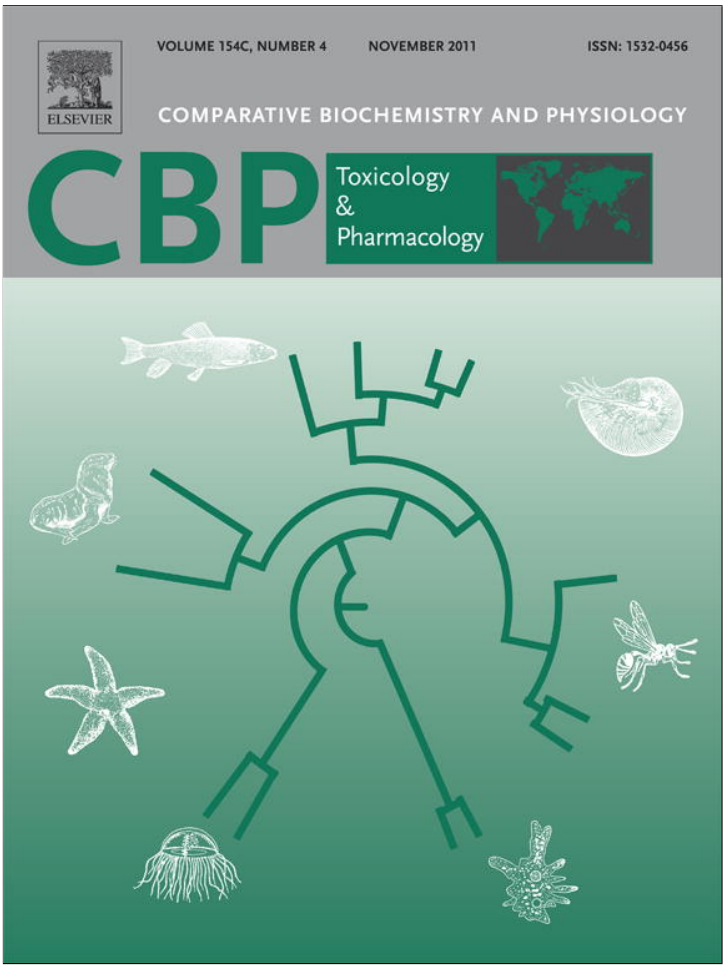

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Oxidative stress and histological alterations produced by dietary copper in the fresh water bivalve *Diplodon chilensis*

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ABSTRACT

The aim of this work was to study the oxidative stress effects and histological alterations caused by dietary copper on the filter-feeding freshwater mussel *Diplodon chilensis*. Bivalves were fed during 6 weeks with the green algae *Scenedesmus vacuolatus* previously exposed to copper. Metal concentration in algae cultures and bivalve digestive gland was measured by TXRF. A maximum accumulation of 0.49 μg Cu/mg protein was detected at week 6. Also at this week, the hepatosomatic index (HSI) showed the highest decrease (50%) in response to Cu exposure. SOD and GST activities were significantly increased at weeks 4, 5 and 6, reaching an activity on average 50% higher than in controls for GST. CAT activity and GSH increased significantly at weeks 5 and 6. Despite this response, oxidative damage measured as TBARS and carbonyl groups contents increased significantly at weeks 4, 5 and 6, respectively. Digestive tubule and duct atrophy and cell-type replacement in treated mussels were observed by histological studies. The presence of intracellular rhodanine-positive granules, suggests copper accumulation in intracellular vacuoles of digestive cells.

1. Introduction

The presence of industrial, domestic and agricultural wastes in the aquatic system has been widely recognized as a potential threat to the environment. Among the variety of contaminants present in water, trace metals can reach high concentrations in sediments and also in aquatic organisms by bioaccumulation through the food chain (Gumgum et al., 1994). Metal environmental persistence is directly related to the chemical speciation, which plays a key role in the bioavailability and toxicity on aquatic organisms (Van Ginneken et al., 1999).

Exposure and effects of several pollutants on living organisms can be studied through the use of biomarkers, i.e. specific changes on biological parameters, such as the evaluation of biological molecule oxidation and/or determining changes in antioxidant levels and in detoxification mechanisms (Boelsterli, 2003).

Aquatic invertebrates accumulate different trace metals at varying concentrations, whether essential or not for their metabolism (Eisler, 1981), in relation to the physiological condition of the organism, its metal uptake-release rate and environmental factors (Rainbow, 1987, 1990; Connell et al., 1999). Metal bioaccumulation also depends on the exposure time and on accumulation/elimination rate (Landrum et al., 1994). Bivalves have seasonal changes in weight, which would add another variable to consider when using this variable to estimate the metal accumulation. Some authors recognize the great variability of body weight as a limitation of this expression, and instead have expressed metal concentrations in terms of less variable parameters such as body length (Al-Yousuf et al., 2000) or protein content (Sabatini et al., 2009b).

Metals uptake in bivalves depends on their availability in the water column, if they are bound to sediment or not, and if they are present in the phyto-bacterio plankton filtered by these organisms (Lee and Louma, 1998; Griscom et al., 2000).The freshwater mussel *Diplodon chilensis* (d'Orbigny, 1835) is abundant in a great number of lakes and rivers of West Patagonia, Argentina and Chile (Bonetto, 1973; Parada and Peredo, 2002; Parada et al., 2007). *D. chilensis* is a filter-feeder which consumes phyto-plankton, phyto-benthos as well as bacteria and particulate organic matter. In a field study, this bivalve has been shown to accumulate heavy metals, even when these are present in

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the environment at very low concentrations (Ribeiro Guevara et al., 2004). Two recent papers highlighted the important ecological role that this species plays in Chilean and Argentinean lakes and the alarming population decrease caused by eutrophication (Valdovinos and Pedreros, 2007; Sabatini et al., in press).

Most experimental studies on trace metals toxicity using bivalves have been carried out by adding the contaminant to water (Ringwood et al., 1998; Raftopoulou and Dimitriadis, 2011; Maria and Bebianno, 2011). However, in nature, especially in not heavily polluted water bodies, trace metals are not abundant in water but can be accumulated in sediments and in phytoplankton and bacterioplankton. Thus, the food chain seems to be the most important source of trace metals for filter feeding organisms such as *D. chilensis*.

The goal this study was to assess whether copper can be accumulated, causing oxidative stress and histological damage in the digestive gland of *D. chilensis* by trophic transference from microalgae exposed to low free Cu ions concentrations. We exposed *D. chilensis* to dietary copper, by feeding the animals with the green algae *Scenedesmus vacuolatus* previously cultured in the presence of Cu. Metal accumulation in the digestive gland was measured by TXRF. The antioxidant response was evaluated as the glutathione content and the enzyme activity related to oxidative stress (superoxide dismutase, catalase, and glutathione-S-transferase). Damage to proteins and lipids was determined as carbonyl groups and TBARS content. Histological examinations were made in order to detect the presence of Cu within the tissues, histopathological alterations and possible Cu excretion mechanisms.

2. Materials and methods

2.1. Organisms

BAFC CA4 strain of *Scenedesmus vacuolatus* (Chlorophyceae, Chlorophyta) and BAFC CA10 strain of *Chlorella kessleri* are currently kept in the Culture Collection of the Laboratory of Phycology, Department of Biodiversity and Experimental Biology, Faculty of Exact and Natural Sciences, University of Buenos Aires.

Individuals of *D. chilensis* were collected by a diver at Yuco, an unpolluted area of the Lacar Lake with a waterborne Cu concentration of 0.02 mg/L (Rocchetta et al., unpublished results) (40°10′S, 71°31′ 30″W) Neuquén province, Patagonia, Argentina, from the benthos at a depth of 6–8 m. Mussels were transported alive at low temperature to the University of Buenos Aires, where they were used for laboratory studies. Mussel's identification was performed according to Vega Aguayo (1982).

2.2. Algae cultures

Algal cultures were prepared in Bold's basal medium (BBM) (Bischoff and Bold, 1963). The control medium contained 6.2 μM Cu (already present in normal BBM), the high Cu medium was prepared by adding 108 μM Cu as $CuCl_2$.$2H_2O$ to BBM (Sabatini et al., 2009a). Since this medium contains EDTA, we used the MINEQL$^+$ 4.0 software to calculate the free Cu ions concentrations (2.12×10^{-12} mg/L for the control medium and 7.71×10^{-10} mg/L for high Cu medium). The number of cells used to feed the mussels was determined by cell counting in Neubauer's chamber. Total copper content was determined in both cultures of *S. vacuolatus* (control and high Cu) by TXRF, according to Sabatini et al. (2009a), resulting in 0.06 μg Cu/10^6 cells and 1 μg Cu/10^6 cells respectively.

2.3. Filtration rate

A filtration rate experiment was performed with the objective of determining the maximum cell concentration at which *D. chilensis* could filter *S. vacuolatus* cultured in BBM medium with and without copper addition at similar rates. Twelve mussels (6.07 ± 1.56 cm shell length) were put into individual beakers with 500 mL of dechlorinated tap water, with constant aeration and a temperature of 20 ± 1 °C. The possible presence of Cu in the dechlorinated tap water used for the assays was tested by TXRF with negative results.

After 48 h of acclimation with no food, when each individual had the valves open, *S. vacuolatus* was added at a final concentration of 3×10^6 cells/mL. Samples were taken after 3 h and cell number was estimated by direct counting in a Neubauer's chamber, using an Olympus light microscope at 1000×. Filtration rate for each individual, expressed as L/h per dry soft tissue mass (g), was calculated according to Jorgensen (1990). Dry soft tissue mass was measured after dissecting out and drying the soft tissues in an oven for 48 h at 60 °C, until constant mass.

2.4. Experimental design

Prior to the experiments, 72 adult *D. chilensis* (6.13 ± 1.49 cm shell length) were acclimated at constant temperature (20 ± 1 °C), photoperiod 12-h light: 12-h dark cycle and fed with the green alga *C. kessleri* for 3 weeks. Following the acclimation period, two groups of 36 animals were weighed and placed in 5 L containers with aerated dechlorinated tap water. The control group was fed with *S. vacuolatus* cultured in normal BBM. The treated group received the *S. vacuolatus* cultured in the high Cu medium. In both groups, each individual mussel was allowed to filter 3×10^6 algae cells for 24 h, twice a week, resulting in an approximated weekly dose of 0.018 μg Cu/g mussel (considering 0.06 μg Cu/10^6 cells) for the control group and 0.3 μg Cu/g mussel (considering 1 μg Cu/10^6 cells) for the treated group. This procedure was repeated for 6 weeks. Water was changed before adding algae. Six individuals were sacrificed at the end of each week, and their body mass and shell length were recorded. The hepatosomatic index (HSI) was calculated as digestive gland mass/fresh soft tissue mass.

Digestive glands were homogenized with 0.134 M KCl (1:5 w/v) containing 0.5 mM PMSF and 0.2 mM benzamidine (protease inhibitors) to study oxidative stress parameters. The homogenate was centrifuged at 11,000×*g* for 20 min. Biochemical determinations were carried out in 11,000×*g* supernatants from total homogenate and results were expressed per mg total protein.

Total soluble protein content was measured by the method of Bradford (1976), using bovine serum albumin as standard. Results were expressed as μg total protein per mL.

2.5. Digestive gland copper content

To determine copper content by TXRF, *D. chilensis* digestive glands were homogenized in 0.134 M KCl (1:5, w/v) and digested in a microwave oven with HNO_3 and H_2O_2 (2:1, v/v) following the protocol described by Sabatini et al. (2009b). Spectrum evaluation and quantitative analysis were performed using the QXAS software package from IAEA, using least square regression analysis and calibration curves within the range of 1–20 ppm. Results were expressed as μg Cu per mg protein.

2.6. Oxidative damage

Lipid peroxidation was determined measuring thiobarbituric acid reactive substances (TBARS) in the digestive glands of *D. chilensis* by the Beuge and Aust procedure (1978). TBARS concentration was estimated using an extinction coefficient of 156 $mM^{-1}\,cm^{-1}$ and absorbance determination at 535 nm. Results were expressed as μmol TBARS per mg protein.

Protein oxidation was quantified as carbonyl groups according to Resnick and Packer (1994). Carbonyl content was calculated from the peak absorbance (355–390 nm) using an extinction coefficient of

22,000 M^{-1} cm^{-1}. Results were expressed as nmol carbonyl per mg protein.

2.7. Reduced glutathione content

Reduced glutathione (GSH) levels were measured in digestive glands of *D. chilensis* following the Anderson procedure (1985). Absorbance at 412 nm was measured after 30 min incubation at room temperature. Results were expressed as nmol GSH per mg protein.

2.8. Enzyme activities

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured using the procedure of Beauchamp and Fridovich (1971). Results were expressed as U per mg protein. One SOD unit was defined as the enzyme amount necessary to inhibit 50% of the reaction rate.

Catalase activity (CAT, EC1.11.1.6) was measured by monitoring the decay of H_2O_2 during 30 s at 240 nm, using an extinction coefficient of 40 M^{-1} cm^{-1} (Aebi, 1984). Results were expressed as U per mg protein. One CAT unit was defined as the enzyme amount that transforms1mmol of H_2O_2 per min.

Glutathione-S-transferase (GST, EC1.11.1.9) activity was measured by the technique of Habig et al. (1974). One GST Unit was defined as the amount of enzyme needed to catalyze the formation of 1 μmol of GS-DNB per min at 25 °C.

2.9. Histology

Histological examinations were carried out after 5–6 weeks of experiment. Control and treated individuals were removed from their shells (4 animals/treatment), and their digestive glands were fixed in 10% phosphate-buffered formaldehyde (pH 7.2). Samples were then dehydrated through an ethanol series, cleared in xylene, and embedded in paraffin. Sections (7 μm thick) were stained with, haematoxylin and eosin. Potassium permanganate $KMnO_4$ (0.25% v/v) and hydrogen-peroxide H_2O_2 (7% v/v) were used to detect melanin-like deposits by bleaching. Selected subsamples were stained for copper using rhodanine (Irons et al., 1977). Observations were performed with a Leica ICC50 light microscope attached to a digital camera.

2.10. Statistical analyses

Copper effects on biochemical variables were analyzed by two-way analysis of variance (ANOVA) followed by a Dunnett's post hoc test, taking treatment and time as factors. Copper accumulation in mussels was compared statistically by two-way analysis of variance (ANOVA) followed by a Dunnett's post hoc test. Normality and homogeneity of variances were tested by Lilliefors' and Bartlett's tests, respectively (Sokal and Rohlf, 1999). Copper content as a function of time and TBARS levels as a function of internal copper content were studied in treated mussels by linear regression analysis (Sokal and Rohlf, 1999). Graph Pad Prism 3 software was used for statistical analysis.

3. Results

3.1. Filtration rate

The filtration rate of *D. chilensis* for a *S. vacuolatus* concentration of 3×10^6 cells/mL was 0.789 ± 0.078 L/h per g of dry tissue, for mussels exposed to high Cu algae (treated mussels) and 0.826 ± 0.093 L/h per g of dry tissue, for mussels fed with control algae (control mussels). These rates were not statistically different ($p>0.05$).

3.2. Copper content

The digestive gland Cu concentration was significantly higher ($p<0.05$) in treated mussels than in controls, at weeks 5 and 6 (Fig. 1). At the end of the experiment, treated mussels accumulated up to 0.48 μg Cu/mg protein, which is 55% higher than the control value (0.31 μg Cu/mg protein). Both control and treated mussels had higher Cu concentrations than freshly collected individuals from the same site (0.08 μg/mg protein) (Rocchetta et al., unpublished results). The total protein content did not differ between treatments through the experimental time ($p>0.05$). Accumulated Cu in the digestive gland of treated *D. chilensis* followed a positive linear regression with time of exposure ($p<0.001$, $r^2=0.721$) (Fig. 1 inset).

3.3. Hepatosomatic index

Copper induced a progressive decrease in the hepatosomatic index (HSI) in treated mussels (Fig. 2) that was significantly lower than the control value at weeks 5 and 6 ($p<0.05$). HSI values of treated mussels diminished 50% compared to control mussels at week 6.

3.4. Oxidative damage

Lipid peroxidation, measured as TBARS content, was significantly increased in treated mussels at weeks 4, 5 and 6. At week 6, treated animals showed 70% higher TBARS levels than control animals ($p<0.001$) (Fig. 3). TBARS increment in treated animals followed a linear relationship with Cu accumulation ($p<0.001$, $r^2=0.791$) (Fig. 3 inset). Protein oxidation as carbonyl groups increased significantly at week 6 ($p<0.05$) in treated mussels compared to controls (Fig. 4).

3.5. Antioxidant defense

In response to dietary Cu the digestive gland of *D. chilensis* showed a significant increase in all the enzymatic and nonenzymatic defenses studied. Significant increases ($p<0.05$) in enzymatic activities were detected at weeks 4, 5 and 6 for SOD and GST. SOD activity was 50% higher than in controls while GST activity was increased by 65% (Figs. 5 and 6). CAT activity in treated mussels showed a significant increase at weeks 5 and 6 ($p<0.001$) (Fig. 7). GSH levels in treated mussels were 50% increased at the last week of experiment with respect to the controls ($p<0.05$) (Fig. 8).

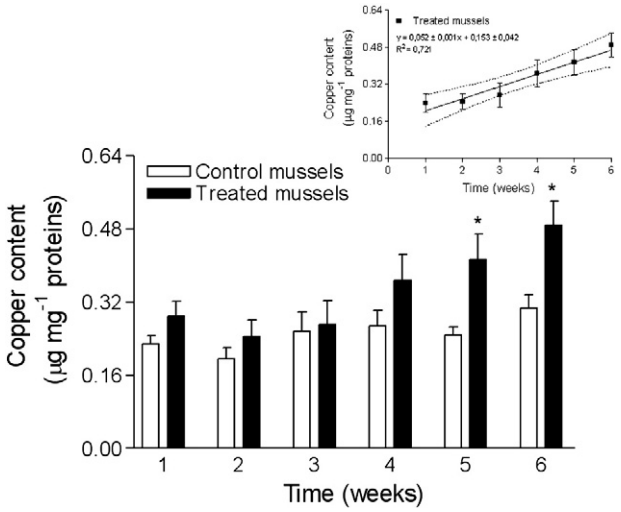

Fig. 1. Copper accumulated (μg Cu mg^{-1} protein) in the digestive gland of *D. chilensis* along 6 weeks of feeding with control and Cu-rich algae. Data are expressed as means ± SD ($n=6$). Significant differences between treatments are indicated by asterisks: * $p<0.05$. Inset: linear regression of μg Cu mg^{-1} protein vs. time of exposure for treated mussels ($p<0.001$, $r^2=0.721$).

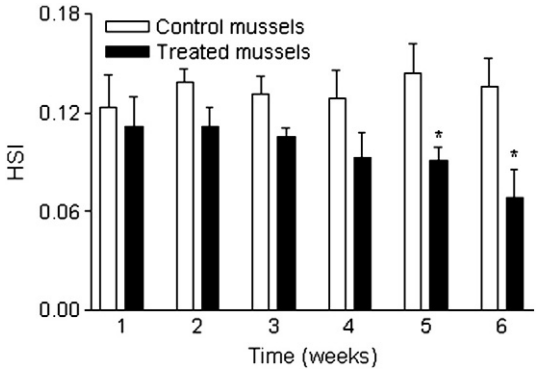

Fig. 2. Hepatosomatic index (HSI) of *D. chilensis* along 6 weeks of feeding with control+ and Cu-rich algae. Data are expressed as means ± S.D. (n = 6). Significant differences between treatments are indicated by asterisks: * $p<0.05$.

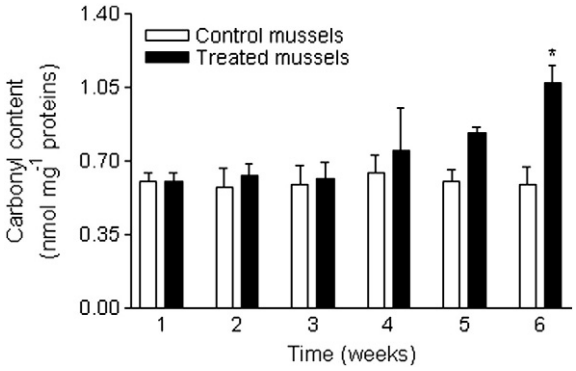

Fig. 4. Protein oxidation (nmol Carbonyl mg^{-1} protein) measured in the digestive gland of *D. chilensis* along 6 weeks of feeding with control and Cu-rich algae. Data are expressed as means ± S.D. (n = 6). Significant differences between treatments are indicated by asterisks: * $p<0.001$.

3.6. Histology of digestive gland

Digestive gland of control animals showed well defined digestive tubules, main and secondary digestive ducts and surrounding connective tissue (Fig. 9a). The main digestive ducts appeared with a large lumen lined by an epithelium composed of ciliated and non-ciliated areas, with well defined cilia roofs. The digestive tubules epithelium was formed by two cell types: digestive cells, which were columnar, with small basal nuclei and conspicuous lysosomal digestive system; and basophilic cells, characterized by their triangular shape, and nuclei with well visible nucleoli, which suggests a protein synthesis function. Digestive cells had prevalence of 72 ± 1.2% in total epithelium compared to the basophilic cells (28 ± 1.1%) (Fig. 9b).

Different histopathological alterations were observed in the digestive gland of treated individuals at the last 2 weeks of treatment. The epithelial cells were disrupted and spilled off into the lumen of digestive tubules and ducts. Surface microerosions of the epithelium, deeper erosive disturbances of tubules and lumen occlusion were observed (Fig. 9c). Vacuolization of digestive cells was increased in treated mussels respect to the controls. Atrophy of digestive tubules was accompanied by changes in cell-type ratios. An increase was observed in the proportion of basophile cells (38 ± 1.8%) at the expense of the digestive cells (62 ± 1.9%), with changes in the cell size (Fig. 9d). Both basophilic and digestive cells were significantly shorter in treated mussels' digestive glands ($p<0.05$ and $p<0.001$, respectively) respect to controls (36.94 ± 2.97 μm versus 45.22 ± 3.24 μm, for basophilic cells and 37.19 ± 5.39 μm versus 53.30 ± 4.82 μm for digestive cells). No changes were observed in the cell width measured in treated (11.17 ± 2.40 μm, for digestive cells and 11.48 ± 2.70 μm, for basophilic cells) and control cells (12.28 ± 1.20 μm and 13.30 ± 1.07 μm, for digestive and basophilic cells respectively).

Distinct intracellular rhodanine-positive granules, which reveal the presence of copper, were observed in the digestive tubules and ducts of treated mussels. These granules appeared to be packed into vacuoles inside the cells and were also detected in the lumen within the cell detachment material (Fig. 9e and f). The connective tissue surrounding the digestive tubules and ducts of treated mussels presented a necrotic aspect and numerous melanin-like deposits (Fig. 9f).

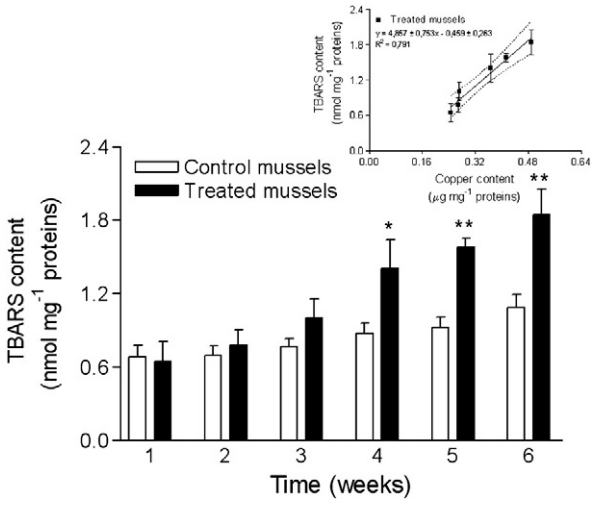

Fig. 3. Lipid peroxidation (nmol TBARS mg^{-1} protein) measured in the digestive gland of *D. chilensis* along 6 weeks of feeding with control and Cu-rich algae. Data are expressed as means ± S.D. (n = 6). Significant differences between treatments are indicated by asterisks: * $p<0.05$. Inset: linear regression of nmol TBARS mg^{-1} protein vs. μg Cu mg^{-1} protein for treated individuals ($p<0.001$, $r^2 = 0.791$).

4. Discussion

We have observed a decrease in the hepatosomatic index (HSI) at weeks 5 and 6 of experiment in mussels exposed to a Cu-rich diet, which correlates with a progressive Cu accumulation. Reduction of HSI has also been reported for *D. chilensis* dietary exposed to microcystin (Sabatini et al., in press), for the crab *Neohelice granulata* exposed to Cu (Sabatini et al., 2009b), and for salmonid fishes exposed to Cd, Zn and Cu (Ricard et al., 1998; Norris et al., 2000). The latter authors proposed that excessive lipid peroxidation caused by metal uptake through the diet could lead to hepatic cell death and thus to reducing the digestive gland size. In accordance, we recorded

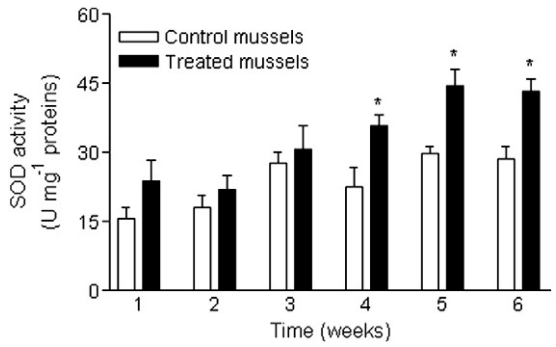

Fig. 5. Superoxide dismutase activity (U SOD mg^{-1} protein) measured in the digestive gland of *D. chilensis* along 6 weeks of feeding with control and Cu-rich algae. Data are expressed as means ± S.D. (n = 6). Significant differences between treatments are indicated by asterisks: * $p<0.05$.

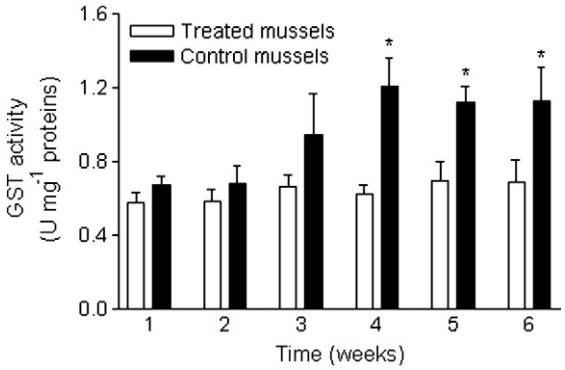

Fig. 6. Glutathione-S-transferase activity (U GST mg^{-1} protein) measured in the digestive gland of *D. chilensis* along 6 weeks of feeding with control and Cu-rich algae. Data are expressed as means ± S.D. (n = 6). Significant differences between treatments are indicated by asterisks: * $p<0.05$.

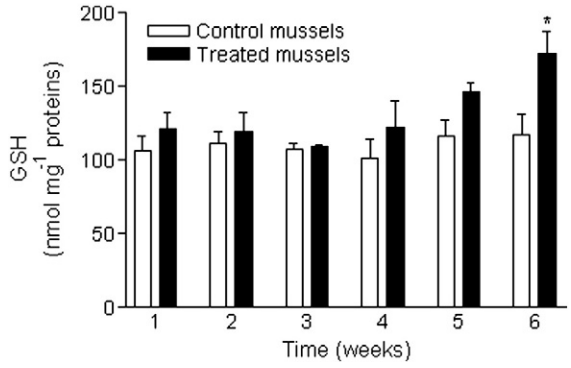

Fig. 8. Reduced glutathione (GSH) content (nmol GSH mg^{-1} protein) measured in the digestive gland of *D. chilensis* along 6 weeks of feeding with control and Cu-rich algae. Data are expressed as means ± S.D. (n = 6). Significant differences between treatments are indicated by asterisks: * $p<0.05$.

oxidative damage to lipids and proteins in *D. chilensis* exposed to Cu, which has probably led to the partial or total detachment of digestive gland epithelial cells, which is evident in the histological micrographs.

The capacity of lipid peroxides to damage cells by changing the fluidity and permeability of the membrane or by directly attacking DNA and other intracellular molecules such as proteins has been previously reported (Mattie and Freedman, 2001).Oxidative stress induced by copper exposure, evidenced by increased lipid peroxidation products such as malondialdehyde and lipofuscins, has also been demonstrated for the mussels *Mytilus galloprovincialis* (Viarengo et al., 1990), *Perna perna* (Almeida et al., 2004), *Ruditapes decussatus* (Roméo and Gnassia-Barelli, 1997; Geret et al., 2002), and for the oyster *Crassostrea virginica* (Ringwood et al., 1998).

Antioxidant defenses may be increased or inhibited by chemical stressors. The occurrence of one kind of response or the other depends on the intensity and duration of the applied stress and the susceptibility of the species that are exposed (Bebianno et al., 2005). There are several reports on increased SOD and CAT activities in bivalves in the presence of excess free radicals (Livingstone et al., 1990; Pellerin-Massicotte, 1994; Fournier et al., 2000). In our work, treated mussels have shown increased SOD, GST and CAT activities after 4–5 weeks of treatment. In particular, GST activity has been used as a biomarker of exposure to anthropogenic compounds such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) (Di Giulio et al., 1993; Fitzpatrick et al., 1997; Park et al., 2009). Recently, Won et al. (2011) have postulated GST as biomarker for cadmiun pollution at the gene expression level. The fact that the antioxidant enzymatic defenses of *D. chilensis* are induced by Cu only after long-term exposure suggests gene expression induction rather than enzyme modulation as the response mechanism.

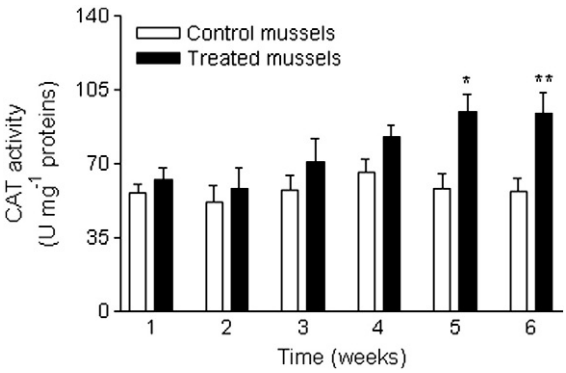

Fig. 7. Catalase activity (U CAT mg^{-1} protein) measured in the digestive gland of *D. chilensis* along 6 weeks of feeding with control and Cu-rich algae. Data are expressed as means ± S.D. (n = 6). Significant differences between treatments are indicated by asterisks: * $p<0.05$ and ** $p<0.001$.

Reduced glutathione (GSH) is a tripeptide involved in transport and metabolic processes, protecting the cell against toxic effects of endogenous and exogenous compounds, including reactive oxygen species and trace metals (Cnubben et al., 2001). Several studies show that GSH concentrations are modified in response to exposure to different pollutants (Hoffman, 2002; Irato et al., 2003; Lehmann et al., 2007). Differences in GSH content in animals exposed to Cu and control animals at the end of the experiment (week 6) suggest that this low-molecular weight antioxidant of the defense system was activated after long-term exposure. Similar results were observed in the digestive gland of the green mussel *Perna viridis* after exposure to lead and mercury (Yan et al., 1997) and on three bivalve species (*Mytilus galloprovincialis*, *Scapharca inaequivalvis* and *Tapes philippinarum*) who live in places with metal-containing sediments (Irato et al., 2003).

Our results indicate that *Diplodon chilensis* is able to accumulate copper through the diet. Several bivalve species have the ability to accumulate trace metals in concentrations proportional to those present in the environment, being the digestive gland the main tissue of metabolism and accumulation (Rainbow, 1990; Walsh and O'Halloran, 1997; Adler-Ivanbrook and Breslin, 1999). Dietary copper appears to be innocuous to the digestive system at low concentrations, as copper is a cofactor of enzymes such as cytosolic SOD (Cu-SOD iso-enzyme) and copper chaperones (Markossian and Kurganov, 2003) and is also part of the hemocyanin molecule. The excess of this metal could be sequestered into vacuoles or immobilized by biological compounds for a possible excretion (Lanno et al., 1987). On the other hand, when the metal quantities provided through the food chain are significantly increased, the digestive gland is the first tissue to suffer damage. Acute waterborne Cu toxicity can also damage other internal tissues, such as gills, muscle and gonads (Domouhtsidou and Dimitriadis, 2000; Rodriguez de la Rua et al., 2005; Zorita et al., 2006; Liu et al., 2010).

Lanno et al. (1987) have observed intrahepatocytic copper-containing granules in rainbow trout reared on diets containing elevated levels of copper. These rhodanine-positive granules, observed as electron-dense bodies, have been shown to contain Cu by electron microprobe analysis. The accumulation of copper within inclusions in the cells appears to be a generalized response mechanism utilized by diverse organisms to sequester copper to protect cell compounds. In the present study, Cu-containing granules (identified as rhodanine-positive) are evident inside the cells and within the lumen of digestive tubules and ducts of treated mussels. Similar results have been observed by Zorita et al. (2006) who detected copper deposits in digestive cell lysosomes and their exocytosis, loading the metal and lipofuscins into the lumen, in mussels located closer to a copper mine.

We have detected a progressive Cu accumulation, up to 0.49 μg/mg protein, in the digestive gland of *Diplodon chilensis* along time with

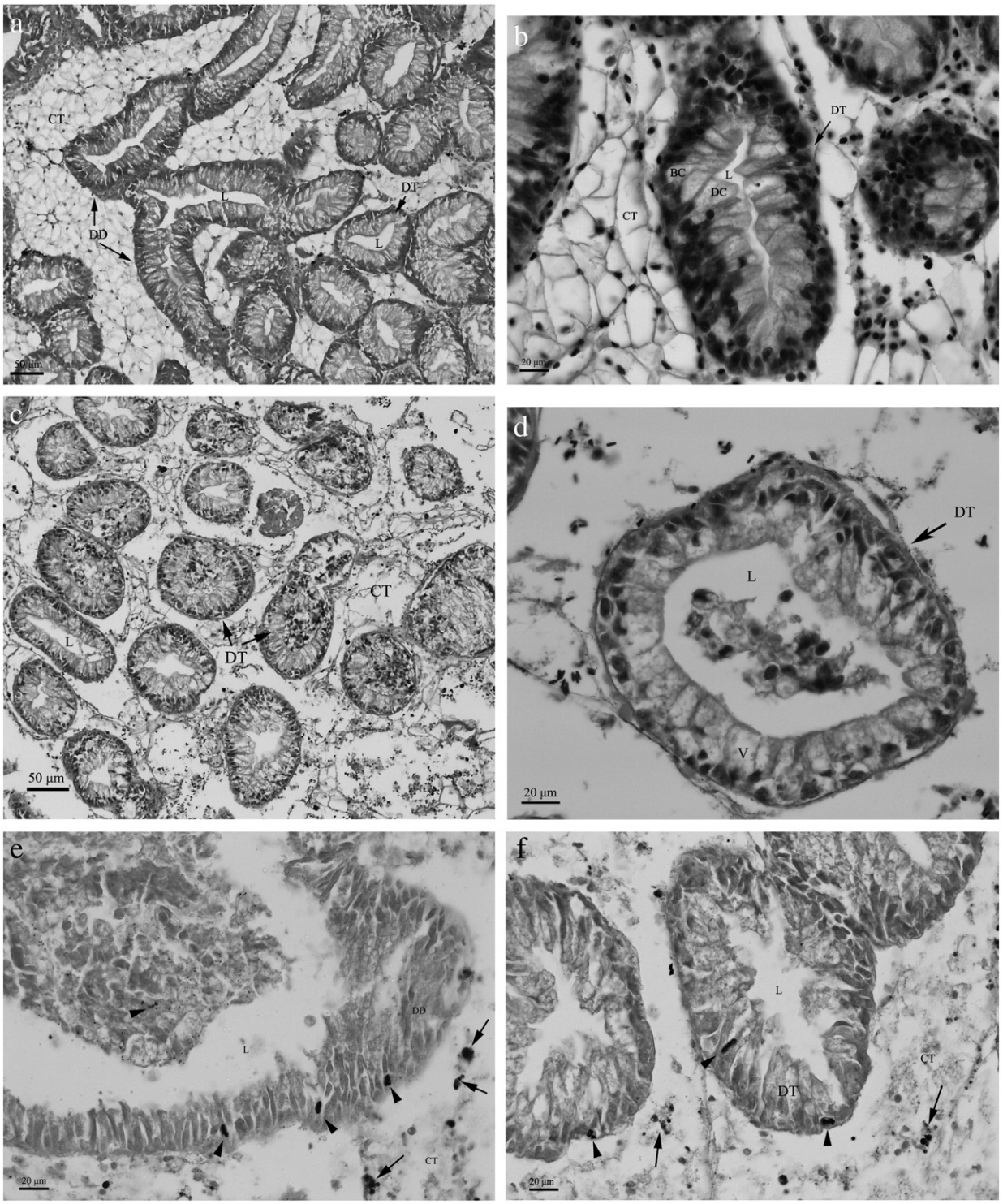

Fig. 9. *Diplodon chilensis* digestive gland sections from a, b, control animals, c, d, e, f, treated animals (fed with Cu-rich algae for 6 weeks). (a) Well defined digestive tubules (DT), main and secondary digestive ducts (DD) and surrounding connective tissue (CT). (b) Detail of control tubule with basophilic (BC) and digestive cells (DC). (c) Digestive gland of a Cu treated individual showing lumen (L) occlusion of some digestive tubules and connective tissue necrosis (d) Detail of an atrophic tubule with detached epithelium in the luminal space and increased vacuolization (V) of digestive cells. (e, f) Distinct intracellular granules revealed as rhodanine-positive (arrowheads) which indicate the presence of Cu, both inside the cells and in the lumen of digestive ducts. Melanin-like deposits (arrows) are evident within the connective tissue.

increased oxidative stress at the end of the experiment. This has probably led to increased lysosome exocytosis with loss of the apical part of digestive cells and, consequently, to the reduction of the epithelium height and the appearance of atrophic digestive tubules. Similar histological effects were observed by Usheva et al. (2006) when they relocated the mussel *Crenomytilus grayanus* into polluted waters with high concentrations of copper, chlorinated pesticides, and phenols. Exocytosis of Cu-loaded lysosome with loss of apical cytoplasm has been reported for mussels by Viarengo et al. (1981) as a mechanism to excrete accumulated Cu. Several authors have proposed the variation of the cell type-ratios as a good biomarker of exposure to organic pollutant and trace metals (Cajaraville et al.,

1990; Rodriguez de la Rua et al., 2005; Sheir et al., 2010). The increase of the basophilic/digestive cell ratio induced by Cu in the digestive tubules of *D. chilensis* is probably due to proliferation of basophilic cells number to enhance synthesis of metal binding proteins. In addition, the reduction of epithelial height as a consequence of the loss of the apical part of digestive cells with Cu-containing lysosomes, the presence of whole cells containing numerous Cu-granules in the tubular lumen and the reduction of HSI suggest the presence of a merocrine/holocrine mechanism for Cu excretion. This mechanism seems to be accelerated by oxidative stress, which in term is delayed by the enhancement of the antioxidant defenses recorded at the last week of the experiment.

5. Conclusions

The algae provided to the treated group were cultured at a free Cu ions concentration of 0.05 mg/L, which is lower than the allowed limit for human consumption, 1.3 mg/L (Fitzgerald, 1998). Cu was biomagnified by algae to 1 μg/10^6 cells (3 mg/L) and transferred to the mussels, which accumulated 0.49 μg/mg protein in the digestive gland, suffering toxic effects. Although enzymatic and non enzymatic defenses were induced at weeks 4–6 of exposure, oxidative damage to lipids and, later, to proteins occurred. This oxidative process could lead to partial or total detachment of epithelial cells and to the accumulation of Cu granules in the tubular lumen and to significant reduction of the HIS. Besides the evident oxidative cell damage observed, this process could also reflect the existence of a merocrine/holocrine mechanism of Cu excretion, accelerated by oxidative stress. Similar bioaccumulation and oxidative-histopathological effects would be expected for bivalves living in phytoplankton-rich environments with mild metal pollution.

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References

Adler-Ivanbrook, L., Breslin, V.T., 1999. Accumulation of copper, chromium, and arsenic in blue mussels (*Mytilus edulis*) from laboratory and field exposures to wood treated with chromated copper arsenate type c. Environ. Toxicol. Chem. 18 (2), 213–221.
Aebi, H., 1984. Catalase in vitro. Methods Enzymol. 105, 121–126.
Almeida, E.A., Miyamoto, S., Bainy, A.C.D., Medeirosm, M.H., Mascio, P.D., 2004. Protective effects of phospholipid hydroperoxide glutathinone peroxidase (PHGPx) against lipid peroxidation in mussels *Perna perna* exposed to different metals. Mar. Pollut. Bull. 49 (5–6), 386–392.
Al-Yousuf, M.H., El-Shahawi, M.S., Al-Ghais, S.M., 2000. Trace metals in liver, skin and muscle of *Lethrinus lentjan* fish species in relation to body length and sex. Sci. Total. Environ. 256, 87–94.
Anderson, M.E., 1985. Determination of glutathione and glutathione disulfide in biological samples. Methods Enzymol. 113, 548–553.
Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: improved assays and an assay applicable to polyacrylamide gels. Anal. Biochem. 44, 276–286.
Bebianno, M.J., Company, R., Serafim, A., Cosson, R.P., Fiala-Medoni, A., 2005. Antioxidant systems and lipid peroxidation in *Bathymodiolusazoricus* from Mid-Atlantic Ridge hydrothermal vent fields. Aquat. Toxicol. 75, 354–373.
Beuge, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. Methods Enzymol. 52, 302–310.
Bischoff, H.W., Bold, H.C., 1963. Some soil algae from Enchanted Rock and related algae species. Phycol. Stud. 6318, 1–95.
Boelsterli, U.A., 2003. Mechanistic toxicology. Taylor and Francis, London and New York, pp. 187–211.
Bonetto, A., 1973. Náyades de la Patagonia. Rev. Asoc. Cienc. Nat. Lit. 4, 177–185.
Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72, 248–254.
Cajaraville, M.P., Díez, G., Marigómez, J.A., Angulo, E., 1990. Responses of the basophilic cells of digestive gland of mussels to petroleum hydrocarbon exposure. Dis. Aquat. Org. 9, 221–228.
Cnubben, N.H., Rietjens, J.M., Wortelboer, H., van Zanden, J., van Bladeren, P.J., 2001. The interplay of glutathione-related processes in antioxidant defense. Environ. Toxicol. Pharmacol. 10, 141–152.
Connell, D., Lam, P., Richardson, B., Wu, R., 1999. Introduction to Ecotoxicology. Blackwell Science Ltd, UK. pp. 71.
d'Orbigny, A., 1835. Synopsis terrestrium et fluviatilium molluscorum in suo per Americam Meridionalem itinere collectorum. Magasin Zoologie 5.
Di Giulio, R.T., Habig, C., Gallagher, E.P., 1993. Effects of Black Rock Harbor sediments on indices of biotransformation, oxidative stress, and DNA integrity in channel catfish. Aquat. Toxicol. 26, 1–22.
Domouhtsidou, G.P., Dimitriadis, V.K., 2000. Ultrastructural localization of heavy metals (Hg, Ag, Pb and Cu) in gills and digestive gland of mussels, *Mytilus galloprovincialis* (L.). Arch. Environ. Contam. Toxicol. 38, 472–478.
Eisler, R., 1981. Trace Metal Concentrations in Marine Organisms. Pergamon Press, Oxford. 687 pp.
Fitzgerald, D.J., 1998. Safety guidelines for copper in water. Am. J. Clin. Nutr. 67, 1098–1102.
Fitzpatrick, P.J., O'Halloran, J., Sheehan, D., Walsh, A.R., 1997. Assessment of a glutathione S-transferase and related proteins in the gill and digestive gland of *Mytilus edulis* (L) as potential organic pollution biomarkers. Biomarkers 2, 51–56.
Fournier, M., Cyr, D., Blakley, B., Boermans, H., Brousseau, P., 2000. Phagocytic activity as a biomarker of immunotoxicity in wildlife species exposed to environmental xenobiotics. Am. Zool. 40, 412–420.
Geret, F., Serafim, A., Barreira, L., Bebianno, M.J., 2002. Response of antioxidant systems to copper in the gills of the mussel *Ruditapes decussatus*. Mar. Environ. Res. 54 (3–5), 413–417.
Griscom, S.B., Fisher, N.S., Luoma, S.M., 2000. Geochemical influences on assimilation of sediment-bound metals in mussels and mussels. Environ. Sci. Technol. 34, 91–99.
Gumgum, B., Unlu, E., Tez, Z., Gulsun, Z., 1994. Heavy metal pollution in water, sediment and fishfrom the Tigris river in Turkey. Chemosphere 29, 111–116.
Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.
Hoffman, D.J., 2002. Role of selenium toxicity and oxidative stress in aquatic birds. Aquat. Toxicol. 57, 11–26.
Irato, P., Santovito, G., Cassini, A., Piccinni, E., Albergoni, V., 2003. Metal accumulation and binding protein induction in *Mytilus galloprovincialis*, *Scapharca inaequivalvis*, and *Tapes philippinarum* from the lagoon of Venice. Arch. Environ. Contam. Toxicol. 44, 476–484.
Irons, R.D., Schenk, E.A., Lee, J.C.K., 1977. Cytochemical methods for copper: semiquantitative screening procedure for identification of abnormal copper levels in liver. Arch. Pathol. Lab. Med. 101, 298–301.
Jorgensen, C.B., 1990. Bivalve filter feeding: hydrodynamics, bioenergetics, physiology and ecology. Olsen and Olsen, Fredensborg.
Landrum, P.F., Dupuis, W.S., Kukkonen, J., 1994. Toxicokinetics and toxicity of sediment-associated pyrene and phenanthrene in *Diporeia* sp: examination of equilibrium-partitioning theory and residue- based effects for assessing hazard. Environ. Toxicol. Chem. 13, 1769–1780.
Lanno, R.P., Brad Hicks, B., Hilton, J.W., 1987. Histological observations on intrahepatocytic copper-containing granules in rainbow trout reared on diets containing elevated levels of copper. Aquat. Toxicol. 10, 251–263.
Lee, B.G., Louma, S.N., 1998. Influence of microalgal biomass on absorption efficiency of Cd, Cr, and Zn by two bivalves from San Francixco Bay. Limnol. Oceanogr. 43 (7), 1455–1466.
Lehmann, D.W., Levine, J.F., Law, J.M., 2007. Polychlorinated biphenyl exposure causes gonadal atrophy and oxidative stress in *Corbicula fluminea* mussels. Toxicol. Pathol. 35, 356–365.
Liu, X.J., Luo, Z., Xiong, B.X., Liu, X., Zhao, Y.H., Hua, G.F., Lv, G.J., 2010. Effect of waterborne copper exposure on growth, hepatic enzymatic activities and histology in *Synechogobius hasta*. Ecotoxicol. Environ. Saf. 73, 1286–1291.
Livingstone, D.R., Garcia-Martinez, P., Michel, M., Ribera, D., Winston, G.W., 1990. Oxyradical production as a pollution-mediated mechanism of toxicity in the common mussel, *Mytilus edulis*, and other molluscs. Funct. Ecol. 4, 415–424.
Maria, V.L., Bebianno, M.J., 2011. Antioxidant and lipid peroxidation responses in *Mytilus galloprovincialis* exposed to mixtures of benzo(a)pyrene and copper. Comp. Biochem. Physiol. C 154, 56–63.
Markossian, K.A., Kurganov, B.I., 2003. Copper chaperones, intracellular copper trafficking proteins. Function, structure, and mechanism of action. Biochem. (Moscow) 68 (8), 827–837.
Mattie, M.D., Freedman, J.H., 2001. Protective effects of aspirin and vitamin E (alpha-tocopherol) against copper and cadmium-induced toxicity. Biochem. Biophys. Res. Comm. 285, 921–925.
Norris, D.O., Camp, J.M., Maldonado, T.A., Woodling, J.D., 2000. Some aspects of hepatic function in feral brown trout, *Salmo trutta*, living in metal contaminated water. Comp. Biochem. Physiol. C 127, 71–78.
Parada, E., Peredo, S., 2002. Estado actual de la taxonomía de bivalvos dulceacuícolas chilenos: progresos y conflictos. Rev. Chil. Hist. Nat. 75, 691–701.
Parada, E., Peredo, S., Valenzuela, J., Manuschevich, D., 2007. Extention of the current northern distribution range of freshwater mussel *Diplodon chilensis* (Gray, 1828) (Bivalvia:Hyridae) in Chile. Gayana 71, 212–215.
Park, H., Ahn, I., Kim, H., Lee, J., Shin, S.C., 2009. Glutathione S-transferase as a biomarker in the Antarctic bivalve *Laternula elliptica* after exposure to the polychlorinated biphenyl mixture Aroclor 1254. Comp. Biochem. Physiol. C 150, 528–536.

Pellerin-Massicotte, J., 1994. Oxidative processes as indicators of chemical stress in marine bivalves. J. Aquat. Ecosyst. Health 3, 101–111.
Raftopoulou, E.K., Dimitriadis, V.K., 2011. Comparative study of the accumulation and detoxification of Cu (essential metal) and Hg (nonessential metal) in the digestive gland and gills of mussels *Mytilus galloprovincialis*, using analytical and histochemical techniques. Chemosphere 83, 1155–1165.
Rainbow, P.S., 1987. Heavy metals in barnacles. In: Southward, A.J. (Ed.), Barnacle Biology. Crustacean Issues 5. A.A., Balkema, Rotterdam, pp. 405–417.
Rainbow, P.S., 1990. Heavy metal levels in marine invertebrates. In: Furness, R.W., Rainbow, P.S. (Eds.), Heavy Metals in the Marine Environment. CRC Press, Boca Raton, Florida, pp. 67–79.
Resnick, A.Z., Packer, L., 1994. Oxidative damage to proteins: spectrophotometric method for carbonyl assay. Methods Enzymol. 233, 357–363.
Ribeiro Guevara, S., Bubach, D., Vigliano, P., Lippolt, G., Arribere, M., 2004. Heavy metal and other trace elements in native mussel *Diplodon chilensis* from Northern Patagonia lakes, Argentina. Biol. Trace Element Res. 101, 1–19.
Ricard, A.C., Daniel, C., Anderson, P., Hontela, A., 1998. Effects of subchronic exposure to cadmium chloride on endocrine and metabolic functions in rainbow trout *Oncorhynchus mykiss*. Arch. Environ. Contam. Toxicol. 34, 377–381.
Ringwood, A.H., Conners, D.E., Di Novo, A., 1998. The effects of copper exposures on cellular responses in oysters. Mar. Environ. Res. 46 (1–5), 591–595.
Rodriguez de la Rua, A., Arellano, J.M., Gonzalez de Canales, M.L., Blasco, J., Sarasquete, C., 2005. Acumulación de cobre y alteraciones histopatológicas en el Ostión *Crassostrea angulata*. Ciencias Marinas 31, 455–466.
Roméo, M., Gnassia-Barelli, M., 1997. Effect of heavy metals on lipid peroxidation in the Mediterranean mussel *Ruditapes decussatus*. Comp. Biochem. Physiol. C 118, 33–37.
Sabatini, S.E., Juárez, A.B., Eppis, M.R., Bianchi, L., Luquet, C.M., Ríos de Molina, M.C., 2009a. Oxidative stress and antioxidant defenses in two green microalgae exposed to copper. Ecotox. Environ. Saf. 72, 1200–1206.
Sabatini, S.E., Chaufan, G., Juárez, A.B., Coalova, I., Bianchi, L., Eppis, M.R., Ríos de Molina, M.C., 2009b. Dietary copper effects in the estuarine crab, *Neohelice (Chasmagnathus) granulata*, maintained at two different salinities. Comp. Biochem. Physiol. C 150, 521–527.
Sabatini, S.E., Rocchetta, I., Luquet, C.M., Guido, M.I., Ríos de Molina, M.C., in press. Effects of sewage pollution and bacterial load on growth and oxidative balance in the freshwater mussel Diplodon chilensis. Limnologica. doi:10.1016/j.limno.2011.04.004.
Sheir, S.K., Handy, R.D., Galloway, T.S., 2010. Tissue injury and cellular immune responses to mercuric chloride exposure in the common mussel *Mytilus edulis:* Modulation by lipopolysaccharide. Ecotoxicol. Environ. Saf. 73, 1338–1344.
Sokal, R.R., Rohlf, F.J., 1999. Introducción a la Bioestadística. Reverté, Barcelona, Spain.
Usheva, L.N., Vaschenko, M.A., Durkina, V.B., 2006. Histopathology of the digestive gland of the bivalve mollusk *Crenomytilus grayanus* (Dunker, 1853) from Southwestern Peter the Great Bay, Sea of Japan. Histology 32, 166–172.
Valdovinos, C., Pedreros, P., 2007. Geographic variations in shell growth rates of mussel *Diplodon chilensis* from temperate lakes of Chile: implications for biodiversity conservation. Limnologica 37, 63–75.
Van Ginneken, L., Chowdhury, M.J., Blust, R., 1999. Bioavailability of cadmium and zinc to the common carp, *Cyprinus carpio*, in complexing environments: a test for the validity of the free ion activity model. Environ. Toxicol. Chem. 18 (10), 2295–2304.
Vega Aguayo, R., 1982. Anatomía de *Diplodon chilensis chilensis* (Gray, 1828). Informe final proyecto 2.81.1. Comisión de Investigación Pontificia universidad Católica de Chile sede Regional Temuco. 1–41 pp.
Viarengo, A., Zanicchi, G., Moore, M.N., Orunesu, M., 1981. Accumulation and detoxification of copper by the mussel *Mytilus galloprovincialis* Lam.: a study of the subcellular distribution in the digestive gland cells. Aquat. Toxicol. 1, 147–157.
Viarengo, A., Canesi, L., Pertica, M., Poli, G., Moore, M.N., Orunesu, M., 1990. Heavy metals effects on ipid peroxidation in the tissues of *Mytilus galloprovincialis*. Comp. Biochem. Physiol. C 97, 37–42.
Walsh, A.R., O'Halloran, J., 1997. The accumalation of chromium by the mussel *Mytilus edulis* (L.) as a function of valency, solubility and ligation. Mar. Environ. Res. 43, 41–54.
Won, E.J., Kim, R.O., Rhee, J.S., Park, G.S., Lee, J., Shin, K.H., Lee, L.M., Lee, J.S., 2011. Response of glutathione S-transferase (GST) genes to cadmium exposure in the marine pollution indicator worm. *Perinereis nuntia*. Comp. Biochem. Physiol. C 154, 82–92.
Yan, T., Teo, L.H., Sin, Y.M., 1997. Effects of mercury and lead on tissue glutathione of the green mussel, *Perna viridis*. Bull. Environ. Contam. Toxicol. 58, 845–850.
Zorita, I., Ortiz-Zarragoitia, M., Soto, M., Cajaraville, M.P., 2006. Biomarkers in mussels from a copper site gradient (Visnes, Norway): an integrated biochemical, histochemical and histological study. Aquat. Toxicol. 78S, S109–S116.