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Concentrations of Metallothionein-Like Proteins and Heavy Metals in the Freshwater Snail *Lymnaea stagnalis* Exposed to Different Levels of Waterborne Cadmium

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Metallothioneins (MTs) are a group of low molecular weight, soluble, cysteine-rich and heat-stable proteins, which are induced by a variety of trace metals (Roesijadi 1992). Many fish and aquatic invertebrate species possess proteins that have features consistent with MTs, but often these metal-binding proteins are better referred to as metallothionein-like proteins (MTLPs) when they have not been purified and characterized to a sufficiently high level. MTs and MTLPs have important and unique roles in the homeostasis of particular essential metals such as Zn and Cu, and detoxification of excess levels of these and specific non-essential metals including Cd and Hg (Roesijadi 1992; 1996; Dallinger et al. 1997). In addition, they have been proposed as potential biomarkers for metal contamination in the aquatic environment (Viarengo et al. 1999).

Previous laboratory studies on MT or MTLP induction in mollusks have used relatively high concentrations of Cd ranging from 100 to 1000 μg l^{-1} (e.g. Bebianno and Langston 1992, 1993, 1995, 1998; Leung et al. 2000). However, the range of average Cd levels occurring in freshwater environments varies from 0.01 to 0.1 μg Cd l^{-1} (Martin et al. 1980). Therefore the aim of the present study is to compare the concentration of MTLPs in the freshwater snail *Lymnaea stagnalis* exposed to a realistic environmental concentration of 0.01 μg Cd l^{-1} with a high sublethal concentration of 1000 μg Cd l^{-1} under controlled laboratory conditions. Furthermore, the concentrations of various trace metals in the tissues of *L. stagnalis* exposed to these two levels of Cd were quantified and compared.

MATERIALS AND METHODS

Lymnaea stagnalis, which are common pond snails in Europe, have been increasingly employed for toxicity studies (e.g. Pyatt et al. 1997; Jumel et al. 2002). It has been shown that they are very sensitive to Cd toxicity (Gomot 1998). Adult snails of *L. stagnalis* used in the present study originated from laboratory cultures maintained in the School of Biological Sciences, Royal Holloway, University of London. Prior to experimental procedures, all glassware was acid washed (10% nitric acid), and then rinsed twice with distilled water. A total of 84 snails were randomly divided into 12 groups of 7 individuals, and placed into 500 ml beakers which were then covered with a fine mesh. Four replicate groups of 7

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adult snails (Shell length: 26.9 ± 3.1 mm) were exposed for 10 days to a control (without addition of Cd) and to nominal concentrations of cadmium at 0.01 and 1000 μg l^{-1} in artificial hard water. Each beaker contained 450 ml of the test solution. The hard-water (250 mg l^{-1} $CaCO_3$, pH 8.00) was prepared using procedures described by HMSO (1969). All exposures were undertaken in an environmental chamber with continuous aeration (> 80% dissolved oxygen), constant temperature (20 ± 2°C), and a 16/8 h light/dark regime. No food was provided during the exposure period. Mortality was checked daily and dead animals removed. Test solutions were renewed once every two days. At the end of the exposure period, viable snails were collected and stored at –25°C.

For each individual, shell length was measured with a caliper (± 0.1 mm). The soft body was removed from each snail after carefully breaking open the shell with a vice. The soft-tissue was blotted dry using absorbent tissue and the wet weight determined to the nearest 10 mg (Sartorius, MCI electronic balance, Laboratory LC 2200 P). Weighed tissue samples were analyzed for trace metals (Cd, Cr, Cu, Ni, Pb and Zn) and metallothionein-like proteins (MTLPs) using Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) and the silver saturation method (Scheuhammer and Cherian 1991), respectively.

Weighed tissues were homogenized with 0.5 ml of 0.25 M sucrose at 4°C using an Ultraturax homogenizer (T25 Janke & Kunkel, IKA Labortechnik). For each sample, 0.50 g of the homogenate was centrifuged at 20,000× *g* at 4°C for 20 min (Eppendorf Centrifuge 5417R, Eppendorf-Netheier-Hinz). The supernatant was collected and weighed and 300 μl aliquots of supernatant analyzed for MT content using the silver saturation method (Scheuhammer and Cherian 1991) as modified by Leung and Furness (1999). Briefly, samples were incubated with 0.4 ml glycine buffer (0.5 M, pH 8.5) and 0.5 ml of 20 mg l^{-1} Ag solution for 20 min at 20°C to saturate the binding sites of MTs. Excess Ag ions were removed by the addition of 100 μl of the ovine red blood cell haemolysate (which was freshly prepared from ovine blood supplied by Oxid Ltd, Basingstoke, UK) to the assay tubes followed by heat treatment in a water bath at 100°C for *ca.* 2 min. The heat treatment caused precipitation of Ag-bound hemoglobin and other proteins, except for MTs, which are heat-stable. Denatured proteins were removed by centrifugation at 1,200 × *g* for 10 min. The hemolysate addition, heat treatment and centrifugation were repeated 3 times for each sample. Finally, the supernatant was centrifuged at 20,000 × *g* for 10 min. The amount of Ag in the final supernatant fraction, which was proportional to the amount of MTs present, was determined using an atomic absorption spectrophotometer (UNICAM 929 AAS, Analytical Technology Inc., TJA solutions, UK) with deuterium background correction. Calibration in the concentration range 2 to 20 μg was achieved using purified horse kidney MT standards (Sigma Chemicals) for MT quantification. Results were expressed as μg MT g^{-1} dry tissue wt.

The remaining homogenate was dried at 60°C for at least 96 h until a constant mass was achieved. The dry weight of the tissue was obtained by the difference

between the total dry weight and the amount of sucrose added. Homogenates were than digested in concentrated nitric acid for 24 h at room temperature followed by boiling for at least 2 h until a clear solution was obtained. Concentrations of trace metals (Cd, Cr, Cu, Ni, Pb and Zn) were determined using an ICP-AES (Perkin Elmer Optima 3300RL) and the accuracy was regularly checked by including a standard reference material (dogfish muscle, DORM-1, from the National Research Council, Canada) within batches (Table 1).

The normality of the data was checked using normal probability plots and Kolmogorov-Smirnov tests whilst the homogeneity of variance was checked with the Bartlett's test. Mortality data were compared using a Kruskal Wallis test. Differences in the concentration of each element (or MTLPs) between controls and treatments were compared using one-way analysis of variance (ANOVA), followed by post hoc Dunnett's multiple comparison tests. As data of Cd concentrations in the tissues were on a log scale and not evenly distributed along the x-axis, Spearman rank correlation analysis was used to test if there was any significant correlation between the concentrations of Cd and MTLPs in the tissues of *L. stagnalis* across all samples. The significance level was defined at $p < 0.05$.

Table 1. Comparison of metal concentrations ($\mu g\ g^{-1}$ dry wt; means ± 95% CI) in standard reference material, dogfish muscle (DORM-1) certified by the National Research Council of Canada, and analytical results from the current study.

Metal	Certified	Current study (n = 5)
Cd	0.086 ± 0.012	0.109 ± 0.054
Cr	3.60 ± 0.40	3.36 ± 0.34
Cu	5.22 ± 0.33	5.28 ± 0.19
Ni	1.2 ± 0.30	1.05 ± 0.23
Pb	0.40 ± 0.12	0.47 ± 0.09
Zn	21.3 ± 1.0	21.7 ± 1.02

RESULTS AND DISCUSSION

No significant differences in mortality of *Lymnaea stagnalis* were observed in the snail groups exposed to the three different Cd concentrations (Kruskal-Wallis statistic = 1.583, $p > 0.05$; overall median cumulative mortality = 7.1%), except for one replicate of the highest Cd concentration where a high mortality of 57% was recorded. Nevertheless, *L. stagnalis* exposed to 1000 μg Cd l^{-1} contained significantly higher concentrations of Cd, Cr and Ni in their tissues but there was no significant difference in Cu, Zn and Pb concentrations among all three groups (Fig. 1). There was a significant induction, almost threefold, of MTLPs in *L. stagnalis* exposed to the highest concentration of Cd when compared to the control (Fig. 2; ANOVA: $F_{2, 11} = 6.156$, $p < 0.05$; Dunnett's multiple comparison test: $p < 0.05$). However, *L. stagnalis* exposed to a Cd concentration of 0.01 $\mu g\ l^{-1}$ did not show significantly elevated MT concentrations in their tissues. Across all

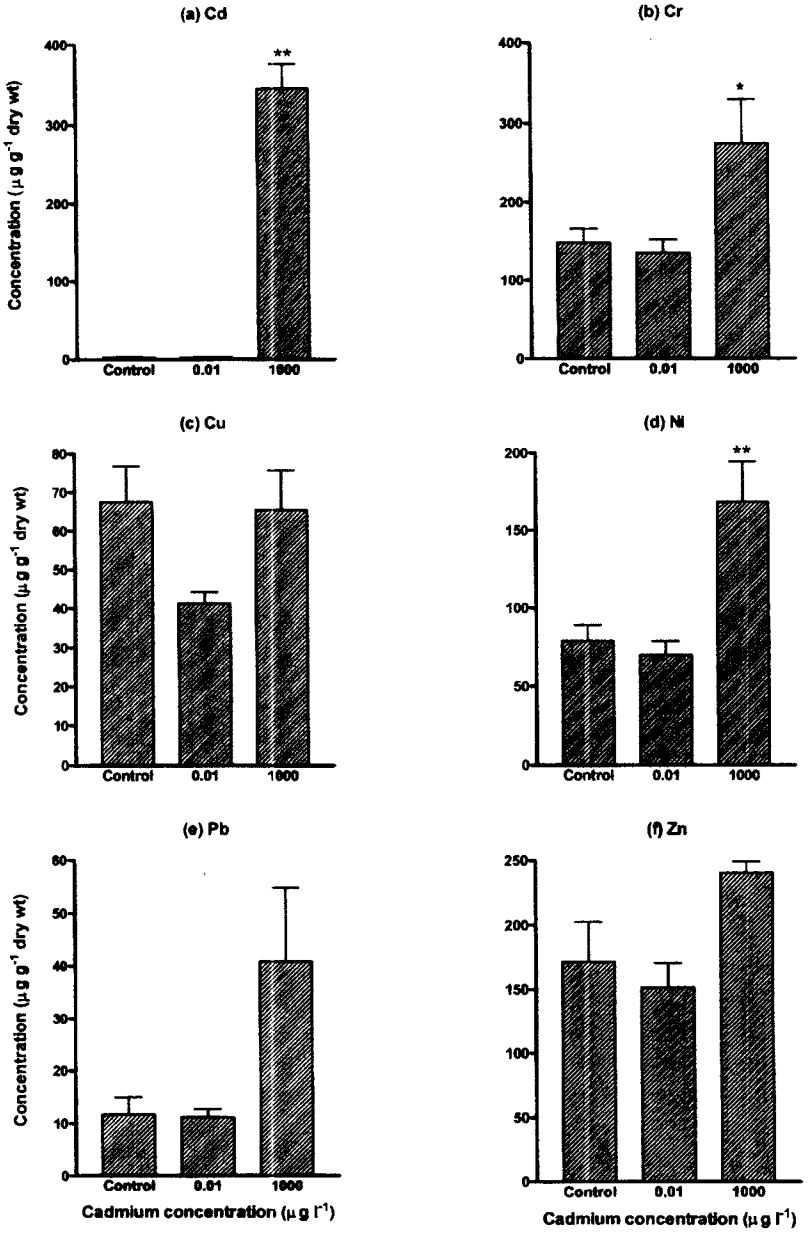

Figure 1. Concentrations of (a) Cd, (b) Cr, (c) Cu, (d) Ni, (e) Pb and (f) Zn in the tissues of *Lymnaea stagnalis* following exposure to different concentrations of Cd for 10 days. Mean and standard error of mean are presented. Asterisks indicate a significantly different mean when compared with controls, based on Dunnett's multiple comparison tests (* $p < 0.05$; ** $p < 0.01$).

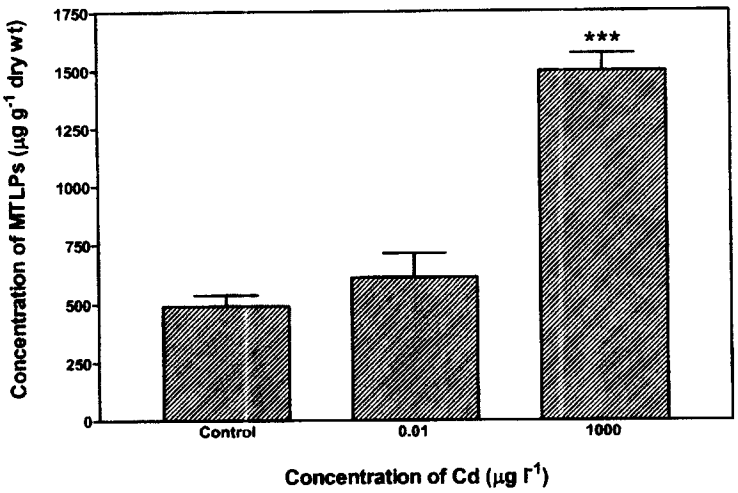

Figure 2. Concentrations of metallothionein-like proteins (MTLPs) in the tissues of *Lymnaea stagnalis* following exposure to different concentrations of Cd for 10 days. Mean and standard error of mean are presented. Asterisks indicate a significantly different mean when compared with controls, based on a Dunnett's multiple comparison test (*** $p < 0.001$).

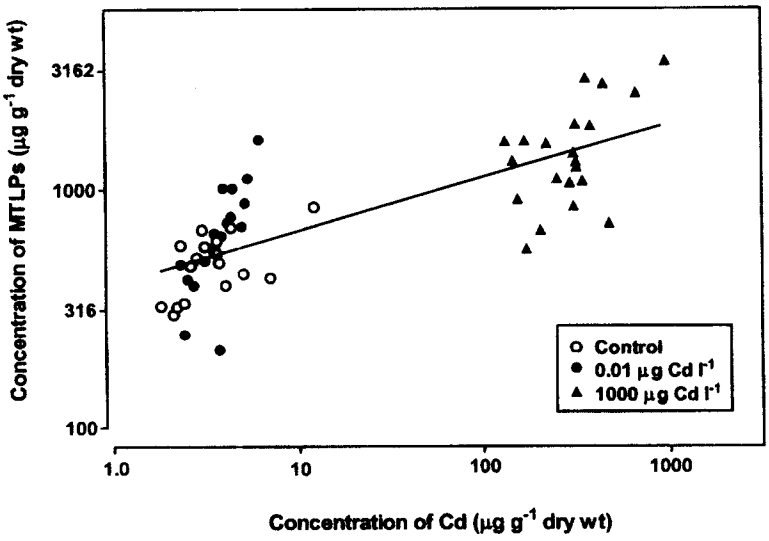

Figure 3. The relationship between concentrations of Cd and metallothionein-like proteins (MTLPs) in the tissues of *Lymnaea stagnalis* following exposure to different concentration of Cd for 10 days. The equation of the linear regression is log [MTLPs] = (0.222 ± 0.026) log [Cd] + (2.608 ± 0.041), where mean and standard error of mean of each constant are presented.

treatment groups there was a highly significant positive correlation between the concentrations of Cd and MT in the tissues of *L. stagnalis* (Fig. 3; $r_s = 0.782$, $p < 0.0001$, n = 73).

These results confirm the induction of MTLPs in the snail *L. stagnalis* exposed to high Cd concentrations (1000 μg Cd l^{-1}). However, the rate of Cd-induced MTLPs induction in *L. stagnalis* exposed to 0.01 μg Cd l^{-1} was much slower than that in snails exposed to the highest Cd concentration (Fig. 2 and 3). Furthermore, concentrations of Cr and Ni in the tissues were significantly increased in *L. stagnalis* exposed to 1000 μg Cd l^{-1}. Such changes in metal concentrations could be attributed to an increase in induction of metal binding proteins including MT or MTLPs, and other physiological changes such as weight loss mediated by Cd toxicity (Leung and Furness 2001).

Recently, Roesijadi (1999) has demonstrated that the rate of MT synthesis in the oyster *Crassostrea virginica* under laboratory exposure to Cd is much higher than that of animals under field conditions. Although high MT concentrations were measured in a field population of *C. virginica* from a Cd-contaminated area, MTs were actually synthesized at basal rates, and were stabilized by the presence of Cd in the tissues (Roesijadi, 1999). These observations are in agreement with the present results on MTLP concentrations in *L. stagnalis*. Results in the present study also suggest that exposure to a high Cd concentration can increase the concentration of other trace metals in tissue, and in turn accelerate the rate of MT or MTLP synthesis. However, these phenomena were not observed in snails exposed to a realistic environmental concentration of Cd.

Laboratory studies on MT or MTLP induction should avoid using high sublethal concentrations of MT-inducing metals. In order to understand the mechanisms of MT or MTLP expression and to evaluate their usefulness as biomarkers in the field, further investigations under laboratory conditions should consider exposure levels of trace metals within a realistic range of environmental concentrations over longer exposure periods.

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