



## MONITORING OF TOXIC RESIDUES IN BIVALVE MOLLUSCS ALONG THE ADRIATIC COASTAL LINE OF ALBANIA

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### SYNOPSIS

#### Key words:

PSP biotoxins, lysosome, membrane destabilization test.

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*. Data on the level of contamination in Albanian seashore with PSP biotoxins (saxitoxin and derivatives) as well as heavy metals Hg and Cr are given. Lysosome membrane destabilization test has been the method of choice in the present study.

### 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, 2000b; ICES, 2004). Biomarkers, for example, mussels *Mytilus* spp., are early warning biological tools able to detect pre-pathological 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 processing 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 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 MEDWET, it was selected transect Vjosa-Seman for the implementation and evaluation of the destabilization experiment of webbed lysosome in the target type of *Mytilus galloprovincialis*.

For this purpose, samples of mussels *Mytilus galloprovincialis* in process of natural increase were taken by the transect Vjosa-Semani respectively in the distances 50, 100 and 200 m from the shore. Championships were carried out twice in the period April-May 2009 and twice in the period August-September 2009.

Totally were withdrawn samples by 3-point of samplings according to respective transects, by using respective GPS surveys carried out by the Zoophylactic Institute of Teramos, Italy. The number of samples analyzed was 18. For the elaboration of mussel samples were used elastic gloves and knives. After the mussel was taken they were placed in plastic pails and were held and ventilated,

until they were sent to the laboratory. In all samples it has been marked the prelevation point, the water temperature, the saline, pH, the date and relevant GPS coordinates.

**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 of ice. We have conducted shell's 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.

It was performed the dissection of the dissolvent gland (hepatopancreas), foreign external tissues were cut and disjoint 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 muscular case of bivalve mollusk.

**BASE MATERIALS AND CHEMICALS. Needed Solution:**

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

**TEST PROCEDURE:**

1. It was performed the length and height measuring of the mussel, the definition of gonads index, it was cut and separated the solvent gland.  
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 cell's cultures plates.  
3. The samples were shaken in a magnetic wave with 120 frequency nutation in minute.  
4. It was Added 400 ml of dissolved trypsin in physiological solution in each sample, the composite was mixed for 20 minutes and then it was kept in cold.

5. It was treated in micro- centrifugal 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 °C.
8. To the sample it was added stock solution in report 1:1. It was performed the mixing and incubation in a humid and dark glass room for 60 minutes.
9. By means of a optical microscope with 40X lens there were accounted the hepatocytes 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 lizosomes. Control accuracy procedures and quality control of laboratory analysis. To confirm the validity of hepatocyte counting it were performed microscopic pictures of stable and destabilized cells. There were performed two successive counting for the same area and was determined the average.

## RESULTS AND DISCUSSION

In tables 1-4, data appear 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.

**Table 1: The first samples, April 2009.**

<i>Mytilus galloprovincialis</i>	Distance 50 m from the coast	Distance 100 m from the coast	Distance 200 m from the coast
Retention time (in minutes)	46	113	117
Hg (ug/kg)	1,225	0,20	0,07
Cr (ug/kg)	0,123	0,130	0,113
Saxitoxin and derivations (ug/100g)	395	38	-
Cs-134 and Cs-137 (Bq/kg)	310	108	108

**Table 1A:**

Station no.	Bottle no.	ToC	pH	Salinity 0/00	O <sub>2</sub> %	Colour	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

What's noticeable from table 1, is a very low retention of the red neutral dipper in the level of lysosome membrane to the withdrawn samples in distance 50 m from the shore.

The determined time of 46 minutes of retention of red neutral color to the samples *Mytilus 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.

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

High concentration of toxic phytoplankton during this period is owed by favorable climatic conditions and physical-chemical indicators of sea water expressed in the following table. It is noticed the relatively low percentage of wasted oxygen in this period in comparison with the other periods of monitoring.

In the table 2. data on the stability of lysosome membranes of mussel *Mytilus galloprovincialis* for the second period of sampling May 2009 are presented. From the table it is noticeable that in the distance 50 m from the coast continues to be preserved a low retention of the red neutral dipper. In essence it expresses a pronounced damage of membranes and is connected significantly even with the respective values of residuals in heavy metals for this distance from the coast of sampling.

**Table 2: Second samples, May 2009.**

<i>Mytilus galloprovincialis</i>	Distance 50 m From the coast	Distance 100 m From the coast	Distance 200 m From the coast
Retention time (in minutes)	54	113	116
Hg (ug/kg)	1,123	0,17	0,07
Cr (ug/kg)	0,096	0,115	0,113
Saxitoxin and derivations (ug/ 100g)	43	n.d.	n.d.
Cs-134 andCs-137 (Bq/kg)	338	100	110

**Table 2A:**

Station no.	Bottle no.	ToC	pH	Salinity 0/00	O <sub>2</sub> %	Colour	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

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

**Table 3: Third samples, August 2009.**

<i>Mytilus galloprovincialis</i>	Distance 50 m From the coast	Distance 100 m From the coast	Distance 200 m From the coast
Retention time (in minutes)	60	115	117
Hg (ug/kg)	0,977	0,10	0,08
Cr (ug/kg)	0,044	0,101	0,084
Saxitoxin and derivations (ug/ 100g)	n.d	n.d.	n.d.
Cs-134 andCs-137 (Bq/kg)	213	112	124

**Table 3A:**

Station no.	Bottle no.	ToC	pH	Salinity 0/00	O <sub>2</sub> %	Colour	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, it was shown a tendency 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 monitoring. During this period there weren't noticed in mussels' concentrations of sea toxin PSP.

**Table 4: Fourth sample, September 2009.**

<i>Mytilus galloprovincialis</i>	Distance 50 m From the coast	Distance 100 m From the coast	Distance 200 m From the coast
Retention time (in minutes)	58	112	118
Hg (ug/kg)	1,075	0,15	0,10
Cr (ug/kg)	0,093	0,102	0,093
Saxitoxin and derivations (ug/ 100g)	n.d	n.d.	n.d.
Cs-134 andCs-137 (Bq/kg)	193	110	107

Table 4A:

Station no.	Bottle no.	ToC	pH	Salinity 0/00	O <sub>2</sub> %	Colour	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 retention of neutral dipper, correlate significantly with 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, were noticed damages expressed in the level of hemocytes which expresses a high degree of biological stress. The concentration of marine biotoxins PSP (saxitoxin and derivate) in the kind *Mytilus galloprovincialis* is of the seasonal character and is significantly influenced by water temperature and dissolved oxygen.

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