	<LOD	2.59	<LOD	<LOD

Comparison of phycotoxin profiles of the studied wild and cultivated mussels (Fig. 1) showed the presence of all detected toxins in both types of samples. In cultivated mussels YTX was dominating, followed by domoic acid and PTX-2sa/ epi-PTX-2sa. The phycotoxin profile of wild mussels was characterized prevalently by domoic acid, followed by PTX2. Morton *et al.* (2009) [20] also investigated the presence of toxins in mussels from the Black Sea. The authors found the dominance of PTX2 and PTX-2sa/ epi-PTX-2sa in the phycotoxin profile. Another study from the Black Sea [21] showed that the majority of the toxin load is due to YTX and its analogues. To characterize the change of the phycotoxin profile in the three studied seasons, the ratio between the detected toxins was calculated (Fig. 2). In spring 2017 the richest variety of toxins - DA, YTX, PTX2 and GTX2/3 was registered. The dominating toxin was domoic acid, followed by YTX and PTX2. DA and PTX2 were determined in the samples until the beginning of May, while YTX was detected throughout the whole season. The presence of GTX2/3 in the samples of both seasons was scarce.

As domoic acid was only detected in spring 2017, a comparison of mean DA levels (t-test) was reasonable. It showed a statistically significant difference (Fig. 3) ($p=0.014 \leq 0.05$) between the mean levels in cultivated (181.1 ± 209.5 ng.g^{-1} hp) and in wild mussels (396.8 ± 119.7 ng.g^{-1} hp). The large standard deviations are explained by the variation in DA level throughout the season, as well as with the number of negative samples.

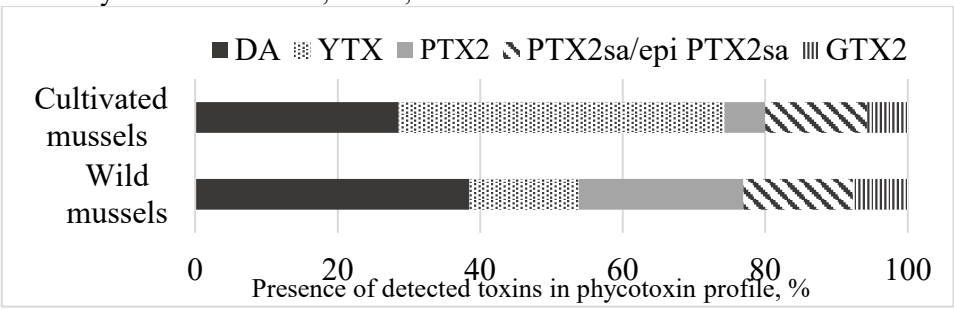

Figure 1. Phycotoxin profile of wild and cultivated mussels

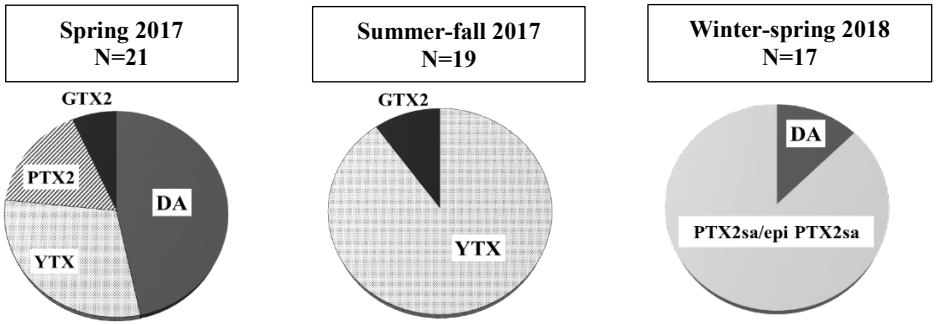

Figure 2. Seasonal phycotoxin profiles of investigated mussel samples

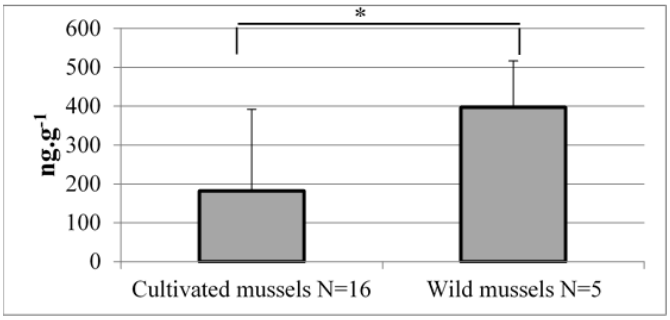

Figure 3. Comparison of domoic acid levels in mussel samples from spring 2017

In summer-fall YTX was the prevalent toxin. Its highest level (14.806 $ng.g^{-1}$ hp) was registered in July 2017. The only toxin that appeared in the samples from two subsequent seasons – spring and summer-fall 2017 was YTX. The range of YTX levels in spring 2017 was much wider than in summer-fall 2017, but the *t-test* showed no statistically significant difference between the mean seasonal levels (Fig. 4) ($p = 0.93 \geq 0.05$). But a large standard deviation of YTX levels was determined, which is due to fluctuations in the YTX level throughout the season and the YTX levels below the LOD. Only results of cultivated mussels were subjected to statistical processing because in these samples more positive results were registered (Table 3).

Interestingly, in the third studied period- winter-spring 2018, a new toxin emerged in the samples - the epimeric pair PTX2sa/ epi-PTX2sa and domoic acid in only one sample. Since there is evidence of PTX2 presence in plankton samples from other investigation seasons [9] and conversion of PTX2 to PTX2sa is well documented in the literature [22-24], it is reasonable to assume that PTX2sa/ epi-PTX2sa also resulted from PTX2 through metabolic conversion in mussels. A comparison of the spatial differences between the phycotoxin profiles was also made (Fig. 5). Although there were much more samples from Sozopol than from Ravda and Primorsko/Tsarevo, sampling was performed throughout the whole investigated period at both locations. Results from Primorsko and Tsarevo were combined due to their close proximity.

It is obvious that phycotoxin diversity is higher in samples from Sozopol. All detected toxins were determined in the samples from this location. The quantities of DA, YTX and PTX2sa/ epi-PTX2sa were similar. In contrast, the phycotoxin profile of samples from Ravda only contained DA and YTX. In samples from Primorsko/Tsarevo predominant were also YTX and DA, but in some samples also PTX2 was registered.

DA and YTX are the phycotoxins that were determined in the samples from the three locations (Fig. 6). Mean DA level in samples from Sozopol was much lower than in the other two locations, whereas mean YTX level was decreasing from north to south. Nevertheless, no statistically significant differences ($p \geq 0.05$) were established between the mean concentrations for both DA and YTX for the three sampling sites.

CONCLUSION

Phycotoxin profiles of investigated mussel samples contained DA, YTX, PTX2, PTX2sa/ epi-PTX2sa and GTX2/3. The temporal profile changed each season, whereas only in Spring 2017 all the toxins were detected in the samples. The spatial profile differs at the sampling locations. The samples from Sozopol contained all the detected toxins. In the current perspective of climate change, any variation in the environmental factors could contribute to a change of the toxin load. Although hereby reported levels are much lower than the legislative limits further surveillance on phycotoxins content in mussels is required in order to protect consumers' health.

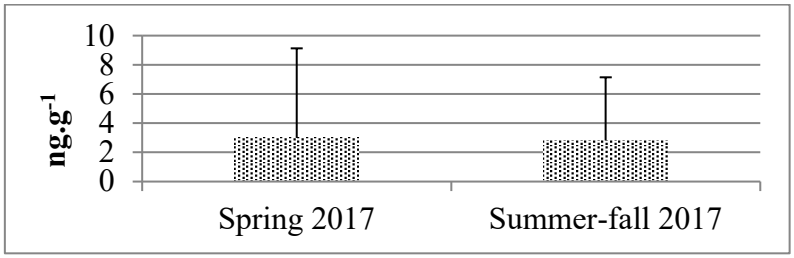

Figure 4. Comparison of mean seasonal YTX levels of cultivated mussels

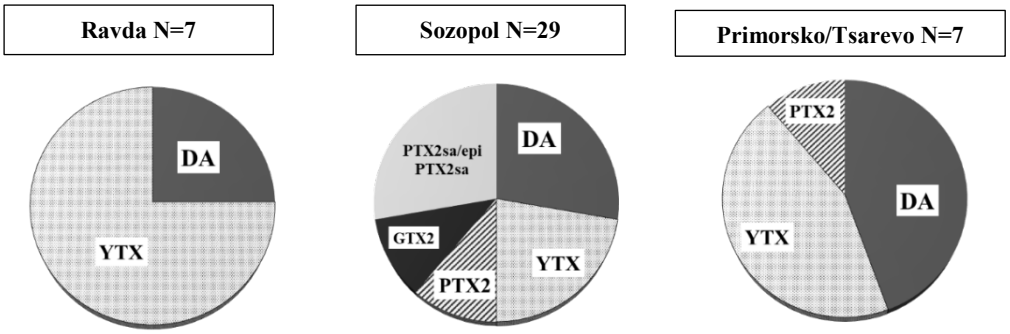

Figure 5. Spatial phycotoxin profiles

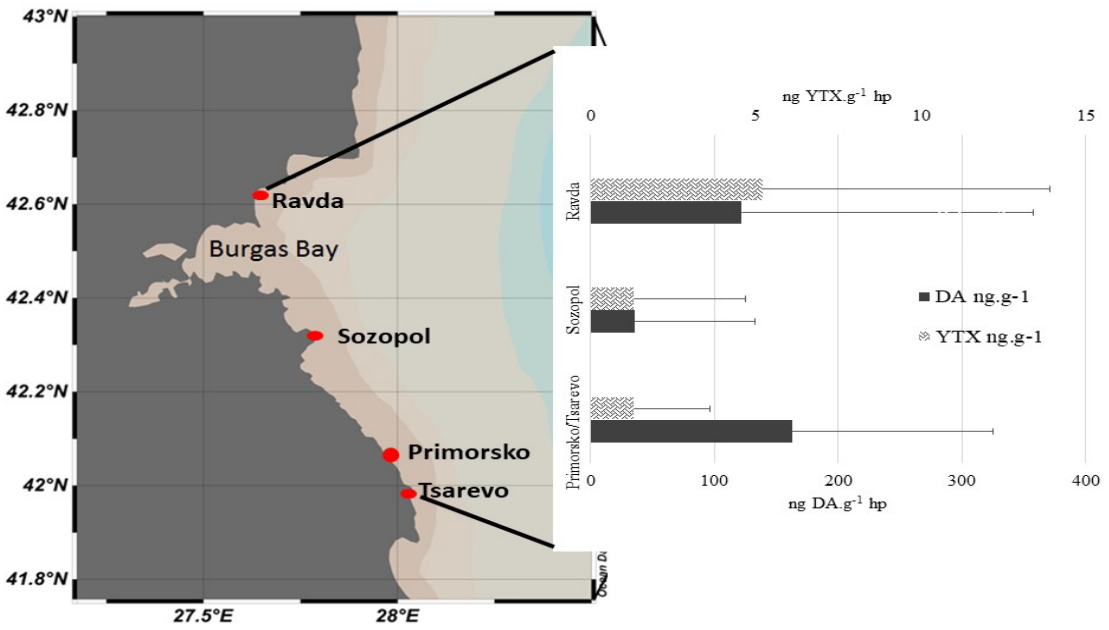

Figure 6. Spatial distribution of DA and YTX

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