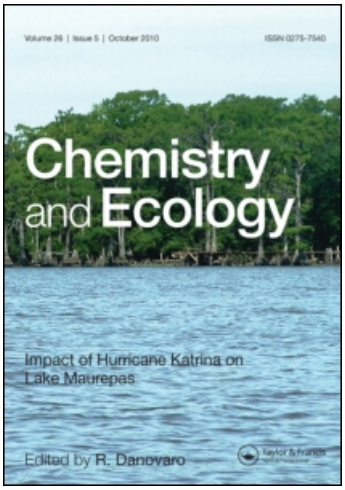

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Heavy-metal effects on lipid peroxidation and antioxidant defence enzymes in mussels *Mytilus galloprovincialis*

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Heavy-metal effects on lipid peroxidation and antioxidant defence enzymes in mussels *Mytilus galloprovincialis*

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The use of lipid peroxidation and antioxidant defence enzyme activities in aquatic organisms as molecular biomarkers of environmental pollution has been proved to be of great diagnostic value. Toxicities of Cu(II), Cd(II), Pb(II), and Fe(II) were investigated in mussels *Mytilus galloprovincialis* with a 24-h short-term bioassays (lethal concentration 50%, LC_{50}) using end-point mortality. Results showed that the LC_{50} values were: 0.35 mg l^{-1} for Cu, 1.6 mg l^{-1} for Cd, 4.5 mg l^{-1} for Pb, and >6 mg l^{-1} for Fe. The same metals were used at sub-lethal concentrations to study the modulation of lipid peroxidation and antioxidant enzyme activities in the gills and the mantle of *M. galloprovincialis* (exposed for a period of 10 d) and compared with a control (unexposed) group. Superoxide dismutase activities increased for Cu and displayed a high variability for Cd, Pb, and Fe, compared with control. Catalase (CAT) activities decreased for Cu, Cd, and Pb, but increased for Fe in both tissues. Lipid peroxidation (measured as MDA levels) increased for all metals in both tissues and were associated with equivalent decreases in antioxidant enzyme activities (especially CAT). These results indicated that mussels stimulated the increase in antioxidant enzyme activities as an adaptive response to metals' oxidative damage. However, these increases were not adequate to prevent oxidation of membrane lipids of soft tissues.

Keywords: Antioxidant enzymes; Toxicity; Superoxide dismutase; Catalase; Lipid peroxidation; Heavy metals; *Mytilus galloprovincialis*

1. Introduction

All aquatic organisms have antioxidant enzymes in their tissues, reflecting a physiological adaptation to effects of oxidative metabolism and exposure to pollutants with oxidative potential in their natural environment. Organisms can adapt to increase the production of reactive oxidative species (ROS) by upregulating antioxidant defences to detoxify excess oxyradicals and to reduce oxidative damage to biomolecules. Also, antioxidant defences can be inhibited, depleted, or saturated [1].

Bivalve molluscs, especially *Mytilus galloprovincialis*, have the ability to accumulate high concentrations of heavy metals in their tissues, thus providing a useful tool for monitoring metal pollution in coastal environments [2]. This ability is principally due to the functioning

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of a coordinated complex biochemical system [3]. Bivalves withstand high levels of pollution, such as heavy metals, polycyclic aromatic hydrocarbons (PAHs), and other xenobiotics, by inducing enzymatic antioxidant defence mechanisms that allow protection from oxidative damage [4–6].

Heavy metals are involved in toxic redox mechanisms through the generation of ROS, associated with oxidative damage to important biomolecules [7, 8], and molecular mechanisms of metal toxicity and carcinogenicity have been reviewed in the scientific literature [9, 10].

Mussels are widely used as sentinel aquatic organisms in the investigation of heavy metals in the marine environment [11, 12]. Antioxidant enzymes, like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and other ancillary enzymes are of great diagnostic value for environmental pollution indices [5, 13]. Failure of antioxidant defence enzymes to detoxify excess ROS production can lead to significant oxidative damage (protein degradation, DNA damage, enzyme inactivation and lipid peroxidation). Lipid peroxidation (LP) by ROS is considered to be a major mechanism by which oxyradicals can cause tissue damage, leading to impaired cellular function and alteration in physicochemical properties of cell membranes [14]. Lipid peroxidation has been proved as a useful biomarker of oxidative damage by metals [15, 16].

Bivalves are sessile filter-feeders of large volumes of water and are widely used in biomonitoring programmes [17]. The mussel *Mytilus galloprovincialis* is widely distributed in many areas of the Mediterranean Sea and is used for many years as a sentinel marine biomarker of environmental pollution in Greek coastal areas [18].

In order to investigate the effects of several heavy metals (*i.e.* Cu, Cd, Pb, and Fe), on the antioxidant enzyme activities (SOD and CAT) and the lipid peroxidation, short-term bioassays at sub-lethal concentrations were performed in sea water and in the gills and mantle of *M. galloprovincialis*. We also investigated, using short-term toxicity tests (24 h), the lethal concentration 50% (LC_{50}) of the same metals (Cu, Cd, Pd, and Fe) in other *M. galloprovincialis* individuals.

2. Material and methods

2.1 *Mussel specimens and acclimation*

Black mussels *Mytilus galloprovincialis* were collected, during March–May, from a mussel marine farm in a 'clean' area of Greece (as reported in our previous experimental work) and transferred to the laboratory for an acclimation period of 3 d. Mussels (selected for size, 4–5 cm length) were divided in five groups of 10 individuals ($5 \times 10 = 50$) and were placed in five aerated glass tanks (30 cm radius and 15 cm height) with 3 l of clean sea water. A group of 10 mussels was kept as the unexposed group (control).

2.2 *Determination of LC_{50} of metals*

First, short-term toxicity tests with metals (Cu, Cd, Pb, and Fe) were performed in order to establish the range of their lethal concentrations (LC_{50}) for this particular type of mussel and were very informative for our subsequent bioassay at sub-lethal concentrations. Mortality of mussels was used as the end-point. Toxicity tests were carried out as described by Yap *et al.* [19]. Briefly, 10 healthy test mussels were selected for each treatment of metal exposure in 3-l glass tanks of sea water. For each metal, different concentrations (mg l^{-1}) were used: $CuSO_4$: 0.10, 0.25, 0.50, and 1.00 mg l^{-1}; $CdCl_2$: 1.00, 1.50, 2.00, and 3.00 mg l^{-1}; $Pb(CH_3COO)_2$: 3.50, 4.50, 5.50, and 6.00 mg l^{-1}; $FeSO_4.7H_2O$: 1.00, 4.00, 6.00, 8.00, and 10.0 mg l^{-1}.

The exact values of LC_{50} were determined by Probit analysis, while those plotted in the graphs were just estimated values. A control group of 10 mussels without metal exposure was simultaneously set up for each experiment. The test solutions were kept static but constantly aerated and were held at room temperature ($25 \pm 2\,^{\circ}C$), salinity at 35‰ and dissolved oxygen at 7–8 mg l^{-1}. After 24 h of exposure, mussels were considered dead if they gaped with shell valves wide and showed no response to tactile stimulus.

2.3 *Antioxidant enzyme activity assays for ten day exposure*

Metal concentrations used in our experiments were much lower than LC_{50} concentrations found in the toxicity experiments. Values were: $CuSO_4$ 4 $\mu g\,l^{-1}$; $CdCl_2$ 100 $\mu g\,l^{-1}$; $Pb(CH_3COO)_2$ 125 $\mu g\,l^{-1}$; $FeSO_4.7H_2O$ 150 $\mu g\,l^{-1}$, respectively.

Fifty healthy mussels in five groups (5×10) were tested, and a control group of 10 mussels (without metal exposure) were kept under similar conditions. Mussels (10 healthy specimens) were kept for 10 d in well-aerated fresh sea water ($25 \pm 2\,^{\circ}C$, salinity 35‰). Aquarium sea water was exchanged every second day to keep test concentrations of metals at constant levels. SOD, CAT activities, and lipid peroxidation (as MDA levels) were measured in tissue homogenates after 1, 3, 6, 8, and 10 d of exposure, respectively. During experiments, no dead mussels were found. Mussels were removed, their gills and mantle were excised, and measurements were performed on the same day. Tissues were homogenized in ice-cold buffer (Tris HCl 50 mM, 1 mM EDTA, 0.15 KCl, pH 7.4), and the homogenate was centrifuged at 10 000 *g* for 15 min at 4 °C to remove cell debris. Separated supernatant fractions were collected and split onto two equal parts. The first part was used for SOD and CAT activities, and the second for MDA and protein quantification.

2.4 *Determination of Enzymatic assays and protein quantification*

Superoxide dismutase activity (EC 1.15.1.1) was determined by the degree of inhibition on the reduction of cytochrome *c* by superoxide anion generated by the xanthine oxidase/hypoxanthine system. Measurements were recorded at a wavelength of 550 nm [20]. The reaction was carried out in 0.05 M $Na_2PO_4/NaHPO_4$ buffer, pH 7.4, with 48 μM xanthine/0.2 units of xanthine oxidase and with 96 μM EDTA. One unit of SOD activity is defined as the amount of sample that inhibits the reduction in cytochrome *c* (19.2 μM) by 50%. The SOD activity is expressed, as it is found in many other similar studies, as SOD units/g of wet tissue.

Catalase activity (EC 1.11.1.6) was measured following the decrease in absorbance at 240 nm due to H_2O_2(0.036% w/w) consumption [21]. The reaction was carried out in phosphate buffer (50 mM at pH 7.0 at 25 °C containing 50–100 units of catalase per millilitre). The CAT activity is the difference in the absorbance, at that wavelength, per unit of time. The CAT activity is expressed as CAT units/mg of total protein concentration [21, 22].

2.5 *Determination of lipid peroxidation*

The extent of lipid peroxidation was measured as thiobarbituric acid reactives (TBARs), such as malondialdehyde (MDA), measured at 535 nm [23]. Two millilitres of the reaction mixture (thiobarbituric acid 0.375%, trichloroacetic acid (15%) and hydrochloric acid (0.25 N) were mixed in 1:1:1) were added to 1 mL of the heat-denatured supernatant. TBAR levels were estimated at 535 nm using MDA as standard. The concentration of lipid peroxidation compounds is expressed as nanomoles of MDA per gram of wet tissue.

2.6 *Total protein concentrations*

Total proteins were measured on the supernatants (10 000 *g* for 20 min at 4 °C) of the homogenized gills and mantle tissues [24]. The protein concentration is the difference in absorption at 280 and 260 nm. Protein concentration is calculated from the $(1.55 \times A_{280}) - (0.76 \times A_{260})$ in mg mL^{-1}. Bovine serum albumin (BSA) was used as a standard. Protein concentrations are expressed as mg g^{-1} of wet tissue.

2.7 *Statistical analysis*

Data are expressed as means ± standard deviation (SD) of four independent experiments, each performed in triplicate. Data of our results were previously tested for normality and homogeneity, and analysed using a one-way analysis of variance (ANOVA) to determine significant differences between the levels of antioxidant enzyme activities and the LP of the control group and the test groups (SPSS software). The level of significance was set at 5%. A p value of <0.05 was considered significant, whereas $p < 0.01$ and $p < 0.001$ were considered less significant.

3. Results and discussion

3.1 *24-h toxicity tests of metals in mussels*

The results of our short-term toxicity tests (24-h) with mussels *Mytilus galloprovincialis* suggested that the method was rapid, inexpensive, and practical. LC_{50} toxicity values, calculated from Probit analysis, were: Cu(II): 0.35 ± 0.02 mg l^{-1}; Cd(II): 1.7 ± 0.1 mg l^{-1}; Pb(II): 4.5 ± 0.2 mg mg l^{-1}; Fe(II): >6 mg l^{-1}.

High mortalities were observed in mussels at high concentrations of Cu, Cd, and Pb concentrations, whereas Fe exposure did not induce a high mortality, even for exposures higher than 6 mg l^{-1}. These results suggested that *M. galloprovincialis* mussels were most sensitive to the toxic effects of Cu, and the decreasing order of toxicity was Cu > Cd > Pb > Fe.

There are no similar short-term toxicity investigations with *M. galloprovincialis* mussels, as far as we know from the scientific literature. In order to compare the results of our investigation with other studies, we consider the toxicity bioassays (for LC_{50}) performed on mussels *Perna perna* and *Perna viridis*. The LC_{50} values for *Perna perna* were: Cu 0.25 mg l^{-1}, Cd 1.53 mg l^{-1}, Cu 0.25 mg l^{-1}, Pb 4.12 mg l^{-1}, and Zn 3.20 mg l^{-1} [19]. The ranges of LC_{50} values for *Perna viridis* were: Cu 0.08–0.25 mg l^{-1}, Cd 1.50–1.53 mg l^{-1}, Pb 2.3–4.46 mg l^{-1}, Zn 2.8–6.0 mg l^{-1} [25–27]. Eisler [28] reported that the values on acute toxicities (LC_{50}) of metals in the softshell clam *Mya arenaria* were: Cu 0.035 mg l^{-1}, Cd 0.15 mg l^{-1}, Zn 1.55 mg l^{-1}, Pb 8.8 mg l^{-1}, Ni ∼ 50 mg l^{-1}, and Mn 300 mg l^{-1}. These data suggest that mussels display a high feeding rate and can be more sensitive to metal toxicity for short-term toxicity tests. Preliminary measurements of the concentrations of metals in the gills and the mantle of mussels, for the 10-d short-term toxicity tests, show that accumulation is slower for metals with a higher toxicity. Fe concentrations in the gills and mantle are higher than Cu, Cd, and Pb. According to Yap *et al.*, this is an indication that mussels' valves close in response to metal toxicity levels [19].

3.2 *Exposure to sub-lethal concentrations of metals*

The results of LC_{50} for the four metals (Cu, Cd, Pb, and Fe) were used to establish the sub-lethal concentrations for the 10-d exposure experiments. We observed that indigenous mussels in a

typical polluted coastal area of the Saronikos Gulf (Greece) showed concentrations of these metals (in their gills and mantle) 5–7 times lower than the concentrations used in our 10-d experiments [29].

3.3 *SOD activities in mussels*

The levels of SOD activities in the mantle and gills of *M. galloprovincialis* exposed to Cu, Cd, Pb, and Fe(II), at sub-lethal concentrations for 10 d and compared with unexposed control group of mussels (10 mussels for each group), are shown in figure 1. Results for metals were placed in order of decreasing toxicity (with Cu the most toxic). Our results suggest that sub-lethal trace metal levels appear to influence the antioxidant enzymatic defences when compared with the unexposed control group.

Our results showed that SOD activity increases with time of exposure, compared with the control group. Small decreases were observed for Pb and Fe at the end of the 10-d exposure period. In the case of Cu, the SOD activities (in gills and mantle) increase and reach the maximum on the sixth day.

The results for Cd showed that SOD activity fluctuated within the test period, in both gills and mantles. After the third day, SOD activity decreased and subsequently, after the sixth day, increased again until the end of the experiment. This high variability can be an indication that mussels, after the initial loss of SOD activity, stimulate the production of SOD to contrast the effects of oxidative stress. Pb, which is a non-essential and toxic metal for marine organisms, causes an initial decrease in both the gills and mantle, but subsequently the SOD activity increases to the initial level. Fe, which is less toxic and essential for marine organisms, increases the SOD activity from the beginning of the experiment, but subsequently after the sixth day of exposure, the activity decreased, compared with the control group.

Our results can be related to the modulation, inhibition, or depletion of these antioxidant enzymes as the organisms respond to the increase in oxidative stress by metals [30]. It is inevitable that in the process, some of the enzymatic activity decrease and the biochemical metabolism of the organism requires some time to respond and recover its enzymatic activity in order to protect tissues from oxidative damage [31, 32].

Our results are comparable with other short-term toxicity studies in the scientific literature. Antioxidant enzyme activity levels were found to be significantly higher in mussels *Perna viridis* (mantle, digestive gland, and gills) when exposed to Cd, Pb, and Al at sub-lethal concentrations [11]. Also, it is known from other studies in aquatic organisms that high levels of antioxidant enzyme activity are observed in polluted areas with high concentrations of heavy metals, compared with non-polluted areas [33]. A decrease in SOD activity was reported in the digestive gland of *M. galloprovincialis* when exposed to Cd for 21 d [34].

3.4 *CAT activities in mussels*

Results showed that CAT activities decreased in the mantle of mussels when exposed to Cu, Cd, and Pb and in the gills for exposure to Cu and Cd, compared with the control group. Results for CAT are shown in figure 2.

Cu causes the CAT activity (mantle) to fluctuate but then if decreases after the sixth day of exposure, whereas in the gills the CAT activity showed a permanent decrease throughout the exposure period, probably as an indication for the highly toxic effects of the metal. Cd effect was a permanent decrease in the levels of CAT activity (mantle and gills) throughout the 10-d exposure period. Pb decreased CAT activity in the mantle, but in the gills there was some decreased and increased activity. The CAT activity increased during the 10-d exposure to Fe (gills and mantle).

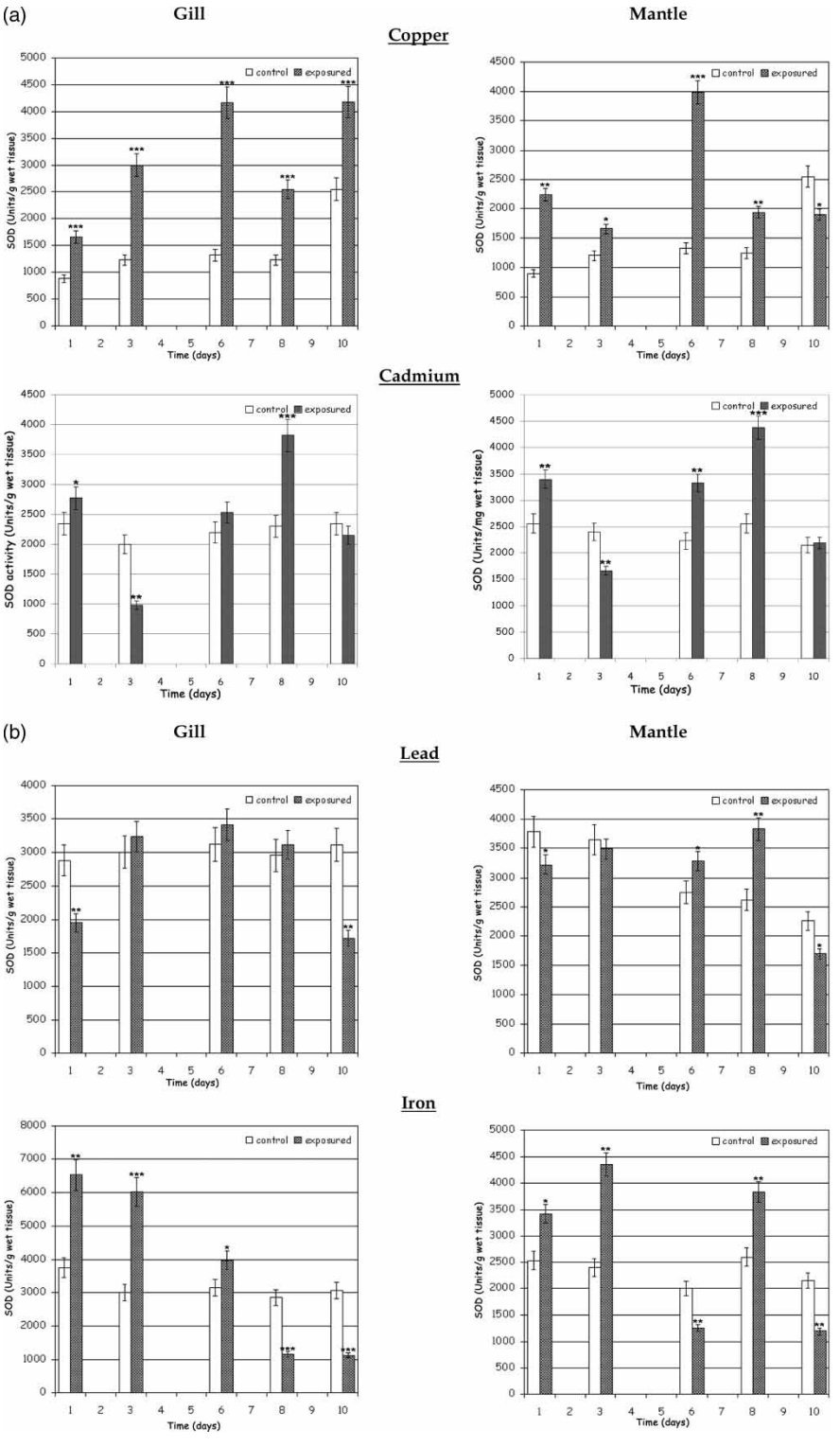

Figure 1. Levels of SOD activities in the mantle and gills of mussels exposed to Cu, Cd, Pb, and Fe, and control. Values of SOD activities are expressed as SOD units/g of wet tissue. Test significance differences between exposed and control $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

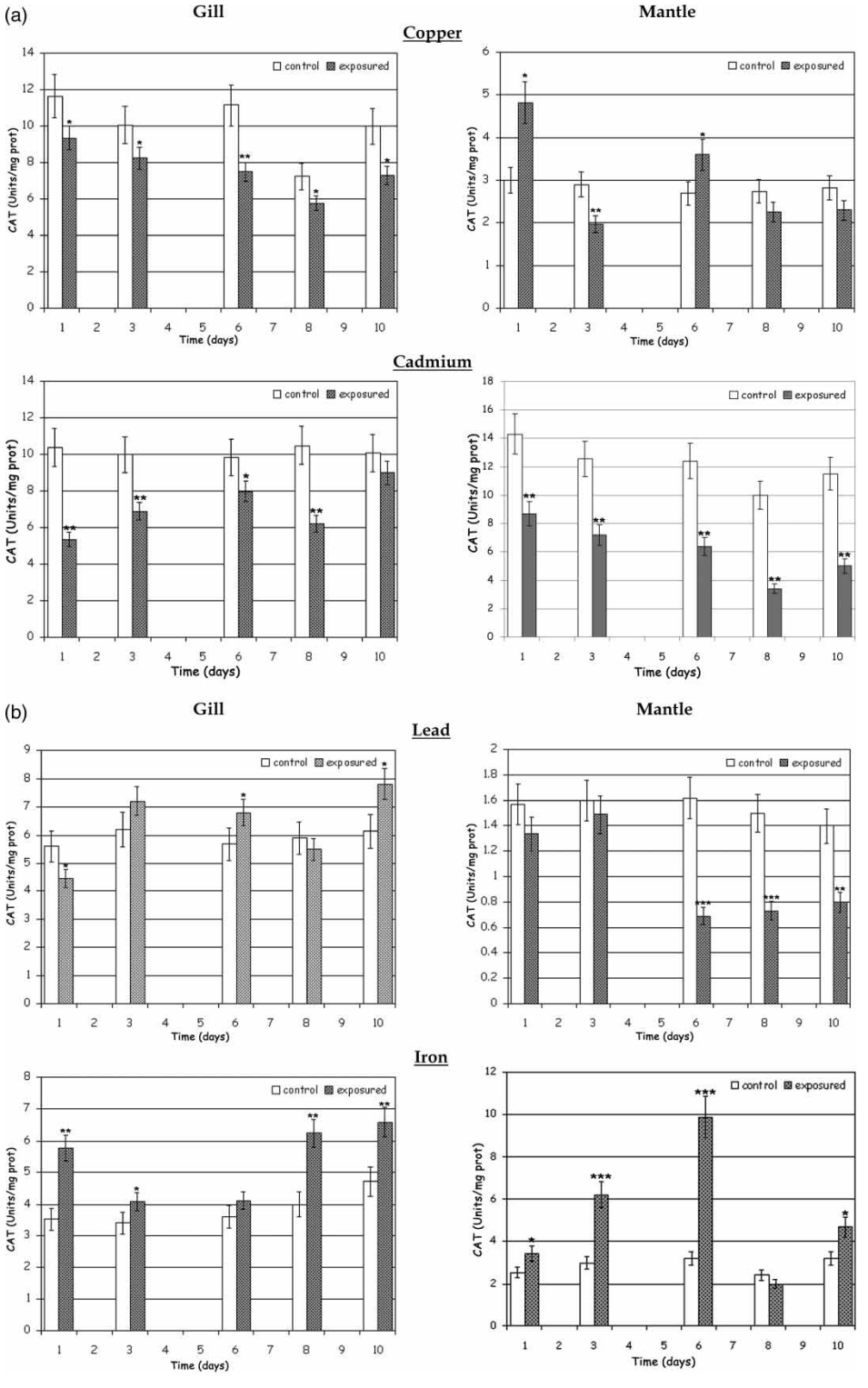

Figure 2. Levels of CAT activities in the mantle and gills of mussels exposed to Cu, Cd, Pb, and Fe compared with the control group. Values are expressed as units/mg of total protein concentration. Test significance differences between exposed and control $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Our results can be compared with previous findings by several studies. Fluctuations of CAT activity were observed in the mussel *Perna perna* exposed to Cu and Cd for 120 h. Increased levels of CAT activity can be accounted for the protection against lipid peroxidation [12]. Viarengo *et al.* [35] observed increased levels of CAT in *Mytilus galloprovincialis* exposed to $CdCl_2$ ($0.2\,mg\,l^{-1}$) for 7 d, but negligible variations for SOD and GPX enzymes. The authors suggested that exposure to Cd, detoxification (lasting 28 d), and subsequent exposure to 0.3–0.6 $mg\,l^{-1}$ $FeCl_3$, produced a strong and persistent metallothionein (MT) increase in the mussel's digestive gland [35].

3.5 *Lipid peroxidation in mussels*

Results of lipid peroxidation (LP) showed that all metals caused an increase in MDA levels in both tissues with time of exposure. The results are presented in figure 3. Results of LP increases are expected, since all investigated metals have a redox potential contributing to the production of ROS and subsequently to oxidative damage to membrane lipids.

Cu increased LP from the first day of exposure (only a temporary drop on the sixth day) in both the gills and mantle of mussels. Cd showed an almost linear increase in LP with time of exposure. Also, Pb and Fe increased LP levels in both tissues. In particular, Fe exposure caused a substantial increase in LP in gills. Most of these increases are correlated with a decrease in CAT activity in both tissues, compared with the control group in which the levels of LP remained almost the same with small variations.

There are similar results in the scientific literature on LP with exposure to metals. Viarengo *et al.* [15] observed elevated levels of MDA in the gills and the digestive gland of *M. galloprovincialis* for six day exposure to Cu, Cd and Zn, with Cu being the most toxic metal. Similarly, increased levels of LP were found for Cu and Cd exposure [12]. Significant increases of MDA levels were observed in the gills of *Perna viridis* [16]. Prakash and Rao [11] observed high levels of LP in the bivalve *Perna viridis* for 7-d exposure to Cd. Geret *et al.* [36] observed increases in the cellular MDA levels of the clam *Ruditapes decussatus* after 14 d of exposure to Cd. Finally, the effects on phospholipase C activity (derived from lipid peroxidation) of heavy metals Hg and Cu were observed in the gills and the digestive gland of *M. galloprovincialis* [37]. In the last decade, lipid peroxidation, as well as antioxidant defence enzymes, in aquatic organisms have become useful biomarkers for biomonitoring of environmental pollution [38].

3.6 *Protein concentrations in gills and mantle*

Total protein concentrations in the supernatants of the homogenized gills and mantle tissues (10 mussel specimens) were found in the range: gills 1.82–2.09 mg/g (min–max) wet weight tissue; mantle 1.80–2.18 mg/g wet weight tissue.

3.7 *Conclusion*

This study has been carried out to provide information on heavy-metal effects on antioxidant enzyme activities and lipid peroxidation levels of mussels after 10-d exposure at sub-lethal concentrations. The initial results of the short-term toxicity assay (24 h) in *M. galloprovincialis* suggest that Cu is the most toxic to mussels, followed by Cd, Pb, and Fe. The results of LC_{50} are very similar to values reported in other studies based on *Perna perna* and *Perna viridis*. Our results on the antioxidant enzymes in both the gills and the mantle show that SOD increases compared with the control group, whereas CAT mostly decreases for exposure to Cu, Cd, and Pb, but increases when exposed to Fe. As expected, higher levels of LP products (as MDA) are

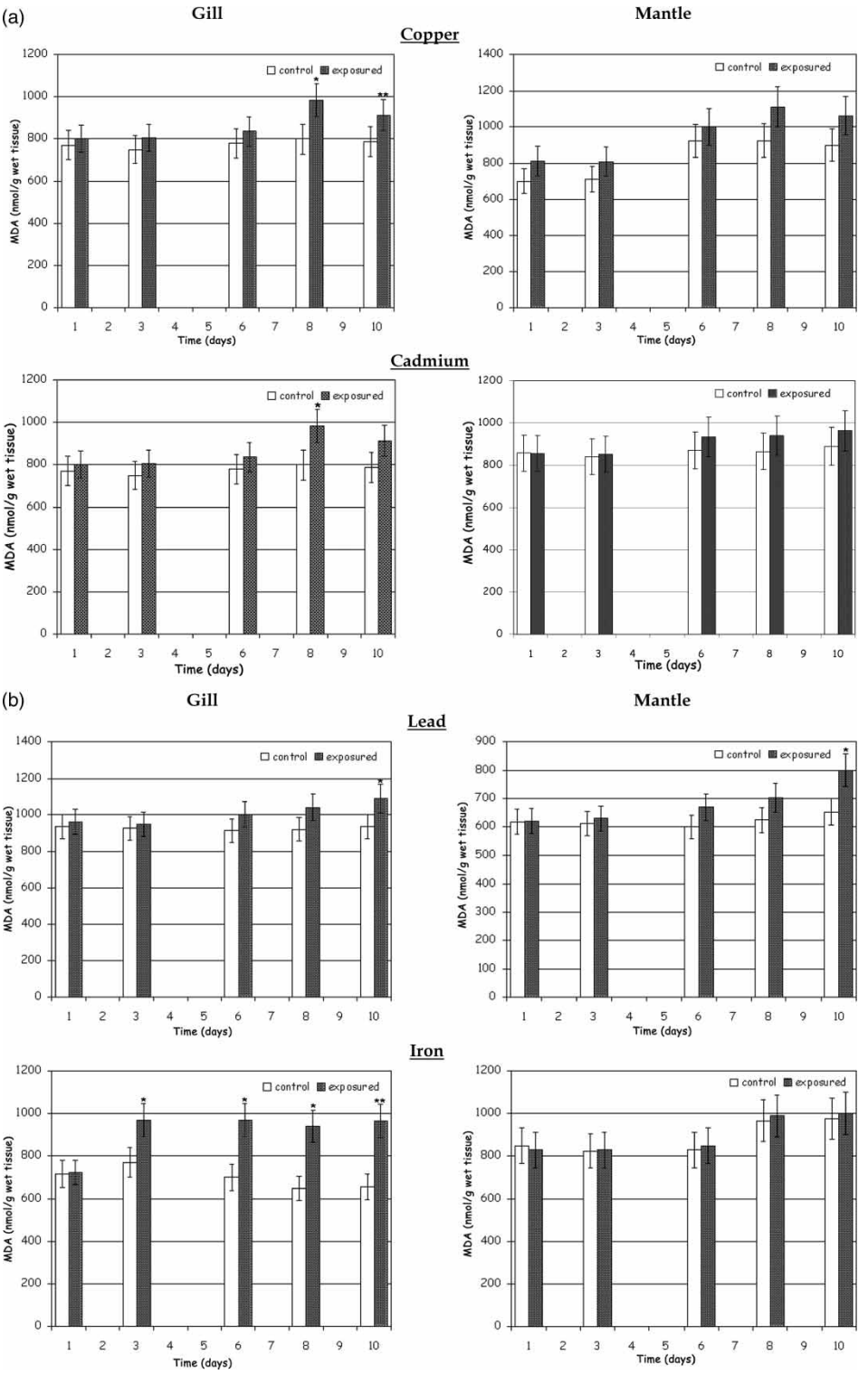

Figure 3. Levels of lipid peroxidation (as MDA) in the gills and mantle of mussels exposed to Cu, Cd, Pb, and Fe, and the control group. Values are expressed as MDA nmol/g wet tissue. Test significance differences between exposed and control $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

observed for all metals. The increasing levels of LP are correlated with the decrease in CAT activity observed at the same period. Marine organisms increase their antioxidant defences and scavenging activity towards oxyradicals for the protection of tissues from oxidative damage when they are subjected to the high levels of heavy-metal pollution.

Acknowledgements

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