

Occurrence and Variability of Marine Biotoxins in Mussel (*Mytilus Galloprovincialis*) and in Plankton Samples from Bulgarian Coast in Spring 2017

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Abstract

Shellfish aquaculture has become an increasingly important factor in Bulgarian economy in the recent years. Marine biotoxins, produced by some phytoplankton species, may accumulate in mussels and present an important challenge in commercialization of shellfish.

The aim of this study was to determine the occurrence and variability of hydrophilic toxins – paralytic shellfish poisoning (PSP) and amnesic shellfish poisoning (ASP) as well as lipophilic toxins – including diarrhetic shellfish poisoning (DSP), pectenotoxins (PTXs) and yessotoxins (YTXs) in plankton, wild and farmed mussel samples from the Southern Black Sea coast, an important shellfish farming area in spring 2017. PSP toxins were determined by HPLC with postchromatographic oxidation and fluorescence detection and domoic acid and lipophilic toxins by liquid chromatograph coupled to tandem mass spectrometry.

DA and pectenotoxin-2 were detected in plankton, wild and farmed mussel samples. Yessotoxins were detected only in cultivated mussels and no PSP toxins were detected.

The occurrence of phycotoxins differed over both space and time. Toxin profile includes prevalent domoic acid, scarce presence of pectenotoxin-2 and yessotoxins in the samples.

Keywords : phytoplankton, mussels, domoic acid (DA), pectenotoxin-2 (PTX-2), yessotoxin (YTX), Bulgaria

I. INTRODUCTION

The Mediterranean mussel *Mytilus galloprovincialis* is a popular sea food in Bulgaria [1]. In shops, markets and restaurants cultured and wild mussels are offered. In addition, people harvest mussels on a recreational basis. In these two ways, mussels are reaching the table [2].

Shellfish aquaculture is widespread in Bulgaria along its entire coastline. Along the Bulgarian coast

there are 32 sampling sites for *Mytilus galloprovincialis* farming [2]. To our knowledge there is no register of wild mussels harvesting areas as well as for recreational harvesting.

The production of cultivated mussels in Bulgaria has increased in the recent years and Mediterranean mussel is becoming more popular aquaculture species in Bulgaria with a production of 2520 t in 2014, overtaking traditional species such as rainbow trout (2322 t), carp (2142 t) and silver carp (226,5 t). In 2015 the Bulgarian mussel production reached 3100 t and mussels form over 28% of the total aquaculture production in Bulgaria [1]. Compared to other European countries such as Spain (200000 t), France (80000 t) or Italy (65000 t) [3], it is still a small volume, but already exceeded the production of its neighbouring countries – 20 t in Rumania and 2100 t in Turkey. In the same year the wild catch reached 18,1 t [1].

Despite of its recent growth, mussel aquaculture still has a potential for further expansion within the 375 km of Bulgarian coastline. More places became attractive for recreational catch (Galata on the north coast, Kavatsi and Fishermen villages in the south) (recreational harvesters, oral communication).

Marine biotoxins constitute, at present, the most important challenge for shellfish commercialization as mussels are filter-feeding organisms that tend to accumulate different biological and chemical agents in their tissues [4]. Especially marine biotoxins may pose a risk of food-borne diseases and poisonings representing a serious threat to consumer health ([5], [6], [7]). Approximately 60 000 human intoxications yearly with overall mortality of approximately 1.5% are related to toxins produced by algae (including freshwater cyanotoxins) [8]. In EU countries, according to European Agency for Food Safety (EFSA), molluscs were the source of 3 % of the food-borne outbreaks in 2016 [9].

Marine biotoxins are produced by some phytoplanktonic species (and accordingly also named phycotoxins) can be categorized in two groups according to their solubility: hydrophilic and

lipophilic toxins. Hydrophilic marine toxins include paralytic shellfish poisoning (PSP) and amnesic shellfish poisoning (ASP). Lipophilic toxins include diarrhetic shellfish poisoning (DSP), yessotoxins (YTX), and pectenotoxins (PTX).

Currently there are at least 22 known PSP toxin derivatives that are grouped into three categories: carbamoyl, N-sulphocarbamoyl, and decarbamoyl toxins. Saxitoxin (STX), a carbamoyl toxin, is considered the most potent variant [10]. Symptoms of intoxication with PSP include numbness of the fingers and extremities, tingling, nausea and vomiting; but at higher doses PSP intoxication can result in muscular paralysis and death by respiratory paralysis and cardiovascular shock ([11], [12]).

Domoic acid (DA), causing ASP, is found in a variety of shellfish species. Symptoms of ASP include gastrointestinal effects (nausea, vomiting, diarrhoea or abdominal cramps) and/or neurological signs (confusion, loss of memory, or other serious signs such as seizure or coma) occurring within 24 to 48 h after ingestion, respectively [13].

DSP is caused by okadaic acid and its variants called dinophysistoxins (DTXs). Yessotoxins (YTXs) and pectenotoxins (PTXs) were initially included in the DSP group as they often co-occur in natural microplankton assemblages and in filter-feeding molluscan shellfish species exposed to them and the all lipophilic toxins are extracted together by standard methods. However, now it has been well established that these three toxin groups have different biological effects and that only OA and DTXs are diarrhoeagenic ([3], [14], [15]).

DSP is characterized by symptoms such as diarrhoea, nausea, vomiting, and abdominal pain [16].

Pectenotoxins have been reported to be highly hepatotoxic after intraperitoneal (i.p.) injection to mice [17] and have also attracted attention due to their cytotoxicity against several human cancer cell lines [18].

Symptoms of intoxication caused by YTX in humans are still unknown due to the fact that no human intoxication has been reported to date [19]. YTX is known to induce endoplasmic reticulum stress [20], apoptosis [21], and endocytosis inhibition [22].

Toxin profiles of plankton and shellfish from the Bulgarian Black Sea coast have been very poorly characterised so far. Most research of shellfish toxins has been carried out in the field of cultivated bivalves. Recent studies showed that some samples had toxin (PSP and ASP) values below the respective regulatory limits ([23], [24]).

PSP toxin variants that were reported in Bulgarian shellfish in a study from 2015 comprised saxitoxin (STX), decarbamoyl gonyautoxins 2/3 (dc-GTX 2/3) and traces of B1 with a total PSP toxicity range between 89.3 and 428.7 µg STX.2HCL equivalent/kg [23]. A study from 2017 reported gonyautoxin 2/3 (GTX 2/3) and STX with a total toxicity of 51.1 µg STX.2HCL equivalent/kg [25]. An earlier study

reported DA causing ASP in the range of 0.02 - 0.55 mg/kg in a study [24]. According to the authors, the ASP and PSP results are not representative because farms provided samples for toxicity testing very randomly. Furthermore, lipophilic toxins were not tested because of the lack of equipment [26].

The occurrence of ASP and PSP in Bulgarian shellfish is in line with results of the national seawater monitoring program that confirmed the presence of potentially PSP producing *Alexandrium* spp. and *Gymnodinium* spp., ASP producing *Pseudo-nitzschia* spp., OA/DTXs producing *Prorocentrum* spp., OA/DTXs and PTXs producing *Dinophysis* spp. and YTXs producing *Protoceratium reticulatum*. The presence of these potentially toxicogenic plankton species suggests a high risk of occurrence of marine toxins in Black Sea mussels ([27], [28]).

Data of marine toxins from the other areas of the Black Sea are also scarce. YTX intoxication of mussels from Caucasian Black Sea Coast of the Russian Federation was reported in 2007 [29]. and STX and related derivatives in 2006 [30]. Analyses of mussel and plankton samples from the period 2001-2005 showed the presence of okadaic acid (OA) and the related congener dinophysistoxin-1 (DTX-1) along with pectenotoxins (PTX-2 and PTX-2sa) [31].

A species of *Pseudo-nitzschia* isolated from Sevastopol Bay, Black Sea, was examined for its toxicity. The species was identified as *P. calliantha* and DA was detected in a batch culture throughout the growth cycle of this species [32].

The overall aim of this study was to determine the occurrence and variability of regulated hydrophilic toxins – PSP and ASP as well as lipophilic toxins – DSP, PTXs and YTXs in plankton, wild and farmed mussel samples from the South Black Sea coast in the spring 2017 and to relate the results with environmental parameters – salinity and temperature and water exchange.

Research on occurrence and variability of marine toxins in the Black Sea would be of interest because of some characteristics of the sea that could influence the result.

The Black Sea has a positive freshwater balance as four European rivers flow into it – Danube, Dniester, Dnieper and Southern Bug. The average positive balance of the freshwaters does not lead to a refresh of the seawater due to the flow of salty seawater through the Bosphorus from the Sea of Marmara. But the mean yearly salinity of the upper layer remains low - 16-18 psu that is increasing from south to north [33].

Surface water reaches a temperature of 28 °C in the summer months. In the rest of the year, a huge effect on the seawater temperature has the Cold Intermediate Layer (CIL) formed due to autumn-winter convection. Surface water decreases its temperature to 6 °C. In the summer months (July and August) water is warmed up but the heating does not disperse the CIL, only its upper level is moved in a higher depth. The advection (horizontal flow) of cold water from the north-western

Black Sea in the winter also affects the water temperature on the Bulgarian coast [33].

The Black Sea is the world’s largest marine anoxic basin ([34], [35], [36]) decoupled in oxygenated surface layer and a sulfide-containing deep layer [37].

It is worth to mention that changes of temperature determine the concentration of dissolved oxygen and in this way the availability of phytoplankton and other organisms. The effect of salinity is also of interest as many studies reveal a potential relation between salinity and toxin production of phytoplankton species ([38], [39], [40], [41]) as well as with depuration process in mussels [42]. Therefore, temperature and salinity correlation with toxin production of toxic phytoplankton in both field and culture samples is often discussed ([41], [43], [44], [45], [46], [47])

II. MATERIALS AND METHODS

A. Sampling Location and Procedures

Burgas and Kavatsi Bay are the most important shellfish farming areas in South Bulgaria with an about 10-year tradition. The culturing areas are

situated as follows: nine sites near Sozopol, four sites near Nessebar and one site near Tsarevo (Fig. 1).

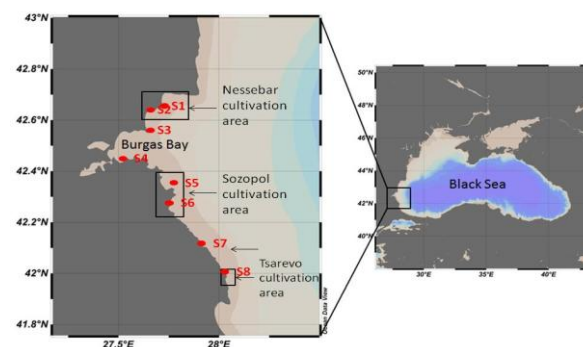


Figure 1. Sampling Sites

The sampling sites are located on the south coast. The selection of the sampling sites was based on different criteria related to the exploitation of the area as aquaculture zone, for wild mussels catch or recreational mussel harvesting (Table I). Additional information about water temperature and salinity of the sampling sites is in the table provided.

Table I. Description of Sampling Sites (nd - no data)

Sampling site	Nearest town	Criteria and conditions				Samples
		Localization of shellfish cultivation farms/other	Fresh water discharge River*	Water temperature °C in 1- 3 m depth in April; May	Salinity [psu] in 1-3 m depth April; May	
S1	Nessebar	Shellfish farm within 5 km/ recreational mussel harvesting area		13.850-13.852; 18.579-18.316	17.066 - 17.066; 17.245-17.275	Plankton Wild mussels
S2	Ravda	Shellfish farm		nd	nd	Cultivated mussels
S3	Pomorie	recreational mussel harvesting area		14.309 – 14.313; 19.721-18.361	16.940 – 16.941; 17.099-17.102	Plankton
S4	Burgas Fishermen village	recreational mussel harvesting area	Aytoska River*	14.819-14.754; 17.982-17.661	16.950-16.949; 17.057-17.066	Wild mussels
S5	Sozopol 1	shellfish farm		14.706- 14.545; 18.735-17.941	16.885-16.890; 17.070-17.075	Cultivated mussels
S6	Sozopol 2 (Kavatsi)	Shellfish farm within 5 km/ recreational mussel harvesting area		nd	nd	Plankton Wild mussels Cultivated mussels
S7	Primorsko	Shellfish farm within 5 km/ recreational mussel harvesting area	Ropotamo River Dyavolska River	13.823 - 13.816; 18.027-17.906	16.985 - 16.984; 17.609-17.608	Plankton Wild mussels
S8	Tsarevo	Shellfish farm within 5 km/ recreational mussel harvesting area	Karaagach River	14.674 - 14.678 17.997-17.854	17.045 - 17.044; 17.398-17.397	Plankton Wild mussels Cultivated mussels (Primorsko)

*data on high contamination due to domestic and industrial discharges [72]

In total 5 plankton samples, 5 wild shellfish samples and 9 cultivated shellfish samples were collected and analyzed. Sampling was performed in the period April-May 2017.

Plankton net samples were collected at 5 stations (Near-shore to Sozopol, Primorsko, Tsarevo, Pomorie and Nessebar) located along the coastline of the Black Sea in spring 2017.

Most harmful algal blooms (HAB) originate away from the shore and, for them to endanger human health, they must be first transported to shore where they can be feed upon by filter feeders [48].

In the southern Black Sea coast environmental parameter (temperature and salinity) that may affect the phycotoxin production remain almost unchanged in depth up to 15 m, as temperature difference with superficial layer (1-3 m) is less than 1 °C and salinity difference ranges 0,5-0,01 psu ([28], Table II).

Therefore, by phytoplankton sampling in 1-3 m depth and location near mussel harvesting were preferred criteria.

Phytoplankton samples were collected vertically from depths between one and three meters from the surface with a conical plankton net (20 µm mesh size, 40 cm outer diameter). Sampling was performed in shallow water in the depth where usually cultivated mussels are gathered (Sozopol) and wild mussels are harvested (Nessebar, Pomorie, Sozopol, Primorsko, Tsarevo) – 1-3 m depth. Additionally, there is evidence that, for example, high DA level was recorded in phytoplankton samples from inshore, shallow sites [49].

At least two net hauls were collected at each station – one for hydrophilic and one for lipophilic toxin analysis. Each net hauls were end up to a volume of 200 mL. Each net tow concentrate was collected on a 20 µm pore size plankton sieve to discard the water. After that the pellet was washed into a centrifugation tube to a volume of 50 mL. Plankton was harvested by centrifugation (4000 x g, 10 min at 10 °C) and kept at -20 °C until chromatographic analysis.

Table II. Mean Values of Temperature and Salinity in Investigated Period

	Mean temperature °C in 1-3 m depth	Mean salinity [psu] in 1-3 m depth
April	14.364- 14.326	18.512-18.015
May	18.507- 18.007	17.246-17.254

Up to 1.5 kg specimens of cultivated and wild *M. galloprovincialis* with length 4-9 cm were collected weekly at the south Bulgarian Black Sea coastline.

All *M. galloprovincialis* mussel samples were sorted by location, washed with clean water, kept in portable bags at 4 °C, and transported to the Laboratory of Marine Resources and Aquacultures, Medical University Varna within 12 h.

Hepatopancreas (digestive glands) of at least 1 kg of specimens (with shells) were dissected, homogenized with dispersing instrument (POLYMIX®PT 1200E, KINEMATIKA AG, Germany) for 5 min at 25.000 rpm at ambient temperature and frozen (-20 °C) until further analysis.

B. Extraction of Toxins from Phytoplankton

Phytoplankton pellets were suspended in 1000 µL methanol for lipophilic toxins or 0.03 M acetic acid for hydrophilic toxins and sonicated (40 Hz,

10 min) in order to release the intracellular toxins. Samples were centrifuged (6000 x g, 10 min at 10 °C) and subsequently a certain volume (average = 1100 µL) of the supernatant was filtered through syringe filters (0.45 µm pore size, ø 25 mm, Minisart, Sartorius, Germany). Filtrates were transferred into autosampler vials and kept at -20 °C until chromatographic analysis.

C. Extraction of Toxins from Mussel Samples

Only digestive gland of both wild and farmed mussels was investigated because marine toxins tend to accumulate there.

4.11 ± 0.09 g of the hepatopancreas homogenate was extracted in with 90% methanol and subsequently twice with 80% methanol. After each addition of methanol, the mixture was homogenized with a dispersing instrument (POLYMIX®PT 1200E, KINEMATIKA AG, Germany) for 5 min at 6000 x g. The extracts were combined and centrifuged for 15 min. After that, the methanolic extracts underwent three times liquid-liquid extraction with hexane by means of homogenization with the same instrument for 2 min. An aliquot of the degreased methanolic extract (average = 1360 µL) was filtered through a syringe filter (0.45 µm pore size, ø 25 mm, Minisart, Sartorius, Germany). The extracts were transferred into autosampler vials and kept frozen at -20 °C until analysis.

D. HPLC-FLD Analysis

The plankton extracts were analysed for PSP by reverse-phase ion-pair liquid chromatography coupled to post-column derivatization and fluorescence detection (LC-FLD) following minor modifications of previously published methods [50]. Eluent A contained 6 mM octanesulfonic acid, 6 mM heptanesulfonic acid and 40 mM ammonium phosphate. Eluent B contained 13 mM octanesulfonic acid and 50 mM phosphoric acid. The flow rate was 1 mL/min with a combination of two subsequent isocratic elution steps. Post-column oxidization was performed by addition of 10 mM periodic acid in 555 mM ammonium hydroxide before the reaction coil (50 °C), after which the eluate was acidified with 0.75 M nitric acid. Derivatized PSP toxins were detected by dual-monochromator fluorescence (λ_{ex} 333 nm; λ_{em} 395 nm). In order to test for false positives caused by interference of auto-fluorescent compounds, PSP extracts were analysed with and without derivatization.

E. LC-MS/MS Analysis

LC-MS/MS determination of lipophilic toxins (AZA, DSP, YTX and PTXs) and domoic acid, in plankton and mussel samples was performed according to [51]. Mass spectral experiments were performed on AB-SCIEX-4000 Q Trap, triple quadrupole mass spectrometer equipped with a TurboSpray® interface coupled to an Agilent model 1100 LC. The LC equipment included a solvent

reservoir, in-line degasser (G1379A), binary pump (G1311A), refrigerated autosampler (G1329A/G1330B), and temperature-controlled column oven (G1316A).

The limits of detection (LOD) for lipophilic toxins and DA were determined based on 3:1 signal-to-noise ratio. LODs for the detection of the lipophilic toxins and DA are given in Table III.

Table III. Limits of Detection (LOD, S/N=3) of Phycotoxins Studied in Bulgarian Black Sea Plankton and Shellfish samples (NH- net haul)

Phycotoxins	LOD ng/NH plankton samples	LOD ng/g hepatopancreas shellfish samples
YTX	0.0022	0.0050
DA	3.9612	8.8000
DTX1	0.0098	0.0217
DTX2	0.0007	0.0015
OA	0.0035	0.0078
PTX-2	0.1109	0.2463

LODs for PSP toxins are given in Table IV.

Table IV. LODs of PSP Toxins Studied in Bulgarian Black Sea Plankton and Shellfish Samples

PSP toxins	LOD ng/NH plankton samples	LOD ng/g hepatopancreas mussel samples
C1/2	21.267	9.573
GTX 4	223.667	100.680
GTX 1	222.200	100.019
dc-GTX2	9.533	4.291
dc-GTX 3	9.533	4.291
GTX 2	12.467	5.612
GTX 5	71.133	32.019
GTX 3	16.867	7.592
Neo STX	156.933	70.641
dc-STX	22.000	9.903
STX	22.000	9.903

F. Calculations

Concentrations of phycotoxins in each mussel sample (C_s , ng/g) were calculated according to the following formula,

$$C_s = \frac{A_s}{A_c} \times \frac{C_c}{w} \times \frac{V_{ex}}{1000}$$

where A_s is the average peak area for the sample, A_c is the average area for the calibration standard, V_{ex} is the volume of the extract, μL (range = 1000-1500 μL), C_c is concentration of calibration standard, $\text{pg}/\mu\text{L}$, w - weigh of the sample, g (range = 3,95 – 4,31 g hepatopancreas (hp)).

Concentrations of phycotoxins in each phytoplankton sample (C_{ph} , ng/NH) are calculated according to the following formula,

$$C_{ph} = \frac{A_s}{A_c} \times \frac{C_c}{D} \times \frac{V_{ex}}{1000}$$

where A_s is the peak area for the sample, A_c is the average area for the calibration standard, C_c is concentration of calibration standard, $\text{pg}/\mu\text{L}$, V_{ex} is the volume of the extract, μL (range 1000-1500 μL), D - depth of sampling, m (range 1-3 m).

III. RESULTS AND DISCUSSION

Analysis of toxin composition of plankton and mussel samples were performed. To our knowledge, this is the first attempt to characterize toxin profiles of plankton samples along the Bulgarian coast. In the following tables all results on concentrations of cultivated (Table V), wild mussels (Table VI) and plankton samples (Table VII) are reported because of the huge variations in the results from different sampling sites and dates of sampling. Mean calculation was only for DA in mussel samples appropriate as here the concentrations are in a narrow range.

A. Toxin Composition of Phytoplankton Samples

A summary of potentially toxic phytoplankton species reported by the Bulgarian Academy of Science-Institute of Oceanology (BAS-IO) along the Bulgarian south coast in the investigated period (April-May) [28] and the toxins they may produce is presented in Table VIII.

It is known that e.g. DSP already can occur even at low cell densities ($<10^3$ cell/L) of *Dinophysis* present in the water [52].

This leads us to hypothesize that toxins responsible for PSP, ASP, DSP, but also PTXs and YTXs could be present in the samples, given that their producers were also found at levels above this threshold (Table VIII). Plankton samples from five different locations (Near-shore to Sozopol, Primorsko, Tsarevo, Pomorie and Nessebar) were analysed for PSP toxins, but did not contain PSP toxins at detectable levels (Table IV). This is consistent with own previous work (unpublished) and a literature review that reported rare and only low PSP toxin levels ([23], [25]).

In addition, plankton samples from the locations mentioned above were analysed for the presence of domoic acid and lipophilic toxins. Results are presented in Table VII.

Relatively high sea temperatures (14–17 °C) tend to be associated with increased abundance of *Pseudo-nitzschia*, respectively increased domoic acid production according to [53]. We could only suppose

Table V. Concentrations of Detected Marine Toxins in Cultivated Mussels (N = number of samples; nd = not detected)

Samples (N=9)	Period	Marine biotoxins detected			Location
		ng DA/g hp	ng YTX/g hp	ng PTX-2/g hp	
F20	April	523.7	2.645	1.840	Kavatsi
F21		618.9	1.839	nd	Ravda
F12		314.0	nd	0.629	Sozopol
F13		275.6	nd	nd	Primorsko
F31		108.3	nd	nd	Sozopol
F22.2	May	466.7	nd	nd	Pomorie
F23		229.9	nd	nd	Sozopol
F24		144.2	0.009	nd	Primorsko
F25		233.1	0.055	nd	Ravda
Mean			209.1		

Table VI. Concentrations of Detected Marine Toxins in Wild Mussels (N = number of samples; nd = not detected)

Samples (N=5)	Date	Marine biotoxins detected		Location
		ng DA/g hp	ng PTX-2/g hp	
F4	April	370.1	122.4	Sozopol
F7		428.2	1.8	Primorsko
F10		247.4	nd	Tsarevo
F11	May	362.5	597.6	Burgas
F19		576.0	nd	Nessebar
mean		396.8	-	

Table VII. Concentrations of Detected Marine Toxins in Plankton (N - number of samples; nd - not detected; NH - net haul)

Samples (N=5)	Date	Marine biotoxins detected		Location
		ng DA/NH	ng PTX-2/NH	
F3	April	222.2	nd	Sozopol
F6		963.0	nd	Primorsko
F9		44.4	0.862	Tsarevo
F15	May	166.7	0.862	Pomorie
F17		150.0	nd	Nessebar

Table VIII. Potentially Toxin Producing Phytoplankton Genera from the Southern Bulgarian Black Sea coast [28] (nd - not detected) [28]

Toxins	Producer	Density cells/L	Density cells/L
		April 2016	May 2016
PSP	<i>Alexandrium</i>	3416	3081
	<i>Gymnodinium</i>	48272	3133
		432115	1499010
ASP	<i>Pseudo-nitzschia</i>	9647	56568
DSP and PTXs	<i>Prorocentrum</i>		
	<i>Dinophysis</i>	481	4362
		5683	5376
YTXs		52	nd
	<i>Protoceratium</i>		
	<i>Lingulodinium</i>	nd	469
	<i>Gonyaulax</i>	6288	3133

the same association as to our knowledge the toxicity of *Pseudo-nitzschia* strains from the Bulgarian coast have never been tested for toxicity.

It is worth to mention that another factor, which has been reported to induce *Pseudo-nitzschia* blooming is rainfall [54], occurred during the sampling season. It events in April 2017 and for 8 rainy days rainfall reached a mean of 40,6 L/m² and in May 2017 for 12 rainy days – 60,9 L/m² [55].

Domoic acid was detected in all plankton extracts from spring 2017 varying from 44.4 to 963,0 ng DA/NH. The highest concentration was observed at Station Primorsko in April, 2017.

LC-MS/MS experiments for the determination of lipophilic toxins (including YTXs, PTX-2, OA, DTX-1 and DTX-2) revealed only the presence of PTX-2 in two of the samples from spring 2017 both with concentration 0.862 ng/NH.

The finding of DA, YTX and PTX-2 is expected as phytoplankton responsible for these toxins is present along the Bulgarian coast of the Black Sea ([28], [56], [57], Table II). Interestingly OA and DTX-1 were not detected although OA, DTX-1 and PTXs are known to be produced by members of the genus *Dinophysis* and may even co-occur within the same species ([58], [59], [60], Table II). The fact that there was no correlation between their distributions may indicate differential production by *Dinophysis* strains or species similar to the study of [61], where geographical isolates of the same species produced different phycotoxins.

B. Toxin Composition of Wild Mussels

Parallel to plankton sampling wild mussel sampling was performed. A new sampling location was added – Burgas. It is known as “the fishermen village”. It is popular for recreational mussel harvesting without using special equipment because of shallow water up to 3 m. Oral interviews with recreational harvesters revealed that it had happened very often that they felt sick after having eaten harvested mussels (not published).

The location Pomorie is missing in the list because our sampling campaign happened to be just after harvesting and no samples were found.

Table VI presents the results of toxin content of wild mussels. Domoic acid was prevalent in the toxin composition detected in all samples. It was ranging from 247 to 576 ng/g hp. The highest DA level was found in a sample from Nessebar (the most northern station). The mean domoic acid abundance in wild mussels in this study was about two times less than in another study from the same period and year but from the Bulgarian north coast (unpublished). A study from the Krka River estuary in the Central Adriatic Sea which is also characterized by low salinity showed scarce domoic acid levels. DA was estimated above LOD in only one sample with concentration 0,2 µg/g out of 32 samples studied [62].

The levels of PTX-2 were much lower and only present at three stations. The highest level was found

at the above-mentioned station Burgas with 597.6 ng/g hp.

C. Toxin Composition of Cultivated Mussels

Cultivated mussels were sampled weekly in the investigated period. The toxin profiles were dominated by domoic acid, which was present in all samples. YTX was detected in four samples and PTX-2 in two samples. Results are presented in Table V.

Domoic acid varied from 108 to 619 ng/g hp whereas the mean value almost equaled the mean domoic acid concentration in wild mussels. A monitoring study from Bizerte Lagoon, Tunisia revealed comparable domoic acid levels of 0.13 µg/g tissue in March 2008 and 0.86 µg/g tissue in November 2009 [63]. Compared to the Bulgarian study cited above [24] our results have similar values.

PTX-2 was detected only at two stations (Sozopol 1 and Sozopol 2) at the beginning of the investigated period.

YTX was only detected in cultivated mussel samples. Four samples were positive with a huge difference in abundance depending on location and time of sampling. Two of the samples were from Ravda from the beginning and from the end of the sampling period whereas the estimated YTX concentration at the end of the sampling period is about 30 times lower than in the beginning. According to [64] YTX depuration rate remained consistent over a 3-month period during which the temperature remained between 13 and 16 °C. This corresponds with our findings that YTX-concentration decreased, no YTXs were detected in plankton samples and mean water temperature in the period in 1 m and 3 m depths is 14.364 – 14.326 °C (April); 18.512 - 18.015 °C (May). So, we could assume that the farmed mussels were exposed to toxic phytoplankton before the sampling campaign and we detected the YTXs at the end of the depuration period.

The sampling sites are situated on the south coast of the Black Sea. Therefore, the investigated area is affected by inflow from the north Black Sea and Mediterranean Sea.

The results – domination of DA and scarce presence of YTXs and PTX-2 come in agreement with other studies from the Black Sea and Mediterranean Sea.

A previous LC-MS/MS study from the Black Sea reported the presence of pectenotoxins (PTX2, PTX2sa PTX1/11, PTX1/11sa, and epiPTX2sa) in hepatopancreas of cultivated mussels (Morton *et al*, 2009). In contrast, the majority of the toxin load of shellfish hepatopancreas harvested from the Caucasian Black Sea Coast of the Russian Federation was shown to be yessotoxin (YTX), 45-hydroxy-yessotoxin (45-OH-YTX), and homoyessotoxin (homoYTX) [31].

An investigation of the toxin composition of Greek mussels showed no presence of DA, YTXs or PTXs [65].

D. Association of the Toxin Content with Environmental Factors – Water Temperature and Salinity

Phytoplankton biomass and production are highly variable both in space and time [66]. The investigated period was about 2 months. The temporal trend in DA (Figure 2) and PTX-2 (Figure 3) concentrations are shown. Results show that there was a decrease in the presence of DA in mussels and plankton in the middle of the investigated period. DA was present and in the range of 108.3 – 571.3 ng/g hp for both wild and cultivated mussel. Highest concentrations were observed in the beginning and in the end of the period. It is known that domoic acid production is decreased at a salinity of less than 20 psu ([66], [67]). However, the decreasing DA levels observed in this study cannot be caused by salinity effects, because salinity remained constant during the investigated period (Table II). Laboratory experiments shown that highest DA production is observed between 13.5 and 18.6 °C [69]. We observed the same water temperature increase (Table II) but fluctuations in DA levels. Therefore, differences of DA concentrations of this study cannot be related to the water temperature.

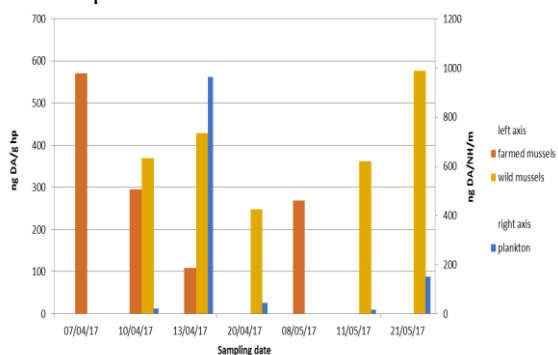


Figure 2. DA levels of Farmed Mussels (N=9), Wild Mussels (N=5) and Plankton (N=5) Samples by Sampling Date (N-number of samples)

PTX-2 production is also reported to increase as water temperature increases [71]. Our results indicate an obvious increase (up to 560 times) in the PTX-2 concentration by the end of the period (Figure 3).

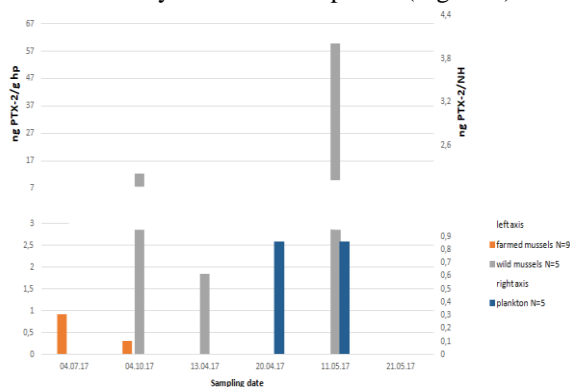


Figure 3. PTX-2 Levels of Farmed Mussels, Wild Mussels and Plankton Samples (N-number of samples) by Sampling Date

This also may be due to the fact that concentrations were measured in the accumulation phase, mussels have not been depurated as certain concentrations are detected in the beginning of the investigation and additionally PTX-2 was also found in plankton samples. Investigations on the PTX-2 concentrations in a prolonged period will be helpful to emphasize this hypothesis.

E. Association of the Toxin content with Water Exchange

As the sampling was performed in a very short period of time (2 months) a spatial distribution of marine biotoxins cannot be representative. Overall, two distinct patterns of distribution can be observed (Figure 4). First, the southernmost (Tsarevo and Primorsko) and northernmost (Nessebar/Ravda and Pomorie) sampling locations showed a similar trend of marine biotoxin contamination – prevalent DA presence. The second pattern includes sampling sites located in Burgas Bay (Burgas and Sozopol/Kavatsi) – presence of both DA and PTX-2. The obvious difference in the concentration of registered phycotoxins in Burgas Bay can be due to the fact that sampling site Burgas is situated inside the bay and Sozopol/Kavatsi on an outlying part of the bay. The presence in YTX in all sampling locations was negligible.

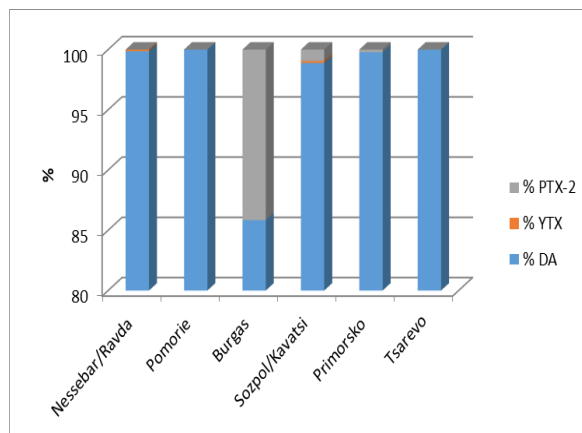


Figure 4. Spatial Distribution of Toxin Profile (N = 14)

Toxin-forming organisms are known to occur periodically, and the toxins are prone to accumulation in shellfish. Seasonal variations in the presence and levels of microalgae toxins in the phytoplankton and shellfish are strongly related [71].

The spatial correlation of domoic acid concentration in wild mussel and plankton samples is presented in Figure 5. The graphical presentation is based on measurements of plankton and mussel samples harvested on the same day. In the location Sozopol plankton, wild and cultivated mussels were sampled. In Burgas only wild mussels are sampled and in all other locations plankton and wild mussels were sampled.

An overall decrease in DA concentrations in both plankton and mussel samples is observed from north to south. Everywhere DA is registered in plankton samples was also detected in mussel samples. Same tendency is followed - highest values were registered in the most northern station and a slight decrease in the next to the last most southern station.

The phytoplankton dynamics in coastal systems is very complex, particularly in the areas affected by freshwater discharge from the rivers [54]. The preference of *Pseudo-nitzschia* species for enclosed water bodies with nutrient loading is reported [73] and a hypothesis that ASP events tend to be more frequent in bays strongly influenced by riverine inputs has been developed [74].

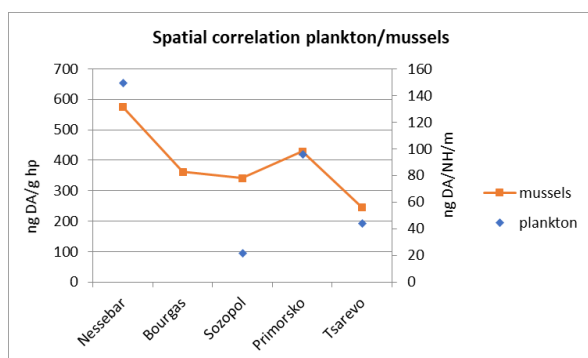


Figure 5. Spatial Distribution Plankton/Mussel Samples

The small rivers Hadjiska ($0.64 \text{ m}^3/\text{s}$), Aytoska ($0.57 \text{ m}^3/\text{s}$), Ropotamo ($1.128 \text{ m}^3/\text{s}$), Dyavolska (no data on annual runoff) and Karaagach (no data on annual runoff) flow near the sampling locations Nessebar, Burgas, Primorsko and Tsarevo (Table I) [75]. Respectively DA is detected, which is consistent with a study from the estuary of Krka river [62]. The fact that some of the rivers are affected by industrial and domestic discharges might also have influence on domoic acid production by the phytoplankton and accumulation in mussels.

The linkage of DA production and accumulation to fresh and seawater input suggest that the control of toxin production is complex and likely influenced by a suite of environmental and anthropogenic factors that may be unique to a particular region during the sampling period.

IV. CONCLUSION

Although the latitudinal span of the coastal zone in Bulgaria is relatively short, the occurrence of phycotoxins differed over both space and time. The study was conducted in spring 2017 and domoic acid, PTX-2 and YTXs were detected in plankton net, wild and farmed mussel samples. Although the lag between the seasonal conditions and outbreaks of shellfish toxin poisoning is compatible with presence of same toxins in plankton samples, predicting when and

where shellfish will be contaminated remains difficult. It is planned the study to be extended by the end of the year and hydrophilic and lipophilic toxins to be monitored.

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