BioMetals (2006) 19:399–407
DOI 10.1007/s10534-005-4064-4

Metallothionein response to cadmium and zinc exposures compared in two freshwater bivalves, *Dreissena polymorpha* and *Corbicula fluminea*

Véronique Marie, Patrice Gonzalez, Magalie Baudrimont*, Jean-Paul Bourdineaud & Alain Boudou

*Laboratoire d'Ecophysiologie et Ecotoxicologie des Systèmes Aquatiques (LEESA), Université Bordeaux 1/UMR CNRS 5805, Place du Dr Peyneau, 33120 Arcachon, France; *Author for correspondence (Tel: +33-556-223-927; Fax: +33-556-549-383; E-mail: m.baudrimont@epoc.u-bordeaux1.fr)*

Received 22 July 2005; accepted 13 October 2005

Key words: metallothionein, freshwater bivalves, cadmium, zinc, gene expression

Abstract

Metallothionein (MT) response to cadmium (Cd) and zinc (Zn) bioaccumulation after single or combined direct exposure was compared in two freshwater bivalves, *Dreissena polymorpha* (zebra mussel) and *Corbicula fluminea* (Asiatic clam). Bivalves were exposed to 0.133 μM Cd and/or 15.3 μM Zn, with metal and MT concentrations analysed in the whole soft body after 1, 3, 10 and 24 days of exposure and compared with controls. Results showed significant increase in MT concentrations in both species exposed to Cd and Cd + Zn with a higher accumulation of the protein compared to the control in *D. polymorpha* for nevertheless similar Cd levels accumulated with time. Exposure to Zn alone led to a significant increase in MT concentrations only in *C. fluminea*, whereas there was a lack of MT gene induction in the zebra mussels which was confirmed by MT mRNA quantification in gills (RT-PCR). Mussel mortality after 10 days of exposure to Zn and Cd + Zn is discussed with regard to detoxification mechanisms, which include metallothioneins.

Introduction

Chronic anthropogenic contamination of freshwater systems by heavy metals can induce structural and functional disturbances in living organisms, from the cellular and molecular basis to higher biological levels, such as populations or communities. For the assessment of metal contamination at organ or individual level, metal-binding metallothioneins (MTs) have been proposed as exposure biomarkers (Roesijadi 1994a). These low-molecular-weight cytosolic proteins are a ubiquitous element, characterized by a large proportion of cysteine residues (around 30%) arranged inside globular domains or clusters, with differential binding affinity between metal ions: zinc < cadmium ≪ copper, silver ≪ mercury (Vasák 1991). MT roles include the homeostatic regulation of essential metals such as zinc (Zn) or copper; detoxification *via* the chelation of harmful heavy metals such as cadmium (Cd) or mercury (Hg); the protection of tissues against various forms of oxidative injury (Davis & Cousins 2000; Haq *et al.* 2003). In aquatic environments, invertebrates and in particular bivalves are often used as biological models to study metal contamination and MT response because they are sessile, widely distributed geographically, and characterized by high bioaccumulation capacities, related to their filtration activity for respiratory and nutritional purposes (Boening 1999). In Europe, two invasive species currently cohabit in freshwater rivers and lakes: the zebra mussel *Dreissena polymorpha*, attached to natural or artificial substrates within the water column; the Asiatic clam *Corbicula fluminea*, buried in the superficial sediment layers. Several

field studies have reported significant increases in MT concentrations in response to metal bioaccumulation in these two species (Baudrimont *et al.* 1999; de Lafontaine *et al.* 2000; Gagne *et al.* 2002). In laboratory experiments, only the effects of single metal exposure on bioaccumulation and MT response have been studied, usually under unrealistic environmental contamination pressures (Doherty *et al.* 1988; High *et al.* 1997; Engelken & Hildebrandt 1999). To our knowledge, only one experimental study has been based on the combined effects of cadmium and copper on MT response in *D. polymorpha* (Lecoeur *et al.* 2004). However, at the field level, metal mixtures are commonly present as a result of mining, industrial and domestic effluents (Baudrimont *et al.* 1999; Gagne *et al.* 2002). In this context, metal additive or synergic effects can be expected and must be assessed.

The aims of the present experimental study were to compare the effects of Cd, Zn and the two metals in interaction on metal bioaccumulation and MT response in *D. polymorpha* and *C. fluminea*. Cd and Zn metals are known to be simultaneously present in the environment, notably from extraction and treatment of Zn ores. In this study, they were added individually or in combination to the water. Metal concentrations and MT protein levels were analysed at the whole soft body level, after 1, 3, 10 and 24 days of exposure and MT gene expression in the gills of *D. polymorpha* in response to metal contamination was quantified after 10 days.

Material and methods

Bivalve collection and experimental design

Zebra mussels were collected from a reference-sampling site on the Garonne River, near Agen (southwest France). A batch of 500 individuals was sorted in order to select homogeneous specimens according to shell length: 27 ± 4 mm ($n = 240$). Around 600 *C. fluminea* were sampled from the Isle River, a tributary of the Dordogne River (southwest France); the organisms selected for the experiment were 28 ± 5 mm ($n = 288$). The bivalves were collected in early autumn, when MT levels are least affected by reproductive activities and body mass variations (Baudrimont *et al.* 1997a; Lowe and Day 2002). Specimens of the two species were randomly allocated to 48 experimental units (EU: glass aquaria, $12 \times 12 \times 30$ cm), with 5 mussels and 6 clams per EU. Each EU was based on a mixed biotope: a pure sand compartment (3 kg/EU, 5 cm deep – 98% silica – granulometry: 0.8 to 1.4 mm – Silaq) and the overlying water (3 l, 20 cm deep). The clams were able to burrow in the sand, their filtration activity taking place at the sediment/water interface. A plastic horizontal grid was placed in the water column, 12 cm above the surface of the sand, in order to support the zebra mussels. During the acclimation (10 days) and experimental (24 days) periods, bivalves were maintained in dechlorinated and permanently aerated tap water at 20 ± 0.5 °C. Daily measurements of pH and dissolved oxygen showed very small variations throughout the entire experiment: 8.5 ± 0.3 and 9.5 ± 0.3 mg l^{-1}, respectively. The artificial photoperiod was 12h light per day. The bivalves were fed every two days with a small amount of fresh green algae (*Scenedesmus suspicatus*) (approximately 10^5 algae ml^{-1} per unit – Baudrimont *et al.* 1997b).

Metal contamination procedure

Three metal exposure conditions were tested against a control: Cd contamination, with a nominal concentration of 0.133 μM (15 μg Cd l^{-1} added as $CdCl_2$); Zn contamination, with a nominal concentration of 15.3 μM (1 000 μg Zn l^{-1} added as $ZnCl_2$); Cd + Zn contamination (0.133 μM Cd + 15.3 μM Zn). Contamination levels were chosen with reference to dissolved metal concentrations measured in a tributary of the Lot River, the Riou-Mort in southwestern France which has been polluted by mining effluents (Audry *et al.* 2004). For each exposure condition, there were three replicates per sampling time (1, 3, 10 and 24 days), thus 48 experimental units were set up simultaneously. Metal contamination pressures were maintained throughout the 24 days of experiment *via* daily Cd and Zn inputs from stock aqueous solutions (0.266 mM for Cd and 15.3 mM for Zn), after metal determinations on water samples from each EU, to compensate for metal concentration decreases: adsorption on the tank walls and sand surface; accumulation in bivalves, etc. Using this procedure, variations in Cd and Zn concentrations were very small throughout the

whole experiment: 0.133 ± 0.025 and 0.133 ± 0.015 μM Cd for Cd and Cd + Zn exposure, respectively; 15.3 ± 2.1 and 15.3 ± 1.5 μM Zn for Zn and Cd + Zn exposure, respectively. At each sampling time, bivalves were collected and immediately frozen at – 80 °C under nitrogen atmosphere to ensure anoxic conditions and avoid MT oxidation and polymerization (Dutton *et al.* 1993).

Cadmium and zinc determinations

Metal determinations were made on three replicates (one bivalve per EU) per species and experimental condition. The soft bodies were dissected using Teflon coated instruments, dried on absorbent paper and weighed (fresh weight, fw). For each sample, a classical digestion procedure was carried out with 3 ml of concentrated nitric acid (Merck 65% HNO_3) added in a pressurized medium (borosilicate glass tube) at 100 °C for 3 h. The digestates were diluted up to 20 ml with ultra-pure water (MilliQ plus). 10 μl of water samples or soft body digestates were mixed before atomization with 4 μl of a 0.2 g l^{-1} Pd and 0.5 g l^{-1} $Mg(NO_3)_2$ mixture to avoid interference. Cd determinations were performed with a polarized Zeeman atomic absorption spectrophotometer (Varian AA400) equipped with a graphite tube atomizer (model GTA 96, Varian). The detection limit was 0.1 μg Cd l^{-1} (DL: 3x SD of the reagent blanks). Zn concentrations were determined on the same samples by flame atomic absorption spectrophotometry (Varian AA220FS). The detection limit was 5 μg Zn l^{-1}. The analytical methods were simultaneously validated for each sample series by the analysis of standard biological reference materials (TORT-2, Lobster hepatopancreas; DOLT-2, Dogfish liver; NCR/CRNC). Values were consistently within the certified ranges (data not shown). Bioaccumulation levels were expressed in nmol Cd or Zn g^{-1}, fw ± standard error (SE).

Metallothionein determination

Total MT proteins were determined in the whole soft bodies by mercury-saturation assay using cold inorganic Hg (Dutton *et al.* 1993; Baudrimont *et al.* 1997a). MT analysis was conducted on three replicates per experimental condition, each corresponding to a pool of two individuals collected in each EU. Soft bodies were homogenized in 20 ml-polypropylene tubes with a tissue grinder (Ultra-Turrax T-25) in an ice-cold Tris–HCl 25 mM buffer (Sigma, pH 7.2 at 20 °C). This step was performed in a glove bag filled with nitrogen (Atmosbag, Aldrich Chemical Co.). The homogenized samples were kept on ice, to inhibit protease activity. Aliquots of 1.5 ml were placed in microtubes (Eppendorf) and centrifuged at 20,000*g* for 60 min, at 4 °C (Sigma 3K12). To 200 μl of supernatant was added a 200 μl $HgCl_2$ solution (Merck) at 50 mg Hg l^{-1} with trichloroacetic acid (10%, Sigma) for the precipitation of non-MT proteins. The Hg excess not bound to MTs was removed using lyophilised pig hemoglobin (Sigma) at 0.2 g Hb ml^{-1} in Tris–HCl 30 mM buffer (pH 8.2 at 20 °C). The final supernatant was then quantitatively recovered and used for Hg determination by cold vapour and atomic absorption spectrometry (CETAC M 6000, Varian). The detection limit was estimated at 0.01 μg Hg l^{-1}.

Reference samples or "blanks" (Baudrimont *et al.* 1997a) were prepared to monitor the Hg chelation efficiency of the hemoglobin. Under our experimental conditions, an average burden of 1.76 ± 0.11 ng Hg ($n = 14$) was measured in these reference samples, corresponding to 0.01% of added total Hg. The mean of the three blank values measured in each analytical run was removed from the Hg burdens measured in each sample.

At the same time, a standard of purified rabbit liver MT (Sigma) enabled us to check the validity of the saturation assay by determining the ratio between the binding sites measured after Hg-saturation and the potential binding sites indicated by the supplier and previously verified by Hg, Cd, Zn and Cu determinations on purified MT solution (10 μg MT ml^{-1} in the homogenization buffer). In our experimental conditions, the average value of the recovery percentage was $104 \pm 3\%$ ($n = 14$).

MT concentrations were expressed in nmol of Hg-binding sites $g^{-1} \pm$ SE (fresh weight, fw). Since the exact quantity of Hg binding sites per MT molecule was unknown for these two bivalve species, MT concentrations cannot be expressed in nmol MT g^{-1}.

Determination of MT gene expression

The *D. polymorpha* cDNA MT sequence has been described (Engelken and Hildebrandt 1999), but no MT sequence is known for *C. fluminea*. We

therefore used the RT-PCR technique to quantify MT gene expression in gills of zebra mussels after 10 days of exposure to Cd, Zn and Cd + Zn.

Total RNA extraction

Total RNAs were extracted from 40 mg of mussel gills using the Absolutely RNA RT-PCR Miniprep kit (Stratagene), according to the manufacturer's instructions. The gills cannot be considered as being strictly representative of the organs of the bivalve in general, but as they are a respiratory epithelium in direct contact with the water in the palleal cavity, they do have a high bioaccumulation capacity for metals and also for MT biosynthesis (Gundacker 1999; Lecoeur *et al.* 2004). The quality of all RNAs produced was evaluated by electrophoresis on a 1% agarose-formaldehyde gel, and their concentration determined by spectrophotometry. For each exposure condition, samples were carried out in triplicate. Each sample was obtained by mixing gills from two individuals.

Reverse transcription of total RNA

First-strand cDNA was synthesized from 5 µg total RNA using the Stratascript First-Strand Synthesis System (Stratagene). RNA was then adjusted to 38 µl with DEPC-treated water. After the addition of 2 µl of oligo(dT) and 1 µl of random primers, the reaction was incubated at 65 °C for 5 min. 40 units of RNAse block Ribonuclease Inhibitor, 2 µl of 100 mM dNTPs, 5 µl of 10× first-strand buffer and 50 units of Stratascript reverse transcriptase were then added. The reaction was incubated for 1 h at 42 °C in an Eppendorf Mastercycler. The cDNA mixture was maintained at –20 °C until it was needed for the RT-PCR reaction.

Primer design

A specific primer pair (5′-CCA TGC AAC TGC GTT GAA-3′ and 5′-TGC ACT GGG CCA GAT TCG TGC-3′) was selected within the coding region of the MT cDNA of *D. polymorpha*, from base pairs 151 to 313 (GenBank accession number: U67347).

Reverse polymerase chain reaction (RT-PCR)

RT-PCR reactions were performed in a LightCycler (Roche Diagnostics) following the manufacturer's instructions (one cycle at 95 °C for 10 min, and 50 amplification cycles at 95 °C for 5 s, 60 °C for 5 s and 72 °C for 20 s). Each 20 µl reaction contained 2 µl of reverse transcribed product template, 1 µl of master mix including the SYBR Green I fluorescent dye, enabling the monitoring of the PCR amplification, and the primer pair at a final concentration of 300 nM for each primer.

Reaction specificity was determined from the dissociation curve of the PCR product obtained by following the SYBR Green fluorescence level during a gradual heating of the PCR products from 60 to 95 °C. MT mRNA quantifications in the zebra mussel gills were expressed from induction factor compared with control values.

Statistical analysis

Statistical comparisons between mean values of metal and MT concentrations and a number of mRNA copies were made with the non-parametric Mann–Whitney U-test (Statistica 5.1, StatSoft Inc., Tulsa, OK, USA). Differences were considered statistically significant at $P \leq 0.05$.

Results

No mortality was observed for *C. fluminea* during the 24 days of exposure. In contrast, all the *D. polymorpha* individuals treated with Zn (Zn and Zn + Cd conditions) died during the last period of exposure (10–24 days). The soft body weight measurements at time zero and at the end of the experiment did not reveal any significant change in either species.

Metal bioaccumulation

Exposure to Cd alone showed a progressive and constant accumulation of the metal in the soft bodies of the two species, with similar maximal concentrations after 24 days: 134 ± 22 nmol Cd g^{-1} (fresh weight) for *D. polymorpha* and 130 ± 16 nmol Cd g^{-1} (fw) for *C. fluminea* (factors 70 and 175 compared to background levels, respectively – Figure 1a.). After the combined exposure to Cd and Zn, Cd concentrations were markedly lower during the first 10 days (Cd/Cd + Zn ratio close to 2.3) and similar for the two species. At the end of the experiment, the mean Cd

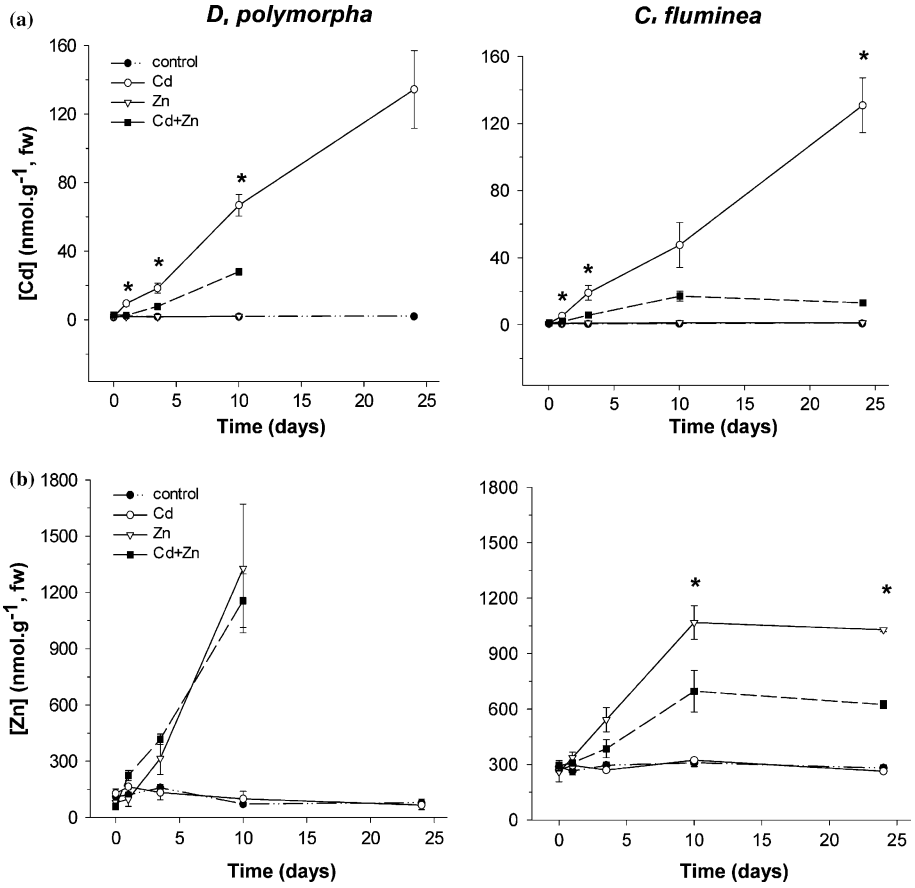

Figure 1. Cadmium (a) and zinc (b) concentrations (nmol g^{-1}, fresh weight) in the whole soft body of *Dreissena polymorpha and Corbicula fluminea* exposed for 24 days to cadmium and/or zinc (0.133 μM Cd and 15.3 μM Zn in the water). Control: no added metal in the experimental units. Data are means with standard errors ($n = 3$). Asterisks indicate the significant differences between single (Cd or Zn) and mixed (Cd + Zn) exposures for each exposure duration (Mann–Whitney U-test, $P < 0.05$).

concentration in *C. fluminea* was 12.5 ± 0.7 nmol g^{-1} (fw), with a Cd/Cd + Zn ratio close to 10.

Zn background level in the whole soft bodies of *C. fluminea* was about 4-fold higher than in *D. polymorpha* (287 ± 8.5 and 74.5 ± 7.8 nmol g^{-1}, fw – Figure 1b). In the soft bodies of *D. polymorpha*, the combined Cd + Zn exposure did not significantly modify Zn concentrations compared to the exposure to Zn alone, with a quasi-linear increase during the 10 days of exposure, with an average value around 1250 nmol Zn g^{-1} (×17/control). In *C. fluminea*, Zn accumulation was significantly higher in individuals exposed to Zn alone (Zn/Zn + Cd ratio close to 1.6); after 10 days of exposure, an equilibrium was reached for the Zn and Zn + Cd conditions, at around 1050 ± 24 and 660 ± 54 nmol Zn g^{-1} respectively (about 3.7-fold and 2.3-fold the control values).

Metallothionein levels

For *D. polymorpha*, the background MT level in the whole soft body at time zero was 15 ± 4 nmol site Hg g^{-1}, fw (Figure 2). Significant increases in MT concentrations were observed after 10 and 24 days of exposure to Cd (Cd/control ratio of 2.3 and 8, respectively) and after 10 days of exposure to Cd + Zn (Cd + Zn/control ratio of 1.9). Unlike Cd, MT levels in zebra mussels showed no significant changes after exposure to Zn alone.

MT quantification in the whole soft bodies of *C. fluminea* were significantly affected by both Cd and Zn after 10 and 24 days of exposure, with accumulation factors of proteins compared to control close to those found in *D. polymorpha* after 10 days of exposure to Cd and Cd + Zn (Figure 2). Nevertheless, no marked increase was observed in

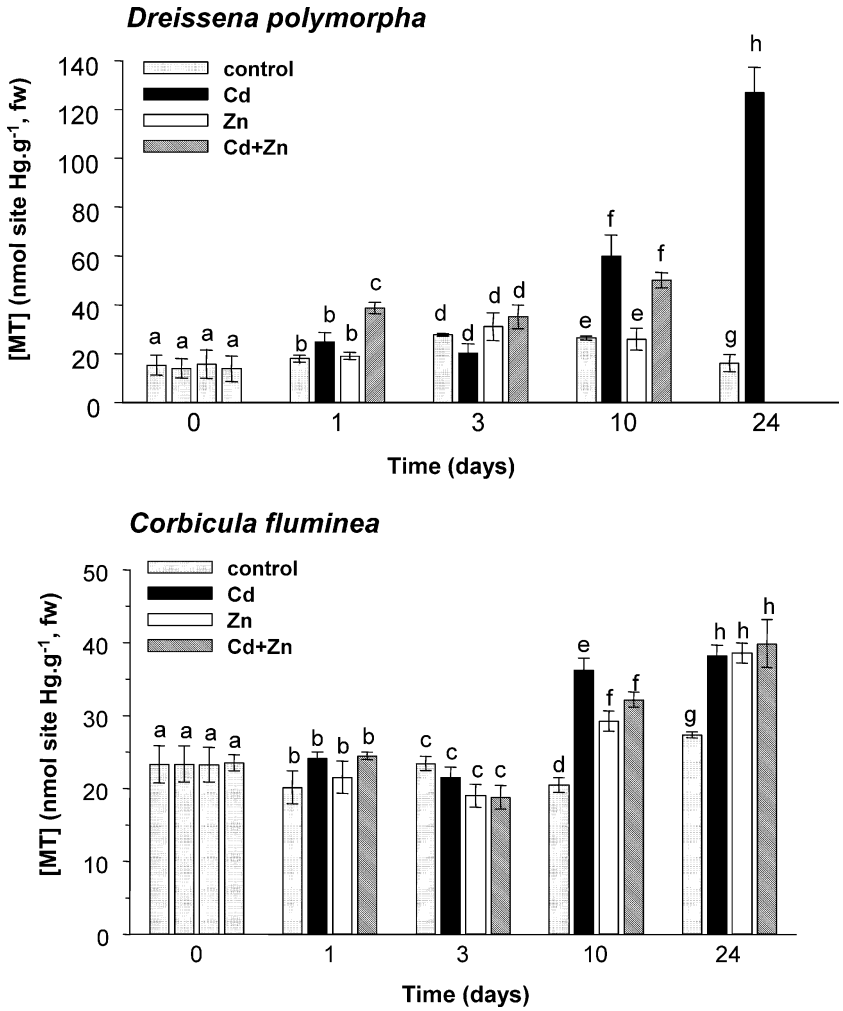

Figure 2. Metallothionein concentrations (nmol site Hg g^{-1}, fresh weight) in the whole soft body of *Dreissena polymorpha* and *Corbicula fluminea* exposed for 24 days to cadmium and/or zinc (0.133 μM Cd and 15.3 μM Zn in the water). Control: no added metal in the experimental units. Data are means with standard errors ($n = 3$). Values noted with the same letter indicate no significant difference between metal exposure conditions for each exposure duration (Mann–Whitney U-test, $P > 0.05$).

these two conditions between 10 and 24 days and similar MT levels were measured for the three exposure conditions (Cd, Zn, Cd + Zn), close to 40 nmol site Hg g^{-1} (fw) and 1.4 higher than the control level.

MT mRNA quantification in D. polymorpha

The lack of increase in MT concentrations in *D. polymorpha* after 10 days exposure to Zn alone, contrasting with the Cd-triggered induction, encouraged us to measure MT gene expression for the three contamination conditions and control. RT-PCR results from gill samples evidenced similarity with MT protein responses at the whole soft body level (Figure 3). MT mRNA amounts in gills revealed that gene expression was significantly up-regulated after exposure to Cd and Cd + Zn (induction factor of 20 and 14, respectively). In sharp contrast, no significant change was observed between control and Zn-contaminated zebra mussels.

Discussion

In this experimental study, the zebra mussel, *D. polymorpha*, and the Asiatic clam, *C. fluminea*,

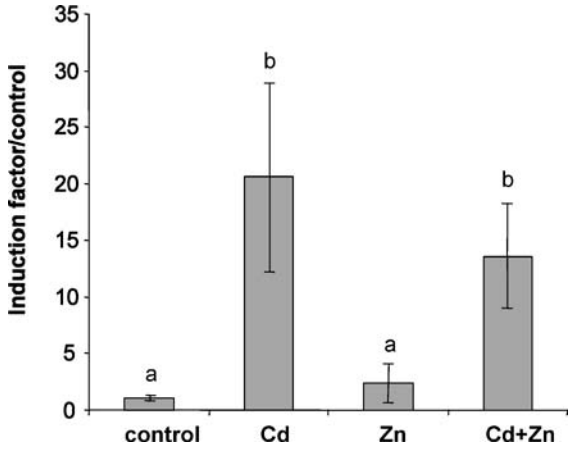

Figure 3. Metallothionein mRNA quantification by RT-PCR in gills of *Dreissena polymorpha* after 10 days of exposure to cadmium and/or zinc (0.133 μM Cd and 15.3 μM Zn in the water). mRNA levels are expressed as induction factors compared with control (control = 1). Control: no added metal in the experimental units. Data are means with standard errors ($n = 3$). Values noted with the same letter indicate no significant difference between metal exposure conditions (Mann–Whitney U-test, $P > 0.05$).

were exposed to Cd and/or Zn water concentrations (0.133 μM Cd and 15.3 μM Zn) similar to those found in numerous freshwater systems located downstream from industrial sites (Audry *et al*. 2004).

Under exposure to Cd alone, the MT responses in the whole soft body differed considerably between the two species that we studied, whereas the metal accumulation levels measured over time were the same. In the zebra mussels, variations in MT concentrations over time remained correlated to the gradual accumulation of the metal and after 24 days of exposure gave values that were 8 times higher than those measured in the controls. In *C. fluminea*, on the other hand, quantities of MT were much less, corresponding to concentrations that were only 1.4 times higher than in the controls over the same time. In *D. polymorpha*, even though the Cd is potentially associated with different intra- and extra-cellular structures (Herwig *et al*. 1989), the pool of MTs measured constitutes a sequestration potential similar to the amount of Cd bioaccumulated in the whole soft body at each exposure time (e.g. after 24 days: 127 nmol site Hg g^{-1} and 134 nmol Cd g^{-1}, fw). For *C. fluminea*, the number of potential sites represents only 30% of the total quantity of Cd accumulated in the whole soft body after 24 days (40 nmol site Hg g^{-1} and 130 nmol Cd g^{-1}). Other Cd sequestration mechanisms therefore predominated in the clam (e.g. glutathion, proteins, metal granules, membranes ...–Langston et al. 1998). As the soluble cellular fraction could represent between 48 and 62% of the total amount of Cd accumulated in the soft body of this species (Baudrimont *et al*. 1997b; Baudrimont *et al*. 2003), the sequestration potential offered by MTs in this soluble fraction could nevertheless represent 59 to 64%.

The differences between species could be accounted for by the different metal distribution between the soluble and insoluble fractions, as observed between *Macoma balthica* and *Potamocorbula amurensis* (Wallace *et al*. 2003). Moreover, MT gene transcription regulation could be divergent (signal transduction cascade, transcription factors, cis-active boxes contained in the MT gene promoters – Andrews 2000).

When *C. fluminea* was contaminated by Zn, MT concentrations increased after 10 days, however, the sequestration capacities of these proteins represented only 3 to 4% of the amount of Zn accumulated in the soft body between 10 and 24 days (30 to 40 nmol Hg g^{-1} and 1050 nmol Zn g^{-1}). In this species, the contribution of MTs in fixing total Zn accumulated at the whole soft body level is therefore limited, as had been observed in other bivalves (*Crassostrea virginica, Hyridella depressa* and *Velesunio ambiguus, Macoma balthica)* (Roesijadi 1994b; Markich *et al*. 2001; Baudrimont *et al*. 2003; Wallace *et al*. 2003). On the other hand, Zn bioaccumulation did not increase between days 10 and 24, which could be interpreted as the consequence of an equilibrium between metal entering and being eliminated from the soft body. This result is compatible with the induction of efflux mechanisms in relation to the metal species, such as exocytosis and the contribution from metal pumps belonging to the ABC transporter family (ATP-binding cassette) (Rensing *et al*. 1997; Achard *et al*. 2004).

In *D. polymorpha*, Zn produced no increase in MT concentration over time. This protein plays therefore only a very small role in the high level of accumulation of this metal. The same result has been observed after copper contamination (Lecoeur *et al*. 2004). Similar results have been reported after Zn contamination in other aquatic invertebrates, such as *Mytilus edulis*, *Orchestia gammarellus* or *Ruditapes decussatus* (George 1990;

George and Langston 1994; Mouneyrac *et al.* 2002; Geret and Bebianno 2004). In the zebra mussel, it can be explained by the absence of MT gene induction by Zn.

The toxicity caused by Zn, which led to the death of the mussels, may be the result of less efficient overall detoxification mechanisms in respect of this metal compared with *C. fluminea*, particularly in relation to the limited role of the MTs in sequestration as MT expression was not induced.

In the combined exposure condition, Cd/Zn interactions may occur at the different stages of absorption, sequestration and excretion (Brzóska and Moniuszko-Jakoniuk 2001). The exposure of the bivalves to cadmium and zinc simultaneously produced a very large decrease in Cd bioaccumulation in the soft body of the two species, over 50% after 10 days of exposure, and over 90% after 24 days in *C. fluminea*. In the zebra mussel, Zn bioaccumulation was not affected, whereas in *C. fluminea* it decreased by about 50% at 10 and 24 days, compared with the exposure to Zn alone. In *D. polymorpha*, these results are compatible with there being a competition mechanism in relation to divalent metal transport mechanisms through biological barriers (divalent metal transporter, calcium channels, ... – Vercauteren and Blust 1999; Zalups and Ahmad 2003) facilitated by a Zn/Cd molar concentration ratio in the water of 115. In *C. fluminea*, competition at entry between Zn and Cd cannot by itself account for the results obtained. The reduction in concentrations of Cd and Zn is compatible with an increased induction in combined contamination condition of active efflux mechanisms for metals with a lower affinity for Cd. In clams, combined and simple exposures produced the same level of MTs, whereas the total amounts of metal ions accumulated differed (672.5 $nmol_{metal}\ g^{-1}$compared with 130 and 1050 nmol g^{-1} for Cd and Zn alone after 24 days). The MT response in *C. fluminea* in the presence of the two metals is therefore not simply a combination of the effects observed under exposure to a single metal. Similarly, in *D. polymorpha*, this response which is attributable to the influence of Cd, does not decrease proportionately with the accumulation of the metal after 10 days. This could be the result of metal interactions on intracellular bioaccumulation. Zn toxicity in the zebra mussels, despite the increased MT biosynthesis in the combined exposure, could be linked with the stronger affinity of Cd for fixing on these proteins (Vasák 1991).

A comparative analysis of the MT gene expression rates measured by RT-PCR in the gill tissue of *D. polymorpha*, after 10 days of exposure, revealed responses comparable with those obtained from MT dosage in the whole soft body. The gills are therefore an appropriate tissue to study in order to analyse the expression of this gene. Cd triggered a MT gene expression rate that was 20 times greater than the level in the control. Zn, on the other hand, did not induce gene expression. These results differ from those obtained in mammals, where Zn concentration of about 100 μM, on cell culture, is a powerful MT gene inducer (Murphy *et al.* 1999). The transcriptional induction of MT genes in mammals and *Drosophila melanogaster* is based on the binding of a transcription factor (MTF-1) to metal regulatory elements (MRE) in their promoters (Egli *et al.* 2003). According to our results, it could be possible that the transcriptional activation of MT gene in *D. polymorpha* may be regulated by the same pathway, but that Zn concentration used in our experiment (15 μM in the water) was not sufficient to induce this activation.

Acknowledgements

We would like to thank Henry Bouillard and Bruno Etecheverria for their helpful assistance during bivalve collection. This study was carried out with financial support from the GIS ECOBAG research programme (Adour-Garonne Water Agency, Aquitaine Region, French Ministry of Ecology and Sustainable Development, National Scientific Research Center). Véronique Marie was supported by a grant from the Aquitaine Region.

References

Achard M, Baudrimont M, Boudou A, Bourdineaud JP. 2004 Induction of a multixenobiotic resistance protein (MXR) in the Asiatic clam *Corbicula fluminea* after heavy metals exposure. *Aquat Toxicol* **67**, 347–357.

Andrews GK. 2000 Regulation of metallothionein gene expression by oxidative stress and metal ions. *Biochem Pharmacol* **59**, 95–104.

Audry S, Schafer J, Blanc G, Bossy C, Lavaux G. 2004 Anthropogenic components of heavy metal (Cd, Zn, Cu, Pb)

budgets in the Lot-Garonne fluvial system (France). *Appl Geochem* **19**, 769–786.

Baudrimont M, Andres S, Durrieu G, Boudou A. 2003 The key role of metallothioneins in the bivalve *Corbicula fluminea* during the depuration phase, after in situ exposure to Cd and Zn. *Aquat Toxicol* **63**, 89–102.

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.

Baudrimont M, Lemaire-Gony S, Ribeyre F, Metivaud J, Boudou A. 1997a Seasonal variations of metallothionein concentrations in the Asiatic clam (*Corbicula fluminea*). *Comp Biochem Physiol* **C 118**, 361–367.

Baudrimont M, Metivaud J, Maury-Brachet R, Ribeyre F, Boudou A. 1997b Bioaccumulation and metallothionein response in the asiatic clam (*Corbicula fluminea*) after experimental exposure to cadmium and inorganic mercury. *Environ Toxicol Chem* **16**, 2096–2105.

Boening DW. 1999 An evaluation of bivalves as biomonitors of heavy metals pollution in marine waters. *Environ Monit Assess* **55**, 459–470.

Brzóska MM, Moniuszko-Jakoniuk J. 2001 Interactions between cadmium and zinc in the organism. *Food Chem Toxicol* **39**, 967–980.

Davis SR, Cousins RJ. 2000 Metallothionein expression in animals: A physiological perspective on function. *J Nutr 2000* **130**, 1085–1088.

de Lafontaine Y, Gagne F, Blaise C, Costan G, Gagnon P, Chan HM. 2000 Biomarkers in zebra mussels (*Dreissena polymorpha*) for the assessment and monitoring of water quality of the St Lawrence River (Canada). *Aquat Toxicol* **50**, 51–71.

Doherty FG, Failla ML, Cherry DS. 1988 Metallothionein-like heavy metal binding protein levels in asiatic clams are dependent on the duration and mode of exposure to cadmium. *Water Res* **22**, 927–932.

Dutton MD, Stephenson M, Klaverkamp JF. 1993 A mercury saturation assay for measuring metallothionein in fish. *Environ Toxicol Chem* **12**, 1193–1202.

Egli D, Selvaraj A, Yepiskoposyan H, Zhang B, Hafen E, Georgiev O, Schaffner W. 2003 Knockout of 'metal-responsive transcription factor' MTF-1 in Drosophila by homologous recombination reveals its central role in heavy metal homeostasis. *EMBO J* **22**, 100–108.

Engelken J, Hildebrandt A. 1999 cDNA cloning and cadmium-induced expression of metallothionein mRNA in the zebra mussel *Dreissena polymorpha*. *Biochem Cell Biol* **77**, 237–241.

Gagne F, Blaise C, Aoyama I, Luo R, Gagnon C, Couillard Y, Campbell P, Salazar M. 2002 Biomarker study of a municipal effluent dispersion plume in two species of freshwater mussels. *Environ Toxicol* **17**, 149–159.

George SG. 1990 Biochemical and cytological assessments of metal toxicity in marine animals. In: Furness RW, Rainbow PS, eds. *Heavy Metal in the Marine Environment*, Boca Raton, Florida: CRC Press Inc., pp. 123–142.

George SG, Langston WJ. 1994 Metallothionein as an indicator of water quality: assessment of the bioavailability of cadmium, copper, mercury and zinc in aquatic animals at the cellular level. In: Sutcliffe DW, eds. *Water Quality and Stress Indicators in Marine and Freshwater Ecosystems: Linking Levels of Organisation (Individuals, Populations, Communities)*, Ambleside: The Freshwater Biological Association, pp. 138–153.

Geret F, Bebianno MJ. 2004 Does zinc produce reactive oxygen species in *Ruditapes decussatus*? *Ecotox Environ Safe* **57**, 399–409.

Gundacker C. 1999 Tissue-specific heavy metal (Cd, Pb, Cu, Zn) deposition in a natural population of the zebra mussel *Dreissena polymorpha* Pallas. *Chemosphere* **38**, 3339–3356.

Haq F, Mahoney M, Koropatnick J. 2003 Signaling events for metallothionein induction. *Mutat Res* **533**, 211–226.

Herwig HJ, Brands F, Kruitwagen E, Zandee DI. 1989 Bioaccumulation and histochemical localization of cadmium in *Dreissena polymorpha* exposed to cadmium chloride. *Aquat Toxicol* **15**, 269–285.

High KA, Barthet VJ, McLaren JW, Blais J-S. 1997 Characterization of metallothionein-like proteins from zebra mussels (*Dreissena polymorpha*). *Environ Toxicol Chem* **16**, 1111–1118.

Langston WJ, Bebianno MJ, Burt JR. 1998. Metal handling strategies in molluscs. In: Langston WJ, Bebianno M.J, eds. *Metal Metabolism in Aquatic Environments*, London: Chapman and Hall, pp. 219–283.

Lecoeur S, Videmann B, Berny P. 2004 Evaluation of metallothionein as a biomarker of single and combined Cd/Cu exposure in *Dreissena polymorpha*. *Environ Res* **94**, 184–191.

Lowe TP, Day DD. 2002 Metal concentrations in zebra mussels and sediments from embayments and riverine environments of eastern Lake Erie, southern Lake Ontario, and the Niagara River. *Arch Environ Contam Toxicol* **43**, 301–308.

Markich SJ, Brown PL, Jeffree RA. 2001 Divalent metal accumulation in freshwater bivalves: an inverse relationship with metal phosphate solubility. *Sci Total Environ* **275**, 27–41.

Mouneyrac C, Amiard JC, Amiard-Triquet C, Cottier A, Rainbow PS, Smith BD. 2002 Partitioning of accumulated trace metals in the talitrid amphipod crustacean *Orchestia gammarellus*: a cautionary tale on the use of metallothionein-like proteins as biomarkers. *Aquat Toxicol* **57**, 225–242.

Murphy BJ, Andrews GK, Bittel D, Discher DJ, McCue J, Green CJ, Yanovsky M, Giaccia A, Sutherland RM, Laderoute KR, Webster KA. 1999 Activation of metallothionein gene expression by hypoxia involves metal response elements and metal transcription factor-1. *Cancer Res* **59**, 1315–1322.

Rensing C, Mitra B, Rosen BP. 1997 The zntA gene of *Escherichia coli* encodes a Zn(II)-translocating P-type ATPase. *Proc Natl Acad Sci USA* **94**, 14326–14331.

Roesijadi G. 1994a Metallothionein induction as a measure of response to metal exposure in aquatic animals. *Environ Health Perspect* **102**, 91–95.

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

Vasák M. 1991 Metal removal and substitution in vertebrate and invertebrate metallothioneins. *Methods Enzymol* **205**, 452–458.

Vercauteren K, Blust R. 1999 Uptake of cadmium and zinc by the mussel *Mytilus edulis* and inhibition by calcium channel and metabolic blockers. *Mar Biol* **135**, 615–626.

Wallace WG, Lee B-G, Luoma SN. 2003 Subcellular compartmentalization of Cd and Zn in two bivalves. I. Significance of metal-sensitive fractions (MSF) and biologically detoxified metal (BDM). *Mar Ecol Progr Ser* **249**, 183–197.

Zalups RK, Ahmad S. 2003 Molecular handling of cadmium in transporting epithelia. *Toxicol Appl Pharm* **186**, 163–188.