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BIOTOXIN LEVELS IN BIVALVE MOLUSCS IN ALBANIA AND THE RELATED HEALTH IMPACTS

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Data on the level of contamination of the Albanian seashore with PSP biotoxins (saxitoxin and derivates) as well as the heavy metals (Hg and Cr) are given. Lysosome membrane destabilization test has been the method of choice in the present study. A relatively low retention time of the used red neutral dye in some selected monitoring areas was detected at the distance of 50 m offshore indicating a high level of the biological stress. Whereas at 200 m distance the retention time the sensibly increases. Comparably higher levels of Hg and Cr were detected at 50 m offshore distance. The high level of PSP biotoxins in some selected monitoring areas, where sampling of water and bivalve molluscs was performed has impacts on the health.

Key words: PSP biotoxins; lysosome; membrane destabilization test

БИОТОКСИЧНИ НИВОА ВО БИВАЛВНИТЕ МЕКОТЕЛИ ВО АЛБАНИЈА И СООДВЕТНОТО ВЛИЈАНИЕ ВРЗ ЗДРАВЈЕТО

Претставени се податоци за нивото на контаминација на албанското крајбрежје со PSP биотоксини (сакситоксин и деривати), како и со тешки метали (Hr и Cr). Тестот на дестабилизација на лизозомната мембрана беше метод на избор во сегашната студија. Релативно кратко време на задржување на употребената црвена неутрална боја во некои селектирани набљудувани области беше детектирано на растојание од 50 m од брегот, укажувајќи високо ниво на биолошки стрес, додека на растојание од 200 m времето на задржување постепено се зголемуваше. Споредбено повисоки нивоа на Hg и Cr беа детектирани на растојание од 50 m од брегот. Високото ниво на PSP биотоксини во некои селектирани набљудувани области, каде што беа земени примероци од вода и од мекотели, имаше влијание врз здравјето.

Клучни зборови: PSP биотоксини; тест на дестабилизација на лизозомна мембрана

INTRODUCTION

Biomonitoring has become one of the ways of predicting changes in the global environment. Many scientific programmes in different Mediterranean countries are taking this approach to the biological effects of contaminants with the aim of promoting a common and integrated strategy of using marine biomarkers in recommended sentinel species (Viarengo et al., 1997; Cajaraville et al., 2000; Viarengo et al., 2000a,c; ICES, 2004). Biomarkers, for example, mussels *Mytilus* spp., are early warning biological tools able to detect prepathological changes or disturbances as responses to environmental pollutants at the cellular and organism levels (Moore, 1985; Amiard et al., 1986; Viarengo et al., 1990; Lionetto et al., 2003; Regoli et al., 2004; Gravato et al., 2005).

The increase in the human population (more than 50% of Albania's population inhabits the littoral zone), and the absence of urban and/or industrial sewage treatment plants have turned the

coastal marine environment into a prime recipient of several forms of pollution.

The present paper describes the monitoring of the biological effects of pollution in living cells by the Neutral Red Retention Time (NRRT) method (general stress), in mussels *Mytilus galloprovincialis*, the most frequently used sentinel organism in Mediterranean marine environmental biomonitoring programmes. As filter feeders, these animals have the capacity to accumulate organic and inorganic xenobiotics present in their environment (Jernelov, 1996).

Lysosomes are subcellular organelles containing hydrolytic enzymes capable of pro cessing damaged or redundant cellular components. They are also able to accumulate and detoxify a wide range of toxic metals and organic pollutants, capable of damaging cells (Moore, 1985; Viarengo et al., 1987). However, the uptake of toxic compounds can affect lysosomal membrane integrity, which may cause lysosomal contents to leak into the cytoplasm. Changes to the permeability of the lysosomal membrane caused by several environmental pollutants can be monitored in vitro by using the NRRT assay (Lowe & Pipe, 1994; Lowe et al., 1995b; Ringwood et al., 1998; Dailianis et al., 2003; Harding et al., 2004; Koukouzika & Dimitriadis, 2005). In an unstressed state, lysosomes will accumulate and retain the cationic neutral red dye for an extended period of time. However, following a stressor, the destabilized lysosomes will coalesce to form larger lysosomal structures and the neutral red dye will leak into the cytosol of the cell across damaged membranes (Moore, 1980; Lowe et al., 1995a). The NRR in mussel haemocytes is one of the most widely recommended biomarkers in marine biomonitoring programmes.

MATERIAL AND METHODS

Samples taking of mollusks and water

Starting from pollution indicators with marine bio-toxin, heavy metals and chlorine-organic insecticide monitored by the definition and the evaluation project of organic and inorganic pollutants in the aquatic fauna of the Adriatic coast, and from some indicators of pollution by heavy metals of bivalve mollusks and sea waters of the Adriatic performed in the framework of the project MED-WET, transection Vjosa-Seman was selected for the implementation and evaluation of the destabilization experiment of the webbed lysosome in the target type of *Mytilus galloprovincialis.*

For this purpose, samples of mussels *Mytilus galloprovincialis* in the process of natural increase were taken by the transect Vjosa-Semani respectively in the distances of 50, 100 and 200 m from the shore. Champions were carried out twice in the period of April-May 2007 and twice in the period of August-September 2007.

Samples by 3-point of samplings were totally withdrawn according to respective transects, by using respective GPS surveys carried out by the Zoo-prophylactic Institute of Teramos, Italy.

The number of samples analyzed was 18. For the elaboration of mussel samples elastic gloves and knives were used. After the mussel had been taken they were placed in plastic pails and were held and ventilated, until they were sent to the laboratory.

In all samples the water temperature, the saline, pH, the date and relevant GPS coordinates have been marked.

Dissection and tissue processing of samples

Usually the tissue processing of mollusks is performed on a cold surface. In our case we have used a Petri plate filled with ice. We have conducted shells' measurements of length and height and their careful opening.

Then we have conducted a subjective assessment with four levels of gonads maturity:

1. Gonads are not noticed.

2. Gonads are present and lie on a small part of hepatopancreas.

3. Great development of gonads which cover most of the hepatopancreas.

4. Great development of gonads which completely cover hepatopancreas.

Dissection of the dissolvent gland (hepatopancreas) was performed foreign external tissues were cut and disjointed carefully.

A small quantity (approximately 0.02 g) of hepatopancreas was isolated carefully by means of a trowel it was cleaned with physiological solution and immediately shifted to examination with the test of destabilization of the lysosome membrane.

Homogenization of samples

In the process of sample homogenization of *Mytilus galloprovincialis*, we used a glass cankerous AZT-300W, which provides a good homogenization of the muscular case of bivalve mollusk.

Base materials and chemicals

Needed solution: Physiological solution without ion Ca and Mg (containing 20 mM HEPES, 360 mM NaCl, 12.5 mM KCl and 5 mM EDTA). Tripsin (1.0 mg tripsin was added to 1.0 ml physiological solution and freezing and melting were performed within a session). Neutral red solution (basic solution was prepared adding 4 mg red neutral powder to 1 ml DMSO. Then stock solution was prepared by adding 20 ml from the basic solution to 1.98 ml CMSF and 10 ml to the stock solution).

Test procedure

1. The length and height measuring of the mussel, the definition of gonads index were performed, the solvent gland was cut and separated.
2. The solvent gland tissue was rinsed out by physiological solution, it was cut into small pieces, and it was rinsed again and was passed to cells' cultures plates.
3. The samples were shaken in a magnetic wave with 120 frequency nutation in a minute.
4. 400 ml of dissolved tripsin was added to the physiological solution in each sample, the composite was mixed for 20 minutes and then it was kept in cold.
5. It was treated in microcentrifugal filter apparatus for 5 minutes.
6. Cells were passed in suspension in 1 ml physiological solution.
7. There were performed two irrigations and centrifugal in 200–220 g for 5 minutes at 15 ^{o}C.
8. Stock solution in report of 1:1 was added to the sample. Mixing and incubation were performed in a humid and dark glass room for 60 minutes.
9. By means of an optical microscope with 40× lens hepatocites were accounted by classifying them as cells in the presence of color in lysosome and citosol.
10. They were calculated, in % to the total, cells with destabilized lysosomes.

Control accuracy procedures and quality control of laboratory analysis.

To confirm the validity of hepatocyte counting microscopic pictures of stable and destabilized cells were performed. There were performed two successive countings for the same area and the average was determined.

RESULTS AND DISCUSSION

In the following tables data on the degree of instability of the webbed lysosomes expressed as retentions time (min), compared with respective values of environmental pollutants as well as physical-chemical indicators of water at the monitoring points.

What's noticeable from Table 1 is the review of data with a very low retention of the red neutral dipper in the level of lysosome membrane to the withdrawn samples in distance of 50 m from the shore. The determined time of 46 minutes of retention of the red neutral color to the samples *Mytilis galloprovincialis* for this distance from the shore, expresses in essence a high degree of lysosome's membrane damage and is an indirect indicator of their poor health state, but also of the ecosystem aggravation of their natural growth.

The very high levels of too high sea biotocsins PSP (saxitoxin and derivatives) are very impressive which have captured values of 395 μg/100 g mollusk from 40 μg/100 g which is regarded as the maximum limit allowed.

One sample of mollusk and a water sample withdrawn in humidity of 50 m distance from the coast have been sent for analysis at the Research Institute for Fish in Cuxhaven, in which the high concentration of sea bio-tocsins PSP and toxic phytoplankton were confirmed, mainly with high presence of the Alexandrium tamarense type.

High concentration of toxic phytoplankton during this period is due to the favorable climatic conditions and physical-chemical indicators of the sea water expressed in Table 2. Relatively low percentage of wasted oxygen is noticed in this period in comparison with the other periods of monitoring. In Table 2 data on the stability of lysosome membranes of mussel *Mytilus galloprovincialis* are presented for the second period of sampling (May 2007).

Table 1

The first samples, April 2007

Mytilus galloprovincilalis	Distance from the coast (m)		
	50	100	200
Retention time (in minutes)	46	113	117
Hg (μg/kg)	1.225	0,20	0,07
Cr (μg/kg)	0.123	0.130	0,113
Saxitoxin and derivations (μg/100 g)	395	38	–
Cs^{134} and Cs^{137} (Bq/kg)	310	198	108

Station number.	Bottle number.	*T* °C	pH	Krip. 0/00	O_2 %	Kol	Width dms east	Length dms north
1	1	14.7	8.15	27	74.1	norm	20 01 33	39 45 09
2	2	14.6	8.25	28.2	76.1	norm	20.02.15	39.45.10
3	3	14.1	8.23	26.8	80.7	norm	20.00.09	39.46.09
4	4	14.1	7.41	21.1	81.4	norm	20.01.08	39.47.02

Table 2

The secodnd samples, May 2007

Mytilus galloprovincilalis	Distance from the coast (m)		
	50	100	200
Retention time (in minutes)	54	113	116
Hg (μg/kg)	1.123	0.17	0.07
Cr (μg/kg)	0.096	0.115	0.113
Saxitoxin and derivations (μg/100 g)	43	n.d.	n.d.
Cs^{134} and Cs^{137} (Bq/kg)	338	100	110

Station number	Bottle number	*T* °C	pH	Krip. 0/00	O_2 %	Kol	Width dms east	Length dms north
1	1	16.7	8.14	28.22	82.1	norm	20 01 33	39 46 23
2	2	16.2	8.23	27.72	84.1	norm	20.02.24	39.45.19
3	3	16.1	8.16	28.81	87.7	norm	20.01.08	39.45.28
4	4	15.7	8.08	27.22	86.4	norm	20.01.08	39.45.10

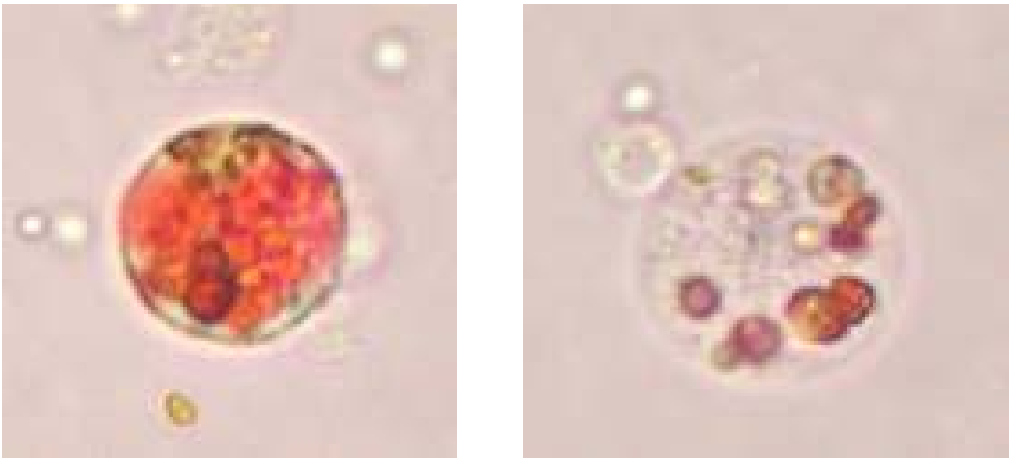
From the table data it is noticeable that in the distance of 50 m from the coast a low retention of the red neutral dipper continues to be preserved. In essence it expresses a pronounced damage of membranes and koleron significantly even with the respective values of residuals in heavy metals for this distance from the coast of sampling.

What is worth mentioning from the data from this table is that in this period of monitoring, the level of PSP toxin respectively saxitoxin and derivates to the mussel *Mytilus galloprovincialis* greatly descends. This should be linked with the reduction of concentration of the toxic phytoplankton in the sea water, as a result of changes in physical-chemical indicators expressed in the overview of the data which belong to this period

In the Pcture 1, the impact of pollutants is clearly indicated in the impenetrable membrane of

lysosomes to the hemocytes of mussels *Mytilus galloprovincialis*. In the first microscopic view it is noticed the dipper "neutral red" to the membranes of lysosomes in hemocytes. There are obvious large sizes of lysosomes in the composition of hemocytes, which have fixed the color.

In the second microscopic field a widespread destruction of lysosomes membrane in the hemocytes is noticed. This deterioration of lysosome membrane is easily identifiable by the massive liberation of the dipper by the membranes in cytoplasm. The dipper reddens all citozolin of the hepatocyte.



Stabilized Destabilized

Picture 1. Fixing and the liberation of the dipper "neutral red" by the webbed lysosome of hepatocyte of the bivalve mollusk *Mytilus galloprovincialis*

Table 3

The third samples, August 2007

Mytilus galloprovincilais	Distance from the coast (m)		
	50	100	200
Retention time (in minutes)	60	115	117
Hg (μg/kg)	0.977	0.10	0.08
Cr (μg/kg)	0.044	0.101	0.084
Saxitoxin and derivations (μg/100 g)	n.d	n.d.	n.d.
Cs^{134} and Cs^{137} (Bq/kg)	213	112	124

Station number	Bottle number	*T* °C	pH	Krip. 0/00	O_2 %	Kol	Width dms east	Length dms north
1	1	20.7	8.07	28.22	84.1	norm	20 02 26	38 48 24
2	2	19.6	8.01	28.26	83.3	norm	20.01.33	39.42.21
3	3	20.1	8.01	28.61	85.7	norm	20.01.09	39.45.29
4	4	20.1	8.18	27.53	84.42	norm	20.01.09	39.45.12

Even during the third sampling of mussel samples, a tendency was shown for high retention for the neutral red, which indicated a high degree of damage for the membranes of hepatocytes of *Mytilus galloprovincialis*. There is no significant change in the levels of heavy metals compared to the first two monitorings. During this period there were not noticed in mussels concentrations of the sea toxin PSP.

Table 4

The fourth samples, September 2007

Mytilus galloprovincilais	Distance from the coast (m)		
	50	100	200
Retention time (in minutes)	58	112	118
Hg (μg/kg)	1.075	0.15	0,10
Cr (μg/kg)	0.093	0.102	0,093
Saxitoxin and derivations (μg/100 g)	n.d	n.d.	n.d.
Cs^{134} and Cs^{137} (Bq/kg)	193	110	107

Station number	Bottle number	*T* °C	pH	Krip. 0/00	O_2 %	Kol	Width dms east	Length dms north
1	1	20.1	8.04	27.94	86.17	norm	20 01 43	38 12 32
2	2	21.6	8.06	28.06	87.30	norm	20.02.16	39.33.20
3	3	21.4	8.04	28.01	87.70	norm	20.01.32	39.32.20
4	4	19.5	8.10	27.64	87.42	norm	20.01.76	39.31.20

CONCLUSIONS

Damage indicators of lysosome and hemocytes membranes *Mytilus galloprovincialis*, expressed in the retention of the neutral dipper, correlate significantly with the monitored respective of pollution with heavy metals Hg and Cr.

To the selected type of bivalve mollusks *Mytilus galloprovincialis*, in the points of sampling of transect Vjosa Semani, damages were noticed expressed in the level of hemocytes which expresses a high degree of biological stress. The concentration of marine biotoxins PSP (saxitoxin and derivates) in the kind *Mytilus galloprovincialis* is of the seasonal character and is significantly influenced by the water temperature and the dissolved oxygen.

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