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DETECTED MARINE TOXINS IN DIFFERENT TROPHIC LEVELS – STUDY FROM BULGARIAN SOUTH COAST OF THE BLACK SEA

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Abstract: Marine toxins originate in unicellular algae. About 2% (60-80 species) of the estimated 3400-4000 known phytoplankton taxa are toxic. In response to favorable conditions in their environment, toxic microalgae (dinoflagellates and diatoms) may proliferate and/or aggregate to form dense concentrations of cells called "harmful algal blooms" (HABs). Such cases, but also low cell concentration of toxic phytoplankton species may result in contamination of the whole food chain. Filter-feeding shellfish, zooplankton, and herbivorous fishes ingest these algae and act as vectors to humans either. The aim of this study was to summarize and review the marine toxin content along the Black Sea food chain levels (phytoplankton and mussels), to discuss the metabolic changes they may undergo as they move to higher trophic level and the potential impact to the apex consumer – the human. Plankton and mussels were sampled in the period 2016-2018 on the South Black Sea coast of Bulgaria. Lipophilic toxins and domoic acid were detected on LC-MS/MS and saxitoxins and its analogues on HPLC-Fl. Domoic acid (DA) was the toxin with the highest detected concentrations: in plankton - 962963.0 pg/net haul and in mussels – 618859.8 pg/g. In addition, yessotoxins (YTX), pectenotoxin-2 (PTX2) and gonyautoxin-2 (GTX2) were also found in the samples. Despite of the low toxin concentrations detected, distribution and mobility of toxin producing algae in harvest waters, toxin accumulation potential of mussels, etc. need to be considered to protect human from contamination.

INTRODUCTION

The Black Sea has unique ecological characteristics, such as positive freshwater balance, presence of the toxic hydrogen sulfide in the deep waters, etc. provide a unique environment (Golemansky, 2007, Trayanov, 2014). The Black Sea ecosystem provides many species as a matter of commercialization -fishes, mussels, rapana etc. (Ministry of Agriculture and Food, 2016) due to their nutritional quality (Dobreva, *et*

al., 2018; Merdzhanova, *et al.,* 2016; Stancheva, *et al.,* 2012). Nevertheless, marine food webs are strongly influenced by the accumulation of hazardous compounds (Islam & Tanaka, 2004). Contaminants with anthropogenic origin (e.g. pesticides, oils, heavy metals, etc.) from the Bulgarian coast of the Black Sea have been studied in the last ten years in details. Organochlorine pesticides (such as 1,1,1-trichloro- 2,2 - bis (4-chlorophenyl) ethane (DDT) and its metabolites) and polychlorinated biphenyls (PCBs) were detected in fish and mussel species in concentrations, comparable to those found in other marine ecosystems (Peteva, *et al.,* 2018a, Stancheva, *et al.,* 2017; Georgieva, *et al.,* 2012). Likewise, heavy metals such as Cu, Zn, Pb, As etc. were detected in selected fish and shellfish species (Makedonski, *et al.,* 2017; Stancheva, *et al.,* 2012).

Other harmful substances, such as marine toxins have biogenic origin (Gregg, *et al.,* 2018; Fabro, *et al.,* 2017). Primary marine toxins producers are phytoplankton species, mainly diatoms and dinoflagellates (Van Dolah, 2000) that are also detected in European coastal waters (Table 1). About 2% (60-80 species) of the estimated 3400-4000 known phytoplankton taxa are toxic (Van Dolah, 2000). Constantly, the list of harmful species on a world scale is growing rapidly. A sudden increase in the microalgae population due to suitable growth and physical conditions and adaptive strategies in considered as harmful algal bloom (HAB) (originally called "red tide") (Maso & Garces, 2006). The phytoplankton concentration can reach 10^4-10^5 cell dm^{-3} during certain periods of time. Nevertheless, even low microalgae concentrations may result in toxic events, e.g. 10^2–10^4 *Dinophysis* cell dm^{-3} concentration on the Galician coast was associated with shellfish poisoning episode (Reguera, *et al.,* 1993).

Table 1. Main marine toxins and their known producers

Marine toxins	Producers	Occurrence in Europe
Saxitoxin (STX) and analogues	Alexandrium spp. Gymnodinium sp.	The Black Sea (BAS-IO, 2017) Mediterranean Sea (Fertouna-Bellakhal, et al., 2015; Laabir, et al., 2013;) Baltic Sea (Salgado, et al., 2015)
Domoic acid (DA)	Pseudo-nitzschia spp. Nitzschia spp.	The Black Sea (BAS-IO, 2017) Mediterranean Sea (Pugliese, et al., 2017 Marmara Sea (Balkis, et al., 2016)
Okadaic acid (OA)	Prorocentrum lima (Ehrenberg) Stein 1878 Dinophysis spp.	The Black Sea (BAS-IO, 2017) Mediterranean Sea (Ben-Gharbia, et al., 2016) Baltic Sea (Hällfors, et al., 2011)
Pectenotoxins (PTXs)	Dinophysis spp.	The Black Sea (BAS-IO, 2017) Baltic Sea (Hällfors, et al., 2011)
Yessotoxins (YTXs)	Prorocentrum reticulatum Faust 1997 Lingulodinium polyedrum (Stein) Dodge 1989 Gonyaulax spinifera (Claparède & Lachmann) Diesing 1866	The Black Sea (BAS-IO, 2017) Mediterranean Sea (Rubino, et al., 2010)

Marine toxins can be transferred via several trophic pathways. First orders consumers that accumulate toxins are bivalves (Reizopoulou, *et al.,* 2008; Vale & Sampayo, 2002), polychaetas (Abbott, *et al.,* 2003) and ascidians (Reizopoulou, *et al.,* 2008), planktivorous fishes (Vale & Sampayo, 2001), crabs (Vale & Sampayo, 2002) etc. Contamination of higher-order consumers include octopuses (Lopes, *et al.,* 2018), seals (Jansen, e*t al.,* 2015), dolphins (Schwacke, *et al.,* 2010), whales (Doucette, *et al.,* 2006) and even cases of endangered species mortalities, e.g. shortnose sturgeon (*Acipenser brevirostrum* Lesueur*,* 1818) (Fire, *et al.,* 2012). Marine toxins are known to have long-term effects on health of aquatic animals, involving increased susceptibility to diseases, immunosuppression, abnormal developments, development of tumors (Vasconcelos, *et al.*, 2010; Landsberg, 2002) etc.

Consumption of toxin-contaminated seafood results in poisoning syndrome in human. Among these are amnesic, diarrheic and paralytic shellfish poisoning, caused respectively by domoic acid, okadaic acid, saxitoxins and their analogues.

Therefore, the fate of the marine toxins in marine food webs is essential for the evaluation of their associated risks. The mussel *Mytilus galloprovincialis* was proven to be an appropriate indicator for a safe warning of marine toxins contamination (Reizopoulou, *et al.,* 2008).

The aim of this study was to summarize and review the marine toxin content along the Black Sea food chain levels (phytoplankton and mussels), to discuss the metabolic changes they may undergo as they move to higher trophic level and the potential impact to the apex consumer – the human.

MATERIALS AND METHODS

Collection sites and samples

Sampling was conducted along the south Bulgarian Black Sea coast (Nessebar – Tsarevo) in autumn 2016 (self-funding) and in spring, summer and autumn of 2017 and in spring of 2018 within the framework (work package 2) of Project № M09/1, 48/05.12.2016, funded by National Science Fund, Bulgaria. Phytoplankton was sampled with 20 μm mesh size plankton net in depths 1 to 3 m. Wild mussels *Mytilus galloprovincialis* were harvested from rocks. Farmed mussels were sampled from cultivation ropes.

Analytical methods for phycotoxins determination

Paralytic toxins were extracted from plankton samples with 0.03 M acetic acid, domoic acid and lipophilic toxins with methanol. Mussel sample homogenate was processed with 0.2 M acetic acid for paralytic toxins and with methanol and subsequently with hexane for domoic acid and lipophilic toxins. Detailed extraction procedures are provided by Peteva, *et al.,* 2018c.

Paralytic toxins were determined via liquid chromatography with fluorescence

detection and domoic acid and lipophilic toxins via liquid chromatography tandem mass spectrometry according respectively Krock, *et al.,* 2007 and Krock, *et al.,* 2008.

RESULTS AND DISCUSSION

In total 16 plankton and 24 mussel samples were investigated for the presence of paralytic toxins (Table 2) and 26 plankton and 58 mussel samples for domoic acid, okadaic acid, dinophysis toxins, yessotoxins, and pectenotoxins (Table 3).

Among plankton samples studied only PTX2 and DA were detected. Seven were the positives for both toxins out of 26 samples investigated (**Figure 1**). No paralytic toxins, YTXs, OA and DTXs were detected (Table 2 and Table 3), although phytoplankton potentially responsible for these toxins production was registered in 2017 in the investigated area by the Institute of Oceanology (BAS-IO, 2017) and e.g. YTXs were detected in the mussel samples (Peteva, *et al.,* 2018b, 2018e).

GTX2, DA, PTX2 and YTX were detected in the studied mussel samples. DA and YTX were the most prevalent toxins, but both were present in less than 50% of the samples (Figure 1). The presence of the paralytic toxin, gonyautoxin - 2 (GTX2) in mussel samples was scarce, with only 3 positives in 2017 (Peteva, *et al.,* 2018d) (Table 2) and of PTX2 in mussel samples being available only in spring 2017 (Peteva, *et al.,* 2018a) (Table 3) (**Figure 1**).

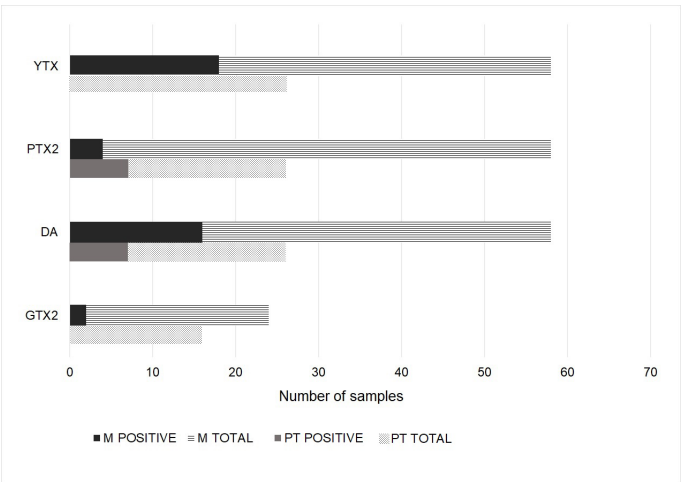

Fig. 1. Number of positive samples among all samples investigated (YTX- Yessotoxin; PTX2- Pectenotxin-2; DA-domoic acid; GTX2- Gonyautoxin-2) .

Table 2 Samples investigated and level ranges of detected GTX2 via LC-FD phycotoxins (M- mussels, PT-plankton, NH- net haul)

Period of sampling	Type of sample	Number of samples	GTX2 PT[ng/NH] M[ng/g]
spring 2016	PT	*not sampled*	
	M	2	nd
spring 2017	PT	5	nd
	M	7	3,7
summer 2017	PT	3	nd
	M	6	5.3-5.5
autumn 2017	PT	*not sampled*	
	M	4	nd
spring 2018	PT	8	nd
	M	5	nd

Table 3 Samples investigated and level ranges of detected DA, PTX2 and YTX via LC-MS/MS phycotoxins (M- mussels, PT-plankton, NH- net haul)

Period of sampling	Type of sample	Number of samples	DA PT[pg/NH] M[pg/g]	PTX2 PT[pg/NH] M[pg/g]	YTX PT[pg/NH] M[pg/g]
spring 2016	PT	*not sampled*			
	M	2	nd	nd	nd
spring 2017	PT	5	44444.4 - 962963.0	nd - 862.1	nd
	M	22	108270.8 - 618859.8	628.8 - 59785.5	9.5 - 24558.8
summer 2017	PT	3	nd	19962.5 - 45953.9	nd
	M	15	nd	nd	1596.5 - 14805.6
autumn 2017	PT	*not sampled*			
	M	4	nd	nd	nd
spring 2018	PT	18	nd - 1265.1	3028.1- 7562.8	nd
	M	15	nd	nd	nd

In plankton samples DA level reached a peak of 962963.0 pg/NH in spring 2017 whereas the detected DA level in spring 2018 was significantly lower (Table 3). For a similar period of investigation in 2011-2012 (Dursun, *et al.,* 2017) also registered DA variation in the Sea of Marmara, but to a lower extend. Respectively, DA was detected in mussel samples only during spring 2017, which event was already described by Peteva (Peteva, *et al.,* 2018b)

Interestingly, PTX2 was registered in plankton samples in all investigated seasons but detected in few mussel samples only in spring 2017 (Table 3, Figure 1). Similar results were reported by MacKenzie (MacKenzie, *et al.,* 2002). It the latter study a predominance of PTX-SA (seco acid) within the mussel tissue was registered. The authors suggested a rapid hydrolysis of the lactone bridge of PTX2 within the shellfish tissue. The transformation of PTX2 to PTX-SA might be an explanation for the absence of PTX2 in hereby studied mussel samples. Therefore, further investigations on PTX-SA in collected mussel samples is planned.

YTX level varied widely in mussel samples from 2017 but reached its maximum level (24558.8 pg/g) in May 2017, remained almost constant in summer and was not detected in spring 2018 (*not published*) (Table 3). The YTX presence in the mussels in spring and summer 2017, but not plankton was probably a residue from a previous YTX contamination event.

The dominance of DA and YTX in the mussel samples (**Figure 1**) could be explained by the ubiquitous distribution of potentially toxin producing phytoplankton species, resp. *Pseudo-nitzschia* sp. and *Protoceratium* sp., Lingulodinium sp. and Gonyaulax sp. These data indicated that the proliferation of several toxin-producing species is favored by the same environmental conditions and suggest a possible direct mutualistic/eco-physiological interaction between these species (MacKenzie, *et al.,* 2002). The involvement of the planktonic dinoflagellate *L. polyedra* was also suspected to be a source of YTX and homo-YTX in contaminated shellfish from the Adriatic Sea though this species was not present in the detected bloom (Draisci, *et al.,* 1999).

Marine toxins determination in the samples investigated concluded in different toxin profiles of the producer microalgae and the filter-feeding bivalves (**Figure 2**).

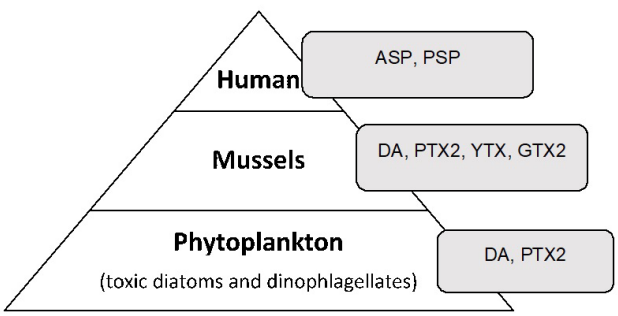

Fig. 2. Possible marine toxins trophic transfer

Chemical properties of the toxins, like epimerization and acid hydrolysis during extraction can explain some of these differences (Shimizu, 2000). Even more, plankton sampling reflects the instant situation, whereas mussel accumulate toxins for longer periods (Cembella, *et al.,* 2001).

If toxin contaminated mussels are ingested by human, severe illness can occur. Threshold levels of phycotoxins are legislated in the European Union. Concentrations in mussels above them are considered not safe for consumption. Table 4 provides information on the highest detected levels in mussel samples. DA, PTX2 and GTX2 highest detected level were beneath the legislative limit, hence, no intoxication by consumption of investigated shellfish might be expected.

Table 4 Comparison of highest level of detected phycotoxins and EU reference limits

Detected toxins	Shellfish poisoning syndrome	Reference EU limit	Highest level detected	Risk of intoxication
DA	Amnesic shellfish poisoning	20 mg/kg	0.62 mg/kg	not expected
YTXs	No human intoxications registered	3.75 mg/kg	0.02 mg/kg	not expected
PTX2	Diarrheic shellfish poisoning	160 µg OA eq/kg	59.79 µg OA eq/kg	not expected
GTX2	Paralytic shellfish poisoning	800 µg STX eq/kg	2.07 µg STX eq/kg	not expected

CONCLUSION

This study reviewed the marine toxin analysis of plankton and wild and farmed mussel samples, harvested along south Black Sea coast. DA and PTX2 were detected in plankton and DA, PTX2, YTXs and GTX2 – in mussel samples. Toxin levels differed significantly in the investigated period. Different toxin profile of producers and first order consumers might be explained by metabolic interactions, timespan of analysis etc. DA, PTX2 and GTX2 highest detected levels were much lower than the EU legislative limit. No potential risk of shellfish intoxication for second order consumers (human) was concluded.

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CONFLICT OF INTEREST DECLARATION

The authors declare that there is no conflict of interests regarding the publication of this article.

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