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PHYCOTOXIN PROFILE OF PLANKTON NET AND SHELLFISH SAMPLES FROM BULGARIAN BLACK SEA SOUTH COAST: A CASE STUDY

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Abstract

Photosynthetic microorganisms play an important role in aquatic ecosystems. Among them about 300 are involved in harmful events by producing potent and persistent natural toxins (phycotoxins) that can be harmful or even lethal to humans and animals. These chemically diverse compounds synthesized by toxic phytoplankton species have been associated with different syndromes in humans put together in diagnosis shellfish poisoning. As shellfish are filter feeding they can be a possible vector of phycotoxins. Therefore, in EU there are legislative levels of phycotoxins in shellfish above that seafood is not safe for consumption. The dynamics and toxicity of harmful phytoplankton species is unpredictable and variable, influenced by complex of factors. For this reason, an instantaneous phycotoxins determination can be representative when monitoring in not possible. The aim of this research was to study the case of occurrence and concentration of phycotoxins in plankton and mussel samples in a relatively short area on the Bulgarian Black Sea South coast. Sampling was performed during a two-day field trip in June 2017. Phycotoxins determination was performed with liquid chromatography coupled to mass spectrometry (LC-MS). In total plankton, wild and farmed mussel samples were analyzed. Pectenotoxin-2 (PTX-2) was detected in only plankton samples and yessotoxin (YTX) only in mussel samples. As plankton and mussel sampling was performed in same locations, results indicate that PTX-2 accumulation can be expected in mussel samples in perspective. Detection of YTX in only mussel samples demonstrates mussels are in depuration phase. The research provides an evidence for presence YTX and PTX-2 producing phytoplankton species in the investigated area. Determination and isolation of these species would be valuable to better understand and manage the impact of potential harmful algal blooms and in order to prevent human intoxication and losses to aquaculture.

Keywords: toxic phytoplankton species, mussels, LC-MS, PTX-2, YTX

1. INTRODUCTION

Mussels *M. Galloprovincialis* are sedentary, long-lived, and easily identifiable and sampled organisms. They are fairly abundant and available throughout the year, reasonably tolerant to environmental change and pollution, and they have good net accumulation capacities (Stankovic, et al., 2011).

Mussels (*Mytilus galloprovincialis*) are the only bivalves cultured on the Bulgarian coast of the Black Sea (Ministry of Agriculture and Food 2016). They are mainly cultured on ropes suspended in the water column and attached to rafts. Aquacultured mussels are usually suspended in 3-4 m above the sea bottom (Excecutive agancy for fish and aquacultures (IARA) 2017). Mussel production is based on an extensive culture and depends entirely on spat, food and space availability (Smaal 2002).

Some researches provide evidence that there is a difference in metabolism of wild and cultivated mussels (Schmidt, et al., 2012). This is reasonable as their natural habitats are usually close to estuaries and they are exposed to contaminants from land-based activities (Jovic 2011) as the farmed mussels are mostly cultured in protected areas (Otero, et al., 2013). Shellfish feed by straining suspended algae and food particles from large volumes of water through their bodies and through the gills, where tiny food particles

are caught in the gill and assimilated into nutrition by the organism. Hence they have the potential to concentrate both bacterial pathogens and phycotoxins at dangerous levels (Huss 1997). In all cases, the toxins are *de novo* produced by certain photo- or mixotrophic microalgae not by the shellfish (Landsberg 2002; Lewitus, et al., 2012; Scheuer 1996;Wright 1995).

Phycotoxins are potent marine toxins, responsible for human intoxications. Depending on the involved toxins symptoms range from nausea over paralysis and amnesia to death, include gastrointestinal disorders, muscular paralysis, memory loss etc. (García 2016; Pulido 2016). Consequently, commercial shellfish harvesting (fisheries or aquaculture) are subject to extensive monitoring of in situ concentrations of causative algae and/or toxicity of harvested shellfish.

Therefore, in EU there are legislative levels of phycotoxins in shellfish above that seafood is not safe for consumption (European comission (EC) 2013; EC 2004). By now in investigated mussels from Bulgarian farms either no phycotoxins are detected (Peteva, et al., In press) or the detected concentrations are much lower than the allowed threshold (Krumova-Valcheva and Kalinova, 2017; Peneva 2010). Among phycotoxin producing phytoplankton species, on the |Bulgarian coast are detected potentially toxic microalgae. Table 1 summarizes the detected potentially toxic microalgae from the south Bulgarian coast (Bulgarian academy of Science – Institute of Oceanlology (BAS-IO) and Slabakova, 2016), the possible toxins they produce (Luckas, et al., 2005), the symptoms of intoxication (Pulido 2016) and the legislative norm of phycotoxins concentration (EC 2013; EC 2004).

Table 1. Potentially toxic marine microalgae and possible phycotoxins they produce

Potentially toxic marine microalgae	**Possible phycotoxins they produce**	**Legislative norm in shellfish meat**	**Symptoms of intoxication**
Pseudonitzschia sp.	Domoic acid (DA)	20 mg/kg (EC 2004)	Nausea, vomiting, diarrhoea, abdominal cramps
Dynophysis sp. ***Prorocentrum sp.***	Okadaic acid (OA); dinophysistoxins (DTX) etc. Pectenotoxins (PTXs)	160µg OA eq/kg (EC 2004) 160µg PTX-2 eq/kg (EC 2004)	Diarrhea, nausea, vomiting, abdominal pain
Gonyaulax sp. ***Lingulodinium polyedrum***	Yessotoxins (YTXs)	3,75 mg/kg (EC 2013)	Considered not to pose any health risk; induce endoplasmic reticulum stress (Rubiolo, et al. 2014), apoptosis (Kornes 2012), and endocytosis inhibition (Callegari and Rossini 2008)

The dynamics and toxicity of harmful phytoplankton species is unpredictable and variable, influenced by complex of factors (Yasakova 2013). For this reason, an instantaneous phycotoxins determination (over short period of time) can be representative when monitoring in not possible. The aim of this research was to study the case of occurrence and concentration of phycotoxins in plankton and mussel samples in a relatively short area on the Bulgarian South coast during a two-day field trip.

2. METHODS

In this research a case study design (Suman 2014) (Razlog, et al., 2016) (Bermejo, et al., 2018) was used. A 2-day field trip along relatively short area (70 km coastline, 5 sampling stations) was taken. Plankton and wild mussels were sampled, cultivated mussels were provided from mussel farms from the same area (3 sampling stations). The case analysis evaluated phycotoxin profile of the samples with only spatial distribution as variable. Fluctuations in time can be neglected as the investigated period is only 2 days. These results could indicate the instantaneous phycotoxin profile as water quality parameters (oxygen content, temperature, salinity) affecting the toxin production (Basti, et al., 2015) (Alvarez, et al., 2011) of the Black Sea coast the are variable in time (Pavel, et al., 2012) especially in the summer time because of anthropogenic influence are strongly negatively affected (Negreanu-Pirjol, et al., 2014)

2.1. Sample collection and site description

Field samples were collected on in June 2017 from 5 locations (Figure 1) . Sampling sites, located at the South Bulgarian Black Sea coast, were selected after a detailed study of their particular characteristics. Among them: anthropogenic inputs, availability of wild mussels, punctual pollution sources (presence of industrial areas, large cities, etc.), upwelling, farming activities, urban discharges, etc. The studied area extends for more than 70 km, from 42°39'40'' to 42°24'13'' (coordinates) . The seaborne-related activities there hold main economical relevance, which justifies the continuous monitoring of the pollution of the coastal water and marine organism intended for human consumption.

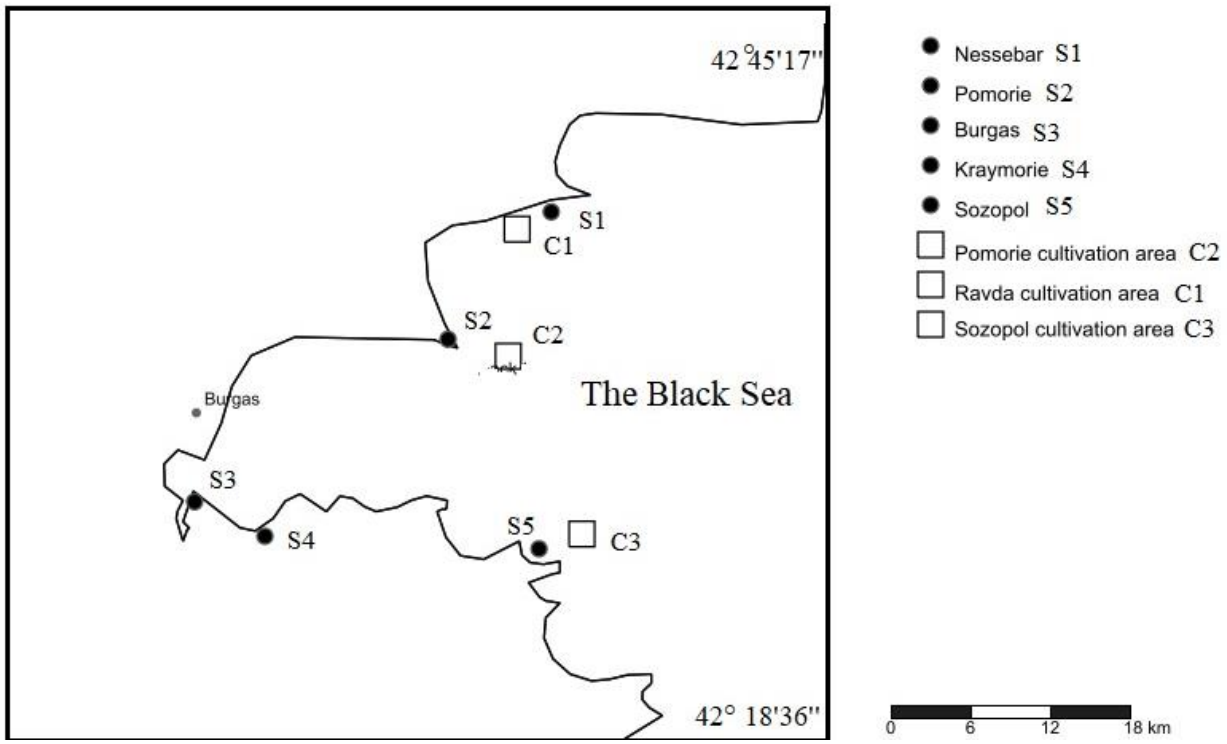

Figure 1. Map of sampling area

Concentrated water samples were taken with a 20µm mesh size plankton net from shallow (3 m depth) (Figure 2a), well mixed system within freshwater discharge from 3 locations (S1, S2, S5). Each net haul was end up to a volume of 200 mL(Figure 2b). Each net tow concentrate was collected on a 20 µm pore size plankton sieve to discard the water (Figure 2c). 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 further processing.

a- plankton sampling with plankton net **b** - collecting net haul **c** - plankton collecting

Figure 2. Plankton sampling at the coast of Pomorie (S2)

Wild mussels (*Mytilus galloprovincialis*) were collected manually, during low tides, at the same time in samplings sites S1-S5. As well, live mussels were purchased at the local seafood market. The mussels came from an aquaculture site off Ravda (C1), Pomorie(C2) and Sozopol (C3) , approximately 5 km away from the wild sites. Parallel mussel sampling aimed to minimize variations caused by differences in the mussels' physiology and avoid significant seasonal environmental variations, as far as possible (Carro,et al. 2004).

2.2. Sample preparation

Phytoplankton pellets were suspended in 1000 μL methanol for lipophilic toxins 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 = 1000 μL) of the supernatant was filtered through syringe filters (0.45 μm pore size, ⌀ 25 mm, Minisart, Sartorius, Germany). Filtrates were transferred into autosampler vials and kept at -20 °C until chromatographic analysis.

In order to minimize natural variability in following procedures, each sample of mussels was prepared from 50 or more individuals representing the available size range (mean weight 6.22 g; shell length 5.02 cm) existing in the selected area.

The digestive gland (hepatopancreas) of each mussel was separated from the soft tissues, homogenized and frozen.

An aliquot of the homogenized sample (average 4.08 g) of the hepatopancreas homogenate was extracted in with methanol three times. After each addition of methanol, the mixture was homogenized with a dispersing device (POLYMIX®PT 1200E, KINEMATIKA AG, Germany) for 5 min at 25.000 rpm. The extracts were combined and centrifuged for 15 min. After that, the methanolic extract was degreased three times with hexane by means of homogenization with the same instrument for 2 min. An aliquot of the methanolic extract (average = 1125 μL) was filtered through a syringe filter (0.45 μm pore size, ⌀ 25 mm, Minisart, Sartorius, Germany). The extracts were transferred into autosampler vials and kept frozen at -20 °C until analysis.

2.3. LC-MS determination

A wide array of phycotoxins (domoic acid and lipophilic toxins) was surveyed. 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 according (Krock, et al., 2008). The limits of detection (LOD) of studied toxins were determined based on 3:1 signal-to-noise ratio. LODs, mass transition (m/z) and retention time (min) of studied lipophilic toxins and DA are given in Table 2 .

Table 2. LODs, mass transition (m/z) and retention time (min) of studied phycotoxins (hp – hepatopancreas, NH-net haul)

Toxin	LOD pg/g hp	LOD pg/NH	mass transition (m/z)	Retention time (min)
YTX	1.9	2.3	1160/695	13,46
DA	3307.6	4000.0	312/266	11,02
DTX1	8.3	10.0	836/237	12,57
OA	3.0	3.7	822/223	11,85
PTX-2	925.8	1119.7	876/213	12,24

3. RESULTS AND DISCUSSION

Studies on phytoplankton and/or mussel samples mostly report on simultaneous production and/or contamination of shellfish when a long period of time is investigated. Analyses of mussel and plankton samples from the Black Sea 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) (Morton, et al, 2009) . Toxin profiles of phytoplankton cell concentrates and Greenshell mussels (Perna canaliculus)

revealed high levels of yessotoxins (YTXs) and pectenotoxins (PTXs) and low levels of okadaic acid (OA) (MacKenzie, et al., 2002). Mussels from Caucasian Black Sea coast of the Russian Federation contained YTXs along with OA and PTXs (Morton et al, 2007). In another study (Schirone, et al., 2018) specimens of mussels (*Mytilus galloprovincialis*) were collected along the coasts of the central Adriatic Sea during the years 2015–2017. Some of the samples were exposed to a multi-toxin mixture with regards to okadaic acid, yessotoxin and 1-homo yessotoxin.

The present study aimed the instantaneous toxin profile of plankton and mussel samples. In total 3 plankton, 5 wild and 3 cultivated mussel samples were investigated. PTX2 was detected in plankton samples only, as YTX was detected in wild mussel samples only (Table 3). DA, DTX1 and OA were not detected.

Table 3. Detected toxins values in plankton and mussel samples from Bulgarian Black Sea south coast (nd - not detected)

Sample number	Sample type	pg PTX2/NH	pg YTX/g hp	Sampling sites
1	plankton	nd	nd	S1
2	plankton	45953.9	nd	S2
3	plankton	19962.5	nd	S5
4	wild mussels	nd	1596.5	S1
5	wild mussels	nd	3926.0	S2
6	wild mussels	nd	5827.9	S3
7	wild mussels	nd	8137.3	S4
8	wild mussels	nd	2156.2	S5
9	farmed mussels	nd	nd	C1
10	farmed mussels	nd	nd	C2
11	farmed mussels	nd	nd	C3

Shellfish toxicity is a result of algal abundance and toxicity as well as accumulation and depuration kinetics in mussels (Nielsen, et al., 2016). The presence of PTX-2 in only plankton samples indicate that phytoplankton responsible for pectenotoxins production (e.g. Table 1) was present in the samples. Consequently PTX-2 accumulation can be expected in mussels in perspective. Nielsen et al (2016) reported PTX-2 accumulated rapidly in the mussels whereas the net retention appeared to decrease during a seven-day intoxication period, from nearly 100% after the first day to 25% at the end of the seven-day intoxication period. Mafra et al (2015) detected PTX-2 seco acid in mussels, following the period when the maximum levels of PTX-2 were found in the plankton.

In general, measured PTX-2 concentrations are lower than those detected in plankton samples from the North Sea. Reported abundance of PTXs was highly correlated with the occurrence of the marine dinoflagellate Dinophysis spp (Krock, et al., 2008).

This only pectenotoxin-2 containing toxin profile of plankton samples is comparable with the unique toxin profile found in the first bloom widespread over that part of Chile and persisted for months (Blanco, et al., 2007). A bloom produced by *Dinophysis acuminata* , in autumn of 2005, resulted a closure of the scallop harvesting in Bahía Inglesa, in the Chilean III region. Isolated cells of this Dinophysis species were shown to contain 180 pg/cell of pectenotoxin 2 but neither okadaic acid nor any of its analogs or derivatives (at least at a detectable level) (Blanco, et al., 2007).

Yessotoxin are detected in only wild mussel samples. The concentration is obviously increasing from north to south whereas the highest is in S4.

YTX levels in mussel samples (average = 4328.8 pg/g hp) were much lower than the legislative threshold of 3,75 mg/kg shellfish meat (sm) and levels reported by the Italian monitoring program in mussels *Mytilus galloprovincialis* harvested from the Adriatic Sea (Mudadu, et al., 2017) and by Haddouch et al (2017) in *Mytilus galloprovincialis* from the North Atlantic coast of Morocco.

Bacchiochi et al (2015) reported yessotoxins (YTXs) content in mussel increased sharply in the autumn-winter periods even exceeding the legal limit; although this accumulation did not always correlate with the YTX-producers in water (such as *Lingulodinium polyedrum* and *Protoceratium reticulatum*). This conclusion is similar to our findings that YTXs were not detected in plankton samples, but in mussel samples only.

Detection of YTX in only mussel samples demonstrates mussels could be in depuration phase.

An experiment with mussels fed by YTX producing dinophlagelate (Röder, et al., 2011) showed no strong increase of YTXs in the tissue directly after start of the inoculation. The amount of YTXs increased significantly after 16 days. After a break of 4 days, the concentration of the YTXs did not decrease. Subsequently, the levels of YTX declined during the detoxification period (Röder, et al., 2011).

The absence of toxicity in farmed mussel can be explained with the dynamics of harmful phytoplankton species (Yasakova 2013). Cultivation areas do not overlap with wild mussel harvesting ground and absence of toxic phytoplankton is possible hypothesis. Other explanation could be the specificity of mussel metabolic processes. In the final depuration phase mussels are free of toxins (Mafra, et al., 2010).

The results of this research – presence of toxins in both plankton and wild mussel samples – points out two aspects that should be considered.

I) there are toxin producing species in the investigated area;
II) mussels accumulate toxins in detectable concentrations;

The phenomenon of massive algae development due to anthropogenic activities is well known. But also some new dimensions of algae usage are considered (Dragomir, et al., 2014) so there could be benefited from this proliferation. Phytoplankton blooming in the Black Sea is also described (Moncheva, et al., 2001). If toxic phytoplankton species are those with increasing concentrations than it also should be considered how could be favored from this event (Alfonso, et al., 2016).

If constant and regular proliferation of toxin phytoplankton that is leading to accumulation of toxins in mussels meant for consumption is proven than different solutions are possible. Among them are e.g. relocation of the mussel farms, following the example in Gulluk Bay (Arisoy, et al., 2012) or contemporary ban on mussel harvesting (Higgins, et al., 2013) (Reich, et al., 2015).

4. CONCLUSIONS

Toxic algae blooms are a worldwide phenomenon, which appear to be increasing in frequency and severity. These natural events cause mussel contaminations that often have significant economic consequences, including supply interruptions due to closed cultivation grounds, losses from human illness, and losses due to a decline in demand for the affected products. This paper evaluates the occurrence and concentration of phycotoxins in plankton and mussel samples in a relatively short area on the Bulgarian South coast of the Black Sea during a 2-day field trip. Results indicated presence of PTX-2 in plankton and YTX in mussel samples. This shows that more investigations are required in order to prevent human intoxication and closures to aquaculture farms.

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