ACTA MICROBIOLOGICA BULGARICA Volume 33 / 1 (2017)

Escherichia coli and Paralytic Shellfish Poisoning Toxins Contamination of Mussels Farmed in Bulgarian Black Sea Coast

Gergana Krumova-Valcheva, Ginka Kalinova

National Diagnostic and Research Veterinary Medicine Institute, Bulgaria, 1606 Sofia, 15 Pencho Slaveikov Blvd.

Abstract

Mussels are filter-feeding organisms that could be a potential source of food-borne diseases and poisonings. Consumption of molluscs, contaminated with bacterial contaminants or marine biotoxins, may lead to severe food-borne diseases. Considering the importance of the health problem connected with *Escherichia coli* and paralytic shellfish poisoning (PSP) toxins and also the requirements of the European legislation, the aim of this study was: to evaluate the level of *E. coli* and PSP contamination in cultivated mussels produced in farms along the Bulgarian Black Sea coast.

The study was performed on 55 samples of live mussels (*Mytilus galloprovincialis*), from 20 aquaculture farms, during the period 2012 - 2015, in the warm season (May - October) and in the cold season (November- April). The determined concentration of *E. coli* varied between <20 MPN/100 g and 8.2 x 10^2 MPN/100 g. The level of *E. coli* in 5 samples tested in the warm season and in 2 samples tested in the cold season was higher than the permissible quantity. All samples met the health standards referring to the content of PSP toxins in bivalve molluscs. No connection was established between the concentration of *E. coli* and PSP toxins in the tested samples and the level of contamination with *E. coli* and PSP toxins in cultivated mussels, from the Bulgarian Black Sea coast, was minimal.

Key words: bivalve molluscs, *Escherichia coli*, PSP toxins

Резюме

Мидите са организми, които се хранят чрез филтруване на водата и могат да бъдат потенциален източник на хранителни заболявания и отравяния. Консумацията на мекотели, замърсени с микроорганизми или морски биотоксини може да доведе до тежки хранителни заболявания. Оценявайки важността на здравния проблем, свързан с *Escherichia coli* и паралитичните (PSP) токсини, както и изискванията на европейското законодателство, целта на това изследване беше: да се оцени нивото на *E. coli* и PSP замърсяване на култивираните миди, произведени във ферми от българското Черноморие.

Проучването е проведено с 55 проби от живи миди (*Mytilus galloprovincialis*) от 20 ферми в периода 2012-2015 г., през топъл сезон (май-октомври) и студен сезон (ноември-април). Определената концентрация на *E. coli* варира между <20 MPN / 100 g до 8.2 x 102 MPH / 100 g. Нивото на *E. coli* в 5 проби, тествани през топъл сезон и 2 проби от студения сезон е по-високо от допустимото количество. Всички проби отговарят на здравния стандарт за съдържание на PSP токсини в двучерупчестите мекотели, посочен в европейското законодателство. Не беше установена връзка между концентрацията на *E. coli* и PSP токсини в тестваните проби, като нивото на замърсяване с *E. coli* и PSP токсини в култивирани миди от българското черноморско крайбрежие беше минимално.

Introduction

The main species of marine aquaculture grown in the coastal waters of the Black Sea is the black mussel – *Mytilus gallop*rovincialis. The production of cultivated mussels in Bulgaria has increased in recent years. There are 25 mussel farms along the Bulgarian Black Sea coast. The annual production of mussels increased by 12 % and reached 18 t in 2016 (Ministry of Agriculture and Food, 2016).

*Corresponding author: e-mail: dr.krumova_vulcheva@abv.bg

Mussels are filter-feeding organisms that tend to accumulate different biological and chemical agents in their tissues (Oliveira *et al.,* 2011). They could be a potential source of food-borne diseases and poisonings. According to the EFSA report, in 2016 in the EU countries molluscs were the source of 3 % of the food-borne outbreaks (EFSA, 2016). The main source of contamination of mussels is marine pollution caused by untreated household and industry sewage in the absence of treatment plants. During a two-year period (2012–2014), the water quality of the Black Sea was evaluated and a presence of pathogenic microorganisms such as *E. coli*, *Vibrio* spp., rotaviruses was detected (Panaiotov *et al*., 2015).

Stoica (2014) analyzed wild mussels harvested from the north-western coast of the Black Sea (Romania). She proved a close relation between the quantity of *E. coli* in sea water and in the mussel tissues. Her investigations confirmed the interdependence between environmental conditions and the levels of *E. coli* in wild mussels and surface shellfish waters. She detected levels of *E. coli* which exceeded the European requirements in 16.7 % of the Romanian shellfish growing coastal zones. A study carried out in the Eastern Aegean Sea showed a maximum fecal coli form count of $2.4x10^5$ MPN/100 g (Kacar, 2011). Yilmaz *et al.* (2005) found *E. coli* values between <10 and $5.9x10^2$ cfu/g in *M. galloprovincialis* harvested in the Marmara Sea. Pomykala *et al.* (2012) isolated *E. coli* ranging from $<2.0 \times 10^1$ up to $>1.8 \times 10^4$ MPN/100g from raw mussels originated from Spain, the Netherlands and Norway.

Contaminated with bacteria molluscs may lead to severe serious health problems for consumers. Regulation (EC) No 2073/2005 lays down the microbiological criteria for certain microorganisms, including *E. coli* in bivalve molluscs, which must be less than 230 in 100 g of flesh and intra-valvular liquid to be safe for human consumption. Another serious health risk for humans consuming mussels are marine biotoxins. The most common and dangerous is paralytic shellfish poisoning (PSP), which in extreme cases can lead to death due to respiratory distress and muscle paralysis. This type of poisoning is caused by a group of naturally occurring potent neurotoxins produced by Alexandrium, Gymnodinium and Pyrodinium marine dinoflagellates, which are on the list of established phytoplankton species in the Black Sea. The seawater monitoring performed by the Institute of Oceanology-Varna, included investigation of phytoplankton at 13 stations along the Bulgarian coast during 2015 – 2016. The reports confirmed a presence of *Alexandrium spp., Gymnodinium spp., Prorocentrum spp.* up to 6% in the phytoplankton in Bulgarian Black Sea waters (18). These data suggest a high risk of occurrence of PSP toxins in Black Sea mussels.

Having regarded the commercial and nutritional importance of bivalve molluscs, Regulation (EU) No 853/2004 has introduced specific requirements for growing, harvesting, storage conditions and set health standards for maximum levels of marine biotoxins in bivalve molluscs. The Health Standards for live bivalve molluscs indicate that live bivalve molluscs placed on the market for human consumption must not contain marine biotoxins in total quantities that exceed 800 micrograms per kilogram for paralytic shellfish poison. The sampling frequency for toxin analysis in molluscs was established in Regulation (EU) No 854/2004.

Considering the importance of the health problem connected with *E. coli* and PSP toxins and also the requirements of the European legislation, the aim of this study was: to evaluate the level of *E. coli* and PSP-contamination in cultivated mussels produced in farms from Bulgarian Black Sea.

Materials and Methods

Sampling

The study was performed on 55 samples of live mussels (*M. galloprovincialis).* They were originated from 20 aquaculture farms located in Bulgarian waters of the Black Sea. One sample was around 1 kg of mussels. After collection, the samples were immediately delivered to the laboratory at refrigeration temperature. The studies were carried out two seasons: warm (May - October) and cold (November - April) in the period 2015-2016. All of the samples were used for enumeration of *E. coli* and determination of the level of PSP-toxins.

Sample preparation

The microbiological analysis started not later than 24 hours after collection. At least ten individual organisms (meat and intra-valvular liquid) were used for preparation of microbiological laboratory samples. The selected mussels were opened with a flame-sterilized shucking knife and the meat and intra-valvular liquid were placed into a sterile stomacher bag.

Determination of MPN/100g of E. coli

The most probable number (MPN/100 g) of *E. coli* was evaluated according to the ISO TS 16649-3/2005 reference method.

PSP toxin analysis

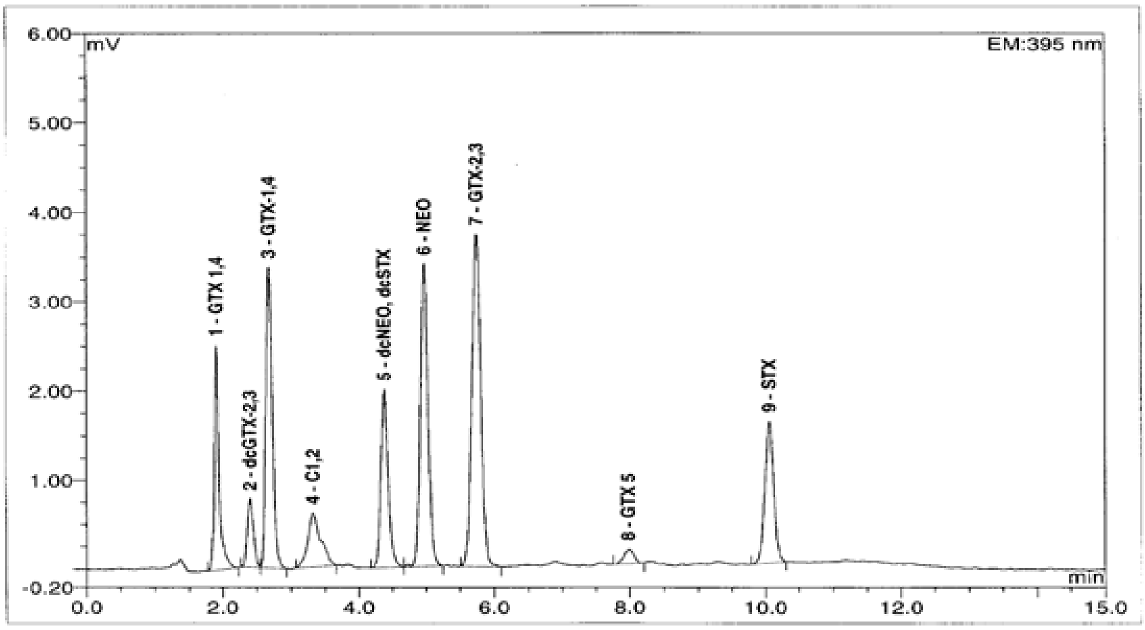


Fig. 1. Chromatogram with 9 paralytic toxins (certified reference materials), obtained by HPLC-FLD method after periodate oxidation.

The sample preparation and analysis of mussels tissue extract were performed in accordance with the method described in AOAC OMA 2005.6.

Chromatographic conditions and equipment

HPLC analysis was carried out by UltiMate 3000 Thermo Fisher Scientific Dionex, equipped with a quaternary pump UltiMate 3000, fluorescent detector Dionex RF 2000 with EX:340 nm and EM:395nm; chromatographic column Kinetex: C18, 150 mm x 4.6 mm, 5µm particle size; flow rate of the mobile phase 1ml/min.

Calibration curves

PSP toxins were identified by comparing chromatograms of certified reference materials (dc-GTX2,3; C1,2, dc-STX, GTX2,3; GTX5, STX, NEO, GTX1,4 and dc-NEO). The quantification of each PSP toxin in the samples was performed by calibration graphs with five concentration levels. Figure 1 shows a chromatogram of 9 certified reference materials obtained by a HPLC-FLD method after periodate oxidation.

The linearity of nine calibration graphs (r > 0.99) was established for the concentrations in the range of (20 to 800) µg STX.2HCL equivalent/kg. The limits of quantitation (LOQ) of the nine toxins were in the range of 20 to 89.3 µg STX.2HCL equivalent/kg. The total amount of paralytic toxins was presented as µg STX dihydrochloride equivalent/kg.

Results

Microbiological analysis

The MPN/100 g of *E. coli* determined in samples is presented in Table 1. The results varied between less than 2.0 x 10^1 MPN/100 g and 8.2 x 10^2 MPN/100 g. The samples with a content of *E. coli* over the limit (> 230 MPN/100 g) dominated in the warm season. The highest result of 8.2 x 10^2 MPN/100 g was registered in July 2016. The number of *E. coli* determined in samples during the cold period was in the range less than 2.0 x 10^1 MPN/100 g to 4.0 x 10^2 MPN/100 g.

Four samples with a content of *E. coli* higher than the limit were from mussels originated from the north coast of the Bulgarian Black Sea and three samples from the south part of the Bulgarian Black Sea.The distribution of the samples of live shellfish depending on the *E. coli* content is presented in Table 2.

The majority of samples (48 out of a total of 55 samples) contained *E. coli* in quantities not exceeding the acceptable limit of 230 MPN/100 g. The numbers of samples (24) with undetectable values of *E. coli* (below 20 MPN/100 g) were equal with those up to 230 MPN/100 g. The samples with over the limit content of *E. coli* during the warm season were twice as many as those found in the cold season.

PSP toxin analysis

All 28 samples from 2015 were negative for presence of PSP toxins – the quantities were below

Table 1. Quantity of *E. coli* (MPN/100 g) in Black Sea cultivated mussels, 2015 – 2016

Season	Number of the tested samples	MPN/100g		
		< 2.0 x 10^1	Up to 2.3 x 10^2	> 2.3 x 10^2
Warm	28	6	17	5
Cold	27	18	7	2
Total	55	24	24	7

Table 2. Distribution of the samples cultivated mussels according to the content of *E. coli*

2015				2016			
№	Warm season	№	Cold season	№	Warm season	№	Cold season
1.	1.5 x 10^2	15.	2.1 x 10^2	1.	5.4 x 10^2	15.	4.0 x 10^2
2.	9.2 x 10^1	16.	4.0 x 10^2	2.	1.6 x 10^2	16.	< 2 x 10^1
3.	9.3 x 10^1	17.	9.3 x10^1	3.	2.3 x 10^2	17.	2.3 x 10^1
4.	2.4 x 10^1	18.	1.1 x10^2	4.	8.0 x 10^2	18.	< 2 x 10^1
5.	2.4 x 10^1	19.	< 2 x 10^1	5.	1.4 x 10^2	19.	< 2 x 10^1
6.	2.3 x 10^2	20.	1.1 x 10^2	6.	9.0 x 10^1	20.	< 2 x 10^1
7.	< 2.0 x 10^1	21.	< 2 x 10^1	7.	2.3 x 10^2	21.	< 2 x 10^1
8.	2.3 x 10^2	22.	< 2 x 10^1	8.	< 2 x 10^1	22.	6.0 x10^1
9.	8.0 x10^2	23.	< 2 x 10^1	9.	< 2 x 10^1	23.	< 2 x 10^1
10.	< 2.0 x10^1	24.	< 2 x 10^1	10.	2.4 x 10^1	24.	< 2 x 10^1
11.	3.5 x 10^2	25.	2.0 x 10^1	11.	< 2 x 10^1	25.	< 2 x 10^1
12.	1.6 x 10^2	26.	< 2 x10^1	12.	8.2 x 10^2	26.	< 2 x 10^1
13.	1.6 x 10^2	27.	< 2 x10^1	13.	< 2 x 10^1	27.	< 2 x 10^1
14.	9.2 x 10^1	28.	< 2 x10^1	14.	9.2 x 10^1		

the limit of detection. A small quantity of PSP toxins was identified in July 2016 in a sample from a farm on the northern coast. The chromatogram of the sample is shown in Fig. 2.

The positive sample contained the following PSP toxins:

- gonyautoxin 2,3 (GTX 2,3) with quantity 21.3 µg STX.2HCL equivalent/kg

- saxitoxin (STX) with quantity 29.8 µg STX.2HCL equivalent/kg.

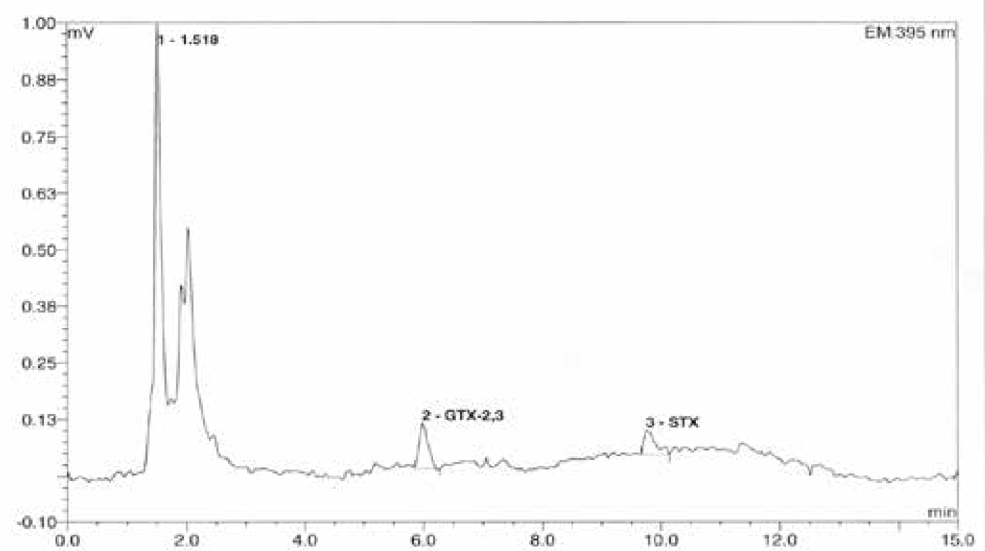
The total toxicity of the sample was 51.1 µg STX.2HCL equivalent/kg. The content of *E. coli* determined in the sample was < 2.0 x 10^1 MPN/100 g.

Discussion

Data from our study showed that 12.7% of all 55 analysed samples exceeded the microbiological criteria for *E. coli,* listed in Commission Regulation (EC) No 2073/2005. The highest levels of contamination were in the mussels tested in the warm season. Significantly better was the hygiene status of molluscs during the cold period – 93% of the samples complied with the requirements.

Our results are in agreement with the findings of other authors. Panaiotov *et al*. (2012) identified toxigenic E. coli in the Black Sea waters during summer and early autumn. Stoica (2014) established that 16.7% of the mussel samples from the Romanian Black Sea coast exceeded the levels permitted by the European legislation. She registered the highest concentrations (up to 490 MPN/100 g) during the summer months. Soegianto and Supriyanto (2008) found a very low MPN of *E. coli* in October and December and a greater content of these bacteria during the summer months. Contrary of our results, Kacar (2011) reported a maximum faecal coliform level in mussels from Eastern Aegean Sea during the cold months (November and March) and the lowest value in July.

The summarised results for *E. coli* content in tested mussels during the period 2015–2016 showed a minimum risk for consumers of cultivated mussels, especially in the summer months, when the pollution of the sea water was greater. Food



No.	Ret.Time (detected) min	Peak Name	Height mV	Area mV*min	Rel.Area %	Amount	Type
2	5.98	GTX-2,3	0.098	0.017	57.22	0.011	BMb*
3	9.75	STX	0.054	0.013	42.78	0.009	MB*
Total:			0.153	0.030	100.00	0.020	

Fig. 2. Chromatogram of sample № 586 from July 2016, farm on the northern coast, obtained by HPLC-FLD method after pre-column derivatisation

business operators have to perform strictly the Hygiene Requirements for The Production and Harvesting of Live Bivalve Molluscs Set in Regulation (EC) 853/2004, Annex III, Section VII.

The results of the analyses for the detection of biotoxins in cultivated mussels from Bulgarian Black Sea for the period 2015 – 2016 showed that there was no risk of paralytic shellfish poisoning for consumers. PSP toxins were detected only in one sample of all tested mussels. The detected quantities were close to the limits of quantitation of PSP toxins. The total toxicity (51.1 µg STX.2HCL equivalent/kg) of the sample was significantly lower than the health standard for PSP toxins – 800 µg STX.2HCL equivalent/kg.

The toxic profile of the sample from the present study, which contained saxitoxin (STX) and gonyautoxin 2,3 (GTX 2,3), complied with the *Alexandrium spp*. profile. This corresponds fully to the type of phytoplankton found in the Bulgarian Black Sea waters during the monitoring in 2015 - 2016. The presence of dinoflagellates of the genus *Alexandrium* was proven by Vershinin *et al.* (2006) in a 2001–2004 monitoring of the northeast Black Sea coast. They identified *Alexandrium tamarense* and *Alexandrium minutum* in the waters of the Caucasus coast and also small amounts of saxitoxin (STX), gonyautoxin 2,3 (GTX 2,3) and gonyautoxin 5 (GTX 5) were determined in extracts of mussel hepatopancreas, applying HPLC method and fluorescence detection.

The established lack of contamination with PSP toxins of Bulgarian cultivated mussels for the studied period 2015 – 2016 confirmed the results of our previous investigation (Kalinova *et al*., 2015). Of all 57 analysed samples for the period 2011-2014, PSP toxins were determined in 5 samples in quantities 2 – 3-fold less than the legal limit.

Regardless of the minimum risk, it was assumed that these results do not fully reflect the actual level of contamination with biotoxins, as manufacturers control the mussels once a year and there were no data for contamination in the remaining months. They do not perform the inspection required by Regulation (EC) No 854/2004, Annex II, Chapter II, point B (5): "The sampling frequency for toxin analysis in the molluscs is, as a general rule, to be weekly during the periods at which harvesting is allowed".

Conclusion

This was the first parallel study of the amount of *E. coli* and paralytic toxins in mussels farmed along the Bulgarian Black Sea coast. No connection was established between the concentration of *E. coli* and PSP toxins in the tested samples. The level of contamination with *E. coli* and PSP toxins in cultivated mussels from the Bulgarian Black Sea coast was minimal and their concentrations met the requirements of the European legislation.

Considering the conclusion of the EFSA report (2016) that bacteria and marine biotoxins were the causative agents in 11.9 % and 2.9 %, respectively, in food-borne outbreaks in EU countries, the Bulgarian competent authorities should reinforce the hygiene control of cultivated mussels.

Reference

Annual report on the condition and development of agriculture (2016), Bulgaria, Ministry of Agriculture and Food.

AOAC OMA 2005.6. (2006). Method 2005.06: Paralytic Shellfish Poisoning Toxins in Shellfish. Prechromatographic Oxidation and Liquid Chromatography with Fluorescence Detection (First Action 2005). *AOAC INTERNATIONAL, Gaithersburg, MD, U.S.A*. 18th Ed.: pp. 83-96.

European Food Safety Authority (2016). The community summary report on trends and sources of zoonoses, zoonotic agents and foodborne outbreaks in the European Union in 2015. *EFSA Journal,* **14**: 12.

ISO 16649-3 (2015). Microbiology of the food chain - Horizontal method for the enumeration of beta-glucuronidase-positive *Escherichia coli* -- Part 3: Detection and most probable number technique using 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide.

Kacar, A. (2011). Some microbial characteristics of mussels (*Mytilus galloprovincialis*) in coastal city area. *Environ. Sci. Pollut. Res.* **18**: 1384-1389.

Kalinova, G., P. Mechkarova, M. Marinova (2015). A study of paralytic toxins in cultured mussels from Bulgarian Black sea. *Trakia J. Sci.* **13**: 303-308.

Oliveira, J., A. Cunha, F. Castilho, J. L. Romalde, M. J. Pereira (2011). Microbiological contamination and purification of bivalve shellfish: crucial aspect in monitoring and future perspectives – a mini review. *Food Control* **22**: 805-816.

Panaiotov, S., I. Simeonovski, V. Levterova, V. Karamfilov, N. Brankova, K. Tankova, K. Campbell, P. Jacob, A. Helmi Bas Boots, Em. D·Ugo, S. Marcheggiani, L. Manchini, U. Breirenbach, E. Mielke, T. Kantardjiev (2015). Two-year monitoring of water samples from Dam of Iskar and the Black Sea, Bulgaria by molecular analysis: focus on Mycobacterium spp. *Int. J. Environ. Res. Public Health* **12**: 7330-7443.

Pomykala, R., M. Michalski, A. Jozwik, J. Osek (2012). Microbiological and marine biotoxins contamination of raw bivalve molluscs commercially available in Poland. *Bull. Vet. Inst. Pol.* **56**: 563-568.

Regulation (EC) No 853/2004 of the European parliament and of the council of 29 April 2004 laying down specific hygiene rules for on the hygiene of foodstuffs. *Off. J. Eur. Union* **L139**, 55-205.

Regulation (EC) No 854/2004 of the European parliament and of the council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption.

Regulation (EC) № 2073/2005 of microbiological criteria for foodstuffs.

Soegianto, A., A. Supriyonto (2008). Concentration of pathogenic bacteria and trace metals in bivalve mollusks *Anadara granosa* (Bivalvia Arcidae) harvested from East Java Coast, Indonesia. *Cah. Biol. Mar.* **49**: 201-207.

Stoica, E. (2014). Presence of fecal indicator organisms (FIOs) in shellfish waters and mussels *Mytilus galloprovincialis* (Lam.) from the Romanian Black Sea coast. *Sci. Ann. Danube Delta Institute* **20**: 65-70.

Vershinin, A., S. Morton, T. Leighfield, S. Pankpov, L. Smith, M. Qualliam (2006). *Alexandrium* in Black Sea – identity, ecology and PSP toxicity. *Afr. J. Mar. Sci.* **28**: 209-213.

Yilmaz, I., B. Bilgin (2005). Occurrence of *Vibrio* and other pathogenic bacteria in *Mytilus galloprovincialis* and *Venus gallina* harvested from the Marmara Sea. *Turk. J. Vet. Anim. Sci.* **29**: 409-415.

http://phyto.bss.ibss.org.ua/wiki/List_checked

http://www.bgodc.io-bas.bg/Monitorig