Arch. Environ. Contam. Toxicol. 41, 163–170 (2001)
DOI: 10.1007/s002440010234

ARCHIVES OF
Environmental
Contamination
and Toxicology

Effects of Copper and Cadmium Exposure on Functional Responses of Hemocytes in the Clam, *Tapes philippinarum*

V. Matozzo, L. Ballarin, D. M. Pampanin, M. G. Marin

Department of Biology, University of Padova, Via Ugo Bassi 58/B, 35121 Padova, Italy

Received: 6 November 2000/Accepted: 6 March 2001

Abstract. The effects of Cu and Cd on functional responses of hemocytes in *Tapes philippinarum*, a Veneridae clam widespread in the lagoon of Venice, were evaluated. Bivalves were exposed for 7 days to various concentrations of $CuCl_2 \cdot 2H_2O$ (0, 10, 60, and 110 μg/L) and $CdCl_2 \cdot H_2O$ (0, 150, 300, and 450 μg/L). Hemocytes were collected from exposed clams, and the effects of Cu and Cd on phagocytosis, Neutral Red retention capacity, superoxide dismutase, and cytochrome oxidase activities were investigated. Hemocytes from animals exposed to Cu showed a significant decrease in phagocytic activity, whereas no inhibition was observed in cells from Cd-exposed animals. Diffusion of Neutral Red into the cytosol, an index of lysosomal membrane alteration, was enhanced by high concentrations of both metals. Different results in biochemical parameters were observed. Exposure to 60 and 110 μg Cu/L caused a significant reduction in hemocyte superoxide dismutase activity, whereas no changes resulted after Cd exposure. Increased cytochrome oxidase activity was observed in hemocytes from mollusks exposed to 60 μg Cu/L and 300 μg Cd/L; no significant differences were found in cells from bivalves exposed to 110 μg Cu/L and 450 μg Cd/L. These results suggest a relationship between heavy metal exposure and alterations in functional responses of hemocytes in *T. philippinarum* and indicate that the type of observed effects vary with the nature and concentration of heavy metals. Moreover, the data obtained in the analyzed clam support the possibility of using it as sentinel organism in biomonitoring studies, even if used biomarkers will be further evaluated in field conditions.

It is known that xenobiotics may alter functional parameters in molluscan hemocytes, such as viability (Alvarez and Friedl 1992), phagocytosis (Fries and Tripp 1980; Anderson 1988; Cima *et al.* 1998a), aggregation (Auffret and Oubella 1997), adherence (Chen and Bayne 1995), lysosomal enzyme activity (Cima *et al.* 1999), and lysosomal membrane stability (Lowe *et al.* 1995; Grundy *et al.* 1996). In the last few years, these functional responses have been used in the field of ecotoxicology as biomarkers or stress indexes to assess the effects of pollutants on environmental quality (Anderson 1988; Cajaraville *et al.* 1996). In this regard, bivalve mollusks are commonly used in monitoring programs to study environmental pollution of coastal marine ecosystems (Goldberg 1986; Kraak *et al.* 1992). Several factors contributed to this choice: Bivalves are sessile, have a high filtration rate, may accumulate a wide range of contaminants, play an important ecological role in aquatic environments, and are a widespread commercial product (Goldberg 1986). Among pollutants that may have detrimental consequences on the physiological responses of bivalve mollusks, heavy metals are one of the most frequently studied groups.

Molluscan hemocytes play a key role in several functions, such as wound and shell repair, digestion, excretion, and internal defense (Cheng 1981). In cell-mediated immune responses, phagocytosis by circulating hemocytes is the main defense against pathogens and foreign materials (Cheng 1981). Consequently, toxic effects on hemocytes potentially affect the survival of these animals. Alterations of the immunosurveillance have been reported for bivalve mollusks exposed to metals (Cheng and Sullivan 1984; Cheng 1988; Pipe *et al.* 1999) and xenobiotics (Fries and Tripp 1980; Alvarez and Friedl 1992; Beckmann *et al.* 1992; Coles *et al.* 1994; Cima *et al.* 1998a). Lysosomes play an important role in immune responses of bivalve mollusks: On phagocyte stimulation, lysosomal hydrolases are released out of cells to degrade foreign materials (Mohandas *et al.* 1985) or into phagosomes, thus participating in the degradation of internalized foreign particles (Cheng 1981). It is known that the hemocytes of bivalve mollusks may accumulate high levels of metals, mainly in lysosomes (Pauley and Nakatini 1968; Viarengo *et al.* 1981; Moore 1990; Bordin *et al.* 1996). Alteration of the integrity of lysosomal membranes may cause undesired release of hydrolases into the cytosol, with consequent damage for cells themselves (Lowe *et al.* 1995). In this respect, a reduction in lysosome membrane stability has been reported in mussels and oysters exposed to heavy metals and proposed as an indicator of cell damage (Regoli 1992; Ringwood *et al.* 1998).

It has also been observed that exposure of bivalves to heavy metals induces peroxidation of membrane lipids (Viarengo *et al.* 1990; Ringwood *et al.* 1998). This kind of damage is probably mediated by reactive oxygen species (ROS), as heavy

Correspondence to: M. G. Marin; *email*: mgmar@civ.bio.unipd.it

metals are known to enhance ROS levels (Pipe *et al.* 1999). ROSs may initiate a sequence of reactions that produce compounds, such as free lipid radicals and hydroperoxides, extremely toxic to cells (Esterbauer *et al.* 1982; Slater 1984; Viarengo *et al.* 1990). Antioxidant agents, such as glutathione, glyoxalase, superoxide dismutase, and catalase, act against ROS-mediated toxicity. Variations in their concentrations or activities have been proposed as biochemical biomarkers to assess the toxic effects of metals in both field and laboratory conditions (Regoli and Principato 1995; Cossu *et al.* 2000).

Recently, the activities of enzymes of the aerobic pathway, such as cytochrome oxidase, have also been used as biomarkers of exposure to pollutants in aquatic organisms, although only a few data are available in the literature concerning this point. Altered cytochrome oxidase activity has been found in crabs exposed to sublethal concentrations of Cd (Reddy and Bhagyalakshmi 1994).

In the present study, we evaluated the effects of Cu and Cd on functional responses of hemocytes in the Manila clam *Tapes philippinarum,* common in the lagoon of Venice and of great ecological and commercial importance. After *in vivo* exposure, the effects of metals on phagocytosis, Neutral Red retention capacity, superoxide dismutase, and cytochrome oxidase activities were investigated; the use of these cell responses as biomarkers of exposure in future researches is discussed.

Materials and Methods

Animals

Specimens of *T. philippinarum* were collected from the lagoon of Venice, maintained in the laboratory in large aquaria provided with a sandy bottom and aerated sea water at salinity of 35% ± 1% and temperature of 17°C ± 0.5°C, and fed with microalgae (*Phaeodactylum tricornutum*). Bivalves were kept in the laboratory for 5 days before exposure to metals.

Copper and Cadmium Exposure

In two different series of experiments, bivalves were exposed to 0, 10, 60, and 110 μg Cu/L (as $CuCl_2 \cdot 2H_2O$) and 0, 150, 300, and 450 μg Cd/L (as $CdCl_2 \cdot H_2O$) for 7 days. These concentrations were chosen on the basis of reported data on metal toxicity in bivalves (Chan 1988; Coles *et al.* 1995; Gnassia-Barelli *et al.* 1995; Ringwood *et al.* 1998; Pipe *et. al.* 1999; Bebianno *et al.* 2000). In all experiments, a total of 60 animals, 15 for each concentration, were used. During exposure, animals were maintained in aquaria containing aerated sea water (1 L/animal) to which metal solutions and microalgae were added. Water was changed every day.

Blood Cell Collection

In each experiment, three pools of hemocytes, obtained from control and exposed clams (five per pool), were used. Hemolymph was collected from the anterior adductor muscle with a plastic syringe (1 ml) containing 0.3 ml of 10 mM L-cysteine in filtered sea water (FSW), pH 7.5, to prevent clotting, placed in Eppendorf tubes, and centrifuged at 780 × *g* for 10 min. Hemocytes were resuspended in FSW to prepare short-term cell cultures.

Hemocyte Cultures

Short-term hemocyte cultures were prepared according to Ballarin *et al.* (1994). Sixty-microliters of hemocyte suspension were placed in the center of culture chambers made by a Teflon ring (15 mm internal diameter and 1 mm thick), smeared with petroleum jelly, glued to a siliconized glass slide, and covered with a coverslip. Chambers were kept upside down for 30 min at 25°C to allow hemocytes to settle and adhere to coverslips.

Phagocytosis Assay

After adhesion of hemocytes, FSW was discarded from each culture chamber and replaced with equal volumes of a suspension of yeast (*Saccharomyces cerevisiae*) in FSW (yeast:hemocyte ratio = 10:1). Cells were incubated for 60 min at 25°C; after incubation, hemocyte monolayers were washed several times in FSW to eliminate uningested yeast cells, fixed in a solution of 1% glutaraldehyde (Fluka) and 1% sucrose in FSW at 4°C for 30 min, washed in 0.1 M phosphate buffer saline (PBS: 1.37 M NaCl, 0.03 M KCl, 0.015 M KH_2PO_4, 0.065 M Na_2HPO_4, pH 7.2), for 10 min, stained with 10% Giemsa (Fluka) for 5 min, mounted on glass slides with an aqueous medium (Acquovitrex, Carlo Erba), and observed with a Leitz Dialux 22 light microscope at a magnification of 1,250× The phagocytic index was expressed as the percentage of cells containing ingested yeast particles.

Neutral Red Retention Assay (NRR)

Lysosomal membrane stability was assessed using a modification of the NRR assay described by Lowe *et al.* (1995). A stock solution of 0.4% Neutral Red in FSW was prepared. Working solution was obtained by diluting 10 μl stock solution in 5 ml FSW. After adhesion of hemocytes to the coverslips, FSW was discarded from culture chambers and 60 μl working solution was added. The slides were then observed after various time intervals (5, 30, 60 min). The lysosomal stability index was expressed as the percentage of hemocytes showing dye loss from lysosomes into the cytosol, which, consequently, appears reddish-pink.

Superoxide Dismutase (SOD) Assay

SOD activity was measured according to the method of Flohé and Ötting (1984), modified, based on the reduction of cytochrome *c* by superoxide radical (O_2^-) generated by the xanthine-xanthine oxidase system. Hemolymph, obtained from both control and exposed bivalves, was collected without L-cysteine and centrifuged at 780 × *g* for 10 min; the resulting pellets were resuspended in 1 ml FSW. Hemocytes were then sonicated for 5 min at 0°C with a Braun Labsonic U sonifier at 50% duty cycles and centrifuged at 12,000 × *g* for 15 min at 4°C. Supernatant was collected for SOD assay. Fifty microliters of cell lysate were added to 900 μl of solution A (5 μM xanthine [Sigma] in 1 mM NaOH, 2 μM cytochrome *c* [Sigma] and 0.1 mM EDTA in 50 mM phosphate buffer, pH 7.8) and to 50 μl of solution B (0.45 U xanthine oxidase [Sigma], 1 ml 0.1 mM EDTA in 50 mM phosphate buffer, pH 7.8). Changes in absorbance were measured at 550 nm with an Uvikon 930 spectrophotometer. One unit of SOD is defined as the amount of enzyme inhibiting by 50% the reduction of cytochrome *c* (Flohé and Ötting 1984). Protein concentration into cell lysate was quantified according to Bradford (1976).

Cytochrome Oxidase Assay

The diaminobenzidine method proposed by Novikoff and Goldfischer (1969) was used. After adhesion to coverslips, hemocytes were fixed in a solution of 1% glutaraldehyde plus 1% sucrose in FSW at 4°C for 30 min, washed in 0.1 M acetate buffer (pH 5.5) for 10 min, and incubated for 4 h at 37°C in a reaction mixture containing 20 mg 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma), 9 ml 0.1 M acetate buffer (pH 5.5), 1 ml $MnCl_2$ 1%, and 0.1 ml H_2O_2 0.1%. Slides were then washed in distilled water and mounted with Acquovitrex. Hemocytes were observed under the light microscope, and the cytochrome oxidase index was expressed as the percentage of cells showing positivity for cytochrome oxidase (reaction products appear brown).

Statistical Analysis

The results of all assays were compared using a one-way analysis of variance (ANOVA) and expressed as means ± standard error.

Results

Phagocytosis

No significant effect on phagocytic activity was observed in hemocytes of clams exposed to Cd. Conversely, Cu exposure resulted in a significant decrease in phagocytosis with respect to controls, at all the concentrations assayed ($p < 0.01$ at 10 μg/L, $p < 0.001$ at 60 and 110 μg/L) (Figure 1).

Neutral Red Retention

Exposure to both Cd and Cu resulted in the reduced capability of lysosomes to retain Neutral Red when compared with controls. A significant difference ($p < 0.01$) of the lysosomal stability index with respect to controls was already present at the two higher concentrations of both Cd and Cu after 5 min (Figure 2). This difference was detected after 30 min in the hemocytes of bivalves exposed to 60 and 110 μg Cu/L ($p < 0.01$) and after 60 min in those exposed to 110 μg Cu/L ($p < 0.01$). No differences were observed after 30 and 60 min in the hemocytes of Cd-exposed bivalves.

Superoxide Dismutase

No statistically significant effects were observed in the SOD activity of hemocytes from bivalves exposed to Cd, although a slight but not significant decrease in hemocytes from clams exposed to 150 and 300 μg Cd/L was noted (Figure 3a). Hemocytes from animals exposed to 60 and 110 μg Cu/L significantly decreased their SOD activity ($p < 0.05$ and $p < 0.01$, respectively) (Figure 3b).

Cytochrome Oxidase

A significant increase ($p < 0.05$) in the percentage of hemocytes showing positivity for cytochrome oxidase was observed in bivalves exposed to 300 μg Cd/L and 60 μg Cu/L (Figure 4).

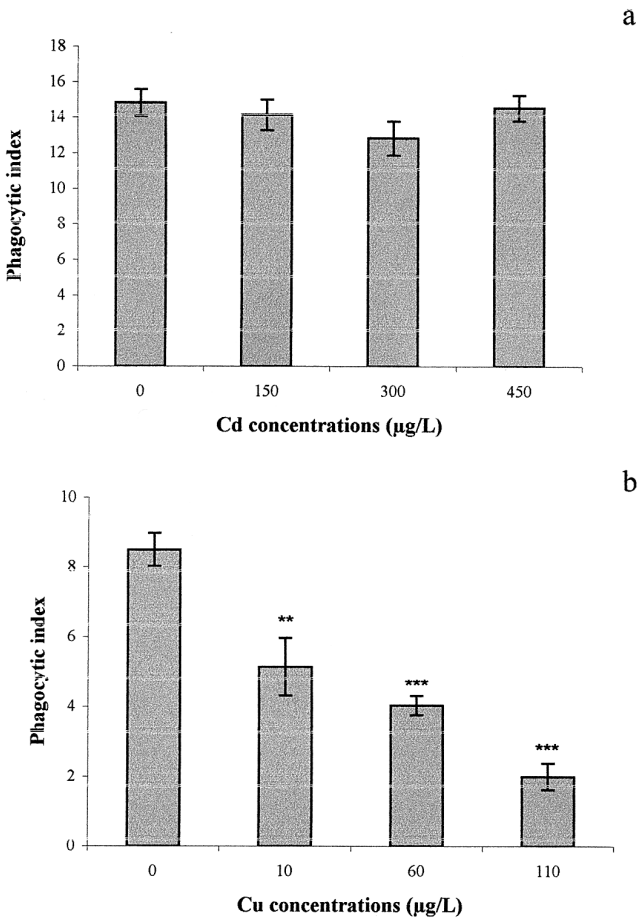

Fig. 1. Phagocytic index expressed as percentage of hemocytes containing ingested yeast particles after exposure of *T. philippinarum* to Cd (a) and Cu (b). Significant results, in comparison with controls, are indicated by asterisks. Values are means ± standard error ($n = 3$); ** $p < 0.01$, *** $p < 0.001$

Discussion

Our results demonstrate that *in vivo* exposure of the clam *T. philippinarum* to sublethal concentrations of Cu and Cd has various effects on the functional responses of hemocytes. In agreement with results reported by Cheng (1988) and Coles *et al.* (1995) for *Crassostrea virginica* and *Mytilus edulis*, in our experiments exposure to Cu caused a significant dose-dependent decrease in the phagocytic activity of hemocytes, whereas Cd had no significant inhibitory effects. Pipe *et al.* (1999) observed significant stimulation of phagocytic activity after *in vivo* exposure of *M. edulis* to 200 μg Cu/L, whereas no significant reduction was observed at 500 μg Cu/L. The differences in phagocytic index between controls of Cu and Cd-exposed clams observed in our study could be interpreted as a consequence of seasonal fluctuations of immune parameters in used clams. Both our unpublished data for *T. philippinarum* and those reported for *M. galloprovincialis* (Pipe *et al.* 1995) demonstrate that phagocytic activity of bivalves may have seasonal variations owing to various factors, mainly the reproductive cycle of organisms.

Alterations in phagocytic activity have been described in

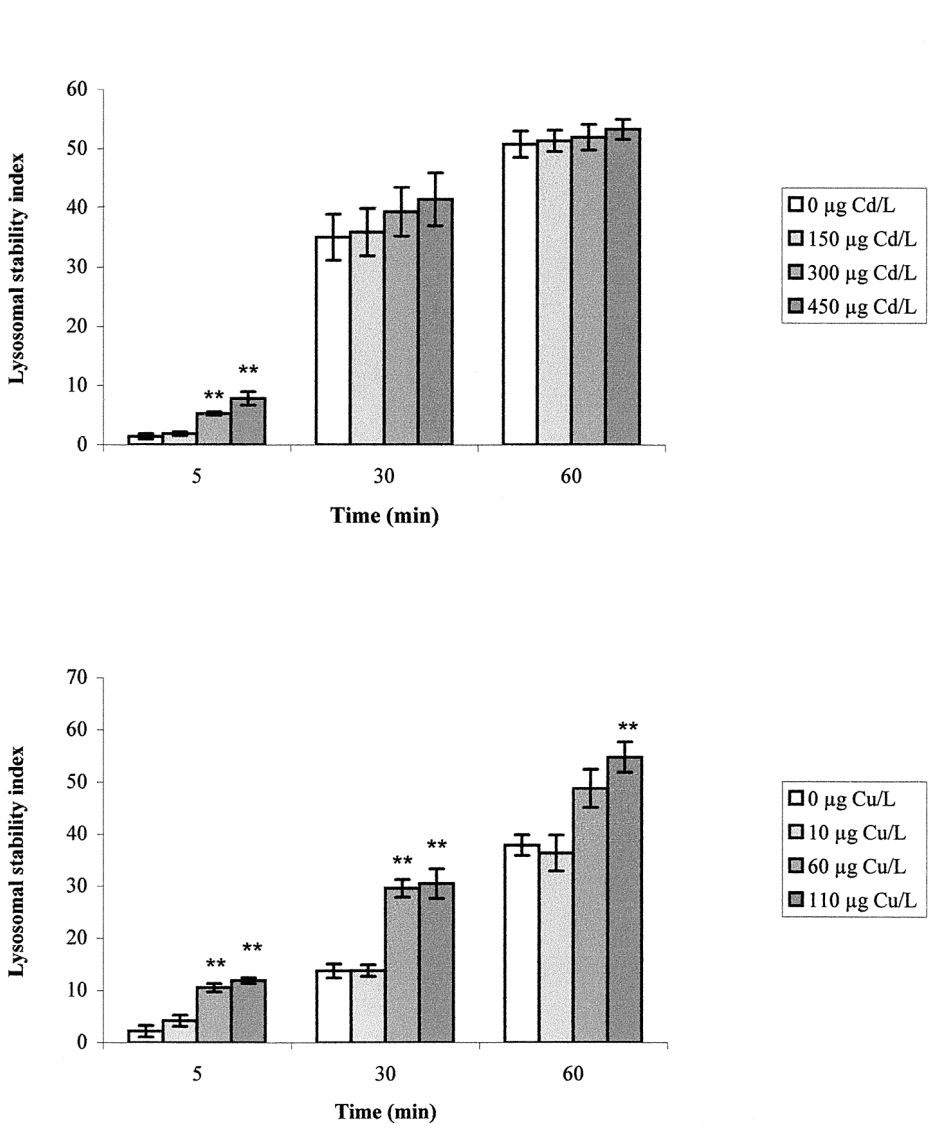

Fig. 2. Lysosomal stability index expressed as percentage of hemocytes showing red cytosol after exposure of *T. philippinarum* to Cd (a) and Cu (b). Times refer to observation intervals under light microscope. Significant results, in comparison with controls, are indicated by asterisks. Values are means ± standard error (n = 3); ** $p < 0.01$

bivalve mollusks exposed to xenobiotics (Alvarez and Friedl 1992; Coles *et al.* 1994; Cima *et al.* 1998a). Relationships between changes in phagocytic activity and alterations in the cytoskeletal organization of hemocytes may explain these data (Cima *et al.* 1998b). Cytoskeletal alterations lead to reduced phagocytic activity, due to the decreased ability of hemocytes to adhere to the substrate and to interact with foreign particles. These alterations could be directly mediated by metals bonding with cytoskeletal structural proteins, followed by their denaturation, and indirectly by either alterations in calcium homeostasis mechanisms or oxidation of the SH groups of cytoskeletal proteins mediated by ROSs. Heavy metals, such as Cu, Cd, Zn, and Hg, are known to have a high affinity for the SH groups of proteins, including Ca^{2+}-ATPases (Viarengo and Nicotera 1991). On interaction of metals with Ca^{2+}-ATPase SH groups, inactivation of Ca^{2+}-ATPases may cause long-lasting and diffuse increases in cytosolic Ca^{2+} concentrations (Viarengo and Nicotera 1991; Viarengo *et al.* 1996), which in turn cause alterations in cytoskeletal organization, thus compromising important aspects of cell physiology, *e.g.*, adhesion, motility, and phagocytic activity, as observed in both mammalian thymocytes and tunicate amoebocytes exposed to butyltins (Chow and Orrenius 1994; Cima *et al.* 1998b). In hemocytes of *M. edulis*, Viarengo *et al.* (1994) demonstrated that heavy metals can also impair Ca^{2+} homeostasis by affecting Ca^{2+} channels. Moreover, higher involvement of Cu in ROS production (Halliwell and Gutteridge 1984) could explain the different results in phagocytic index between Cu- and Cd-exposed clams.

Results of the NRR assay indicate that exposure of clams to metals may cause lysosomal membrane instability. Lysosomes of hemocytes from control animals retain the dye for a long time after uptake, whereas Neutral Red appears in the cytosol of cells from animals treated with higher metal doses. The percentage of hemocytes showing red cytosol significantly increased in clams exposed to higher Cu and Cd concentrations. However, the higher values of positive cells in controls at 30 and 60 min with respect to 5 min may be explained by the toxicity of Neutral Red itself, which is known to produce changes in lysosomal structure (Ohkuma and Poole 1981;

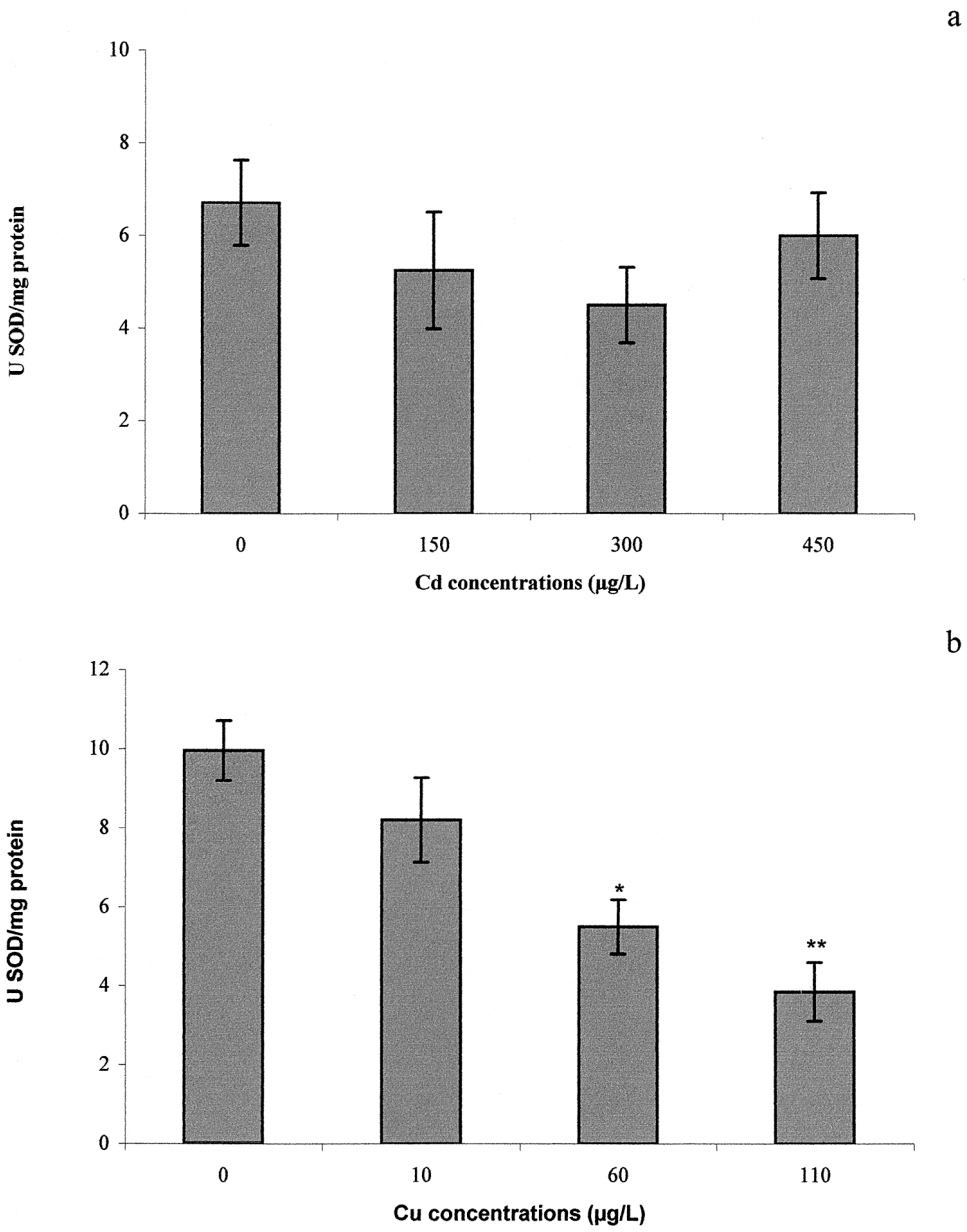

Fig. 3. SOD activity, expressed as U SOD/mg protein, in hemocytes of *T. philippinarum* exposed to Cd (a) and Cu (b). Significant results, in comparison with controls, are indicated by asterisks. Values are means ± standard error (n = 3); * $p < 0.05$, ** $p < 0.01$

Lowe and Pipe 1994). Neutral Red represents an additional stress for hemocytes, so that it may disguise metal effects on Neutral Red retention. According to results of Hauton *et al.* (1998) for *Ostrea edulis*, in our previous observations (data unpublished) we have noted that lysosomes of unstressed specimens of *T. philippinarum* retained Neutral Red for a shorter period of time than that reported for *M. galloprovincialis* collected from clean sites of the lagoon of Venice (Lowe *et al.* 1995). Thus, a distinction between metal- and dye-mediated toxicity must be made. In clams, this condition is probably better achieved by studying the effects of metals on lysosomal membranes within 30 min. Lysosomal membrane alterations are probably due to the high levels of metals that may accumulate in lysosomes, as proposed by Moore (1990) and Regoli (1992), leading to inhibition of Mg^{2+}-ATPase, a proton pump of lysosomal membranes that maintains the internal environment of lysosomes acid (Lowe *et al.* 1992). Dysfunction of this ATPase allows free passage of lysosomal contents, including Neutral Red, into the cytosol (Lowe *et al.* 1995). Alternatively, metals may induce oxidative damage mediated by ROSs, as suggested by Viarengo *et al.* (1990). It is well known that Cu and Fe are involved in ROS production by Fenton reaction (Halliwell and Gutteridge 1984). Therefore, Cu ions seem participate in lipid peroxidation more than Cd (Viarengo *et al.* 1990). A significant decrease in lysosomal membrane stability was observed in both oysters and mussels exposed to metals (Ringwood *et al.* 1998; Regoli 1992), hemocytes of *M. edulis* exposed to polycyclic aromatic hydrocarbons (Grundy *et al.* 1996) and in those of *M. galloprovincialis* exposed to a range of xenobiotics (Lowe *et al.* 1995).

Inhibition of SOD activity is a risk for cells, as it causes oxidative stress. A relevant decrease in SOD activity was

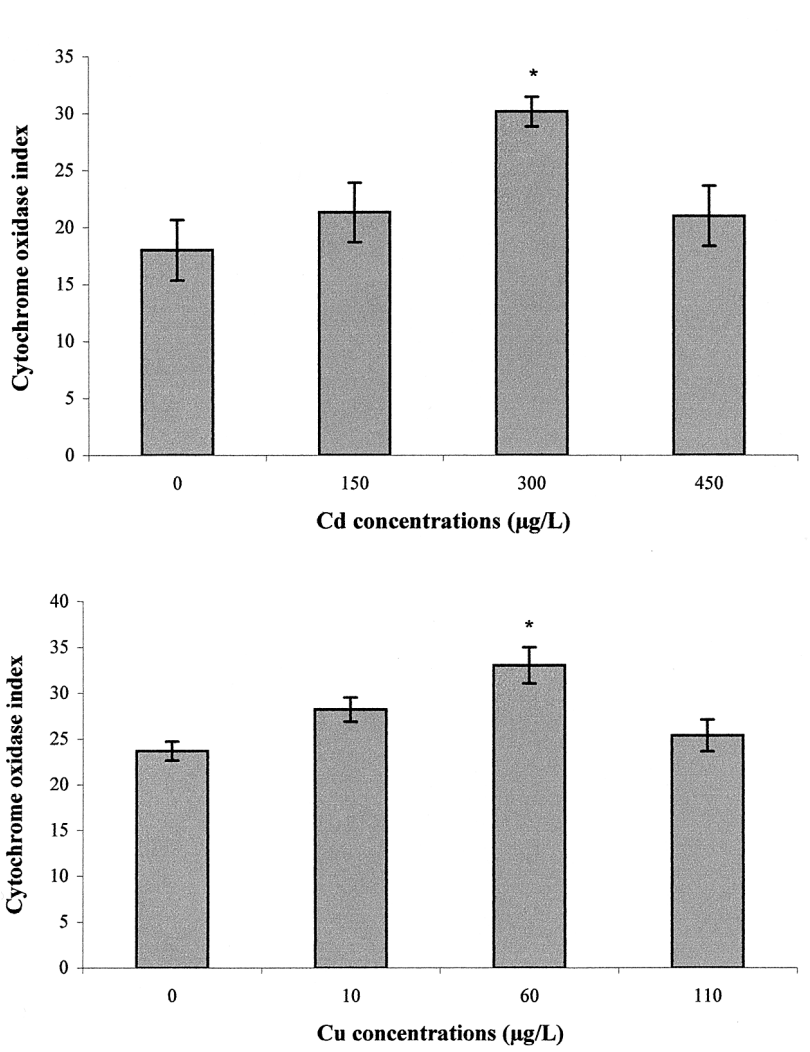

Fig. 4. Cytochrome oxidase index expressed as percentage of hemocytes showing cytochrome oxidase activity after exposure of *T. philippinarum* to Cd (a) and Cu (b). Significant results, in comparison with controls, are indicated by asterisks. Values are means ± standard error ($n = 3$); * $p < 0.05$

observed only in hemocytes from Cu-exposed clams. Regoli and Principato (1995) claim that SOD activity remains constant in the digestive gland and increases in the gills of *M. galloprovincialis* exposed to metals. An increase in SOD activity was reported for gills of mussels collected from a polluted site (Regoli 1998). Induction of antioxidant enzyme activity, such as that of SOD, was also observed in freshwater crabs exposed to Cu but not in Cd-treated animals (Reddy 1999). The different responses of SOD activity between gills and hemocytes of bivalve mollusk may be related to the extraction mechanisms of oxygen from water, which occurs at a higher rate in the gills, through which large quantities of water are filtered. As a consequence, mollusk gills have higher SOD activity than other tissues (Regoli and Principato 1995). The significant inhibition of SOD activity in Cu-exposed clams observed in this study might be due to the oxidation of the enzyme SH groups mediated by ROSs, which production is increased by Cu (Halliwell and Gutteridge 1984). The different involvement of MTs may also explain this result. An increase in MT synthesis is associated with the enhanced capability of MTs to bind metals and to protect cells against metal toxicity (Roesijadi 1994). Positive correlation between Cd, but not Cu, and MT concentrations have been found in bivalves (Wang *et al.* 1999) and lobsters (Canli *et al.* 1997). Moreover, comparing the levels of Cd and Cu bound to MTs with total accumulated concentrations by specimens of a natural population of oysters (*Crassostrea virginica*), Roesijadi (1994) found that about 20% of total tissue Cd, but only 1% of Cu, were bound to MTs. Baudrimont *et al.* (1999) demonstrated that 70% of cytosolic Cd was sequestered by MTs. It has also been observed that, in *Ruditapes decussatus* exposed to heavy metals, Cu content rapidly decreased after a detoxification period of 8 days, whereas Cd levels remained unchanged (Gnassia-Barelli *et al.* 1995). A significant induction of MTs was observed only after 7 days of exposure of *C. virginica* to 80 µg Cu/L (Ringwood *et al.* 1998), whereas in *Macoma balthica* exposed to a mixture of heavy metals (100 µg Cd Cu/L and 600 µg Zn/L) positive relationship between MT levels and metal concentrations, with higher correlation for Cd, was noted (Bordin *et al.* 1997). In the latter study, authors also proposed an important role of MTs in detoxification of Cd, and probably of Cu. Again, in *R. decussatus* multiple correlations between MTs and heavy metal content showed that MTs were significantly correlated with Cd and Zn, with an important coefficient for Cd, but no significant correlation was found for Cu (Hamza-Chaffai *et al.* 2000).

Exposure of *T. philippinarum* to Cd and Cu results in a

significant increase in hemocyte cytochrome oxidase activity only at 300 μg Cd/L and 60 μg Cu/L. These results are difficult to explain, owing to the limited data available dealing with the effects of contaminants on cytochrome oxidase activity in marine organisms. Cytochrome oxidase is an important enzyme of respiratory complexes in mitochondria, and variations in its activity may affect oxidative metabolism, as demonstrated in crabs exposed to Cd (Reddy and Bhagyalakshmi 1994). In the Atlantic salmon *Salmo salar* exposed to petroleum hydrocarbons, cytochrome oxidase activity did not change (Gagnon and Holdway 1999), whereas a relationship between variations in cytochrome oxidase activity and Cd accumulation was observed in the goldfish *Carassius auratus* (Gargiulo *et al.* 1996). The variations in cytochrome oxidase activity observed in our study may be explained by the increased energy requirements of cells from clams exposed to 60 μg Cu/L and 300 μg Cd/L, followed by impairment of mitochondria in hemocytes exposed to 110 μg Cu/L and 450 μg Cd/L.

Conclusions

The results of the present study show that exposure of the clam *T. philippinarum* to Cu and Cd may influence the functional responses of hemocytes. The type of observed effects vary with the nature and concentration of these heavy metals, Cu being more toxic than Cd. The different values between controls of Cu- and Cd-exposed clams obtained in some experiments also suggest seasonal variations of the analyzed physiological parameters. Moreover, different responses of hemocytes from Cu- and Cd-exposed clams might be due to cellular detoxification processes of metal ions in which both metallothioneins and other regulatory molecules are probably involved. This aspect will be investigated in future research. Both physiological and biochemical parameters of hemocytes analyzed in the present work and proposed in previous studies as biomarkers in gills and digestive glands of bivalves are easily reproducible and may be considered useful tools for biomonitoring marine coastal environments. However, their responsiveness would be evaluated in realistic conditions, because metal concentrations used in our study are higher than those found in field. To this aim, further studies will be developed.

References

Alvarez MR, Friedl FE (1992) Effects of a fungicide on *in vitro* hemocyte viability, phagocytosis and attachment in the American oyster, *Crassostrea virginica* Aquaculture 107:135–140

Anderson RS (1988) Effects of anthropogenic agents on bivalve cellular and humoral defense mechanisms. Amer Fish Soc 18:238–242

Auffret M, Oubella R (1997) Hemocyte aggregation in the oyster *Crassostrea gigas: in vitro* measurement and experimental modulation by xenobiotics. Comp Biochem Physiol 118A:705–712

Ballarin L, Cima F, Sabbadin A (1994) Phagocytosis in the colonial ascidian *Botryllus schlosseri*. Dev Comp Immunol 18:467–481

Baudrimont M, Andres S, Metivaud J, Lapaquellerie Y, Ribeyre F, Maillet N, Latouche C, Boudou A (1999) Field transplantation of the freshwater bivalve *Corbicula fluminea* along a polymetallic contamination gradient (river Lot, France): II. Metallothionein response to metal exposure. Environ Toxicol Chem 18:2472–2477

Bebianno MJ, Serafim MA, Simes D (2000) Metallothioneins in the clam *Ruditapes decussatus*: an overview. Analysis 28:386–390

Beckmann N, Morse MP, Moore CM (1992) Comparative study of phagocytosis in normal and diseased hemocytes of the bivalve mollusc *Mya arenaria*. J Invertebr Pathol 59:124–132

Bordin G, McCourt J, Cordeiro Raposo F, Rodriguez AR (1996) Trace metals in the marine bivalve *Macoma balthica* in the Westerschelde estuary. Variability of the role of cytosol in metal uptake by the clams. Sci Total Environ 180:241–255

Bordin G, McCourt J, Cordeiro Raposo F, Rodriguez AR (1997) Metallothionein-like metalloproteins in the Baltic clam *Macoma balthica*: seasonal variations and induction upon metal exposure. Mar Biol 129:453–463

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of the protein-dye binding. Anal Biochem 72:248–254

Cajaraville MP, Olabarrieta I, Marigomez I (1996) *In vitro* activities in mussel haemocytes as biomarkers of environmental quality: a case study in the Abra Estuary (Biscay Bay). Ecotoxicol Environ Saf 35(3):253–260

Canli M, Stagg RM, Rodger G (1997) The induction of metallothionein in tissues of the Norway lobster *Nephrops norvegicus* following exposure to cadmium, copper and zinc: the relationship between metallothionein and the metals. Environ Pollut 96:343–350

Chan HM (1988) Accumulation and tolerance to cadmium, copper, lead and zinc by the green mussel *Perna viridis*. Mar Ecol Prog Ser 48:295–303

Chen JH, Bayne CJ (1995) Bivalve mollusc hemocyte behaviors: characterization of hemocyte aggregation and adhesion and their inhibition in the California mussel (*Mytilus californianus*). Biol Bull 188:255–266

Cheng TC (1981) Bivalves. In: Ratcliffe NA, Rowley AF (ed) Invertebrate blood cells 1. Academic Press, London, pp 233–300

Cheng TC (1988) *In vivo* effects of heavy metals on cellular defence mechanisms of *Crassostrea virginica*: phagocytic and endocytotic indices. J Invertebr Pathol 51:215–220

Cheng TC, Sullivan JT (1984) Effects of heavy metals on phagocytosis by molluscan hemocytes. Mar Environ Res 14:305–315

Chow SC, Orrenius S (1994) Rapid cytoskeleton modification in thymocytes induced by immunotoxicant tributyltin. Toxicol Appl Pharmacol 127:19–26

Cima F, Marin MG, Matozzo V, DaRos L, Ballarin L (1998a) Immunotoxic effects of organotin compounds in *Tapes philippinarum*. Chemosphere 37:3035–3045

Cima F, Ballarin L, Bressa G, Burighel P (1998b) Cytoskeleton alterations by tributyltin (TBT) in tunicate phagocytes. Ecotoxicol Environ Saf 40:160–165

Cima F, Marin MG, Matozzo V, Da Ros L, Ballarin L (1999) Biomarkers for TBT immunotoxicity studies on the cultivated clam *Tapes philippinarum* (Adams and Reeve, 1850). Mar Pollut Bull 39: 112–115

Coles JA, Farley SR, Pipe RK (1994) Effects of fluoranthene on the immunocompetence of the common marine mussel, *Mytilus edulis*. Aquat Toxicol 30:367–379

Coles JA, Farley SR, Pipe RK (1995) Alteration of the immune response of the common marine mussel *Mytilus edulis* resulting from exposure to cadmium. Dis Aquat Org 22:59–65

Cossu C, Doyotte A, Babut M, Exinger A, Vasseur P (2000) Antioxidant biomarkers in freshwater bivalves, *Unio tumidus*, in response to different contamination profiles of aquatic sediments. Ecotoxicol Environ Saf 45:106–121

Esterbauer H, Cheeseman KH, Dianzani MU, Poli G, Slater TF (1982) Separation and characterization of the aldehydic products of lipid

peroxidation stimulated by ADP-Fe^{2+} in rat liver microsomes. Biochem J 208:129–140

Flohé L, Ötting F (1984) Superoxide dismutase assays. Meth Enzymol 105:93–104

Fries CR, Tripp MR (1980) Depression of phagocytosis in *Mercenaria* following chemical stress. Dev Comp Immunol 4:233–244

Gagnon MM, Holdway DA (1999) Metabolic enzyme activities in fish gills as biomarkers of exposure to petroleum hydrocarbons. Ecotoxicol Environ Saf 44:92–99

Gargiulo G, Arcamone N, deGirolamo P, Andreozzi G, Antonucci R, Esposito V, Ferrara L, Battaglini P (1996) Histochemical study of the effects of cadmium uptake on oxidative enzymes of intermediary metabolism in kidney of goldfish (*Carassius auratus*). Comp Biochem Physiol 113C:177–183

Gnassia-Barelli M, Roméo M, Puiseux-Dao S (1995) Effects of cadmium and copper contamination on calcium content of the bivalve *Ruditapes decussatus*. Mar Environ Res 39:325–328

Goldberg ED (1986) The mussel watch concept. Environ Monit Assess 7:91–103

Grundy MM, Moore MN, Howell SM, Ratcliffe NA (1996) Phagocytic reduction and effects on lysosomal membranes by polycyclic aromatic hydrocarbons, in haemocytes of *Mytilus edulis*. Aquat Toxicol 34:273–290

Halliwell B, Gutteridge MC (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J 219:1–14

Hamza-Chaffai A, Amiard JC, Pellerin J, Joux L, Berthet (2000) The potential use of metallothionein in the clam *Ruditapes decussatus* as a biomarker of in situ metal exposure. Comp Biochem Physiol 127C:185–197

Hauton C, Hawkins LE, Hutchinson S (1998) The use of the neutral red retention assay to examine the effects of temperature and salinity on haemocytes of the European flat oyster *Ostrea edulis* (L). Comp Biochem Physiol 119B:619–623

Kraak MHS, Lavy D, Peeters WHM, Davids C (1992) Chronic ecotoxicity of copper and cadmium to the zebra mussel *Dreissena polymorpha*. Arch Environ Contam Toxicol 23:363–369

Lowe DM, Pipe RK (1994) Contaminant induced lysosomal membrane damage in marine mussel digestive cells: an *in vitro* study. Aquat Toxicol 30:357–365

Lowe DM, Fossato VU, Depledge MH (1995) Contaminant-induced lysosomal membrane damage in blood cells of mussels *Mytilus galloprovincialis* from the Venice lagoon: an in vitro study. Mar Ecol Prog Ser 129:189–196

Lowe DM, Moore MN, Evans BM (1992) Contaminant impact on interactions of molecular probes with lysosomes in living hepatocytes from dab *Limanda limanda*. Mar Ecol Prog Ser 91:135–140

Mohandas A, Cheng TC, Cheng JB (1985) Mechanism of lysosomal enzyme release from *Mercenaria mercenaria* granulocytes: a scanning electron microscope study. J Invertebr Pathol 46:189–197

Moore MN (1990) Lysosomal cytochemistry in marine environmental monitoring. Histochem J 22:189–191

Novikoff AB, Goldfischer S (1969) Visualisation of peroxisomes (microbodies) and mitochondria with diaminobenzidine. J Histochem Cytochem 17:675–680

Ohkuma S, Poole B (1981) Cytoplasmic vacuolation of mouse peritoneal macrophages and uptake into lysosomes by weakly basic substances. J Cell Biol 90:656–664

Pauley GB, Nakatini RE (1968) Metabolism of the radioisotope ^{65}Zn in the freshwater mussel *Anodonta californiensis*. J Fish Res Board Can 25:2691–2694

Pipe RK, Coles JA, Carissan FMM, Ramanathan K (1999) Copper induced immunomodulation in the marine mussel *Mytilus edulis*. Aquat Toxicol 46:43–54

Pipe RK, Coles JA, Thomas ME, Fossato VU, Pulsford AL (1995) Evidence for environmentally derived immunomodulation in mussels from the Venice Lagoon. Aquat Toxicol 32:59–73

Reddy PS (1999) Modulations in antioxidant enzymes in the hepatopancreas and gill of the fresh water crab *Oziotelphusa senex senex* during exposure to cadmium and copper. Fresen Environ Bull 8:445–452

Reddy PS, Bhagyalakshmi A (1994) Changes in oxidative metabolism in selected tissues of the crab (*Scylla serrata*) in response to cadmium toxicity. Ecotoxicol Environ Saf 29:255–264

Regoli F (1992) Lysosomal responses as a sensitive stress index in biomonitoring heavy metal pollution. Mar Ecol Prog Ser 84: 63–69

Regoli F (1998) Trace metals and antioxidant enzymes in gills and digestive gland of the Mediterranean mussel *Mytilus galloproyincialis*. Arch Environ Contam Toxicol 34:48–63

Regoli F, Principato G (1995) Glutathione, glutathione-dependent and antioxidant enzymes in mussel, *Mytilus galloprovincialis*, exposed to metals under field and laboratory conditions: implications for the use of biochemical biomarkers. Aquat Toxicol 31: 143–164

Ringwood AH, Conners DE, DiNovo A (1998) The effects of copper exposures on cellular responses in oysters. Mar Environ Res 46:591–595

Roesijadi G (1994) Behavior of metallothionein-bound metals in a natural population of an estuarine mollusc. Mar Environ Res 38:147–168

Slater T (1984) Free radical mechanisms in tissue injury. Biochem J 222:1–15

Viarengo A, Nicotera P (1991) Possible role of Ca^{2+} in heavy metal cytotoxicity. Comp Biochem Physiol 100C:81–84

Viarengo A, Canesi L, Moore MN, Orunesu M (1994) Effects of Hg^{2+} and Cu^{2+} on the cytosolic Ca^{2+} level in molluscan blood cells evaluated by confocal microscopy and spectrofluorimetry. Mar Biol 119:557–564

Viarengo A, Zanicchi G, Moore MN, Orunesu M (1981) Accumulation and detoxication 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 MN, Orunesu M (1990) Heavy metal effects on lipid peroxidation in the tissues of *Mytilus galloprovincialis* Lam. Comp Biochem Physiol 97C: 37–42

Viarengo A, Pertica M, Mancinelli G, Burlando B, Canesi L, Orunesu M (1996) *In vivo* effects of copper on the calcium homeostasis mechanisms of mussel gill cell plasma membranes. Comp Biochem Physiol 113C:421–425

Wang DC, Couillard Y, Campbell PGC, Jolicoeur P (1999) Changes in subcellular metal partitioning in the gills of freshwater bivalves (*Pyganodon grandis*) living along an environmental cadmium gradient. Can J Fish Aquat Sci 56:774–784