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Summer profile of lipophilic toxins in shellfish from the Black Sea, Bulgaria

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Abstract. The presence of phytoplankton responsible for the production of marine biotoxins (phycotoxins) is well recognized globally. Phycotoxins accumulate in filter feeding bivalves and through the food chain find their way to humans. In certain quantities they can cause severe illness. According to the symptoms they cause marine biotoxins are classified as paralytic (*e.g*. saxitoxin), amnesic (*e.g*. domoic acid), which are hydrophilic and diarrheic (*e.g*. okadaic acid) toxins etc. which have lipophilic nature. The aim of this study was to assess the presence of lipophilic toxins in both cultivated and wild mussel (*Mytilus galloprovincialis*) samples, harvested in summer 2017 from the south coast of the Black Sea, Bulgaria. Determination was performed by liquid chromatography coupled to tandem mass spectrometry. Despite of the recent evidence for the presence of a variety of potentially toxigenic producers in the investigated area, only yessotoxins were found in the studied samples. Mean levels of YTX in cultivated mussels were determined as 5832.86 pg YTX/g hepatopancreas (hp) and as 920.42 pg YTX/g hp in wild mussels. In both cases, YTX levels did not exceed the legislative limit of 3.75 mg/kg shellfish meat. These results indicated that the risk through consumption of studied shellfish is low.

Keywords: yessotoxins, mussels, the Black Sea, LC-MS.

1. Introduction

The semi-isolated Black Sea, located between Russia, Georgia, Turkey, Bulgaria, Romania and Ukraine, covers an area of approximately 466,200 km^2 [1] with a maximum water depth of 2245 m [2]. During the last glacial period the sea level was much lower than nowadays, and the Black Sea was separated from the Mediterranean Sea forming an immense lake.

Some unique characteristics of the Black Sea make it interesting for exploring and investigating. Among them are typical freshwater salinity and oxygen and temperature water stratification.

At the Bulgarian coast mean yearly salinity of the upper water layer retains low - 16-18 psu rising from south to north [4]. The characteristically low salinity is a result of positive freshwater budget regulated by an influx of rivers and constant precipitation. Water is exchanged through the narrow and shallow Strait of Bosporus, whereas low-salinity water is exported toward the Mediterranean and denser water flows into from the Sea of Marmara as an undercurrent [3].

Three layers are permanently formed in the Black Sea in depth - sulfidic bottom waters, ~30 m thick suboxic zone and upper ~100 m of oxygenated surface waters. This classifies the Black Sea as the largest anoxic basin in the world [3].

The vertical temperature profile presents a specific stratification in three water layers with original properties. These are the cold intermediate layer (CIL), suboxic layer (SOL) and anoxic layer [1]. Surface water reaches a temperature of 28 °C during summer months [4].

The Black Sea environment is determined by the thin upper layer of marine water (up to 150 m) by forming a unique biological life [5].

All these characteristics makes the Black Sea sensitive to climate-driven environmental changes, and in the same time capable to preserve past continental climate and concurrent hydrologic changes within the underlying sediments [3].

The Black Sea phytoplankton list contains 750 species [6] whereas 544 species are documented for the western part including the Bulgarian shelf. Diatoms (212 species) and dinoflagellates (162 species) constitute to the bulk of the phytoplankton pool [7, 8]. A revision of potentially toxic species found 28 species, where 2 proliferated to blooming densities (*Pseudo-nitzschia delicatissima* and *Prorocentrum cordatum*) [9]. Harmful algal blooms and occurrence of potentially toxic phytoplankton species is important since the Bulgarian coast is a spawning area for mussels (*M. galloprovincialis*). Beds of wild mussels are spread along the entire coastline. Recent research showed a decrease in wild mussel populations because of trawling activities [10]. On the other hand, mussel farming is elevating lately. Both wild mussel catches and cultivated mussel harvests tend to meet the increased consumer demand [11].

To our current knowledge, in Bulgaria there are no reports of human intoxications associated with phycotoxins. No official medical statistics exist on

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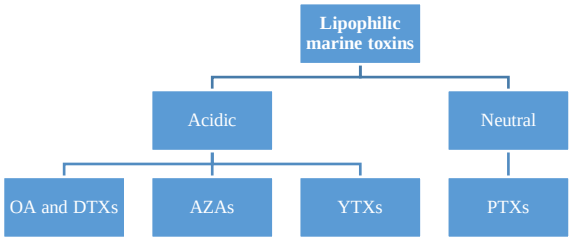
human intoxications caused by HAB toxins. However, recent research on phytoplankton and mussel samples from the Black Sea showed the presence of hydrophilic toxins – domoic acid [12, 13] and paralytic shellfish toxins [14, 15] and lipophilic toxins – yessotoxins and pectenotoxins [13, 16] in the Black Sea. But still data on occurrence of lipophilic toxins along the Bulgarian coast remain insufficient [17].

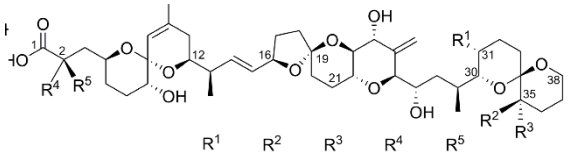
Globally lipophilic toxins are produced by certain species of the genus *Dinophysis* [18], *Prorocentrum* [19], *Lingulodinium* [20] and *Azadinium* [21]. Consumption of seafood contaminated with lipophilic toxins can result in severe gastrointestinal illness [22 - 24]. Therefore, they are mostly known as diarrheic shellfish poisoning (DSP) toxins [25].

Lipophilic toxins can be sorted into two separate groups (Figure 1). The acidic toxins family includes okadaic acid (OA) and its derivatives called dinophysistoxins (DTXs) (the latter are found only in toxic shellfish [24, 26, 27]), azaspiracids (AZAs) [28] [29] and sulphated compounds named yessotoxin and its derivatives (YTXs) and neutral polyether-lactones containing pectenotoxins (PTXs) [30]. YTXs have now been categorized separately because there is no scientific evidence they are diarrhoeagenic [31, 32].

Going out from the unique environment in the Black Sea, a unique toxin profile of the marine organisms is also expected.

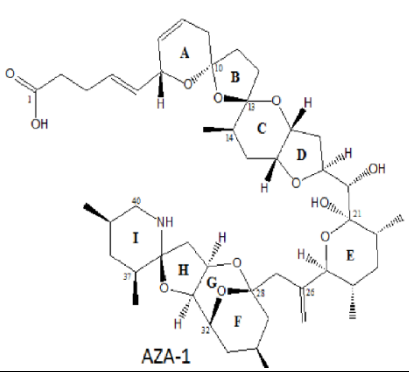


Acidic toxins – OA and DTXs

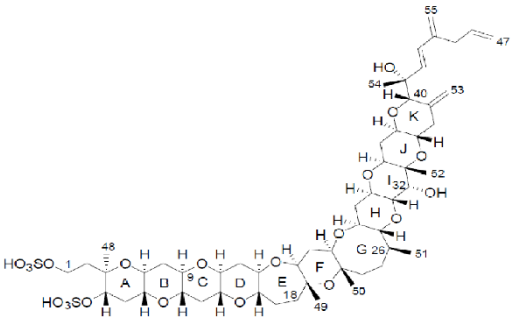


	R^1	R^2	R^3	R^4	R^5
Okadaic acid	CH_3	H	H	CH_3	OH
DTX1	CH_3	CH_3	H	CH_3	OH

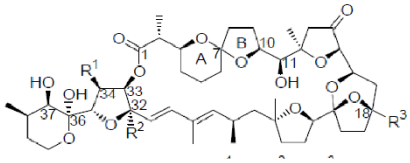
Acidic toxins – AZAs



Acidic toxins – YTXs



Neutral toxins – PTXs



	7	R^1	R^2	R^3
PTX-1	*R*	H	H	CH_2OH
PTX-2	*R*	H	H	CH_3

Figure 1. Classification and chemical structures of lipophilic toxin [33 - 36].

The aim of this study was to assess the presence of lipophilic toxins in both cultivated and wild mussel samples, harvested in summer 2017 from south coast of the Black Sea, Bulgaria.

2. Experimental

2.1. *Study area and sampling methods*

Sampling was conducted in the period June – September 2017. The research included 6 stations situated at major breeding and harvesting areas along the south Bulgarian coast (Fig. 2).

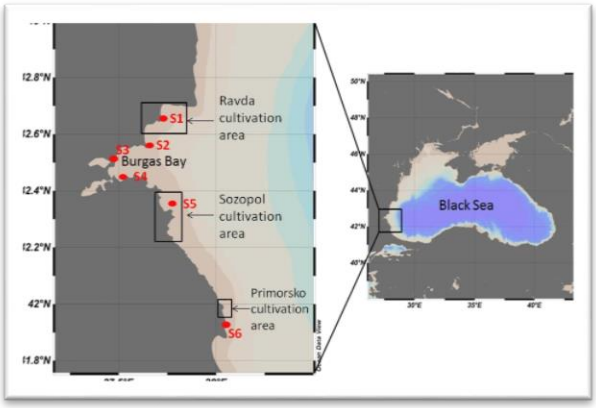


Figure 2. Sampling area

Mussels (*Mytilus galloprovincialis*) were sampled as follows: wild mussel samples (N = 6) were collected at all stations manually; farmed mussel samples (N = 15) were collected directly from ropes. All samples were kept frozen until analyzed.

2.2. *Sample preparation and extraction*

Approximately 1 kg mussels of each sample were dissected to obtain digestive gland tissue (hepatopancreas, hp). Hepatopancreases of each sample were homogenized with dispersing instrument for 5 min at 25.000 rpm at ambient temperature and frozen (-20°C) until further analysis.

Extraction of average 4.02 g hepatopancreas homogenate was performed following the procedure described in Peteva *et al*. [13].

All solvents used were HPLC-grade. Methanol, hexane and water were delivered by Merck, Darmstadt (Germany). Quantitative standards of YTX, DTX-1, OA and PTX-2 were purchased from National Research Council Canada, Marine Analytical Chemistry Standards Program, Halifax, Nova Scotia, Canada.

2.3. *LC-MS/MS analysis of lipophilic toxins*

LC-MS/MS determination of lipophilic toxins (DTX-1, OA, YTX and PTX-2) was performed on, a triple quadrupole mass spectrometer equipped with a TurboSpray® interface (API-4000 QTrap, Sciex, Darmstadt, Germany) coupled to a LC (model 1100, Agilent, Waldbronn, Germany). The LC equipment included a solvent reservoir, in-line degasser (G1379A), binary pump (G1311A), refrigerated autosampler (G1329A/G1330B), and temperature-controlled column oven (G1316A). Basically, a multi-toxin selected reaction monitoring (SRM) method described in Krock *et al*. [37] was followed.

The individual concentration of the analytes (*C*ph) [pg/ul] was quantified according following formula:

$$Cph = \frac{As}{Ac} \text{x } C_c$$

where A_S is the peak area of the sample, A_C is the average area of the calibration standard, *C*c is concentration of the calibration standard. Used standards are listed in Table 1.

Table 1. Concentrations of used calibration standards

Phycotoxin	Standard concentration [pg/µl]
YTX	100
DTX-1	100
OA	100
PTX-2	500

Analyte level (w_{ph}) [pg/g hp] in tissue was calculated using following equation:

$$w_{ph} = \frac{C_{ph} \text{x } V_{ex}}{m_s}$$

where C_{ph} is the individual concentration of the analyte [pg µL]; V_{ex} is the volume of the extract, [µL] (range 1000-1500 µL), m_s - sample weight, [g] (range 3.7 - 4.7 g hp).

Selectivity/specificity was based on retention time (RT) comparison between samples and standard solutions. Confirmation of peaks was assessed by MS/MS fragmentations ratios for each toxin (Table 2).

The limits of detection (LOD) for lipophilic toxins were determined based on a signal-to-noise ratio of 3 (Table 2).

Table 2. Limits of detection (LOD, S/N=3), mass transitions and retention times of investigated phycotoxins

Toxin	LOD	Mass transition *m/z*	RT [min]
	pg/g hp		
YTX	2.02	1160/965	13.46
DTX-1	8.86	836/237	12.57
OA	3.16	822/223	11.85
PTX-2	1.26	876/213	12.24

Robustness of the method was confirmed by analyzing calibration standard solutions and appropriate blanks before and after a set of 10 samples.

3. Results and discussion

Analysis of toxin composition of wild and cultured mussel samples harvested at the south Bulgarian coast in summer 2017 was performed. Despite of the recently published evidence for the presence of a variety of potentially toxigenic phytoplankton species in the investigated area (Table 3), chemical analysis revealed the presence of only YTX above the LOD. Of 21 samples analyzed 14 were positive for YTXs (Table 4).

Although only 2 samples of the wild mussels were positive for YTX it is obvious that YTXs levels in wild mussels were much lower than the lowest YTX level in cultivated mussels (Table 5). This result was consistent with our previous work [13] where YTX were only detected in farmed mussels.

Table 3. Potentially toxin producing phytoplankton genera from the southern Bulgarian Black Sea coast [38, 39]

Toxins	Producer
DSP and PTXs	*Prorocentrum*
	Dinophysis
YTXs	*Protoceratium*
	Lingulodinium
	Gonyaulax

Table 4. Summary of YTXs level in investigated mussel samples

Samples	Number	Positive	Range [pg/g]	Mean [pg/g hp]
cultivated mussels	15	12	2156-24558	5832
wild mussels	6	2	*na*	920
summary	21	14	1596-24559	3376

na - not applicable

Table 5. YTXs level in investigated mussel samples

Cultivated mussels		Wild mussels	
Sample number	YTX level [pg/g hp]	**Sample number**	YTX level [pg/g hp]
1	2355	**1**	1597
2	Nd	**2**	3926
3	Nd	**3**	Nd
4	4418	**4**	Nd
5	6457	**5**	Nd
6	8332	**6**	Nd

Cultivated mussels		Wild mussels	
Sample number	YTX level [pg/g hp]	**Sample number**	YTX level [pg/g hp]
7	24559	**mean**	920
8	5828		
9	4700		
10	5549.2		
11	14806		
12	5018		
13	3315		
14	2156		
15	Nd		
mean	5833		
mean all	3377		

Nd – not detected

The maximum YTX level - 24559 pg/g hp was much lower than detected in reported for mussels from aquaculture farms of the central Adriatic Sea [40] and farms of Sardinia [41]. Detected YTX levels of Bulgarian mussels did not exceed the legislative limit of 3.75 mg/kg shellfish meat (sm). This indicated that the risk for shellfish poisoning through consumption of studied mussels is low.

YTX levels of farmed mussels from the two most frequently sampled sites Ravda and Sozopol showed a sharp decrease from early to mid-May followed by an increase in early June (Fig. 3).

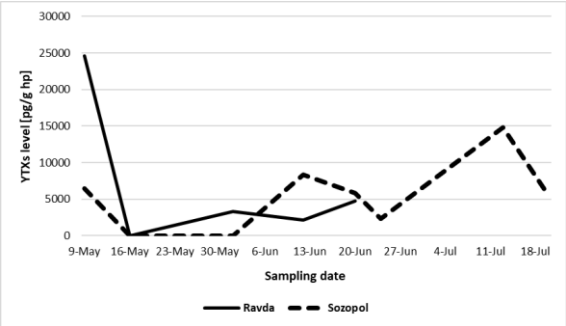


Figure 3. Fluctuations of YTXs levels over time at Ravda and Sozopol.

Although the first measured YTX level in sampling site Ravda (24559 pg/g hp) was about 5-6 times higher than all other levels measured at the same site, the YTX level during the study period remained almost unchanged. This allowed for the calculation of mean concentration, which was 3391 pg/g hp. On the other site of interest – Sozopol, there was one peak – on 13th of July (14806 pg/g hp). This was about 3 times higher than the average – 5516 pg/g hp. Both average values were calculated excluding the peak and the negatives.

In our previous work [13] YTXs were detected in only few samples from the spring of 2017, whereas YTX levels were much lower than reported here. Mean YTX summer levels were approximately 2-3 times higher than the highest in the spring samples. Additionally, these low YTX levels in spring were associated with the depuration phase of mussels, a previous exposure to toxic phytoplankton and specific water temperatures (14-18 °C). So, we could assume that the higher and mostly positive YTX levels in summer could be related to constant exposure to toxic phytoplankton in the whole investigated period and possible accumulation phase of the mussels. Furthermore, the increased water temperature also could be a contributor to the higher toxicity of the farmed mussels [42].

The present YTX values on both sites of interest are in line with IO-BAS investigations on presence of potentially YTX producing phytoplankton species (Table 3). Still this correlation is not sufficient to prove phytoplankton toxicity, which highlight the need for further experiments on phytoplankton toxicity. An interesting aspect of the determination of phytoplankton toxicity is the fact that no other marine toxins are detected in the mussel samples although potentially toxic phytoplankton species were registered (Table 3). This is in agreement with previous work [13] and leads again to the assumption that geographical isolates within the same species have different or no toxin production [43, 44].

4. Conclusions

This is study is a continuation of our previous work [13] and revealed a completely different lipophilic toxin profile of mussels from south Bulgarian coast. The summer toxin profile of mussel samples comprised of only YTXs.

Although the absence of diarrheagenic toxins and of cases exceeding the regulatory limit for YTXs were reported in this study, continuous surveillance for toxin bioaccumulation is necessary in order to predict their harmful effects, prevent human poisoning, and manage the negative consequences for aquaculture operators. Additionally, screening for potentially toxic phytoplankton and testing its toxicity is also needed.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest regarding this article.

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