

Conference Paper

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Experimental study of cadmium bioaccumulation in three Mediterranean marine bivalve species: correlation with selected biomarkers

Abstract: An ecotoxicological study is presented, in which three marine bivalve species (*Mytilus galloprovincialis*, *Callista chione*, and *Venus verrucosa*) living in different habitats were studied for Cd bioaccumulation, under laboratory conditions. The bivalves, originating from a relatively polluted marine area of Greece (Saronicos Gulf), were exposed to 0.5 mg Cd L⁻¹ seawater (4.4 μmol Cd L⁻¹ seawater) for 5, 10, 15, and 20 days. Control animals were kept in metal-free seawater as well. Three or four different parts of the organisms (gills, mantle, body, digestive system) were examined for the bioaccumulation of Cd, as well as the levels of three biomarkers (metallothioneins, acetylcholinesterase, lipid peroxidation). A depuration experiment was also carried out. During the experiment, the initial levels of Cd in the control animal tissues either decreased or remained constant and low. The organisms exhibited different behavior regarding Cd bioconcentration and biomarker responses as well as tissue distribution of Cd. After the depuration period, significant amounts of Cd remained in the organisms' tissues, much higher than the respective levels in control animals.

Keywords: bioconcentration; cadmium; *Callista chione*; environmental chemistry; IUPAC Congress-44; Environmental Chemistry; *Mytilus galloprovincialis*; toxicology; trace elements; *Venus verrucosa*.

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Introduction

Trace metals exist naturally in the earth's crust. Some of them are essential for biological systems, since they participate in numerous enzymatic processes [1, 2]. On the other hand, trace metals such as cadmium, lead and mercury are generally toxic for organisms, even at low concentrations [3, 4]. The accumulation of metals

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in different parts of the organisms has unpredicted results on their health. The scientific research on this topic is of high priority as well as the establishment of effective monitoring networks that shall provide the information needed for minimizing this environmental threat. However, a lot of work is needed towards this direction, as the accumulation of heavy metals is metal- and species-dependent [5, 6], whereas the behaviour of many aquatic organisms, some of them with high commercial value, in heavy metal-polluted environments still remains to be studied.

In this work, the accumulation of Cd is studied in gills, mantles, digestive systems and the remaining bodies of three bivalve species: *Mytilus galloprovincialis*, *Callista chione*, and *Venus verrucosa*, in laboratory conditions. The Mediterranean mussel *M. galloprovincialis* has been widely used as bioindicator for trace metal pollution of coastal areas, but there is not enough information on the distribution of metals in its various parts, as well as on the behaviour of this species in environments polluted with extreme levels of heavy metals [7–9]. On the other hand, two of the less studied bivalves, but very common in the dietary tradition of many Mediterranean countries, are *C. chione* and *V. verrucosa*, living inside coastal mud.

The behaviour of the three bivalve species was studied when transported from their natural, relatively polluted, marine environment, to clean seawater in the laboratory and subsequently exposed in Cd concentrations higher than a polluted environment. The study includes the accumulation of Cd, the correlation of Cd accumulation with Zn levels (biologically related metal which has been found to be affected by Cd [10]), the levels of biomarkers (metallothioneins, acetylcholinesterase, and lipid peroxidation) and the depuration process. Metallothioneins (MTs) are multitask, low-molecular-weight proteins with high cysteine content. In normal conditions they exist at low levels in the organisms, but they become overexpressed under stress [1, 7]. Cd has also been implicated in the formation of reactive oxygen species (ROS), which cause oxidative damage to lipids [11]. The decomposition of lipid hydroperoxides produces low-molecular-weight products, including malondialdehyde (MDA), which reflects the intensity of these processes and can be used as a biomarker [12–14]. Finally, the third biomarker studied is acetylcholinesterase (AChE), indicative of neurotoxicity, since it is responsible for the degradation of the acetylcholine neurotransmitter in cholinergic synapses [8, 15].

Materials and methods

Mussels (*M. galloprovincialis*) were purchased from an aquaculture farm, while both clam species (*C. chione* and *V. verrucosa*) were hand collected by scuba divers. All three species were collected from a relatively polluted marine area, the Elefsis Gulf, in the vicinity of Athens. Organisms were brought alive to the laboratory, where they were put in aquaria filled with fresh seawater collected from an unpolluted area (Chania, Crete, Greece) with an appropriate aeration system. *C. chione* and *V. verrucosa* were placed in a sand layer on the bottom of the aquaria. Care was taken to use animals with the same shell length (6–8 cm for mussels, 5.5–6.5 cm for *C. chione* and 4.5–5 for *V. verrucosa*), since size is related with the age of the organisms [16]. Immediately after their arrival in the laboratory, 10 bivalves of each species were dissected on ice. The dissection procedure for both clam species was carried out in such a way that would allow separation of the gills, the mantle, the digestive system (stomach, digestive gland, intestine, heart, kidney) and the remaining body (adductor muscle and foot). In the case of *M. galloprovincialis*, only the gills and mantle were separated from the remaining animal tissues which were designated as “body”. The separated tissues were kept either at $-24\text{ }^{\circ}\text{C}$ (until heavy metal analysis) or at $-80\text{ }^{\circ}\text{C}$ (for biomarker analysis). The metal levels in these tissues are designated as “Day 0”.

The organisms were transferred to 70 L aquaria and acclimated to laboratory conditions, at $17\text{--}18\text{ }^{\circ}\text{C}$ for 5 days. The number of animals in the aquaria corresponded to 2.5 mussels/L seawater and 1 clam/L seawater. The aquaria were equipped with aeration systems (air pumps). Seawater used for acclimation and subsequent experiments was collected from an unpolluted area in Chania, Crete and was changed every day during acclimation period.

After this 5-day acclimation period the test organisms were exposed to 0.5 mg L^{-1} ($4.4\text{ }\mu\text{mol}\cdot\text{L}^{-1}$) of Cd for 20 days. This concentration of Cd was chosen as a high environmental exposure scenario and has been

previously used in bioaccumulation experiments [17–19]. Half of the water volume was replaced daily by fresh seawater appropriately spiked with Cd.

On the 5th day of exposure, 30 animals were removed and separated to 3 groups of 10 animals for preparation of 3 composite samples (“Day 5”). The organisms were dissected as described above for “Day 0”. The exposure experiment lasted 20 days and collection/dissection of animals was carried out again at the 10th, 15th, and 20th day. After the 20th day, the remaining bivalves were transferred to heavy metal-free seawater for the depuration stage of the experiment. The depuration lasted 10 days for the experiments with *M. galloprovincialis* and *C. chione* and 30 days for *V. verrucosa*. Control organisms were simultaneously kept in metal-free seawater throughout the experiment period. The aquaria were inspected very frequently (several times a day) for dead animals but no mortality was observed, either during acclimation or during exposure and depuration periods.

Tissue samples used for the determination of metal concentrations were lyophilized and homogenized to fine powder. Approximately 0.5 g of the dried tissues was digested overnight with concentrated HNO₃ in closed PTFE beakers on a hot plate at 80 °C. The digests were stored in polyethylene bottles at 4 °C until heavy metal determination. Control samples and blanks (cHNO₃), were also treated as described above. All glassware and plastic ware were pre-cleaned by soaking in 10 % HNO₃ for at least 24 h.

Background metal contamination levels were determined in seawater used in aquaria (Chania, Crete) and in seawater from the organisms harvesting sites (Elefsis Gulf). Seawater samples were filtered through 0.45 µm Millipore filters and dissolved metals were pre-concentrated on Chelex-100 resin columns [20].

Cd concentrations in tissues were determined by graphite furnace atomic absorption Spectrometry with background correction based on the Zeeman effect (on a Varian SpectraAA-640Z GTA 100). Zn concentrations were determined with flame AAS (Varian SpectraAA 200). For quality assurance, blanks and reference materials (Quasimeme QTM 044 BT, *M. edulis* and IAEA 436, tuna fish) were also digested and measured as described above. The recovery for all metals was between 97 and 102 % with very low standard deviation values.

For the determination of metallothioneins 1 g of each tissue was homogenized in 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 using an ultrasonic homogenizer (Hielscher UP 400S Ultrasonic homogenizer). The homogenates were ultra-centrifuged at 50 000 × g for 60 min (Beckman L8-60M ultracentrifuge) at 4 °C. The supernatants were treated with ethanol/chloroform solution [21 with modifications] and MTs were precipitated by centrifugation (Pellet A). 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 8.0, was added at room temperature. Metallothionein content was evaluated spectrophotometrically at 412 nm [22] with GSH as a reference standard. The amount of MTs was calculated assuming a 21 SH/MT [23] and expressed as µmol per gram of wet tissue weight.

For lipid peroxidation and acetylcholinesterase determination, tissue extracts were prepared as follows; 1 g of each tissue of each pooled sample was homogenized at 4 °C using an ultrasonic homogenizer in 5 volumes of 50 mM K-phosphate buffer, pH 7.0, 10 mM PMSF. The homogenates were centrifuged at 6000 × g for 30 min at 4 °C (Sigma 3-18K centrifuge) and the supernatants (SUPER A) were collected and used immediately for determination of enzyme activities. Measurements were carried out with a Shimadzu UV-vis spectrophotometer (UV mini 1240).

For the determination of acetylcholinesterase activity, 10–100 µL of the tissue homogenate (SUPER A) were added to a mixture of 1.5 ml 100 mM K-phosphate buffer pH 8.0, 10 µL 0.01 M acetylthiocholine iodide, 50 µL 0.01 M DTNB. The blank for this test consists of buffer, substrate, and DTNB solutions. The assay was carried out at 25 °C. The yellow colour of the 5-thio-2-nitro-benzoic acid was quantitatively monitored by spectrophotometric absorption at 412 nm (measurement of the rate of colour production) [24]. The method's reference standard was acetylcholinesterase from electric eel. AChE activity was expressed as nmoles min⁻¹ g⁻¹ wet tissue.

The determination of lipid peroxidation was done using TBARS assay as described by Buege and Aust [25]. 100 µL of the tissue homogenate (SUPER A) were combined with 500 µL of an acid reaction mixture (TBA-TCA-HCl) (1:1:1) and 10 µL of 10 mM butylated hydroxytoluene (BHT). After thoroughly vortex, the mixture was heated for 20 min at 95 °C in a boiling water bath, then cooled at room temperature for 10 min and centrifuged at 15 000 × g for 10 min. The absorbance of the supernatant was determined at 535 nm against a blank which consists only of the reaction mixture (TBA-TCA-HCl) and BHT. Lipid peroxidation was estimated using MDA as standard and was expressed as nmoles of MDA per g of wet tissue mass [13].

The determination of biomarkers in each composite sample was conducted in duplicate (both extract preparation and subsequent spectrophotometric analysis).

All statistical analysis was performed using SPSS 17.0. The level of significance for all tests was set at $p < 0.05$. Chemical and biochemical data were checked for homogeneity of variances (Levene's test) and if $p < 0.05$ a one way ANOVA was performed. If not, Mann–Whitney and Kruskal–Wallis tests were performed. When ANOVA was significant, Tukey HSD, LSD and Tamhane's post-hoc tests identified differences between biomarkers, tissues, days and exposure levels. Interrelationships between the selected biomarkers and heavy metal concentrations in different tissues of the test animals were evaluated using Pearson's correlation.

Results and discussion

Metal analysis

The average seawater concentration of dissolved cadmium in Elefsis Bay was $0.06 \mu\text{g L}^{-1}$ or 0.5 nmol L^{-1} far below the U.S. EPA limit for chronic toxicity to aquatic life in saltwater ($8.8 \mu\text{g L}^{-1}$) [26] and the EU maximum allowable yearly average for surface waters ($0.20 \mu\text{g L}^{-1}$) [27], however, it is 6 times higher than the levels in the seawater used for the experiments ($0.01 \mu\text{g L}^{-1}$ or 0.09 nmol L^{-1}). Cd concentrations of the sand samples used in the experimental aquaria were below the detection limit.

The background Cd levels in the test bivalves (total heavy metal content) directly after harvesting (“Day 0”) were $2.60 \text{ mg kg}^{-1} \text{ d.w.}$ ($0.023 \mu\text{mol g}^{-1} \text{ d.w.}$), $0.31 \text{ mg kg}^{-1} \text{ d.w.}$ ($0.003 \mu\text{mol g}^{-1} \text{ d.w.}$) and $0.55 \text{ mg kg}^{-1} \text{ d.w.}$ ($0.005 \mu\text{mol g}^{-1} \text{ d.w.}$) for *M. galloprovincialis*, *C. chione*, and *V. verrucosa*, respectively. The values of *M. galloprovincialis* were higher than corresponding values measured by other researchers in various Mediterranean areas which ranged between $0.4\text{--}2.0 \text{ mg kg}^{-1} \text{ d.w.}$ in the Spanish coastline, and $1.1 \text{ mg kg}^{-1} \text{ d.w.}$ in the French coastline [4], but were similar to levels reported in animals harvested in the Amvrakikos Gulf, Greece (2.9 and $2.0 \text{ mg kg}^{-1} \text{ d.w.}$) [8]. Cd levels measured in *M. galloprovincialis* from the Bosphorus area in Turkey ranged between $8.95\text{--}10.7 \text{ mg kg}^{-1} \text{ d.w.}$ [4], values significantly higher than the values measured in the present study. The total Cd concentration in *M. galloprovincialis* was exactly at the maximum permissible content for edible bivalve molluscs which is set at $1 \text{ mg kg}^{-1} \text{ w.w.}$ ($0.0089 \mu\text{mol g}^{-1} \text{ w.w.}$) (EU 1881/2006).

The average Cd concentrations of the control bivalves from “Day 5” to “Day 20” were compared to the “Day 0” value with one sample t-test. Statistically significant decrease (one sample t-test, $p < 0.05$) was observed for the body and mantle tissues of control *M. galloprovincialis* and *C. chione* remained in unpolluted water for 30 days compared to the initial field concentration. The Cd levels in the other tissues of these two bivalves and all tissues of *V. verrucosa* remained low and constant.

The bioaccumulation factor (BAF), is defined as the ratio of the concentration of a chemical in the tissue of an aquatic organism ($C_{\text{org}} - \text{mg kg}^{-1}$) over its concentration in water ($C_{\text{wat}} - \text{mg L}^{-1}$). BAFs were calculated for all the tissues of the three bivalves during the 20 days exposure period. Maximum BAF values in the case of mussel tissues were calculated at “Day 20” (where maximum Cd concentrations were measured, Fig. 1a), while both clam species exhibited a different pattern. Maximum bioaccumulation in “Day 20” was found only in their mantles, while for the gills the maximum BAF was in “Day 15” and for the body and digestive system in “Day 10”. As for inter-tissue variability *M. galloprovincialis* and *C. chione* maximum BAF values were observed in the gills. A different trend was observed for *V. verrucosa* with higher BAF values in the digestive system and lower in the body.

Figure 1 presents Cd levels in the three test organisms and their various tissues during the entire experimentation period. In *M. galloprovincialis*, Cd was mainly accumulated in the gills (Fig. 1a, Cd gills > Cd body > Cd mantle). This tendency of *M. galloprovincialis* has been reported by other researchers as well [4, 10, 28]. In *C. chione* tissues Cd levels followed a similar order (Cd gills > Cd body \approx Cd mantle \approx Cd digestive system). No clear statistical difference in Cd accumulation between the tissues of *V. verrucosa* was observed except the definite lower values of Cd in its body.

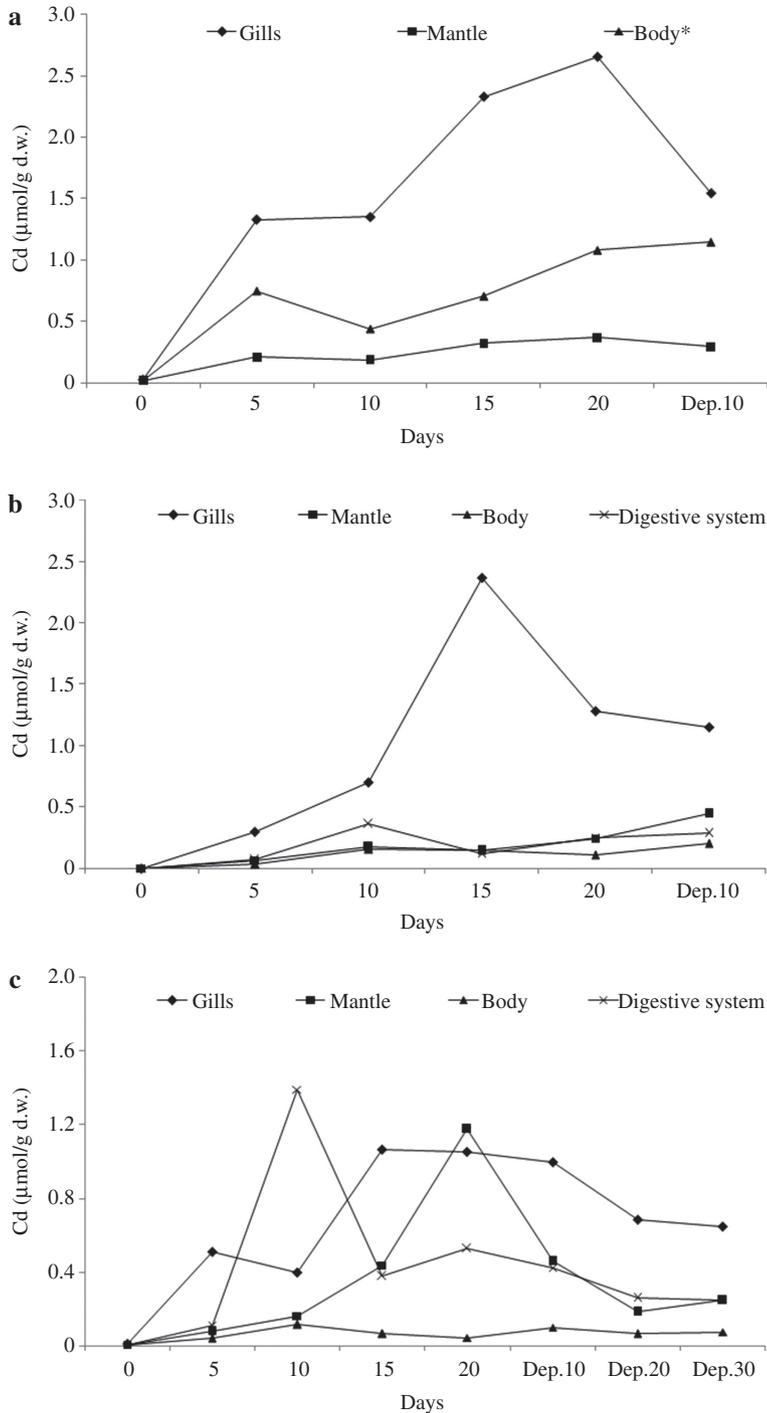


Figure 1 Cd levels ($\mu\text{mol/g d.w.}$) in the tissues of a) *Mytilus galloprovincialis*, b) *Callista chione* and c) *Venus verrucosa* during the experimental period. (Body* means the digestive system and the remaining body.)

The diagrams in Fig. 1 indicate that the Cd levels in the gills of the three species followed the order *M. galloprovincialis* > *C. chione* > *V. verrucosa* and almost the same trend applied to the body (*M. galloprovincialis* > *C. chione* \approx *V. verrucosa*). No statistical differences between the mantle tissues of the test species were observed (one-way ANOVA, $p > 0.05$).

A decreasing trend in Cd concentrations was observed in the gills of all three species during the depuration period. In *M. galloprovincialis* and *C. chione* the gills seem to detoxicate a little more than all other tissues.

However, Cd levels after 10 days of depuration or even after 30 days (*V. verrucosa*) were much higher than pre-exposure levels in every tissue of all test animals (Fig. 1a–c). Thus, metal detoxification is considered to be a slow process, in agreement with observations of other researchers [4, 7, 10, 29]. The slow elimination of Cd from all the organisms' tissues could be rationalized by metallothionein detoxification function: Cd ions are complexed in MT molecules and then sequestered in specific cellular compartments (i.e., vacuoles). Excretion of Cd may follow as a slow procedure due to the efficiency of storage mechanism based on MTs [4]. Fluctuations of Cd concentrations were observed in the tissues of the tested bivalves during depuration period probably due to translocation of Cd from one organ to another, as a part of detoxification procedure. Our statement is in agreement with Serra et al. [17] who exposed the clam *S. inaequalis* for 4 weeks to 0.5 mg Cd L⁻¹ seawater.

Control levels of Zn ($\mu\text{mol g}^{-1}$ d.w.) in the tissues of the three bivalves were: for *M. galloprovincialis* gills: 2.1–6.9, mantle: 0.3–1.3, body: 0.7–3.0, for *C. chione* gills: 0.5–0.6, mantle: 0.3–0.5, body: 0.8–0.9, digestive system: 0.5–0.9 and for *V. verrucosa* gills: 0.4–0.8, mantle: 0.7–3.1, body: 0.3–0.5 and digestive system: 0.4–0.8. The exposure of the three test animals to Cd (0.5 mg L⁻¹ seawater) did not cause any statistical significant changes to the levels of Zn in all the examined tissues.

Metallothioneins (MTs)

MT content ($\mu\text{mol g}^{-1}$ w.w.) in all tissues of the control animals did not change during the experiment. MT levels in *M. galloprovincialis* control tissues were 0.045 in the gills, 0.046 in the mantle, and 0.068 in the body. In *C. chione* tissues MTs were 0.059, 0.034 and 0.063 in the gills, body and digestive system, respectively and between 0.034–0.059 in the mantle. Finally, in *V. verrucosa* tissues the MT levels were 0.016 (gills), 0.011 (mantle), 0.023 (body) and 0.010 (digestive system). It is interesting that although the highest Cd concentrations were measured in the gills of the organisms the highest MT levels were found in other tissues depending on the animal.

In all tissues of the three species, MT levels increased with the time of exposure compared to the control levels. The increase was up to 15-fold in the gills of the test animals. The MTs increase in the mantle and body tissues of *M. galloprovincialis* was up to 63-fold but the most remarkable increase in MT content was found in *V. verrucosa* tissues where it was up to 135-fold.

The variation of MT levels in each animal tissue during the exposure and depuration periods are presented in Fig. 2(a–c). MTs in exposed *M. galloprovincialis* were mainly expressed in the body (MT body > MT mantle \approx MT gills, $p < 0.05$). MT levels in the different tissues of *C. chione* presented no significant differences (Fig. 2b), while for *V. verrucosa* MTs in the gills were statistically lower than in all other tissues (Fig. 2c).

Statistically significant positive correlations of Cd accumulation and concurrent MT levels were found in all three tissues of *M. galloprovincialis* ($R = 0.893$, $p < 0.05$ for gills, $R = 0.900$, $p < 0.05$ for mantle, $R = 0.855$, $p < 0.05$ for body), in the mantle ($R = 0.918$, $p < 0.05$) and body ($R = 0.956$, $p < 0.05$) of *C. chione*, and in the gills of *V. verrucosa* ($R = 0.885$, $p < 0.05$). So, as the Cd concentration in all tissues of *M. galloprovincialis* and selected tissues of *C. chione* and *V. verrucosa* increases, MTs are overexpressed not only as a specific response to metal toxicity but as antioxidant defense in order to sequester the metal cations and lessen metal toxicity [1]. Similar correlations have also been reported in the gills of the clam *R. decussatus* [5], the digestive gland of *M. galloprovincialis* [9, 30] and the mantle of asiatic clam *C. fuminea* [31].

The box plots of Fig. 3(a–d) depict MT variation during the 20 days of exposure and present a graphical comparison between species. One-way ANOVA was performed to verify MT differences between species in the corresponding tissues. The MT content in the gills of the three species (Fig. 3a) followed the order *M. galloprovincialis* \approx *C. chione* > *V. verrucosa* (one-way ANOVA, $p < 0.05$), while the MT content in the mantles of the three bivalve species (Fig. 3b) and the digestive system (Fig. 3c) and body (Fig. 3d) of the two sand-burrowing organisms presented no statistical difference (one-way ANOVA, $p > 0.05$).

After 10 days of depuration, MT content exhibited a tissue-dependent decrease in *M. galloprovincialis* and *C. chione* but for *V. verrucosa* the decrease was most obvious after 20–30 days. It is worth mentioning that the MT levels did not return to the original concentrations (statistical comparison between the values measured during the exposure period and the levels of MTs after depuration period revealed no differences – one-way

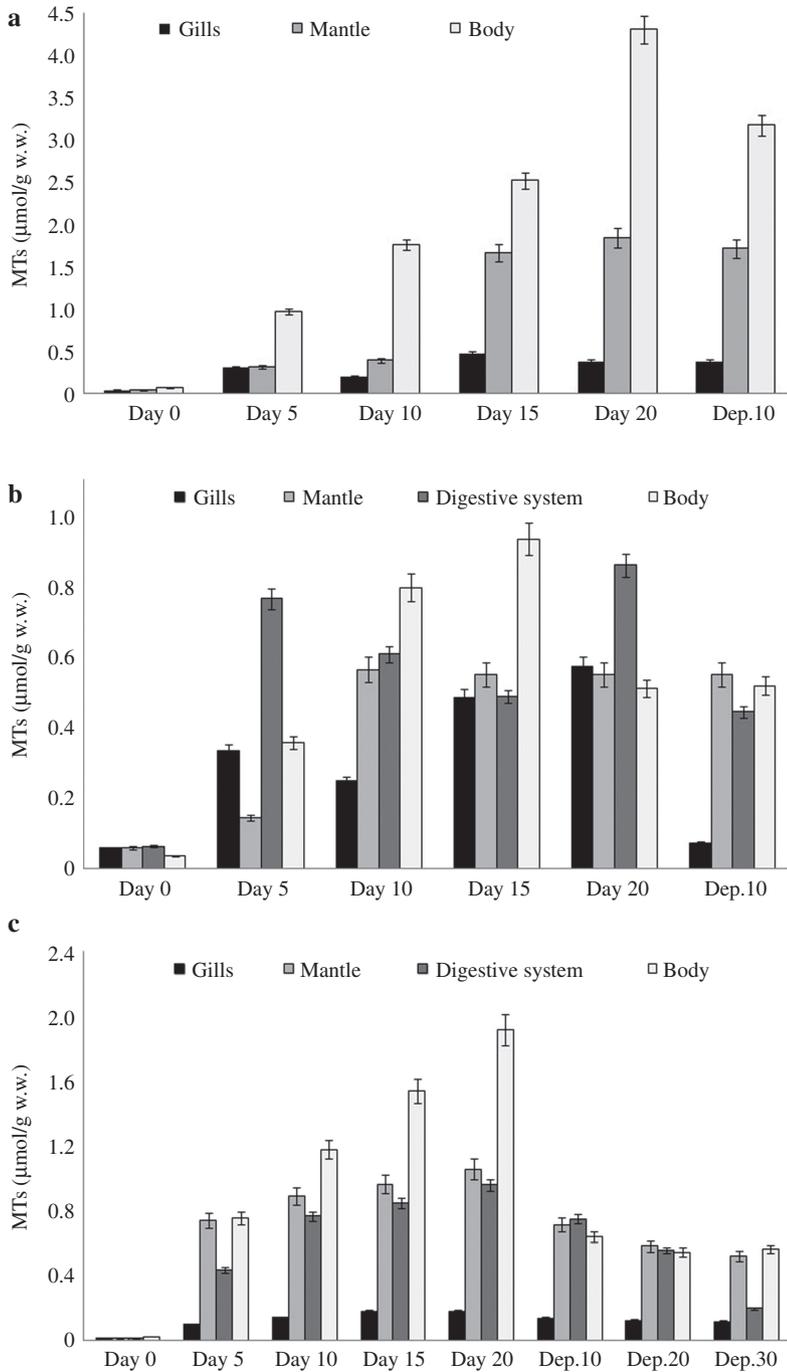


Figure 2 Metallothionein levels in gills, mantle, digestive system and body of a) *Mytilus galloprovincialis*, b) *Callista chione* and c) *Venus verrucosa* during the experimental period (exposure to 0.5 mg Cd/L seawater and depuration). Values are expressed in $\mu\text{mol MT/g w. w.}$ (mean values \pm standard deviations).

ANOVA, $p > 0.05$), not even after 30 days of depuration. This is probably due to high Cd levels persisting during the depuration period [4, 7].

Lipid peroxidation (LPO)

Control levels of MDA (malondialdehyde) for all tissues of the three test organisms were rather constant or slightly decreased (e.g., in all tissues of *M. galloprovincialis* and the gills of *V. verrucosa*) during the 30 days

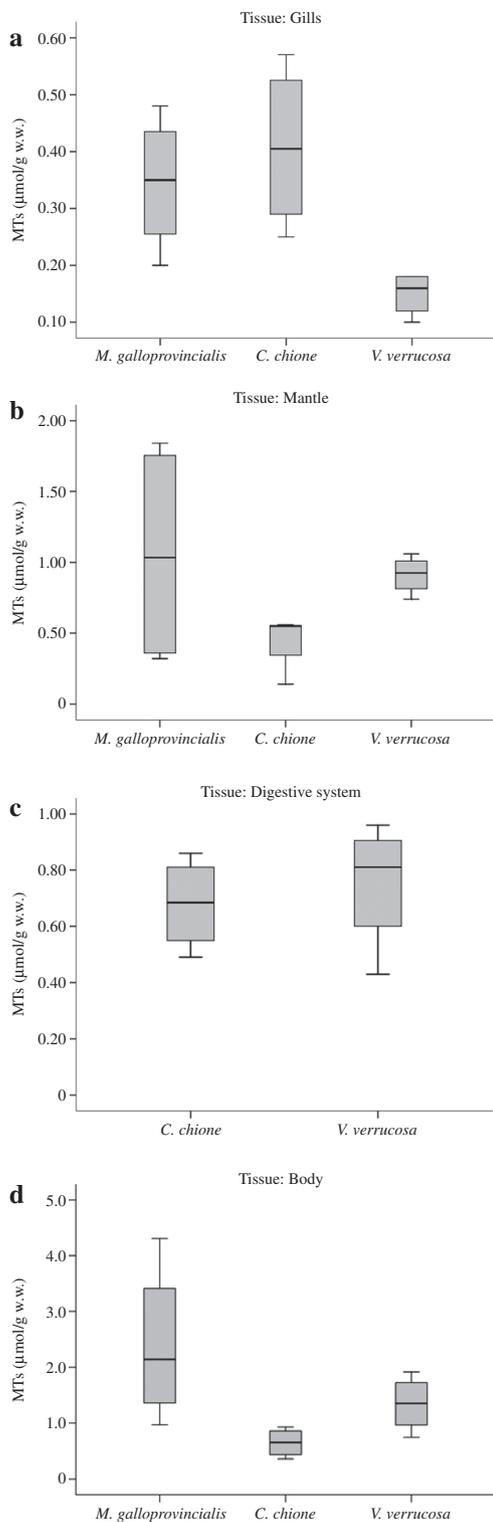


Figure 3 Between species box-plots of metallothioneins ($\mu\text{mol/g w.w.}$) for each tissue of the three examined species a) gills, b) mantle, c) digestive system, d) body.

of the experiment. MDA levels ($\text{nmol g}^{-1} \text{w.w.}$) in *M. galloprovincialis* control tissues were 4507–4747 in the gills, 2820–3153 in the mantle and 5231–5746 $\text{nmol g}^{-1} \text{w.w.}$ in the body. In all *C. chione* tissues MDA levels were lower than *M. galloprovincialis* and rather constant at 501–635 $\text{nmol g}^{-1} \text{w.w.}$ and in *V. verrucosa* tissues

the MDA levels were about 168 (digestive system), 280 (mantle), 205 (body) and decreasing from 1332 to 1202 nmol g⁻¹ w.w. in the gills.

LPO (expressed as MDA levels) in *M. galloprovincialis* control tissues was higher, approximately 5- to 10-fold, than values measured by other researchers, indicatively 500–1000 nmol g⁻¹ w.w. in the gills and 200–360 nmol g⁻¹ w.w. in the mantle of *M. galloprovincialis* from other mussel culture farms of Saronicos [13]. This is not surprising, since the Cd control levels of our test animals were also higher than the ones reported by Vlahogianni et al. [13].

MDA values measured in all tissues of the exposed bivalves were higher (statistical difference verified through one-way ANOVA, $p > 0.05$) than the control's. In *M. galloprovincialis* and *C. chione* tissues MDA levels increased up to 11-fold with the most prominent increase in the gills. On the contrary, the increase in *V. verrucosa* gills and mantle was moderate (3-fold in the gills and 6-fold in the mantle) but more pronounced in the body (16-fold) and the digestive system (10-fold).

Fig. 4(a–c) presents the evolution in LPO levels expressed as nmol MDA g⁻¹ w.w. in the different tissues of the tested organisms throughout the period of Cd exposure and depuration. In all three organisms during the time course of the experiment, gills had the higher MDA levels. The statistical comparisons of MDA content in *M. galloprovincialis* and *V. verrucosa* tissues, revealed that gills compared to the other tissues had the higher MDA content while, as mentioned above, the lowest MT levels for these two organisms were measured at this tissue. Therefore, it can be postulated that when MT levels are not high enough to adequately protect the cells from lipid peroxidation some oxidative damage due to the Cd exposure does occur [12]. Vlahogianni et al. [13] has also observed higher MDA concentrations in *M. galloprovincialis* gills compared to the mantle.

Statistically significant positive correlations between Cd accumulation and concurrent MDA levels were presented in *M. galloprovincialis* gills ($R = 0.889$, $p < 0.05$) and mantle ($R = 0.934$, $p < 0.05$), in *C. chione* gills ($R = 0.995$, $p < 0.05$), mantle ($R = 0.952$, $p < 0.05$) and body ($R = 0.997$, $p < 0.05$). This trend has also been observed by Kamel et al. [32] in the clam *R. decussates*. The rise of MDA content may indicate oxidative damage to lipids due to produced ROS. On the other hand, no MDA-Cd correlations were found in *V. verrucosa* tissues.

The box plots at Fig. 5a, show the comparison of lipid peroxidation levels in the gills of the three bivalves. The trend for MDA content was *M. galloprovincialis* > *C. chione* ≈ *V. verrucosa*. The same trend was also observed in their mantles (Fig. 5b). The MDA content in the gills and mantles of the two clams was 10–12-fold lower than the corresponding values measured to mussels' gills and mantles. As already mentioned, MT levels were higher or similar in the gills and mantle of *M. galloprovincialis* compared to the other two bivalves. So, we observe that with the same, or even higher levels of MTs, *M. galloprovincialis* has suffered larger oxidative damage compared to the other two species. One possible explanation of this observation is that the habitat of each organisms plays an important role to the extent of the oxidative damage that Cd will cause. The two clams live inside the sand, while the mussel is directly exposed to contaminated seawater. This assumption is also supported but the lack of difference between MDA levels in the other two tissues (bodies and digestive systems) of *C. chione* and *V. verrucosa* (Fig. 5c and d).

After the depuration period, MDA concentrations in all test animals exhibited a rather small decrease depending on the tissue, but did not return to initial levels (no statistical difference, one-way ANOVA, $p > 0.05$), similarly to MTs.

Acetylcholinesterase activity (AChE)

AChE activity measured in the control animal tissues (nmol min⁻¹ g⁻¹ w.w.) was about 195, 705 and 442 for *M. galloprovincialis* gills, mantle and body, respectively. For *C. chione* AChE activity levels (nmol min⁻¹ g⁻¹ w.w.) were 84 (gills), 493 (mantle), 344 (body) and 561 (digestive system). For *V. verrucosa* the corresponding values (nmol min⁻¹ g⁻¹ w.w.) were 65 (gills), 61 (mantle), and 530 (body) and 455 (digestive system). During the 20 days of Cd exposure, AChE activity in the test species was statistically lower (one-way ANOVA, $p < 0.05$) than the

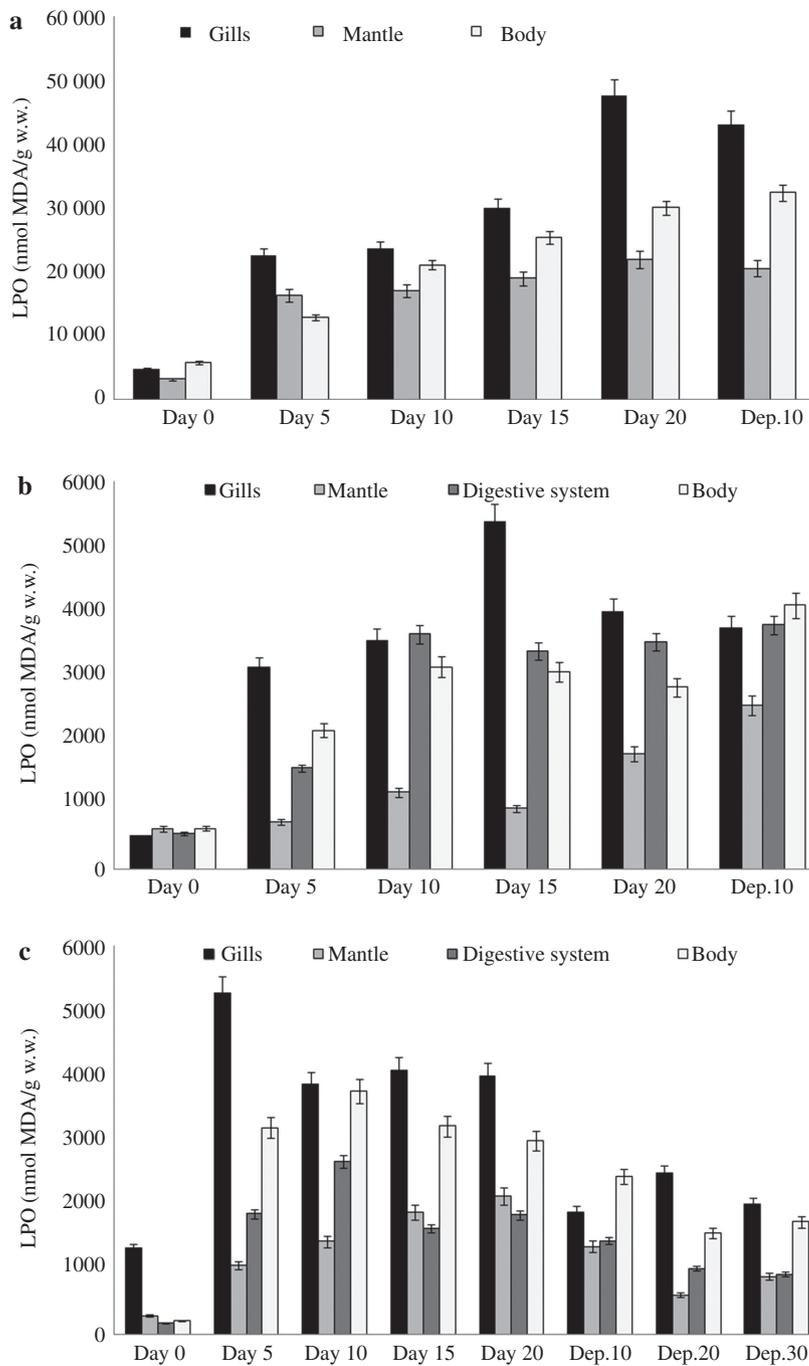


Figure 4 Lipid peroxidation levels in gills, mantle, digestive system and body of a) *Mytilus galloprovincialis*, b) *Callista chione* and c) *Venus verrucosa* during the experimental period (exposure to 0.5 mg Cd/L seawater and deputation). Values are expressed in nmol MDA/g w.w. (mean values \pm standard deviations).

control's corresponding values in all tissues examined except the bodies of both clams. It has been found that AChE levels are lower in animals from polluted sites than the ones from reference areas [8, 33]. Inhibition of AChE activity has also been observed in *P. perna* and *M. galloprovincialis* by Najimi et al. [34]. Schiedek et al. [35] also found significant differences in AChE activity of mussels along a pollution gradient in the German Baltic Sea coastal area.

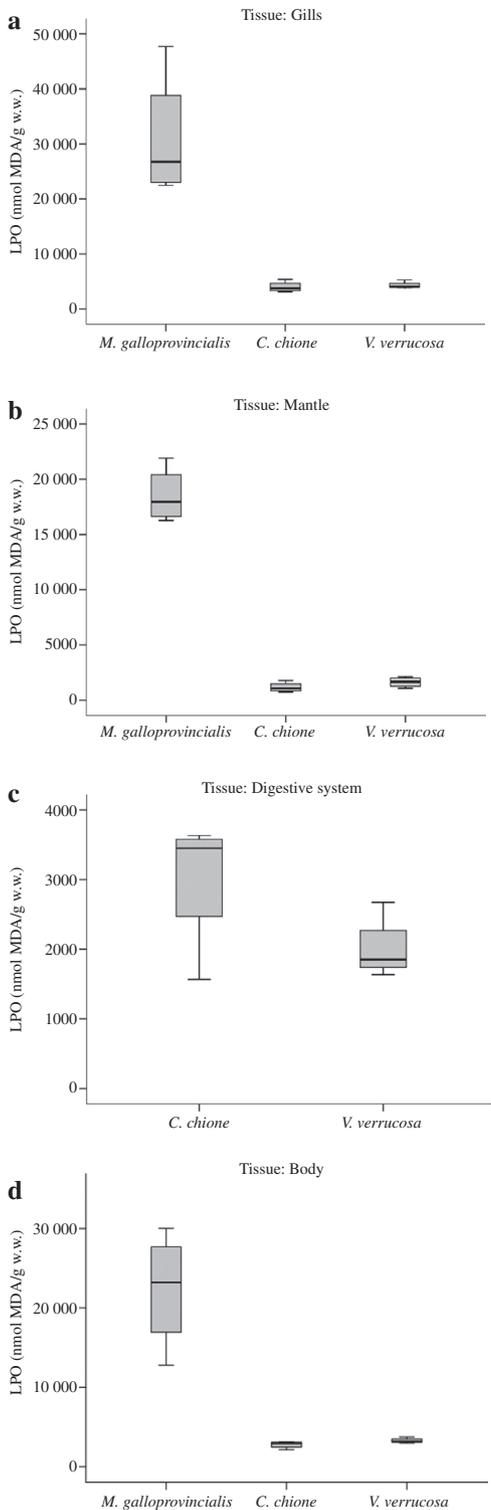


Figure 5 Between species box-plots of lipid peroxidation (nmol MDA/g w.w.) for each tissue of the three examined species a) gills, b) mantle, c) digestive system, d) body.

Figure 6(a–c) presents the effect of Cd exposure on each bivalve’s acetylcholinesterase activity in all the examined tissues. During the exposure period, AChE activity generally decreased in all tissues of all three species examined, except *V. verrucosa* mantle in which AChE increased. A slight increase was observed at all

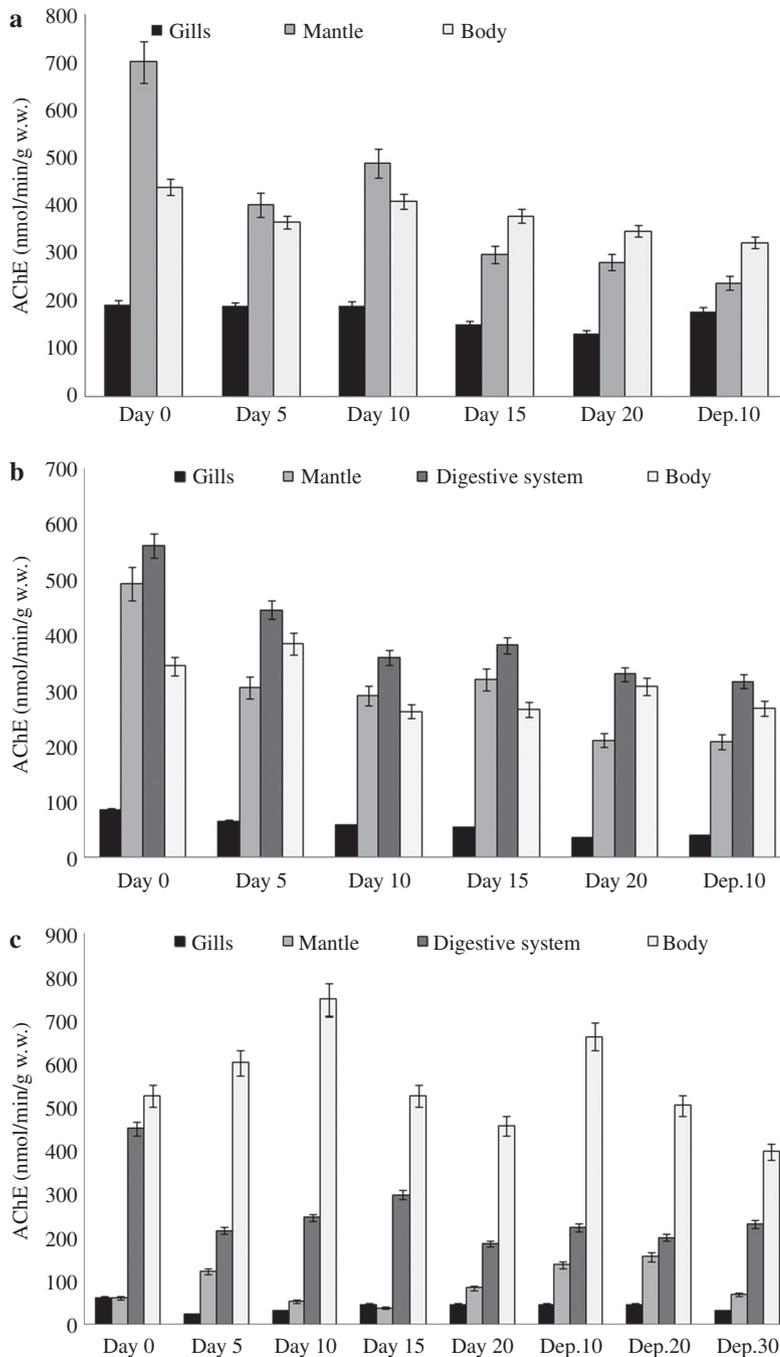


Figure 6 Acetylcholinesterase activity in gills, mantle, digestive system and body of a) *Mytilus galloprovincialis*, b) *Callista chione* and c) *Venus verrucosa* during the experimental period (exposure to 0.5 mg Cd/L seawater and depuration). Values are expressed in nmol/min/g w.w. (mean values \pm standard deviations).

tissues of *M. galloprovincialis* at “Day 10” and in *C. chione* tissues at “Day 15” but the values measured for both bivalves were not higher than the control’s corresponding values. At “Day 10” for the mussel and at “Day 15” for the clam, when the slight increase in AChE activity was observed, Cd concentrations measured were lower than the corresponding values at previous days (except *C. chione* gills, Fig. 6b). At the end of the exposure period, the percentage of the decrease of AChE activity compared to control values for *M. galloprovincialis* was: 31 % for the gills, 60 % for the mantle and 22 % for the body. For *C. chione* the percentages: 59 % for the

gills, 57 % for the mantle, 11 % for the digestive system and 41 % for the body. The corresponding values for *V. verrucosa* were: 28, 0, 13, and 60 %.

M. galloprovincialis gills, mantle and body as well as *C. chione* mantle and body exhibited statistically significant negative correlations between Cd accumulation and concurrent AChE activity (for mussels' gills, mantle and body the Pearson correlation coefficients were $R = 0.996$, 0.953 , and 0.972 , $p < 0.05$, respectively and for *C. chione* mantle and gills the corresponding values were $R = 0.895$ and 0.997 $p < 0.05$). *V. verrucosa* tissues did not exhibit any significant positive or negative trends with Cd levels. The negative correlation between Cd concentration and AChE activity reveals Cd neurotoxicity to organisms, since AChE is an enzyme involved in nerve impulse transmission [34].

AChE activity was lower in the gills of all species compared to the other tissues both in the control and exposed animals. As mentioned above, the gills of all species examined were the target organ for Cd accumulation and at the same time the MT content was low. Thus, it can be concluded, that high concentrations of Cd in a tissue cause AChE inhibition as long as MTs are not overexpressed so the cells are not protected.

Comparing AChE activity in the gills of the three organisms (Fig. 7a) *M. galloprovincialis* had the higher AChE activity (something that was also observed to control animals), while no statistical difference was presented between the other two organisms. That trend has also been observed by Cotou et al. [15] for the same species harvested in the Evoikos Gulf (Greece). The trend for the mantle was: *M. galloprovincialis* \approx *C. chione* $>$ *V. verrucosa* (one-way ANOVA, $p < 0.05$). Statistical comparison of the AChE activity in the digestive system and the body of the two sand burrowing organisms, revealed clear difference for each tissue between the two organisms (Fig. 7c and d).

The observed decrease of AChE levels in all tissues during exposure was inversely correlated with the increase of LPO for *M. galloprovincialis* and *C. chione* tissues. The negative relationship was statistically significant (for the mussels $R = 0.617$ and for *C. chione* $R = 0.486$, $p < 0.05$). This trend has also been observed by Kamel et al. [32] for the gills of clam *R. decussates* and for gonads of freshwater mussels exposed to trace metal rich municipal effluents by Garné et al. [36].

At the depuration period, AChE activity did not show clear increasing trends that would indicate recovery of the test organisms. As mentioned above with Cd levels, MTs and LPO, the depuration period employed in these experiments was not adequate for detoxification of the animals. A slight increase was observed only in the gills of *M. galloprovincialis* and *C. chione* correspondingly to a simultaneous decrease in LPO and Cd levels indicating that the detoxification process is faster which are in the tissue in direct contact with seawater.

Conclusions

Cd levels in bivalve tissues from the harvesting area in our study were generally higher than corresponding levels of other Greek and European marine areas and in the case of *M. galloprovincialis*, the total Cd concentration was at the EU maximum permissible value for human consumption.

After acclimation in metal-free seawater in the laboratory, the test animals exhibited decrease in Cd levels, especially *M. galloprovincialis*.

The response of the test animals to Cd exposure in the laboratory experiments was species- and tissue-dependent but in all cases quite fast and pronounced.

The statistically significant differences of Cd levels between the various tissues as well as the maximum BAF values, indicated that the accumulation of Cd was higher in the gills of *M. galloprovincialis* and *C. chione*. A different trend was observed for *V. verrucosa*, with increased BAF values in the digestive system and no clear Cd accumulation trend in the other examined tissues.

The accumulated Cd was not significantly removed from the tissues of the bivalves during the depuration period indicating that metal detoxification is a slow process. Furthermore, all three studied biomarkers also did not return to pre-exposure levels.

Despite the fact that Cd mostly accumulated in the gills, MT levels were much higher in the body. In most of the tissues of the three organisms examined in our research, statistically significant positive correlations

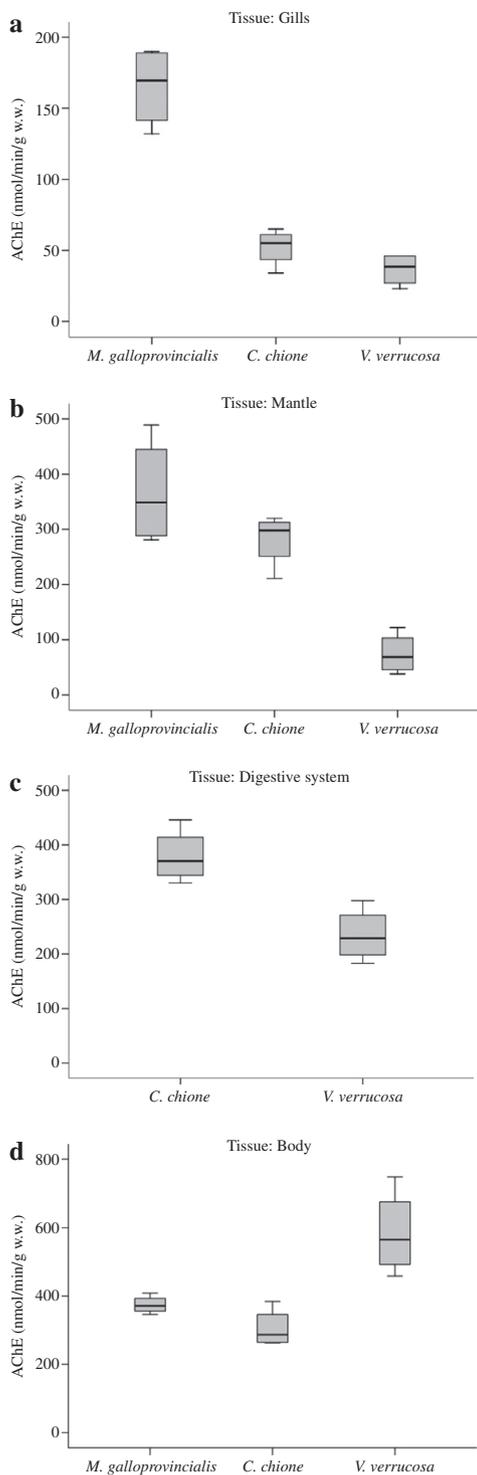


Figure 7 Between species box-plots of acetylcholinesterase activity (nmol/min/g w.w.) for each tissue of the three examined species a) gills, b) mantle, c) digestive system, d) body.

of Cd accumulation and concurrent MT levels were found. Thus, as the Cd concentration increases, MTs are overexpressed not only as a specific response to metal toxicity but as antioxidant defense in order to sequestrate the metal cations and lessen metal toxicity. The positive correlation between Cd and MTs was not observed in *C. chione* gills despite the high Cd values measured there. Therefore, it can be argued that

biomarker measurements should be performed not only in the gills which are the target organ for toxicity and bioaccumulation but in the other tissues, as well, in order to fully assess the response to metal exposure.

The highest MDA levels were measured in *M. galloprovincialis* tissues compared to the other two bivalves, indicating greatest oxidative damage due to Cd exposure, despite the fact that in the mussels' tissues the highest MT concentrations were expressed. Probably, for the mussels, MT levels were not high enough to adequately protect the cells from lipid peroxidation caused by Cd exposure. Likely the habitat of each organism plays an important role to this effect, since the two clams live inside the sand, while the mussel is directly exposed to contaminated seawater. This assumption is also supported by the lack of difference between MDA levels in bodies and digestive systems of *C. chione* and *V. verrucosa*. Statistically significant positive correlations between Cd accumulation and concurrent MDA levels was found for *M. galloprovincialis* gills and mantle and *C. chione* gills, mantle and body but not in *V. verrucosa* tissues.

AChE activity during the exposure period generally decreased in all tissues of all three species examined, except the mantle of *V. verrucosa*. However, statistically significant correlations with Cd accumulation were observed only for *M. galloprovincialis* and *C. chione* mantle and body. AChE activity was lower in the gills of all species compared to the other tissues both in the control and exposed animals, which is another indication that the gills were the target organ for Cd accumulation and subsequent toxicity and cellular damage. The observed decrease of AChE levels in all tissues of *M. galloprovincialis* and *C. chione* during exposure seems to inversely correlate to the increase of LPO.

The results and conclusions of the present study indicate that not all organisms and their biomarkers respond to Cd contamination in the same way and in order to fully evaluate the health status of a bioindicator organism, multi-biomarker approach is needed.

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