CEST 2013
Proceedings of the 13th International Conference on Environmental Science and Technology
Athens, Greece, 5-7 September 2013

LEAD ACCUMULATION AND METALLOTHIONEIN INDUCTION IN DIFFERENT TISSUES OF MUSSELS (*M. galloprovincialis*) AND CLAMS (*C. chione*) EXPOSED TO VARIOUS Pb CONCENTRATIONS

O. CHALKIADAKI[1,2], N. LYDAKIS-SIMANTIRIS[2] and M. DASSENAKIS[1]

[1] Laboratory of Environmental Chemistry, Faculty of Chemistry, National and Kapodistrian University of Athens, Panepistimioupoli Zografou, 15771, Athens, GREECE,
[2] Laboratory of Environmental Chemistry and of Biochemical Processes, Department of Natural Resources and Environment, Technological Education Institution of Crete, PO BOX 89, KDS Chania, 73135 Chania, Crete, GREECE
e-mail: *ochalkiadaki@chem.uoa.gr*

EXTENDED ABSTRACT

Heavy metal pollution of coastal areas due to anthropogenic activity has become a global problem with serious environmental consequences. Various marine organisms have been employed as bioindicators for pollution, and expanding the scientific arsenal with such organisms is a continuing research objective. In this study, the effects of Pb pollution in seawater on two bivalves of different habitats were investigated. *Mytilus galloprovincialis* (a nearshore and intertidal rocky substrate inhabitant mussel) and *Callista chione*, (a sand-buried smooth clam), were exposed to a wide range of Pb concentrations in seawater for 20 days, followed by a 10-day depuration period, in a laboratory experiment. Gills, mantles and the remaining bodies of the two species were separated and the accumulated Pb was determined. The synthesis of Pb-induced proteins (metallothioneins and/or other proteins) was also investigated in the tissues of the two species by Ellman assays, Coomasie-stained and fluorimetric SDS-PAGE experiments. Our results show that both species exhibit a relatively high tolerance to even very high Pb pollution levels and they accumulate considerable amounts of Pb in their tissues. Zinc levels in the examined tissues are affected in most of the tested conditions. The 10 days depuration period of our experiments was not enough for the complete removal of Pb from the organisms. Biochemical analysis showed that low molecular weight, sulfhydryl-containing proteins (metallothioneins), as well as proteins with molecular weight of about 50 kDa are synthesized in a species-dependent and tissue dependent way, due to Pb accumulation.

Keywords: Lead, Metallothioneins, *Mytilus galloprovincialis*, *Callista chione*, Bioindicators

1. INTRODUCTION

Lead is a chemical element in the carbon group which has no known biological function in organisms. It competes with essential metals for active enzyme or membrane protein sites. Effects of Pb on the nervous system, blood pressure, fertility in males, female reproductive systems as well as anaemia have been reported (Hsu and Guo, 2002) whereas Zn appears to have a protective effect against Pb toxicity. Lead is used in a wide variety of products including paint, ceramics, pipes, solders, gasoline, batteries, and cosmetics. Due to its toxicity, the use of Pb has been reduced or even banned in several products (i.e. as gasoline additive, paints, water pipes, etc).
Marine organisms are continuously exposed to variable concentrations of metals in the seawater, especially along coasts, influenced by anthropogenic heavy metal contamination. Due to concern over accumulation and toxic effects on marine organisms and, consequently, on humans, and in order to examine whether waterbodies support

survival and reproduction of aquatic species, biomonitoring programs have become widely established.
Mussels accumulate and tolerate high concentrations of heavy metals over time. It has been demonstrated that the concentration of heavy metals in mussel tissues can provide time-integrated measure of the environmental bioavailability of these contaminants. *Mytilus galloprovincialis,* is extensively used as a biomonitoring organism to evaluate the impact of heavy metal pollution in coastal areas. Although a large amount of research work has been published about *M. galloprovincialis* as a bioindicator organism for the assessment of heavy metal pollution in coastal environments, not so much work has been presented regarding the accumulation of heavy metals in other, commercially or ecologically important organisms. For example, publications regarding the behaviour of *Callista chione,* a very common seafood, in heavy metal contaminated environments are scarce, and to our knowledge, none of them includes lead. Moreover, there are limited data on lead accumulation in *M.galloprovincialis* in laboratory experiments.
Mussels, as many other organisms, respond to heavy metal pollution by producing protective, metal binding proteins, called metallothioneins (MTs) (Amiard *et al*., 2006). Metallothioneins are low molecular weight proteins (6-8 kDa) with high cysteine content (20-30%), no aromatic amino acids, heat stability, non-enzymatic nature and strong affinity to several metal cations. MTs play a role in the homeostatic control of essential metals (Cu, Zn) and are overexpressed in organisms experiencing high metal concentrations in their environment. The effects of several metals on the production of MTs have been studied quite extensively, however little is known about the effect of lead.
In this work we examine the sensitivity and the tolerance of *M. galloprovincialis* and *C. chione* to waterborne Pb and we compare the accumulation of lead in different tissues of the two bivalves. For this, the two marine bivalves were exposed to a wide range of Pb concentrations (0.5-20 ppm) in a laboratory experiment. Time dependence and seawater Pb concentration dependence of the metal uptake as well as the distribution in different tissues were examined. Pb-induced metallothionein synthesis in the two bivalves was also studied and conclusions are presented about the potential of using the metallothionein levels in bivalves as bioindicators for Pb pollution in coastal environments.

2. MATERIALS AND METHODS

2.1 Experimental Design-Laboratory Exposure Experiment

Mussels (*Mytilus galloprovincialis*) and soft clams (*Callista chione*) collected from Elefsis Gulf, SW of Athens. Both species were brought alive in the laboratory, where they were put in 70 L aquaria with fresh seawater (36‰±1‰ salinity). Clams were placed in a sand layer at the bottom of the aquaria. Care was taken to use animals with the same shell length (6-8 cm), since size is related with the age of the organisms.
After their arrival at the laboratory, 10 animals were dissected on ice, and the gills and mantles were removed from the remaining bodies. The three separated tissues were washed thrice in deionized water and kept in -24ºC until use. The samples produced from these organisms were designated as “Day 0”.
The organisms were acclimated to the laboratory conditions in aerated seawater for five days and then were transferred to 70L aquaria (2.5 animals/L seawater for *M. galloprovincialis* and 1 animal/L seawater for *C. chione*) with 0.50, 1.0, 2.5 and 20 ppm Pb for 20 days. Lead was added as $Pb(NO_3)_2$ from a stock solution. Half of the water volume was replaced by fresh seawater every second day. Temperature was kept at 17-18ºC. After five days of exposure at each Pb concentration, 30 animals separated to 3 replicates of 10 in order to make three composite samples, which are represented as “DAY5” in the Tables and Figures. The animals were dissected on ice and the gills, mantle, and the remaining body were seperated. This procedure was repeated at the 10^{th}, 15^{th}, and 20^{th} day of the experiment. After the 20 days period, the remaining bivalves

were transferred to fresh seawater for a 10 days depuration experiment. Then, these organisms were dissected as described above. Control animals were kept in metal-free seawater during the experiment.
Tissue samples collected as described above, were divided into two parts: one for metallothionein determination, and one for determination of Pb and Zn which was lyophilized and homogenized to fine powder. 0.3-0.5 g of the dry tissues were digested in $cHNO_3$. The digests were diluted with MilliQ water at 4°C until analysis.

2.2 Chemical Analysis

2.2.1 Seawater and Sediment Analysis

Seawater samples from Chania, Crete used in the aquaria and from the animals' harvesting sites were analyzed for soluble and total metal concentrations. Particulate heavy metals were collected by filtration through 0.45 μm pre-weighed filters, which consequently were dried to constant weight, and digested with concentrated HNO_3 and then were treated as described in Dassenakis *et al.*, 2004. After dilution, the total metal content was measured by Atomic Absorption Spectrometry (AAS). Same treatment was carried out for blank filters.
Dissolved trace metals were pre-concentrated on Chelex-100 resin columns (Scoullos and Dassenakis, 1984) and atomic absorption spectrometry was carried out.
Heavy metal background contamination was checked in the sand used in the aquaria of *Callista chione.* Six replicates were lyophilized, homogenized to fine powder and sieved through 1mm sieve. The total lead content of the sediment was determined after acid digestion with concentrated acids (HNO_3 - $HClO_4$ – HF) (ISO 14869-1:2000 method).

2.2.2 Heavy Metal Determination

Pb and Zn content in tissue fractions were determined by Flame AAS. Two measurements with < 10% difference were averaged for each one of the three repeats of the same sample. Control (unexposed samples), blanks ($cHNO_3$) and reference materials (NIST 2976 mussel tissue, PACS-2 sediment, NASS-6 seawater) were also treated and measured as described above. All glassware was acid-washed in 10% nitric acid for 24 hours before use.

2.3 Biochemical Analysis of Metallothioneins

1 g of each tissue was homogenized 4 mL of 0.3 M sucrose, 20 mM Tris-HCl (pH 7.6), 0.5 mM PMSF, 0.006 mM leupeptine and 5 mM β-mercaptoethanol at 4°C. The homogenates were centrifuged at 50,000 x g for 60 min, 4°C, the supernatants were treated with ethanol/chloroform solution, (Kimura et al., 1979, with small modifications) and MTs were precipitated by centrifugation (Pellet A). This pellet was used for Coomasie SDS-PAGE, spectrophotometric and fluorimetric assays.
For Ellman's reaction, pellet A was resuspended in 300 μL of 5 mM Tris-HCl, 1 mM EDTA pH 7.0. 2 M NaCl in 0.2 M phosphate buffer with 0.43 mM DTNB, pH 8, was added at room temperature. The metallothionein content was evaluated spectrophotometrically at 412 nm (Ellman 1958) with GSH as a reference standard. The amount of MTs was calculated assuming a 21 SH/MT with a M_r of ~7 kDa (Cotou *et al.,* 2001, Viarengo *et al.,* 1997).
For fluorimetric assay, pellet A was resuspended in 50 μL of 5 mM Tris-HCl, 1 mM EDTA pH 7.0 and treated as described in Cotou *et al*., 2001 and Viarengo *et al*., 1997 with small modifications. The reaction of bromobimane with a thiolate converts the non fluorescent agent into a fluorescent derivative. SDS- PAGE bands containing metallothioneins give fluorescent zones when exposed to UV light.

3. RESULTS AND DISCUSSION

3.1 Seawater and Sediment Analysis

Background concentrations of dissolved Pb and Zn were 0.02-0.62 μg/L, and 0.60-15 μg/L, respectively, far below the USEPA limits (8.1 μg/L and 81 μg/L for Pb and Zn, respectively, USEPA, 2009). For particulate lead, the background concentrations varied between 0.01 and 0.28 μg/L, whereas for zinc varied between 0.05 and 4.0 μg/L.
Pb and Zn concentrations in the sand samples were very low (data not shown) and, according to USEPA criteria the sand used in *Calllista chione* aquaria are in the category of the non polluted (<40 mg/kg for lead and <90 mg/kg for zinc) (Nichols *et al.*, 1991).

3.2 Lead Concentration in Different Tissues of *M. galloprovincialis* and *C. chione*

Pb and Zn concentrations in the three tissues of *M.galloprovincialis* and *C.chione* immediately after their arrival in the laboratory (Day 0) are presented in Table 1. Pb values are relatively high, whereas the background Zn in each tissue for both organisms is higher than the corresponding Pb concentration, as it is expected since Zn is essential for the function of biological molecules. Pb and Zn concentrations in gills of *M. galloprovincialis* are higher than the corresponding in *C. chione* whereas in mantle and the remaining body of the two species the concentrations of Pb and Zn are comparable.
Pb concentrations in the three tissues of *M. galloprovincialis* and *C. chione* for all the pollution levels and days of exposure are presented in Table 2. The plots of Pb concentrations in the tissues of both animals vs. the day of analysis are shown in Figure 1 for the different Pb concentrations in seawater.
Mortality was observed only for the animals exposed to the two highest Pb concentrations and it was higher for *M. galloprovincialis* than for *C. chione* (28-38% vs. 8-17%). Interestingly, dead mussels were observed at the beginning of the exposure, while dead clams were observed after the 13th day of the exposure.
In general, there was a positive trend for the accumulation of Pb in the tissues of the two bivalves vs. the days of exposure. For both animals, almost all trend lines showed a statistically significant positive trend (the correlation coefficients of the trend lines were higher than the theoretical values of 0.773 for df (n-2)=3). The accumulation of Pb in the three tissues of *M. galloprovincialis* followed the order gills > body ≥ mantle (Figure 1). Interestingly, gills accumulate Pb more effectively in 0.5 ppm Pb than in 1.0 ppm Pb in seawater. This behavior has been observed in similar studies regarding Ni as pollutant (unpublished data from our laboratory, Attig *et al.*, 2010), and it may indicate a complex mechanism of metal accumulation and subsequent detoxification processes.

Table 1: Concentrations of Pb and Zn (μg/g dry weight) in gills, mantle and the remaining body of *M. galloprovincialis* and *C. chione* at the Day 0

	M. galloprovincialis		***C.chione***	
	Zn (μg/g)	**Pb (μg/g)**	**Zn (μg/g)**	**Pb (μg/g)**
gills	200	10	59	6.7
mantle	38	1.3	33	3.3
remaining body	60	1.5	61	3.1
total	105	4.5	52	4.1

Depuration of Pb in *M. galloprovincialis* appears to be level of exposure-dependent and tissue-dependent: In gills, the depuration percentage increases with the level of exposure (from 3 to 91 %). Mantle exhibits moderate depuration at the first three levels of exposure (0-34 %) whereas at the highest level of Pb pollution there is Pb accumulation, most likely due to translocation of Pb from gills to mantle. Bodies from mussels exposed to the first

three levels of pollution exhibit either moderate depuration or even accumulation of Pb, whereas at the highest level of exposure, bodies show 27 % reduction of the accumulated Pb.

Table 2: Pb concentrations (μg/g dry weight) in gills, mantle and remaining body of *M.galloprovincialis (M.g.)* and *C.chione (C.c.)*

Pb exposure level	**Day 5**		**Day 10**		**Day 15**		**Day 20**		**depuration**	
Gills	*M.g.*	*C.c.*	*M.g.*	*C.c.*	*M.g.*	*C.c.*	*M.g.*	*C.c.*	*M.g.*	*C.c.*
Control	11	6.7	16	6.0	16	6.1	16	6.0	16	5.9
0.50 ppm	381	107	134	215	605	432	805	700	782	501
1.0 ppm	212	246	161	978	143	1945	375	2449	285	1762
2.5 ppm	332	408	1405	177	1934	997	2391	4073	1705	2982
20 ppm	3224	692	7831	531	6281	364	11578	6766	1024	4386
Mantle	*M.g.*	*C.c.*	*M.g.*	*C.c.*	*M.g.*	*C.c.*	*M.g.*	*C.c.*	*M.g.*	*C.c.*
Control	2.8	3.3	5.1	3.0	5.1	3.1	5.1	3.2	5.1	3.2
0.50 ppm	41	96	54	134	48	217	109	283	114	199
1.0 ppm	17	220	22	907	38	1176	30	1717	20	899
2.5 ppm	54	104	102	198	254	902	330	1021	261	823
20 ppm	630	226	714	335	1060	250	1068	1381	2305	759
Body	*M.g.*	*C.c.*	*M.g.*	*C.c.*	*M.g.*	*C.c.*	*M.g.*	*C.c.*	*M.g.*	*C.c.*
Control	5.0	3.1	5.0	3.2	5.0	2.9	5.0	3.1	5.0	3.1
0.50 ppm	115	53	90	85	75	120	140	151	303	122
1.0 ppm	56	619	60	660	80	666	80	626	62	592
2.5 ppm	46	143	265	159	627	149	486	700	610	371
20 ppm	229	307	804	227	1734	122	1440	260	1053	189

For *C. chione*, the accumulation in the three different tissues follows the order gills > mantle > body with the gills having the highest values. In general, higher concentrations of accumulated Pb are observed in *C. chione* tissues exposed to 1 ppm Pb in seawater compared to both higher and lower pollution levels. Especially for the body, almost saturation of the Pb accumulation is observed after the 5th day of exposure to 1 ppm Pb.
Regarding the relative accumulation of Pb in the tissues of the two species, it seems that at the lower levels of exposure, clams are better Pb accumulators, whereas at higher pollution levels more Pb accumulated in mussels' tissues.
Depuration of Pb in *C. chione* was different than in *M. galloprovincialis*: all tissues of *C. chione* exhibit depuration of Pb at a higher or lower percentage, depending on the conditions tested. It needs to be noted, however, that a considerable amount of the pollutant remains in the tissues of both animals after ten days of depuration, a result also reported by other researchers (i.e. Freitas *et al*. 2012, Hédouin *et al*., 2007) for other bivalve species.

3.3 Correlation of Zn Levels with Accumulated Pb

The potential correlation of the Zn concentrations with the levels of Pb in the tissues of the two bivalves was investigated for all the conditions used in this study. The acquired trend lines were evaluated for their statistical significance, and significant negative correlations were observed for the gills of *M. galloprovincialis* exposed to the two higher exposed levels and for the gills and mantle of *C. chione* for all the exposure levels (Figure 2). Positive correlation was observed for the mantle of *M. galloprovincialis*. No statistically significant correlation between Pb and Zn was observed for the bodies of the

two bivalves. In general, accumulation of Pb in the tissues of *C. chione* affects Zn content much more compared to *M. galloprovincialis*.

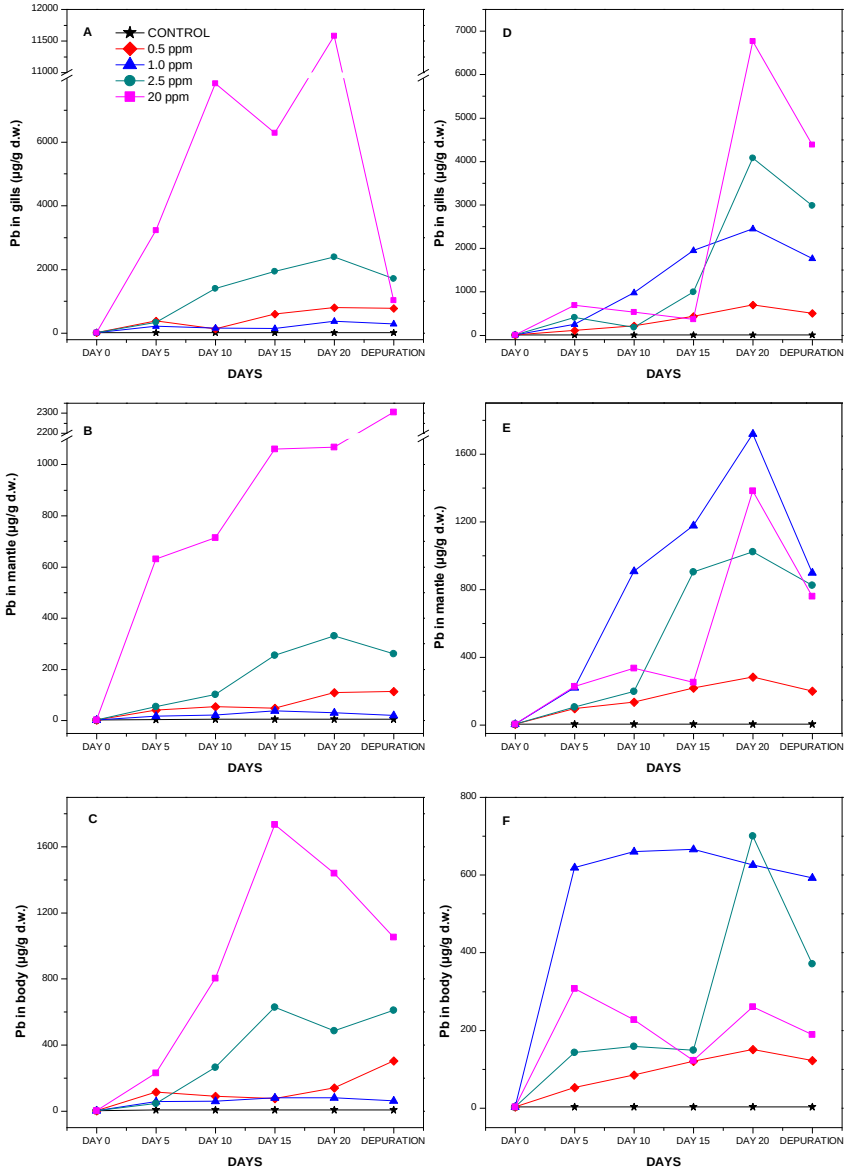


Figure 1. Pb concentration vs. time in gills, mantle and the remaining body of *M. galloprovincialis* (A,B,C) and *C. chione* (D,E,F) in different Pb concentrations in seawater.

3.4 Biochemical Analysis

Preliminary results of the biochemical analysis of the three tissues of *M. galloprovincialis* and of the mantle of *C. chione* are presented in Table 3 and in Figure 3. The induction of specific proteins by the accumulation of Pb and the possibility of the quantitative correlation of these proteins with the accumulation of Pb in the tissues under study were investigated.

The results of Ellman assay reduced to μmol MT's/g of wet tissue are presented in Table 3 and the following plot. Gills exhibit the lowest MT content with relatively small increase with the increase of the pollution level. Mantle shows the highest MT content which seems to saturate at the level of 2.5 ppm Pb in seawater, and the body shows an intermediate content which seems not to be saturated.

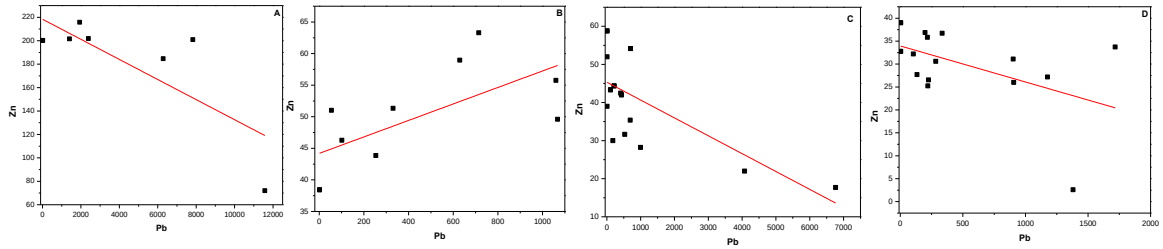
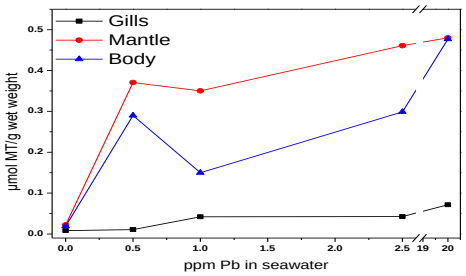


Figure 2. Correlation between Pb and Zn concentrations **A**) in the gills of *M. galloprovincialis* exposed to 2.5 and 20.0 ppm Pb, **B**) in the mantle of *M. galloprovincialis* exposed to 0.5, 1.0, 2.5 and 20.0 ppm Pb, **C**) in the gills and **D**) in the mantle of *C. chione* exposed to 0.5, 1.0, 2.5 and 20.0 ppm Pb

Table 3: MT content in *M. galloprovincialis* tissues

	µmol MT/g wet weight		
	gills M.g. Day 15	mantle M.g. Day 15	body M.g. Day 15
Control	0.00824	0.02198	0.01884
Pb 0.5ppm	0.0105	0.37071	0.28998
Pb 1.0ppm	0.04195	0.35018	0.1499
Pb 2.5ppm	0.04252	0.46073	0.2987
Pb 20ppm	0.07149	0.48013	0.47676



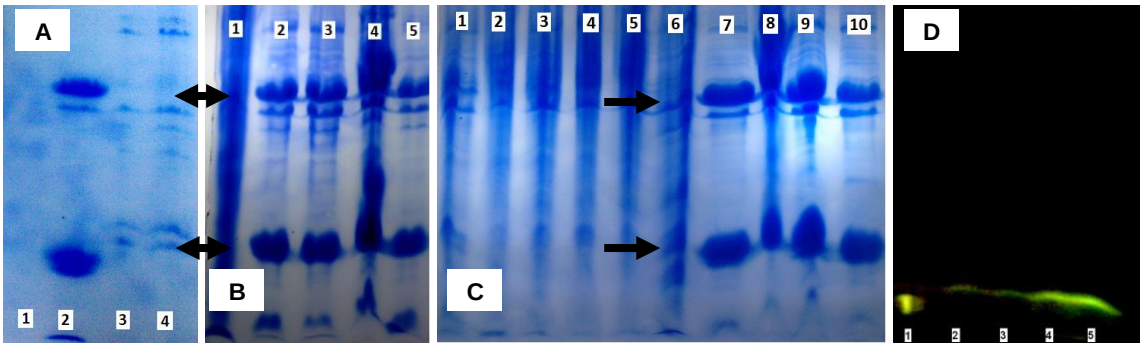


Figure 3. A, B, C, Coomasie-stained SDS-PAGE. **A**: 1 *M. galloprovincialis* (M.g.) mantle no Pb 2. M.g mantle 5 ppm Pb 3. C. chione (C.c) mantle no Pb 4. C.c. mantle 5ppm Pb. **B**. Body M.g. 1. No Pb 2. 0.5 ppm Pb 3. 1.0 ppm Pb 4. 2.5 ppm Pb 5. 20 ppm Pb. **C**. 1-5 Gills M.g.: no Pb, 0.5, 1.0, 2.5, 20 ppm Pb, respectively, 6-10 Mantle M.g.: no Pb, 0.5, 1.0, 2.5, 20 ppm Pb, respectively. **D**. Fluorimetric determination of MTs on SDS-PAGE: Mantle M.g.: 1. No Pb, 2-5: 0.5, 1.0, 2.5, 20 ppm Pb, respectively. M.g. and C.c animals were exposed to Pb for 15 days.

Figure 3 presents results of SDS-PAGE analysis of tissues of *M. galloprovincialis* and *C. chione* exposed to all levels of Pb pollution. The induction of proteins with low (7-8 kDa) and higher M_r (~50 kDa, see arrows), is apparent. Fluorimetric analysis shows that the low molecular weight proteins contain sulfhydryl groups and a good correlation of the intensity of fluorescence with the level of exposure to Pb pollution was observed. Electrophoresis analysis shows that the Pb-induced protein synthesis is species-dependent and tissue-dependent. For example Figure 1A demonstrate that there is a big difference in the expression of proteins in the presence of Pb between the mantles of *M. galloprovincialis* and *C. chione*. Work is in progress to characterize these proteins and to

optimize the experimental set up in order to utilize them for biomonitoring of marine pollution by Pb.

ACKNOWLEDGMENTS

This research has been co-financed by the European Union (European Social Fund – ESF) and Greek national funds through the Operational Program "Education and Lifelong Learning" of the National Strategic Reference Framework (NSRF) - Research Funding Program: Heracleitus II. Investing in knowledge society through the European Social Fund.
Authors acknowledge the excellent technical assistance of Ms. Vicky Paraskevopoulou.

REFERENCES

1. Amiard J.C., Amiard-Triquet C., Barka S., Pellerin J. and Rainbow P.S. (2006) Metallothioneins in aquatic invertebrates: Their role in metal detoxification and their use as biomarkers. Review, *Aquat. Toxicol.*, **76**, 160-202.
2. Attig H., Dagnino A., Negri A., Jebali J., Boussetta H., Viarengo A., Dondero F.and Banni M. (2010) Uptake and biochemical responses of mussels *Mytilus galloprovincialis* exposed to sublethal nickel concentrations, *Ecotoxicol. Environ. Saf.,* **73**, 1712-1719.
3. Cotou E., Vagias C., Rapti T. and Roussis V. (2001) Metallothionein levels in the bivalves *Callista chione* and *Venus verrucosa* from two Mediterranean sites, *Z. Naturforsch* **56c**, 848-852.
4. Dassenakis M., Scoullos M., Rapti K., Pavlidou A., Tsorova D., Paraskevopoulou V., Rozi E., Stamateli A. and Siganos M. (2004) The distribution of copper in Saronikos Gulf after the operation of the wastewater treatment plant of Psitalia, *Global Nest*, **5**, 133-143.
5. Ellman G. (1958) A colorimetric Method for determining low concentrations of mercaptans, *Arch. Biochem. Biophys.,* **74**, 443-450.
6. Freitas R., Ramos Pinto L., Sampaio M., Costa A., Silva M., Rodrigues A.M., Quintino V. and Figueira E. (2012) Effects of depuration on the element concentration in bivalves: Comparison between sympatric *Ruditapes decussatus* and *Ruditapes philippinarum*, *Estuar Coast Shelf Sci,* **110**, 43-53.
7. Hédouin L., Pringault O., Metian M., Bustamante P., Warnau M. (2007) Nickel bioaccumulation in bivalves from the New Caledonia lagoon: Seawater and food exposure, *Chemosphere,* **66**, 1449-1457.
8. Hsu P.S. and Guo Y.L. (2002) Antioxidant nutrients and lead toxicity, *Toxicology*, **180**, 33-44.
9. ISO 14869-1:2000. Soil quality- Dissolution for the determination of total element content, Part 1: Dissolution with hydrofluoric and perchloric acids.
10. Kimura M., Otaki N. and Imano M. (1979) Rabbit liver metallothionein tentative amino acid sequence of metallothionein B. In: Metallothionein ExperientiaSupplementum (Kägi JHR, Nordberg M, eds.). Birkhauser, Basel. **24**, 163-168.
11. Nichols S.J., Manny B.A., Schloesser D.W. and Edsall T.A. (1991) Heavy metal contamination of sediments in the Upper Connecting Channels of the Great Lakes, *Hydrobiologia,* **219**, 307-315.
12. Scoullos M. and Dassenakis M. (1984) Determination of dissolved metals in seawater using the resin Chelex-100. Proceedings of the 1st Symposium on Oceanography and fisheries, 302-309.
13. USEPA, (2009) National Recommended Water Quality Criteria. URL: http://www.epa.gov/ost/pc/revcom.pdf (accessed 18/12/2012).
14. Viarengo A., Ponzano E., Dondero F. and Fabbri R. (1997) A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic mollusks, *Mar. Environ. Res*., **44**, 69-84.