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Metallothionein responses in the Asiatic clam (*Corbicula fluminea*) after exposure to trivalent arsenic

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Abstract
The main objective of this work was to evaluate arsenic effects on metallothionein (MT) induction by exposing a freshwater Asiatic clam (*Corbicula fluminea*) to different concentrations of this metalloid. The presence of MT-like proteins was detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis and compared with a standard rabbit MT. In addition, the polarographic response showed good correspondence between standard MT and MT-like curves from *C. fluminea*, allowing MT quantification. The results show that clams exposed to different concentrations of arsenic are able to induce significant levels of MTs. Although variability was found in MT induction, significant differences in MT levels were found after 28 days of exposure in all treatments in comparison with the controls, suggesting that exposure to arsenic induced MT-like proteins in *C. fluminea*.

Keywords: *Metallothionein, biosynthesis, arsenic,* Corbicula fluminea

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Introduction

Arsenic is a ubiquitous element in the earth's crust that can occur in aquatic environments as the result of natural geochemical reactions (Duker et al. 2005). It has been used in many industrial products, processes and applications (e.g. glass, paints, alloys, pesticides, wood preservation, mining) and is one of the most dangerous pollutants. It takes a number of distinct forms in the environment, with the trivalent form being the most toxic (Aposhian 1997). There is worldwide concern about arsenic contamination of drinking water and the health effects of ingestion of this metalloid, which can cause cancer of the internal organs and skin (Dugo et al. 2005). In addition, arsenic concentrations in wastewater, surface water, groundwater, and geothermal water frequently exceed the drinking water standards (Banerjee et al. 1999).

Several studies have reported elevated levels of arsenic in marine bivalves such as oysters and mussels (Valette-Silver et al. 1999), marine polychaeta (Waring & Maher 2005) and fish (Mason et al. 2000). However, fish and shellfish arsenicals are believed

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to be in relatively non-toxic forms for humans (arsenobetaine, arsenocholine, arsenosugars), although there is recent evidence that biotransformation occurs in arsenosugars converting them to potential carcinogenic compounds (McSheehy et al. 2003).

Corbicula fluminea is a freshwater clam originating from China that is now widely distributed in Asia, North America and Europe (Labrot et al. 1999, Mouthon 2001, Schmidlin & Baur 2007). Usually, the bivalve spreads attached to boats or carried in ballast water, through the aquarium trade and is carried with water currents. This species presents a strong, invasive dynamic in rivers, channels and lakes (Baudrimont et al. 1997, Inza et al. 1997) and rapidly expands its range constituting a serious danger to many native freshwater bivalves (Korniushin 2004). It has high adaptation capabilities and is known to accumulate various pollutants, and is considered as an interesting biological monitor (Doherty 1990, Vidal et al. 2002). In addition, *C. fluminea* is a tool currently used as a biomarker for water contamination, for instance in the national French program ECODYN (Legeay et al. 2005).

The term biomarker is generally used in a broad sense to include almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical or biological (WHO 2001). Induction of metallothioneins (MTs) is usually seen as a potential biomarker of metal exposure in molluscs and fish and has been proposed as a sensitive 'early warning' signal of the potentially detrimental effects of metal contamination (Roesijadi 1992, Carajaville et al. 2000). However, MT is still described as a potential biomarker since its definite functions remain unclear and are still subject to debate more than 50 years after its discovery (Hamilton & Mehrle 1986).

MTs or MT-like proteins are currently used to indicate a series of well-known molecules that present structural and functional similarities to MTs from equine renal cortex (Margoshes & Valee 1957). They are non-enzymatic proteins with a low molecular weight, high cysteine content, no aromatic amino acids and heat stability (Kagi 1993, Stillman 1995). MTs have been reported in a wide range of organisms (e.g. fish, molluscs and crustaceans), presenting a ubiquitous distribution in the animal kingdom (George & Olsson 1994, Langston et al. 1998, Bebianno et al. 2004).

There are, however, few reports on MT induction in the Asiatic clam exposed to heavy metals (Baudrimont et al. 1997, Baudrimont et al. 2003, Marie et al. 2006) and, to our knowledge, there are no studies reporting MT induction in *C. fluminea* after exposure to trivalent arsenic. Thus, the present study investigated the biosynthesis of MT-like proteins in *C. fluminea* exposed to different concentrations of inorganic arsenic. The detection of MT-like proteins was performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and MT quantification by differential pulse polarography.

Materials and methods

Experimental procedure

Asiatic clams (*C. fluminea*) collected from a Portuguese river (the Minho) were acclimatized in the laboratory for 2 weeks in a system of tanks with recirculated, filtered and aerated tap water at a constant temperature ($20 \pm 1^{\circ}$C). Two hundred individuals ($n = 20$ in each tank; 1.4 ± 0.2 g) were randomly distributed in ten polyvinyl tanks (20 l volume) contaminated with different nominal concentrations

of arsenic (100, 300, 500 and 1000 $\mu g\,l^{-1}$) just once at the beginning of the experiments. Stock arsenical solutions were prepared from a standard inorganic arsenic (As) (III) (Aldrich, St Louis, MO, USA) by adding distilled water to a final concentration of 100 $mg\,l^{-1}$ and then different aliquots were taken and added to test tanks according to the final concentration desired. The static assay was performed in duplicate over 28 days using dechlorinated tap water, with continuous aeration and constant photoperiod (12 h light:12 h dark). Temperature and pH were determined using a potentiometer (ORION Model 290; Orion Research Inc., Boston, MA, USA) and conductivity was determined using a conductivity-salinity meter (ORION-Model 140, Orion Research Inc., Germany). The physicochemical parameters were monitored daily. The bivalves were fed on a daily basis with minced fish pellets (Dibaq, Spain). At the beginning of the experiment (time 0) 10 individuals were sampled from non-contaminated tanks. Organisms were sampled 7 and 28 days after exposure and whole soft tissues were taken, dried and weighed (fresh weight and dry weight), and then stored at $-80^{\circ}C$ for further analysis.

Quantification of MT-like proteins

MTs were extracted according to a procedure adapted for MT analysis, as described by Olafson (1981) and Thompson and Cosson (1984). Briefly, the whole soft tissues from *C. fluminea* were homogenized in Tris–HCl (0.02 M, pH 8.6), using a Teflon tip homogenizer (RZR 2100, Heidolph, Schwabach, Germany). Aliquots (3 ml) of each of the homogenates were then placed in an 80°C water bath for 10 min, a process that was followed by ultracentrifugation (30 000*g*). Heat-denaturated homogenates were stored at $-80^{\circ}C$ for later analysis. Differential pulse polarography using the Brdicka procedure was carried out with a Metrohm 694 stand and 693 processor (Metrohm Ion Analysis, Herisau, Switzerland). Analysis was performed on 20 ml of supporting electrolyte using a mercury capillary working electrode, a Ag/AgCl reference electrode and a platinum auxiliary electrode. The Brdicka supporting electrolyte was prepared weekly according to the method proposed by Palecek and Pechan (1971) and Thompson and Cosson (1984); it contained 1 M NH_4Cl, 1M NH_4OH and 2 mM $[Co(NH_3)_6]Cl_3$ and was stored at 4°C. Triton X-100 (Sigma, St Louis, MO, USA) at an appropriate concentration of 2.5×10^{-2}% (v/v) was used to suppress secondary maxima and minima and eliminate baseline noise (Bebianno & Langston 1989). In addition to the electrolyte solution (20 ml), 250 µl of Triton X-100 and 25–150 µl of heat-denaturated homogenates (cytosol) from exposed organisms were added to the polarographic cell. Given the lack of a molluscan MT, a rabbit liver MT (forms I and II; Sigma) was used as a standard, prepared to a working solution of 10 $mg\,l^{-1}$ in deionized water. MTs were expressed as $mg\,g^{-1}$ dry weight whole body homogenate.

Detection of MTs

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was employed to detect the presence of *C. fluminea* MT-like proteins by comparison with an MT standard. In the absence of a molluscan standard, a rabbit liver MT standard (Sigma) was used. The gels (0.75 mm thick) comprised 15% acrylamide in 0.25 M Tris–HCl buffer (pH 8.8) running gel. The stacking gel had 5% acrylamide in 0.125 M Tris–HCl buffer (pH 6.8) and was stained with Coomassie Blue R-250. Cytosolic samples were pretreated with heat (100°C, 2 min) in β-mercaptoethanol-containing

sample buffer (>99%; Merck, Darmstadt, Germany). A broad-range protein standard (BioRad, Richmond, CA, USA) was used to assess protein molecular weight, using the software Quantity One (BioRad), and contained: aprotinin (6.50 kDa), lysozyme (14.4 kDa), trypsin inhibitor (21.50 kDa), carbonic anhydrase (31.00 kDa), ovalbumin (45.00 kDa), serum albumin (66.20 kDa), phosporylase b (97.40 kDa), β-glactosidase (116.25 kDa), myosin (200.00 kDa).

Water concentrations of arsenic

Water samples were collected at the beginning of the study into polyethylene bottles, acidified with 10 $\mu l\,ml^{-1}$ of HNO_3 (SupraPur; Merck) and stored at 4°C. Three replicate analyses for arsenic were performed per experimental condition. Arsenic was directly determined by electrothermal atomic absorption spectrometry (ET-AAS) with Zeeman correction and matrix modifier ($Pd(NO_3)2.2H_2O$; Fluka, Deisenhofen, Germany), using a graphite tube atomizer (AAS, Thermoptec M6Solaar) without filtration, after dilution with ultrapure water. The limits for detection and quantification were calculated with the criteria of 3σ and 10σ, and were 3.1 $\mu g\,l^{-1}$ and 9.3 $\mu g\,l^{-1}$, respectively. To determine As concentrations a standard calibration curve was constructed using a commercial inorganic As (III) standard for atomic absorption (N 206962, 1 $g\,l^{-1}$; Aldrich). Blanks were analysed in triplicate using the same procedure.

Statistical analysis

The statistical analyses were performed with a probability of $p<0.05$, using the software STATISTICA 6.0 (StatSoft Inc., USA). The non-parametric Mann–Whitney *U* test was used to determine significant differences between samples before transformation of the data to percentage of the control values.

Results

The physicochemical results show that temperature (20 ± 1°C), pH (7.1 ± 0.2) and conductivity (539 ± 6 $\mu S\,cm^{-1}$) were constant during the experiment period in all test tanks. The results of total As (III) measured in water sampled from the different exposure concentrations are given in Table I, showing As (III) levels very close to the nominal concentrations. No mortality was observed during the assay for all treatments.

The MT-like proteins were detected by SDS-PAGE electrophoresis (Figure 1) by comparison with a rabbit standard MT. The results showed two bands of apparent molecular weight of approximately 16.4 and 18.2 kDa and were comparable with standard MT. The MT-like proteins were quantified by polarography (Figure 2),

Table I. Total arsenic concentrations ($\mu g\,l^{-1}$, mean ± SD) measured in water samples.

Control	100	300	500	1000
<l.d.	94 ± 3	303 ± 4	535 ± 7	1001 ± 4

l.d., limit of detection ($n = 3$, each replicate).

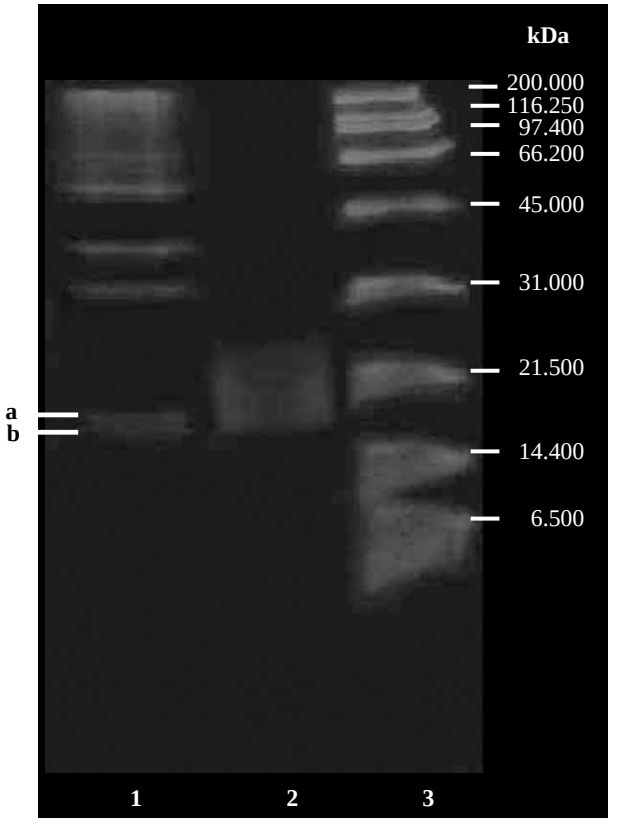

Figure 1. SDS-PAGE electrophoresis from *Corbicula fluminea* whole tissue. Lane 1, MT-like proteins; lane 2, rabbit liver MT (standard); lane 3, broad-range protein standard.

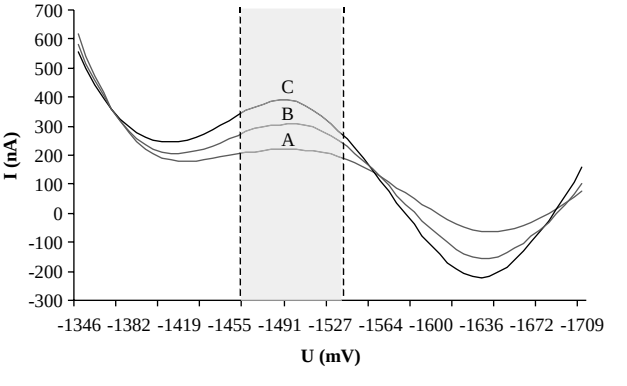

Figure 2. Differential pulse polarography analysis of a cytosolic fraction from Asiatic clam whole soft tissues. Curves: A – sample from a control organism; B and C – rabbit liver MT (standard); — peak tolerance.

showing typical curves that allowed the measurement of this protein at -1500 ± 50 U (mV).

The results show that the lowest average levels of MT-like proteins were measured at time zero (5.4 ± 1.6 mg g^{-1} d.w.) and in the control groups after 7 (7.5 ± 2.8 mg g^{-1} d.w.) and 28 days (5.8 ± 4.5 mg g^{-1} d.w.) of experiment assay. After 7 days of exposure the highest average levels of MT-like (14.4 ± 4.6 mg g^{-1} d.w.) were determined in clams exposed to 300 μg l^{-1} of arsenic and after 28 days of exposure the highest average concentrations of MT-like (38.5 ± 21.5 mg g^{-1} d.w.) were found in clams exposed to 100 μg l^{-1} of arsenic.

The MT analysis results for of clams sampled at 7 days of exposure show a significant increase ($\star p < 0.05$) in organisms exposed to a concentration of 300 μg l^{-1} of arsenic, in comparison to the control group (Figure 3). Nevertheless, the results show that MTs biosynthesis is induced following exposure to arsenic, which was confirmed by the analysis of the organisms exposed for 28 days. Thus, after 28 days of exposure significant differences ($\star\star p < 0.05$) were determined in all organisms exposed to the different nominal concentrations of arsenic, with the highest levels being determined in organisms exposed to 100 μg l^{-1}, as shown in Figure 3.

Discussion

MT induction by metal contaminants (e.g. Ag, Cd, Cu) has been demonstrated in some vertebrate and invertebrate organisms, suggesting the potential use of MT concentrations in organisms as biomarkers of metal exposure (Bebianno & Langston 1992, Bebianno & Machado 1997). In a few species of marine molluscs MTs are fully characterized (e.g. *Mytilus edulis*, *Crassostrea virginica*, *Patella vulgata* and, recently, *Ruditapes decussatus*) (Carajaville et al. 2000), as well as in fish such as rainbow trout, common carp or English sole (Hamilton & Mehrle 1986).

The SDS-PAGE results from cytosolic fractions indicate that MT-like proteins are synthesized by *C. fluminea* cells and are apparently equivalent to standard rabbit MT. These results are comparable to those found by other authors (Baudrimont et al. 2003, Marie et al. 2006) who, however, exposed *Corbicula* spp. to Cd and Zn and identified the presence of MT-like proteins.

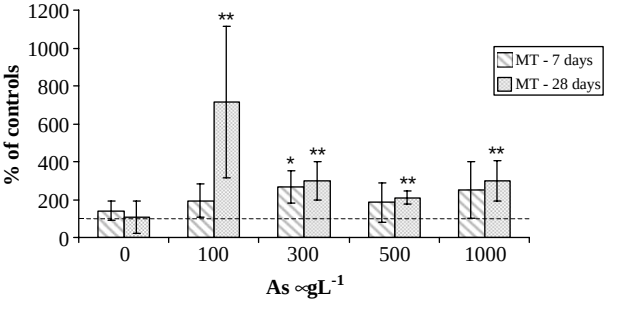

Figure 3. Effects of arsenic (As) on metallothionein (MT) concentrations after 7 and 28 days of exposure. All results are expressed as percentage of control at time zero (mean ± SD). Asterisks indicate significant differences from controls ($p < 0.05$). Dotted line (—) shows percentage of controls at time zero.

It was also established that the polarographic response shows a good correspondence between the rabbit MT standard and *C. fluminea* MT-like curves (see Figure 2), allowing the quantification of MT in the different exposure concentrations.

Although the physiological roles of MTs are still under debate, a fundamental involvement in the homeostasis of essential and non-essential metals seems certain and they are also thought to be involved in the detoxification of excess amounts of both essential and non-essential trace metals (Langston et al. 1998, Amiard et al. 2006).

Most of the studies on arsenic as an MT inducer were carried out on mice injected with arsenical solutions. They show that they are effective inducers of MT *in vivo*, but their potency and efficiency are dependent on the chemical form of arsenic (Kreppel et al. 1993). However, there is no consensus among researchers. Some have claimed that only a small portion of dosed arsenic was found to be associated with the MT fraction (Albores et al. 1992) and, unlike the proposed detoxification of cadmium, MT does not protect the fish against arsenic toxicity by sequestering this metalloid: rather, it may function as an antioxidant against arsenic-induced oxidative injury. Other researchers state that MT might play a role in the detoxification of arsenicals, and have proposed it as an adaptive mechanism for tolerance to arsenic toxicity (Kreppel et al. 1988).

It is known that *C. fluminea* is able to synthesize MTs after exposure to heavy metals such as Cd or Zn and that it presents higher sensitivity for MT response than *Dreissena polymorpha* (Marie et al. 2006). However, to our knowledge, there are no studies of MT induction after exposure to arsenic in bivalve molluscs, except for a single study carried out by Bouskill et al. (2006). This exposed *D. polymorpha* to a single concentration of 80 $\mu g\,As\,l^{-1}$ for 7 days and showed that MTs were synthesized (4.5 ± 0.03 MT $mg\,g^{-1}$), although without significant difference over the time course of the experiment or between control levels for arsenic-exposed mussels. Our findings show an induction in synthesis of MT-like proteins in bivalves exposed to different treatments, after 7 and 28 days of exposure, although considerable variability was found as well. According to Amiard et al. (2006) there are several factors that can influence protein metabolism and are able to influence MT directly; for example, any factor which is known to influence metal uptake and accumulation (e.g. size, sex, sexual maturity) will also be able to influence indirectly variations in MT concentrations.

On the other hand, results after 28 days of exposure indicate that MT were induced in *C. fluminea* whole soft tissues after exposure to arsenic and show significant differences in all treatments ($p<0.05$) in comparison with controls. A possible explanation for the high variability found, principally in organisms exposed to 100 $\mu g\,l^{-1}$ after 28 days of exposure, could be differences in uptake kinetics between exposed organisms or different sensivities to arsenic toxicity at this concentration. For instance, Luoma and Rainbow (2005) showed that variability in Ag bioaccumulation by marine and freshwater invertebrates is a reflection of metal-specific biology (fast uptake rates) and metal-specific geochemistry. Nevertheless, MT induction was lower in organisms exposed to higher concentrations of arsenic (300, 500 and 1000 $\mu g\,l^{-1}$), which can be explained by a reduction in the capacity of the MT-like proteins to sequester arsenic as a consequence of metalloid overload. It is also known that the ubiquity of arsenic in the environment has led to the evolution of arsenic defense mechanisms, from *Escherichia coli* to man (Rosen 2002). Therefore, an additional explanation could be the production of different metal-binding ligands that sequester

metals such as granules, lysosomes and other metal-binding proteins to regulate or neutralize intracellular metal toxicity (Giguère et al. 2003). Moreover, As (III) resistance is conferred by multixenobiotic resistance mechanisms (MXR) that are known to occur in several marine invertebrates and was reported in the the freshwater clam *C. fluminea* after exposure to heavy metals (Achard et al. 2004).

Certain studies also support a relationship between arsenic and MT induction. For instance, hepatic MT induction was found to increase in freshwater teleosts exposed to arsenic, e.g. *Channa punctatus* (Roy & Bhattacharya 2006) and *Ictalurus punctatus* (Schlenk et al. 1997). However, it was only recently fully demonstrated that MTs are capable of binding arsenic. Ngu and Stillman (2006), carried out *in vitro* studies using human MTs and showed that As^{3+} binds to MTs but not in a clustered binding site as observed with other metals (e.g. Cd^{2+}), as only three As^{3+} ions bind to three sulphurs in a trigonal pyramidal coordination with the sulphurs acting as terminal ligands.

Nevertheless, we may hypothesize that even if arsenic acts indirectly it is arsenic toxicity that leads to an increase in MT production. This suggests a protective role against injury, since it is known that the binding of MT to an excess of metals protects the organism against toxicity by limiting the bioavailability of these cations at undesirable sites (Langston et al. 1998). To support the idea of a protective role Liu et al. (2000) exposed MT-null mice to arsenic in drinking water and concluded that the lack of MT rendered MT-null mice more sensitive than MT mice to the nephrotoxicity produced by chronic arsenic exposure.

Independently of the underlying binding mechanisms, our study shows that MT was significantly induced after exposure to different concentrations of arsenic, which suggests an important role for it in arsenic regulation/detoxification processes. The research also shows that MT is important for highlighting the presence of possible mechanisms of action that deserve further research. As the final word, this is the first time that MT induction associated with arsenic exposure has been positively demonstrated in *C. fluminea*.

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