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Lipophilic Toxins in Butrinti Lagoon Mussels and their Relation with Potentially Toxic Dinoflagellates

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Abstract

Diarrheic Shellfish Poisoning (DSP) is a gastrointestinal illness. It is caused by the consumption of contaminated shellfish. These toxins are produced by the planktonic algae (mainly dinoflagellates). The aim of the current study was to evaluate the presence of potential toxic phytoplankton species and marine biotoxins like DSP in Butrinti Lagoon and to analyze the relationship between toxin levels and abundance of possible causative phytoplankton species. Samples were collected in Butrinti Lagoon, known for the cultivation of the, *Mytilus galloprovicialis* and fishing. During January 2011 until December 2012 were analyzed 126 samples of M. galloprovincialis for the presence of lipophilic toxins and dinoflagellates potentially toxic. The toxins were extracted from shellfish tissue using acetone, followed by extraction with dichloromethane and methanol. The final residue is dissolve in 1% (v/v) Tween 60. 19.84% of samples analyzed shown positive results for the presence of toxins that cause DSP with higher concentration of limit sets in EC Regulation No. 853/2004. The presence of different dinoflagellates was observed in the samples during that period in level 120cell/L - 920 cell/L, like *Gonyaulax spinifera, Dinophysis sacculus, Dinophysis acuminata*, that are known to produce algal toxins, including DSP.

Keywords: Butrinti Lagoon, DSP, *Mytilus galloprovincialis*, dinoflagellates

1. Introduction

Successful development and marketing of aquaculture products should be based on compliance with health assurance criteria and water quality, which are very well defined by European Union legislation. The Food Safety and Veterinary Institute, as the National Reference Laboratory, has the duty to implement regularly monitor program on the production area and the mollusks for the compliance of the chemical and microbiological criteria (biotoxins, potentially toxic algae, chemical and microbiological contaminantion).

In recent years, the *M. galloprovincialis* cultivation has been dropped to several hundred tons per year. Low cultivation has been due to the internal organization reasons as well the exportation band of the products for sanitary reasons since October 1994. In order to reach again the exportation criteria the production area should be regularly monitored for the presence of algal toxins such as PSP (Paralytic Shellfish poisoning), DSP (Diarrhetic Shellfish poisoning) and ASP (amnesic Shellfish poisoning), in accordance with EU Regulation No.853/2004.

M. galloprovincialis is the mollusk cultivated in Lake of Butrint and it is well known organism for its filtration abilities. It feeds on fitoplanktonike cells, zooplankton, bacteria, and algae extracts etc. Among existing fitoplanktonike 5000 types, 300 species of algae causing water hues (red tides), and about 40 species produce toxins that by molluscs and fish can pass to humans [6]. According to the literature [6] among 5000 existing species, approximately 300 hundred of them cause the so called water coloring and 40 of them produce toxic substances which thought food chain can reach to the consumer.

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Lipophilic toxins of DSP group are distributed almost all over the world, especially in Japan, North-Western Europe and are found also in Butrint Lagoon. They are a serious problem due to their impact in the public health [13]. The DSP belongs to three different structural groups: the first group is okadaic acid group of toxins (OA-toxins) and its derivates, such as dinophysistoxins (DTXs); the second group includes neutral toxins as pectenotoxins group (PTXs) [4,16] and the third one include yessotoxins (YTX) and its derivatives 45-hidroxi yessotoxin (45-OH-YTX) [4].

Figure 1. Cultivation of mussels (M. galloprovincialis) abandoned in the Butrint growth areas 3. A implant for mussel cultivation in Manastir, Butrint (photo: Kashta, Miho).

DSP toxins are produced by alga of dinoflagjellates genus, *Dinophysis* and *Prorocentrum*. Production of DSP toxins is confirmed in 7 different species of *Dinophysis* (Yasumoto et al., 1980) found in different geographic area, *D. fortii* (in Japan), *D. acuminata* (in Europe), *D. acute*, *D. norvegica* (in Scandinavia), *D. uterus, D. rotundata, D. tripos*, and in benthic dinoflagjellates *Prorocentrum lima*, *Prorocentrum concavum* (or *P. maculosum*) and *Prorocentrum redfieldi*. Also, 3 other *Dinophysis* species are suspected of producing DSP toxins, *D. caudata, D. hastata and D. Sacculus* [5].

Symptoms in people, who are fed with mussels that have accumulated in their digestive apparatus these toxins, are gastrointestinal disturbances such as diarrhea, vomiting, nausea and abdominal pain. These symptoms have an appearance range from 30 minutes to 12 hours after digestion of contaminated mussels [12]. 95% of the toxins are accumulated in hepatopancreas of shellfish without making chemical changes or physiological and organoleptic changes of the mollusks. Although the accumulation of toxin in shellfish requires several days, their elimination requires several weeks and sometimes months [9].

2. Material and methods

The study was conducted during January 2011- December 2012. The samples were taken in three different stations as it is shown in the figure 2. Each of the station represents different micro environmental and they are chosen in base of their geomorphological characteristics according to different source of contamination and water current as well as their impact.

The three stations are listed below:

1. Station **BM1 North (N) - Manastiri Farm Nr 9** geographic coordinate N 39° 48’ 33.2’’ E 20° 1’ 0.88’’;
2. Station **BM1 West (P) - Pallavraqi i vogël Farm Nr 38** geographic coordinate N 39° 46’ 7.23’’ E 20° 1’ 0.34’’;
3. Station **BM1 South (J) - Butrinti Farm Nr 60** geographic coordinate N 39° 45’ 4.08’’ E 20° 1’ 9.69’’.

During this study were taking 126 samples of *M.galloprovincialis* for determination of DSP toxins, 126 water samples for determination of harmful algae with the sequence of twice per month. For the determination of biotoxins the laboratory sample weight was proximately 4 kg of live bivalve mollusk. The DSP presence was determinate by using a biological assay based after the extraction of lipophilic compounds in the sample with acetone. Then in order to separately YTX group from other lipophilic toxins (OA-toxins group, PTX group and AZA group), a dichloromethane/60% methanol solution was used. The extracts where taken to dryness and the residues were reconstituted with an aqueous solution containing 1% Tween 60. One ml of each extract was injected intraperitoneally into albino mice Swiss strain weighing between 17g and 22g.

Figure 2. Map of Butrinti lagoon, Saranda, Albania with the three sampling stations (white circles) (modified after Google Earth, 2008).

For the dichloromethane extract (investigation of OA-toxin group, PTX group and AZA group), the death of 2 out 3 injected mice, within a 24 hours observation period, constitutes a positive result. [10]. In the case of the methanolic extract (investigation of YTX group), the death of 2 out of 3 injected mice during the biological assay, within 6 hours observation period, constitutes a positive result for the presence of YTXs.

Potentially toxic phytoplanktons were analyzed at the same period. Phytoplankton samples were taken on the surface, 1m deep, using dark glass bottles. Samples were preserved in alkaline Lugol's Iodine Solution. The taxonomic list was prepared mainly according to cell counts and photos obtained by the inverted microscope Zeiss Axiovert 25 and Axiovert 40 CFL, equipped with a digital camera. Species determinations were done using different keys [7,6]. Sub samples of 25 ml were analyzed after 24 h of sedimentation [11].

3. Results and Discussion

The presence of DSP toxins group in the Butrint Lagoon during the period January 2011 - December 2012 was estimated by analyzing 144 samples of *M. galloprovincialis*. From the biological test 13 samples of *M.galloprovincialis* positive resulted, for 2011 and 27 samples for 2012 (Table 2). Samples resulted positive for the presence of okadaic acid, pectenotoxins and azaspiracids; which mean that they exceed the limits values set in EU Regulation 2074/2005. The largest percentages of positive cases had resulted during January, February, March, April and December.

Table 1: Results of the DSP with biological test in *Mytilus galloprovincialis* during 2011.
(Pos-positive; Neg-negativ; % Positive test/Total test x 100)

2011	J	F	M	A	M	J	J	A	S	O	N	D
North Butrint	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
West Butrint	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
South Butrint	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
DSP%	100	83.3	66.6	0	0	0	0	0	0	0	0	0

Table 2: Results of the DSP with biological test in *Mytilus galloprovincialis* during 2012.
(Pos- positive; Neg-negativ; % Positive test/Total test x 100)

2012	J	F	M	A	M	J	J	A	S	O	N	D
North Butrint	Neg	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos
	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos
West Butrint	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos
	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos
South Butrint	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos
	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos
DSP%	83.3	100	100	66.6	0	0	0	0	0	0	0	100

Also 144 samples of water lagoon were analyzed for potentially toxic algae. The water samples were sampled at exactly the same time and place as the mussels (Tab.3). The concentrations of *Dinophysis acuminata, D. sacculus, Gonyaulax spinifera* range from 120 cells / L at 920 cells / L. During the same period other species, such as *Lingulodinium polyedrum*, *Dinophysis tripos*, *D. Fortii*, which cause DSP, have been found to be present.

Table 3: Maximum values (cells/L) of potentially toxic dinoflagellates during 2011-2012.

Total (cell/L) Dinoflagellates	J	F	M	A	M	J	J	A	S	O	N	D
2011	200	480	240	120	120	120	120	120	120	120	120	240
2012	720	920	720	120	120	120	120	120	120	120	200	280

Figure 3 : Dinophysis sacculus, D. acuminata, D. fortii (from left to right).

The presence of dinoflagellates DSP during early spring and winter of 2011-2012 was due to the fall of rain, the flow of water and the addition of nutrients, temperatures begin to rise etc. In spring the impact of basin inflows is maximum, which causes not only decrease of the salinity in miksolimn, but also the increase of pollutants from the basin (feeder, bacteria, heavy metals, pesticides). Mollusks as filters organism accumulate these algae over weeks and show the presence of toxins. Reduction of phytoplankton in the late spring - early autumn may be due to the impact of a number of factors. In this period the amount of inflows decreases, while in the summer their impact is minimal; also the communication with the sea decrease at a minimum. On the other hand, in the summer increases the evaporation process by reducing the level of water and their temperature increases. Therefore the nutrients are reduced; abundant winter and spring flows consumed by phytoplankton land at anaerobic water zone, subject for slow decomposition bacteria process or used by the above levels of other organisms as zooplankton, mollusks, fish, etc..

There was presence of DSP group toxins even when concentrations (cells / L) of *Dinophysis acuminata, D. sacculus, Gonyaulax spinifera* were relatively low. Test in mice for DSP had resulted positive also in previous years: in January 2008 (6 of 9 samples) = 6/9, in February 2008 = 12/12, in December 2008 = 1/12, in January 2009 = 4/9, in February 2009= 1/9, in March 2009 = 3/9 (Bregaj *et al*., 2012; Bushati *et al*., 2012, 2012b). In 2010, from 150 tests performed for DSP toxins, only 13 result positive cases, ie. 8.7% of cases. The percentage of positive cases (the death of mice) of DSP in the period 2008-2012, more or less happens in the winter-early spring. Often, the presence of genus *Dinophysis* as in low density (120 cells/L) can cause high level of toxin in mussels and this can lead to human poisoning [2]. On the other hand, in Germany, only blossoms of more than 20,000 cells per liter may result in DSP cases.

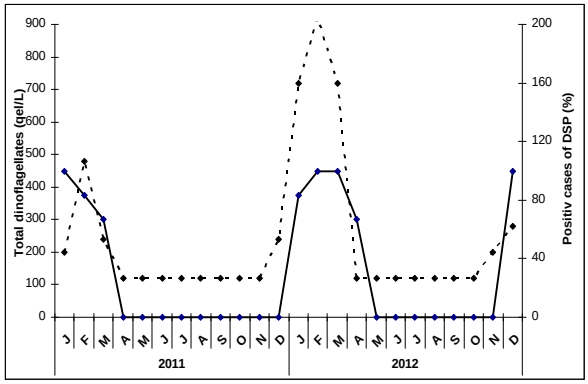

Figure 4. Dynamics of potentially toxic dinoflagellates (maximum values cells/L), compared with dynamics of DSP toxins group (%).

A study in Portugal on genus *Dinopysis* showed that the time required for a mollusk to become toxic depends not only on the presence of toxic algae but also in the abundance of other non-toxic accompanying species [1]. Not all DSP blooming are associated with *Dinophysis* spp. or *Prorocentrum* spp. blooming [13]. Toxicity of *Dinophysis* species varies in space and time and the number of cells per liter required for contamination of shellfish is variable. It has been observed a statistically significant linear relation between the amount of dinoflagellates-DSP (Fig.5) and the value of reported positive / negative result for DSP test in percentage (%), during 2011-2012. ($r = 0.515$, $n = 22$, $p < 0.01$). In our study we found DSP group toxins in mussels about two weeks after we found *Dinophysis* spp., even at low concentrations (240 cells/L).

DSP positive cases in different countries appear in different months of the year. In Japan, on the coast of Spain and France Atlantic the infection ranges from April to September and the highest toxicity cases occurred from May to August, although this may vary by area to area. Otherwise, oysters of Scandinavia have shown DSP toxicity in October.

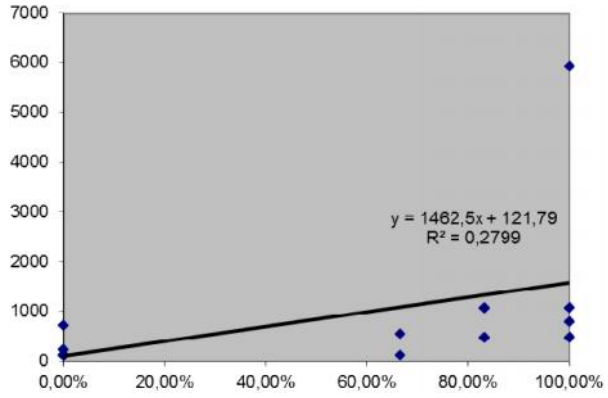

Figure 5. Linear relation between the amount of dinoflagellates-DSP toxins producer (cells/L) and the value of ratio results positive / negative for DSP in percentage (%), during 2011-2012.

Data on the first DSP episode in the Adriatic Sea in 1989 had shown that the period of infestation, in some areas goes from May to November [14]. Positive DSP cases in the Butrint Lagoon appear to be in winter and early spring, mainly from December to late March. In Europe the DSP incidence, or at least the presence of DSP toxins, seems to be growing. Although Albania has positive cases of DSP in mussels have not been reported cases of this syndrome in humans (no official hospitalization) because the symptoms are mild, victims rarely seek medical help and so it is difficult to assess the true incidence of this syndrome.

4. Conclusions

DSP toxic episodes in the Butrinti Lagoon appear generally during winter season and early spring.

Dinophysis spp. even at very low concentrations produces harmful toxins. These toxins are present in mussels about two weeks after the *Dinophysis* spp. presence.

The regular monitoring the production area helps in better understanding the life cycle of *Dinophysis* and the production of its toxins.

Education of medical and public health staff regarding diagnosis, treatment and referral of suspected cases is very important to the success of the monitoring program.

It is important also, the education of the population in relation to preventive measures, such as the consumption of shellfish is not allowed at the time of the toxic algae bloom.

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