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Evaluation of marine water quality along North-Eastern Adriatic coast based on lysosomal membrane stability in mussels *Mytilus galloprovincialis* Lam. - A long term study

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The marine water quality along the north-eastern part of Adriatic coast was assessed by measuring the lysosomal membrane stability in the digestive gland of marine mussel Mytilus galloprovincialis*, as a marker of general stress. Mussels were sampled seasonally at five sites (mariculture Lim, Pula and Rijeka harbours, industrialized site Bakar and reference site Brestova) in the period from March 2002 to October 2007. Mussels from mariculture site Lim and reference site Brestova generally displayed good health conditions. The highest level of general stress in mussels was mostly evident at sites in the inner part of Pula and Rijeka harbours and to a lesser extent at industrialized site Bakar. Temporal changes revealed improvement trends of the mussels' health status at site Lim. The evaluation of lysosomal membrane stability confirmed to be a useful tool for discrimination of stressed from non-stressed marine environments and revealed long - term trends during the assessment of marine water quality.*

Key words: Adriatic Sea, biomonitoring, mussels, lysosomal membrane stability, trends

INTRODUCTION

The ever-increasing anthropogenic pressure along the coastal line results in constant input of wide range of chemical compounds that influence the health of marine environment. These compounds accumulate in the biota and have the potential to induce changes at molecular, cellular and tissue levels thus providing early warning signals of events that might occur at population, community and ecosystem levels (LIVINGSTONE, 1993). The changes at lower level of biological organization are known as biomarkers that indicate the biological effect of pollutants and are widely employed for the assessment of environmental health. Biomarkers are routinely measured in appropriate sentinel species, such as bivalve mussels. Bivalves gained increasing importance for monitoring the quality of coastal waters due to the ability to effectively accumulate various pollutants by filtering large amount of sea water, abundance in coastal and estuarine areas, adaptation to a variety of environmental conditions, wide distribution and ease of collection and maintaining in the laboratory conditions (WIDDOWS & DONKIN, 1992).

Development of biomarkers based on lysosomal responses in digestive gland of mussels has attracted much attention over the last two decades. Lysosomes of digestive tissue are subcellular organelles involved in sequestration, accumulation and detoxification of inorganic and organic compounds (MOORE, 1985; VIARENGO *et al.*, 1987). The integrity of lysosomal membrane upon exposure to a variety of environmental contaminants and chemical compounds is rapidly and significantly reduced and results in the leakage of hydrolytic enzymes into the cytosol. Thus, the method for evaluation of lysosomal membrane stability, based on measuring the activity of lysosomal enzyme N-acetyl-β-hexosaminidase (EC 3.2.1.52) in the digestive gland has been considered as the most sensitive and reliable non-specific marker of general stress in mussels. This fast and low-cost method has been widely employed for the assessment of adverse biological effects of pollution in marine environment (PYTHAROPOULOU *et al.*, 2006; ZORITA *et al.*, 2007) and included in a suite of "core biomarkers" in the Mediterranean Action Plan of the United Nations Environment Program (UNEP/RAMOGE, 1999). Recently, the method for evaluation of lysosomal membrane stability has been proposed for initial tier-1 screening of marine coastal zones in order to detect the development of stress (VIARENGO *et al.*, 2007).

The present study was conducted in the north eastern part of the Adriatic coast, virtually a semi-enclosed bay characterised by highly urbanized and industrialized areas, intensive maritime transport, tourism, fishing and aquaculture activities. Chemical analysis that has been conducted for many years in the sediment, water column as well as in biota revealed elevated concentrations of various types of pollutants indicating significant anthropogenic pressure mainly in the close vicinity to harbours and densely populated zones (UNEP/WHO, 1994, 1999; NATIONAL MONITORING PROGRAMME CROATIA, 2008). The aim of this work was to synthesise the data on lysosomal membrane stability measured in indigenous populations of mussels from five sites over seven years of sampling, to provide information on general stress caused by the effect of pollutants along the north eastern Adriatic coast.

MATERIAL AND METHODS

The analysis of lysosomal membrane stability (LMS) was conducted in the frame of the ongoing National Monitoring Programme Croatia that started in 1998. LMS was determined in digestive gland of mussels collected from natural populations at five sites along the north-eastern Adriatic coast (Fig.1). Samples were collected seasonally (April, June, August and October) in the period from year 2002 to 2007.

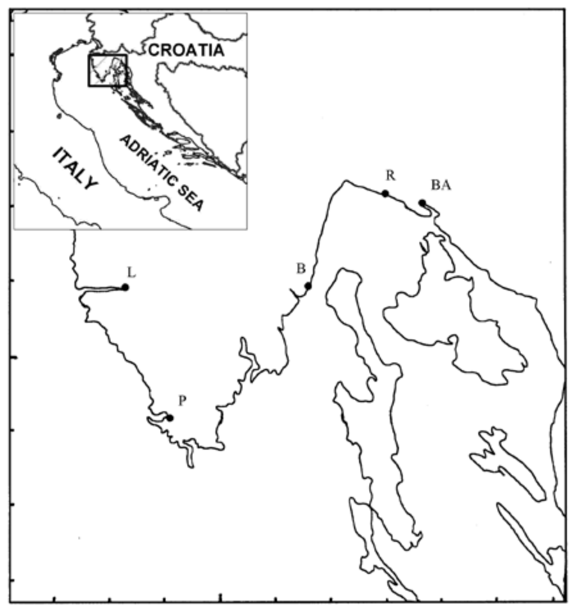

Fig.1. Map of northern Adriatic coast showing the sites of collection. L-Lim Bay, P-Pula, B-Brestova, R-Rijeka and BA-Bakar

Description of sampling sites

The sampling site L (45º08' N, 13º44' E) was positioned in the inner part of fjord-like Lim Bay, a protected area known for mariculture production. Sampling sites P (44º53' N, 13º51' E) and R (45º20', 14º26' E) in the inner part of Pula (P) and Rijeka (R) harbours, respectively, were both located in the close vicinity of highly urbanised areas and industrial facilities, and were exposed to a mixture of pollutants. Site B (45º09' N, 14º14' E) was located near Brestova fishermen settlement, but relatively distant from sources of pollutants. Site B was considered as a reference in accordance with the previous studies (PETROVIĆ *et al.*, 2004) and reports of chemical data (NATIONAL MONITORING PROGRAMME CROATIA, 2008). Sampling site Ba (45º18' N, 14º33' E) was located within a semi-enclosed bay in the vicinity to a former coke-plant that operated in the period from 1976 to 1995, and remediation of the contaminated land has been carried out in the course of the last two decades. This area also receives effluents from town of Bakar.

The overall 6-year temperature pattern was similar at all sites with the lowest values in March ranging from 11.4ºC (B) to 12.9ºC (L) and the highest in August ranging from 19.7ºC (R) to 24 ºC (L). At site P and B, the salinity of above 34 PSU was recorded throughout the whole sampling period. The lowest salinity was detected at site R, ranging from 7.3 PSU in June to 18.5 PSU in October. At site Ba, the salinity varied between 18.4 PSU in March to 26 PSU in June. Low salinity was also observed at site L in March (15.4 PSU) due to influx of fresh water from underwater springs.

Determination of lysosomal membrane stability

LMS was determined according to the cytochemical method of MOORE (1976) in the cryostat sections. Briefly, following sample collection, pieces of digestive gland were excised from five mussels, immediately frozen in cooled n-hexane (-80ºC) and stored. Prior to sectioning, pieces of digestive gland were placed on a chuck, and cut in a cryostat at a cabinet temperature of -30ºC. Multiple cryostat sections of digestive gland were pre-incubated in 0.1 M citrate buffer, pH 4.5 for different times (0, 2, 5, 10, 15, 20, 30 and 40 min) to destabilise the lysosomal membrane. The slides were then submitted to reacting solution containing the substrate AS – BI N-acetyl β-D-glucosaminide for 20 min. Following incubation, the slides were rinsed, stained with Fast Violet B in 0.1 M phosphate buffer and mounted with glycerol gelatine.

The determination of lysosomal membrane stability was based on acid labilization period (LP) necessary to produce the maximum staining intensity of marker enzyme N-acetyl β-D hexosaminidase reaction product (MOORE, 1976). This was assessed under a light microscope (Nikon Microphot-SA) connected to colour CCD camera (Ikegami ICD-803P). Image analysis was performed using image analysis system Lucia 4.60. Values of LP above 20 minutes indicate high lysosomal membrane stability and good health of mussels, whereas LP values from 10 - 20 min and below 10 minutes indicate stressed and severely stressed conditions, respectively (VIARENGO *et al.*, 2007).

Data analysis

Mean values of lysosomal membrane labilization period (LP) were used to determine the significant differences between sites by non-parametric Kruskal-Wallis one-way analysis, followed by non – parametric Mann-Whitney test. A value of $p<0.05$ was considered significant. Spearman's correlation coefficient was calculated to identify the relation between lysosomal membrane LP and environmental parameters.

The cumulative sum of residuals (CUSUM) was performed to establish a trend of general stress over seven years (EPA, 1989). Mean monthly values of lysosomal membrane LP were used for calculation of cumulative sums (S) according to the following equation:

$S_i = S_{i-1} + (\chi_i - \chi)$

Where $i = 1, 2, 3...7$ and $S_0 = 0$.

Positive, flat or negative slope of the CUSUM plot indicates periods when the values of lysosomal membrane LP tend to be respectively higher, similar or lower than the average.

RESULTS

The values of LP for year 2001 were extracted from PETROVIĆ *et al.* (2004) and added to values obtained in the present study from March 2002 to October 2007, to analyse the lysosomal membrane stability for 7-years period from March 2001 to October 2007.

The values of acid labilization period (LP) for each site and all seasons are given in Fig 2. Mussels collected at reference site B displayed the highest values (mostly above 20 min) throughout the whole sampling period with exception of August and October 2002,

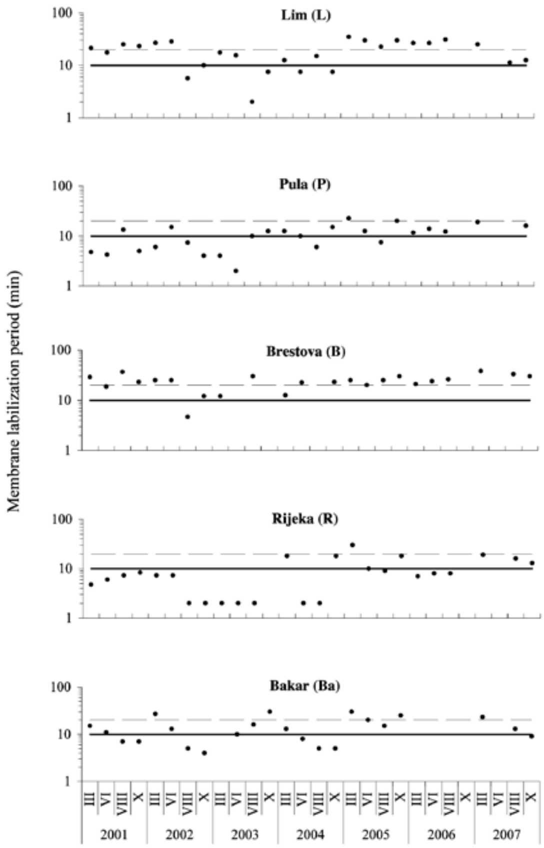

Fig. 2. Lysosomal membrane stability (min) in mussels collected at sites Lim, Pula, Brestova, Rijeka and Bakar from March 2001 to October 2007. Values below solid line (<10 min), between solid and dashed line (10 – 20 min) and above dashed line (>20 min) correspond to severely stressed, stressed and healthy conditions, respectively

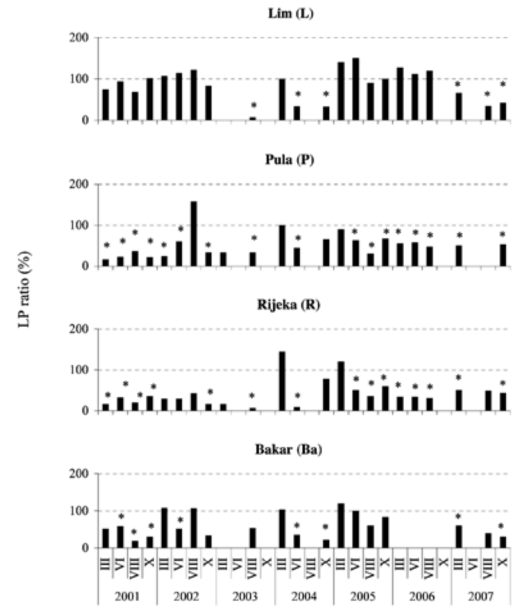

Fig. 3. Lysosomal membrane stability in the mussels sampled from March 2001 to October 2007. The results are expressed as ratio between LP of mussels at reference site B and other sites (L, P, R and Ba). Asterisk above bars indicate significantly lower lysosomal membrane stability with respect to the reference site (p<0.05)*

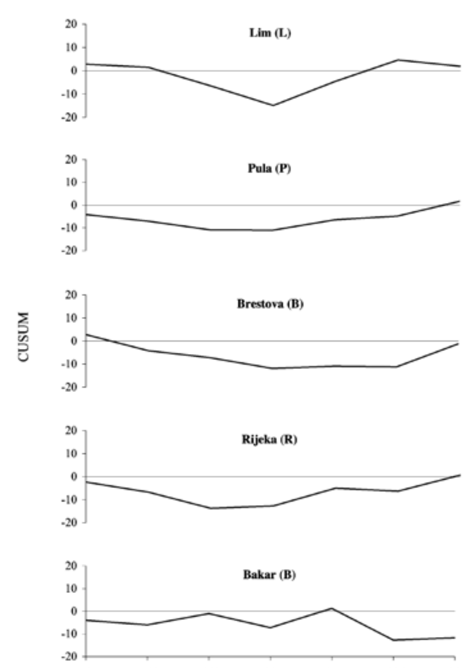

Fig. 4. Trends in lysosomal membrane stability expressed as cumulative sums (CUSUM). Data corresponding to year 2001 was extracted from PETROVIĆ et al. *(2004)*

March 2003 and 2004 when reduced LP (5 - 12 min) was recorded. Most of the values obtained at mariculture site L ranged between 10 and 30 minutes. Reduced values of LP (< 10 min) were occasionally observed from August 2002 to October 2004. At site in harbour P the lowest values of LP (below 10 min) were detected throughout the first three years of sampling. Throughout the rest of the investigation period, LP ranged between 10 – 20 minutes, with exception of August 2004 and 2005 when values below 10 min were found. Site in harbour R displayed LP values that were almost regularly lower than 10 minutes, with minimum values (2 min) observed in the period from August 2002 to August 2004. Values close to or higher than 20 min were found only occasionally (March 2004, 2005 and 2007, August 2007, October 2004 and 2005). The most notable oscillations of LP were found at industrialized site Ba with peaks of LP values above 20 min observed mostly in March and October. Minimum values (5 min and below) were detected in August and October 2002 and 2004 (5 minutes). No correlations were found between LP values and temperature or salinity (data not shown).

In over half of the samples from harbours Pula (P) and Rijeka (R), the lysosomal membrane stability was significantly reduced with respect to reference site B for corresponding season and year of sampling (Fig. 3). At industrialized site Ba, significant difference from reference site B was found in June, August and October '01, June 2002, June and October 2004 and March and October 2007. Mariculture site L was significantly different than reference in August 2003, June and October 2004 and March, August and October 2007.

The trend analysis revealed that for almost the whole investigation period the values for lysosomal membrane stability were relatively steady below average at most of the sites (Fig. 4). The positive values of the slope were detected only at mariculture site L at the beginning and the end of investigated period. Sharp decline of the slope at this site was observed only in 2004.

DISCUSSION

The present investigation revealed site-specific year-to-year oscillations of lysosomal membrane labilisation period (LP) in mussels that reflect a variable long term trend in the pollution input along the investigated area.

The most obvious prevalence of LP values above 20 min observed at reference site B that was in accordance with the previous field study (PETROVIĆ *et al.*, 2001; 2004) indicate overall high lysosomal membrane stability and good health conditions, typically observed at less polluted marine coastal areas (Table 1). Stressed health conditions of mussels detected occasionally in the period from 2002 to 2004 could be related to transient anthropogenic or environmental stress. The trend analysis confirm generally stabile marine water quality at site B.

Lysosomal membrane stability above 20 min displayed by mussels from mariculture site L was also determined in previous field studies (PETROVIĆ *et al.*, 2001; 2004). Slightly elevated incidence of stressed and severely stressed mussels in comparison to reference site B indicates possible influence of aquaculture and occasional boating activities. It could also reflect higher susceptibility to environmental stress due to the slow water circulation in the narrow and shallow fjord-like bay. This is most notable in the period from 2002 to 2004, which is followed by a period of clearly improving trend of marine water quality.

Harbours P and R displayed low values of LP over the whole period of investigation that fall within the range characteristic for polluted harbours along the Mediterranean and Atlantic coast (Table 1) and are in accordance with the results of the previous field study (PETROVIĆ *et al.,* 2001; 2004). The consistently reduced LP values found at sites in harbours P and particularly R indicate low degree of lysosomal membrane stability due to the stressed and severely stressed health conditions of mussels in relation to the long-term trend in the accumulation of chemical compounds such as previously detected chlorinated hydrocarbons and heavy metals (NATIONAL MONITORING PROGRAMME CROATIA, 2008; LIPANOVIĆ LANDEKA, 2010) in the tissues of mussels and sediment. These compounds were found to be related to the reduction of lysosomal membrane stability in the digestive gland of mussels sampled from polluted coastal sites (Table 1). The input of environmental pollutants of industrial and urban origin capable to induce stress in indigenous mussels from above sites was most evident from year 2001 to 2004. The trend analysis indicated no improvement of marine environmental quality at both sites in harbours.

Although high content of chlorinated hydrocarbons and heavy metals was also found in mussels and sediment at industrialized site Ba

Table 1. Overview of lysosomal membrane stability in digestive gland of mussels Mytilus galloprovincialis *Lam. from some European coastal zones*

LMS (min)	Sampling sites with short description	References
> 20 min (Healthy)	Kuagerdi (Bay of Osar, Iceland) – pollutant free Cala Montjoy (Spain) – pollutant free Strymonikos Gulf (Greece) – aquaculture	DA ROS *et al.*, 2007 ZORITA *et al.*, 2007 DOMOUHTSIDOU *et al.*, 2004 RAFTOPOULOU & DIMITRIADIS, 2010
10-20 min (Stressed)	Cortiou (France) – sewage effluents Fangar, Alfacs (Spain) – agriculture, aquaculture Portofino (Italy) –reserve park Dock (Reykjavik harbour, Iceland) – intensive shipping activity Gulf of Corinth – pollutant free Baccarini channel (Italy) – heavy metal input Gulf of Thermaikos (Greece) – heavy metal and organic pollutants Førlandsfjorden (Norway) – small boat traffic	ZORITA *et al.*, 2007 DA ROS *et al.*, 2007 PYTHAROPOULOU *et al.*, 2006 DONNINI *et al.*, 2007 DOMOUHTSIDOU *et al.*, 2004 AARAB *et al.*, 2008
< 10 min (Severely stressed)	Lavera (France), Voltri (Italy) – moderate industrial input, oil refineries and tankers Fos (France), Genova (Italy), Barcelona (Spain) – harbours, heavy metal, PAHs and PCB input Glafkos River estuary (Greece) – agriculture, industrial effluents Patras area (Greece) – factory of cement production Baccarini channel (Italy) – heavy metal, PAHs and PCB input, temperature fluctuations Gulf of Thermaikos (Greece) – outlet tube, heavy metal and organic pollutants Høgevarde (Norway) – discharge from alumina smelter (PAH contamination)	ZORITA *et al.*, 2007 PYTHAROPOULOU *et al.*, 2006 DONNINI *et al.*, 2007 DOMOUHTSIDOU *et al.*, 2004 RAFTOPOULOU & DIMITRIADIS, 2010 AARAB *et al.*, 2008

(NATIONAL MONITORING PROGRAMME CROATIA, 2008; ŽIVKOVIĆ, 2010), located in the vicinity of a former coke plant, slightly lower incidence of severely stressed (LP<10 min) and higher incidence of healthy mussels (LP>20 min) in comparison to harbours P and R, indicate better environmental conditions. However, no improvement trends of environmental quality could be observed which can be attributed to impact of particularly high content of PAHs in the sediment in comparison to harbours (BIHARI *et al.*, 2007).

An important feature of biomarker response in mussels is seasonal variation usually related to fluctuations in abiotic and biotic factors. It is well known that the increase of water temperature in spring is closely associated with the onset of gamete maturation and the triggering of spawning activity in mussels from northern Adriatic (HRS BRENKO, 1971). The thermal stress and consumption of energy resources for the reproductive activity affect the physiology of mussels in order to make them less able to cope with the impact of pollutants, consequently contributing to the higher degree of lysosomal membrane destabilisation, as has been previously documented (DOMOUHTSIDOU & DIMITRIADIS, 2001). In the same study, significant destabilisation of lysosomal membranes was not observed at reference site in the summer period, indicating that non-stressed mussels were more resistant to thermal stress. Relatively high lysosomal membrane integrity throughout the whole year was reported for mussels from reference sites, in good physiological condition (PETROVIĆ *et al.*, 2004). On the other hand, extreme experimental treatment, such as exposure to high water temperature, lead to destabilisation of lysosomal membrane and reduction of LP to values below 3 min (IZAGIRRE & MARIGÓMEZ, 2009) that correspond to those found in the current study particularly within mussels' populations from sites in harbours, under influence of pollutants. Thus, although no correlation with temperature was detected within the frame of this long term study it cannot be excluded that summer temperature increase could contribute to more pronounced destabilisation of lysosomal membranes.

The impact of salinity fluctuations on the stability of lysosomal membranes in digestive gland cells of mussels is less clear. However, previous laboratory experiments showed that acute exposure to low salinity could induce destabilisation of lysosomal membrane in digestive gland cells of mussels (BAYNE *et al.*, 1978; MOORE *et al.*, 1980) and snails (STICKLE *et al.*, 1985). Therefore, lower water salinity that has been recorded predominantly at sites R and Ba could be also considered as factor that might be linked to destabilisation of lysosomal system within mussels sampled at these sites.

CONCLUSIONS

The results reported in the present study confirmed the utility of lysosomal membrane stability measurement for assessment of general stress in mussels and for description of long term pollution trends in marine coastal areas influenced by anthropogenic activity. Values of lysosomal labilization period obtained at sites within industrialized and urbanized harbours generally correspond to other polluted European marine coastal zones. Moreover, the analysis of the 7-years monitoring period revealed an overall stable trend of mussels' health conditions indicating improvement of marine water quality at mariculture site Lim. Furthermore, our results indicate that mussels from sites characterised by no known source of pollutants were subjected to the transient influence of environmental or anthropogenic stress. This study also provides the baseline data on the general stress in mussels that will be useful for the ongoing long-term biomonitoring study of anthropogenic impact along the whole Eastern Adriatic coast.

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Procjena kvalitete morske vode uzduž sjeveroistočne obale Jadrana korištenjem testa stabilnosti lizosomalne membrane u dagnjama *Mytilus galloprovincialis* Lam. - dugoročna studija

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SAŽETAK

Kvaliteta morske vode duž sjeveroistočne obale Jadrana procijenjena je mjerenjem stabilnosti lizosomalne membrane probavne žlijezde dagnje *Mytilus galloprovincialis*, kao markera općeg stresa. Dagnje su sakupljane sezonski na pet postaja (marikultura Lim, luke Pula i Rijeka, Bakar u blizini industrijskog pogona i referentna postaja Brestova) u razdoblju od ožujka 2002. do listopada 2007. godine. Dagnje sa postaje Lim i referentne postaje Brestova generalno su pokazale nisku razinu stresa. Najviša razina stresa u dagnjama bila je uočena na postajama u unutarnjem dijelu pulske i riječke luke i u manjoj mjeri na postaji Bakar. Vremenske promjene pokazale su trend sniženja razine stresa kod dagnji sa postaje Lim. Procjena stabilnosti lizosomalne membrane pokazala se kao koristan alat za prepoznavanje morskog okoliša s niskom i visokom razinom stresa i omogućila prikaz dugoročnih trendova kvalitete morske vode.

Ključne riječi: Jadran, biomonitoring, dagnje, stabilnost lizosomalne membrane, trendovi