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Studies on biomarkers of copper exposure and toxicity in the marine amphipod *Gammarus locusta* (Crustacea): I. Induction of metallothionein and lipid peroxidation

A. D. CORREIA[1]*, G. LIMA[1], M. H. COSTA[1] and D. R. LIVINGSTONE[2]

[1] IMAR – Institute of Marine Research, Department of Environmental Sciences and Engineering, College of Sciences and Technology, Lisbon New University, 2829-516 Caparica, Portugal
[2] Plymouth Marine Laboratory, Citadel Hill, Plymouth, Devon PL1 2PB, UK

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Sublethal exposures of the marine amphipod *Gammarus locusta* to a concentration range of copper (Cu) in water (4 days' exposure; 3, 5 and 10 μg Cu l^{-1}) or spiked sediments (28 days' exposure; 1, 3 and 6 mg Cu kg^{-1} dry weight) were performed, and the resulting bioaccumulation of Cu and effects on putative metallothionein (MT) and lipid peroxidation (LP) were investigated. A time-course exposure study (over 10 days) to a single water-borne concentration of Cu (4 μg l^{-1}) was also carried out. MT and LP were quantified, respectively, by differential pulse polarography and as thiobarbituric acid-reactive malondialdehyde equivalents. The increasing levels of Cu in water and sediment exposures resulted in enhanced uptake of the metal by *G. locusta*. Synthesis of putative MT occurred in response to exposure to water-borne Cu, the levels being higher ($p < 0.05$) over the dose range of Cu compared with controls. A positive correlation was observed between putative MT levels and the Cu body-burden concentration ($p < 0.001$). However, no increase in LP was observed in these animals. In contrast, in the time-course experiment, LP levels increased within 1 day of exposure, subsequently peaking at 4 days (68% greater than control, $p < 0.001$), before returning to control values by day 6. Higher levels of MT were also observed in this exposure, but at days 6 and 10 (55% and 38%, respectively), paralleling the decrease in LP. No increase in MT levels was recorded with exposure to Cu-contaminated sediments, whereas higher levels of LP were seen in comparison with controls ($p < 0.001$). Overall, the inverse relationship between putative MT induction and the occurrence of LP indicates that MT may protect against the pro-oxidant effects of Cu. It is concluded that MT and LP offer potential for application as biomarkers in *G. locusta*.

Keywords: amphipod, copper, *Gammarus locusta*, lipid peroxidation, metallothionein

Abbreviations: DPP, differential pulse polarography; LP, lipid peroxidation; MDA, malondialdehyde; MT, metallothionein; ROS, reactive oxygen species.

Introduction

Studies on the responses of invertebrate species to contaminants have focused extensively on heavy metals, in particular copper (Cu). The increase of Cu in estuarine and coastal areas has resulted from industrial and domestic waste discharge, disposal of mining washings, refineries and the use of this metal as a

* Corresponding author: A. D. Correia, IMAR – Instituto do Mar, DCEA, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal. Tel: (+351) 212 948300; Fax: (+351) 212 948554; e-mail: adc@mail.fct.unl.pt

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base-compound for antifouling paints (Garnacho *et al*. 2000). Recently, McPherson and Chapman (2000) reviewed the available data on the sensitivities to Cu of marine and estuarine organisms, including considerable information on crustacean amphipods. Amphipods merit special attention for toxicity and environmental studies for a number of reasons, including ecological relevance, sensitivity to environmental disturbance, short life cycle and amenability to experimental investigation (Thomas 1993, Conlan 1994, Costa *et al*. 1998, Costa and Costa 2000, Correia *et al*. 2002).

Several toxicity tests have used physiological and behavioural endpoints to study the effects of chronic toxicity of metals on amphipods. However, difficulties of interpretation can occur due to the lack of specificity of the response criteria and to the amphipods being affected by water-sediment geochemical characteristics, metal speciation and metal bioavailability (Correia and Costa 2000). More recently there has been much interest in the development of molecular endpoints as sensitive measures of sublethal impact by different types of contaminants (Schlenk 1996, Walker *et al*. 1996, Livingstone *et al*. 2000) and to understand better pathways of contaminant metabolism, detoxication and toxic action, e.g. for metals (see reviews by Viarengo 1989, Livingstone 1993, Langston and Bebianno 1998).

Essential metals like Cu are needed for normal metabolic function but it can be toxic if intracellular concentrations exceed the organism's requirements and its detoxication capability (Viarengo 1989, Schlenk *et al*. 1999). Copper may exert its toxicity by multiple mechanisms, including the generation of reactive oxygen species (ROS), resulting in various types of oxidative damage, such as peroxidation of unsaturated lipids to cytotoxic lipid hydroperoxides and carbonyl compounds, including malondialdehyde (MDA) (Viarengo *et al*. 1990, Di Giulio *et al*. 1995, Halliwell and Gutteridge 1999, Livingstone 2001).

Of key importance in the homeostasis and detoxication of Cu in aquatic invertebrates and other animals is the role of inducible metallothionein (MT) (Engel and Brouwer 1991, Perkins *et al*. 1997, Schlenk *et al*. 1999), a superfamily of low molecular weight, cysteine-rich, metal binding proteins found in most animal tissues (Roesijadi 1992). MT has shown promise not only as a biomarker of exposure to certain metals, but also as an indicator of cellular stress through its role as a scavenger of organic free radicals and ROS (Sato and Bremner 1993, Wlostowki 1993, Viarengo *et al*. 2000). The potential importance of MT in toxicological responses to metals has been studied in fish and aquatic invertebrates, particularly molluscan species (Livingstone 1993, Langston and Bebianno 1998, Livingstone and Goldfarb 1998). Recent advances in the biochemistry and molecular biology of MT have also facilitated research on MT in crustacean decapods (Schlenk and Brouwer 1991, Moksnes *et al*. 1995, Del Ramo *et al*. 1995, Pedersen *et al*. 1997, Lundebye and Depledge 1998, Francesconi *et al*. 1998, Engel *et al*. 2001), anostnacans (Del Ramo *et al*. 1995, Martínez *et al*. 1991, 1999), amphipods (Stuhlbacker and Maltby 1992, Ritterhoff *et al*. 1996, Correia *et al*. 2001, 2002) and copepods (Barka *et al*. 2001).

It has been argued that a full understanding of ecotoxicological processes must consider an integrated multilevel approach in which molecular impact is linked to higher-order biological consequences at the individual, population and community levels (McCarthy and Shugart 1990, Schlenk 1996). The characterization of these links offers the potential of using molecular and other biomarkers as early warning signals of contaminant-mediated ecosystem deterioration (Di Giulio *et al*. 1995

Ringwood *et al.* 1999; Livingstone *et al.* 2000). Our work on amphipods, in particular on the widely distributed and ecologically relevant *Gammarus locusta* (Costa and Costa 2000), has focussed on such an integrated approach, aiming to link molecular biomarker changes with effects on growth, reproductive performance, survival and recruitment (Correia *et al.* 2001, Costa *et al.* 2002). Copper was chosen as a contaminant because it is environmentally important and knowledge exists on its mechanisms of detoxication and toxicity (see above), although this is limited to amphipods. In the present study, the uptake of Cu and the responses of MT and lipid peroxidation (LP) in *G. locusta* to water- and sediment-exposures to Cu were investigated in relation to a range of Cu doses (both water column and sediment) and exposure time (water column only). MT was selected as being a robust biomarker of metal exposure in aquatic organisms (see above); LP is known to result from exposure to pro-oxidants, including Cu (Livingstone 2001); the water column and sediment are probable major routes of contaminant uptake by *G. locusta*; and both Cu dose and exposure time are important determinants of Cu toxicity (Costa *et al.* 1998, Correia and Costa 2000). The results are discussed in relation to mechanisms of Cu toxicity and the potential usefulness of MT and LP as biomarkers of Cu exposure and toxicity in amphipods, respectively.

Material and methods

Animals, chemicals and sediment

Adult (length *ca.* 10 mm) and juvenile (length class 2–4 mm) *G. locusta* were obtained from laboratory cultures. DORM-1 and DORM-2 dogfish muscle and liver reference material (Canadian National Research Council Standards) were obtained from *Canadian National Research Council of Canada* and rabbit liver metallothionein MT-1 from Sigma, Portugal. All other chemicals were of analytical grade or equivalent and obtained from Merck, Portugal. The sediment used was a sand, with 0.9% fine fraction (<0.063 mm) and 0.9% total volatile solids, collected from the Sado estuary, near Lisbon, Portugal (38° 27′ N, 08° 43′ W), which is the natural site of the *G. locusta* population used in the laboratory studies. The water content of the sediment was determined from the percentage of weight lost after drying for 12 h at 90°C. Sediment total volatile solids (organic content) was measured as the percentage weight loss after ignition of dry sediment at 550°C for 4 h (Correia and Costa 2000).

Water-column exposure experiments

The protocol used followed the general procedure outlined in Costa *et al.* (1998) with some modifications. Temperature was 23°C and salinity 33–34‰. Before the commencement of each experiment, animals were acclimated for 3 days to laboratory test conditions, with unlimited fresh food, macroalgae *Ulva* sp. Adult specimens, both male and female (retained between 1500 and 2000 µm sieves; *ca.* 10 mm length), were placed in test chambers containing 100 ml of filtered seawater alone (control condition) or of seawater containing $CuCl_2$ solution, with one amphipod in each chamber. In the dose–response experiment, animals were exposed to 3, 5 or 10 µg Cu l^{-1} for 4 days. The concentrations were selected according to preliminary lethality tests. In the time–course experiment, the animals were exposed to 4 µg Cu l^{-1} and sampled after 1, 2, 4, 6 and 10 days. Measurements were made on 4 to 5 pools of 4 to 6 equally mixed males and females (pool wet weight *ca.* 0.05 g) per sampling time. During exposures, seawater or seawater containing Cu was renewed every two days, and the organisms were fed with fresh macroalgae *Ulva* sp. on an *ad libitum* basis.

Sediment exposure experiments

Animals were exposed using minor modifications of the chronic sediment toxicity test procedure described in Correia *et al.* (2001). The Cu spiking procedure was as described in Costa *et al.* (1998), the required volume of a stock solution of $CuCl_2$ being added directly to the sediment to achieve concentrations of 1, 3 and 6 mg kg^{-1} dry weight sediment. Five replicates were conducted for control (no Cu added) and each Cu concentration. The 28-day static assay was carried out at 20°C and 33–34‰ salinity with juveniles (2–4 mm length class) that had previously been acclimated for 3 days to the same conditions of temperature and salinity. The exposures were carried out in plastic tanks with a sediment layer of about 1 cm and seawater to a depth of 5 cm. They were left with aeration overnight and on the

next day exactly 70 juveniles were placed in each tank. Seawater renewal took place at 10-day intervals. Aeration was provided with plastic tips placed at least 1 cm above the sediment surface. During the assays, the organisms were fed with macroalgae *Ulva* sp. on an *ad libitum* basis. Test chambers were inspected daily for aeration and feeding needs and to remove dead animals. At the end of the 28-day exposure period, the contents of each chamber were gently sieved through a 1500 µm mesh sieve to collect the survivals from the original cohort. Over the exposure period the juveniles grew to adult size (*ca.* 10 mm length or greater) and were 45 days old compared with approximately 30 to 35 days old for the water column studies. Four to 5 pools of 4 to 6 males (pool wet weight *ca.* 0.05 g) were frozen and stored at −80°C for later quantification of tissue Cu, MT and LP levels.

Chemical and biochemical analyses

Quantification of whole-body Cu and MT levels. Pools of whole animal (pool wet weight *ca.* 0.05 g) were homogenized at 4°C in 4 ml of 0.02 M tris-HCl buffer (pH 8.6) and sub-samples taken for determination of Cu and MT. Whole-body Cu analysis was carried out on dried, HNO_3-digested sub-samples using flame atomic absorption spectrophotometry. Analysis of dogfish muscle (DORM-1) and liver reference (DOLT-1) material was carried out, using the same treatment, in order to validate the metal analysis. The value measured for copper was within the certified range. Copper concentrations were expressed as µg Cu g^{-1} dry weight of whole body homogenate. MT determination was performed by differential pulse polarography (DPP), essentially as described in Bebianno and Langston (1989). An aliquot of the sub-sample homogenate (2 ml) was centrifuged at 30 000 *g* for 1 h at 4°C. The cytosol was heat-treated at 80°C for 10 min to precipitate the high molecular weight proteins, and subsequently centrifuged at 30 000 *g* for 1 h at 4°C. Aliquots (150–250 µl) of the heat-treated cytosol were taken for quantification of heat-stable MT using DPP with a static mercury drop electrode (using a Metrohm 693 VA Processor and the 694 VA Stand). The Brdicka supporting electrolyte containing 1 M NH_4Cl, 1 M NH_4OH and 2 mM $[Co(NH_3)_6]Cl_3$ was prepared weekly and stored at 4°C (Palecek and Pechan 1971). In the absence of a purified amphipod MT, quantification was by reference to standard additions of rabbit liver MT-1. The values obtained were expressed as mg rabbit-MT equivalents g^{-1} dry weight of whole body homogenate.

Lipid peroxidation. MDA was determined by the thiobarbituric acid method of Ohkawa *et al.* (1979) with minor modifications. Pools of whole animal (0.05–0.08 g wet weight) were homogenized at 4ºC in 1:4 wet weight/buffer volume ratio in 50 mM NaH_2PO_4/Na_2HPO_4 pH 7.4 containing 15% glycerol (w/v), and centrifuged at 9000 *g* for 15 min at 4°C. Sub-samples (62.5 µl) of tissue homogenate were treated with 25 µl of 8.1% dodecyl sulphate sodium, 187 µl of 20% tricloroacetic acid (pH 3.5) and 187 µl of thiobarbituric acid. The mixture was made up to 0.5 ml with distilled water and then heated for 60 min in boiling water. After cooling, 125 µl of distilled water and 625 µl of a mixture of *n*-butanol and pyridine (15:1, v/v) were added. The mixture was shaken vigorously before centrifugation at 4000 *g* for 10 min. The organic layer was then recovered and its absorbance measured at 532 nm. MDA concentrations were derived from a standard curve and the values expressed in terms of MDA nmol equivalents g^{-1} wet weight tissue.

Statistical analysis

Analysis of variance was used to determine treatment (one-way ANOVA) and treatment × time (two-way ANOVA)-dependent effects on the studied variables (tissue levels of Cu, MT and LP). Prior to any analysis, the data were checked for normality (Kolmogorov–Smirnov test) and homogeneity of variance (Levene's test). They were log-transformed when necessary. Significant differences were established at $p < 0.05$ level using the least significant difference (LSD) test for multiple range comparisons between pairs of the means. Linear regression analysis was done to study the relationship between MT and whole-body Cu content. The goodness of fit was assessed by determination of *r*. All statistical analyses were performed with the Statistica/W®5.0 (StatSoft™) package using a computer. Graphically (figures 1, 3 and 4), the results are presented as the mean exposed as a percentage of mean control. The statistical significances shown on the figures are for the analyses of the absolute data as described above.

Results

The effects of exposure to Cu on Cu body-burden, MT and LP levels expressed as a percentage of the control values are presented in figures 1, 3 and 4. The absolute data for the same experiments are given in table 1.

Table 1. Absolute values (mean ± sd) of whole body measurements of copper (Cu) body-burden, metallothionein (MT) level and lipid peroxidation (LP) level (in malondialdehyde [MDA] equivalents) in *Gammarus locusta* exposed to Cu in water column (4 days exposure: 3, 5, 10 µg Cu l^{-1} and 10 days exposure: 4 µg Cu l^{-1}) and sediment (28 days exposure: 1, 3 and 6 mg Cu kg^{-1} dry weight).

Cu treatment	Time of exposure (days)	Total [Cu] (µg g^{-1} dry weight)	MT (mg g^{-1} dry weight)	LP (nmol MDA g^{-1} wet weight)
Water-borne exposure: dose-dependent experiment (µg l^{-1})				
Control	4	44.7 ± 4.2	0.27 ± 0.06	12.6 ± 1.4
3	4	42.5 ± 13.1	0.92 ± 0.22	9.8 ± 1.0
5	4	61.8 ± 2.1	0.77 ± 0.10	11.3 ± 1.5
10	4	86.2 ± 18.0	1.56 ± 0.37	12.0 ± 1.9
Water-borne exposure: time-course experiment (µg l^{-1})				
Control	1	58.9 ± 11.1	1.02 ± 0.04	15.4 ± 1.4
4	1	67.5 ± 5.7	1.01 ± 0.08	17.4 ± 4.7
Control	2	57.0 ± 2.2	0.77 ± 0.08	16.6 ± 1.3
4	2	65.0 ± 4.7	1.05 ± 0.09	21.5 ± 3.3
Control	4	59.6 ± 4.6	1.04 ± 0.12	14.8 ± 2.6
4	4	71.5 ± 2.5	0.90 ± 0.08	24.9 ± 6.0
Control	6	53.6 ± 7.1	0.62 ± 0.08	19.0 ± 3.3
4	6	80.6 ± 5.7	0.96 ± 0.15	18.5 ± 1.0
Control	10	75.0 ± 3.5	0.74 ± 0.04	18.9 ± 1.2
4	10	76.2 ± 7.4	1.02 ± 0.06	14.9 ± 4.2
Sediment exposure: dose-dependent experiment (mg kg^{-1} dry weight)				
Control	28	56.0 ± 3.2	1.23 ± 0.10	15.3 ± 1.0
1	28	61.6 ± 1.3	1.25 ± 0.20	19.9 ± 1.2
3	28	72.5 ± 4.8	1.19 ± 0.28	21.0 ± 2.8
6	28	76.1 ± 3.0	1.25 ± 0.13	19.8 ± 1.7

Water-column exposure to Cu

Survival did not differ between control and Cu-exposed conditions in either the dose-dependent or the time-course experiments. The minimum values of average survival in all conditions were *ca.* 80%.

Dose-dependent experiment. Accumulation of Cu was seen in animals after 4 days of exposure at 5 and 10 but not 3 µg Cu l^{-1} (figure 1a). A dose-dependent accumulation was evident, with tissue Cu levels being higher at 10 than 5 µg l^{-1}, i.e. respectively, about 93 and 38% higher than in controls ($p < 0.05$ and $p < 0.01$, respectively). A significant difference was also found between the 5 and 10 µg l^{-1} treatments ($p < 0.05$). The mean whole-body Cu level in control animals was 44.7 g Cu g^{-1} dry weight (table1).

Increased levels of MT occurred in response to water-borne Cu (figure 1b). The values in the 3 and 5 µg Cu l^{-1} treatments were about threefold higher ($p < 0.01$ and $p < 0.05$, respectively) than in control (0.27 mg g^{-1} dry weight), and both were smaller ($p < 0.01$) than the maximum value observed at 10 µg Cu l^{-1}. The latter was over fivefold higher than controls ($p < 0.001$). Using the combined data of individual animals from the controls and the three exposure conditions, a correlation was evident between whole-body Cu and MT levels ($r = 0.83$

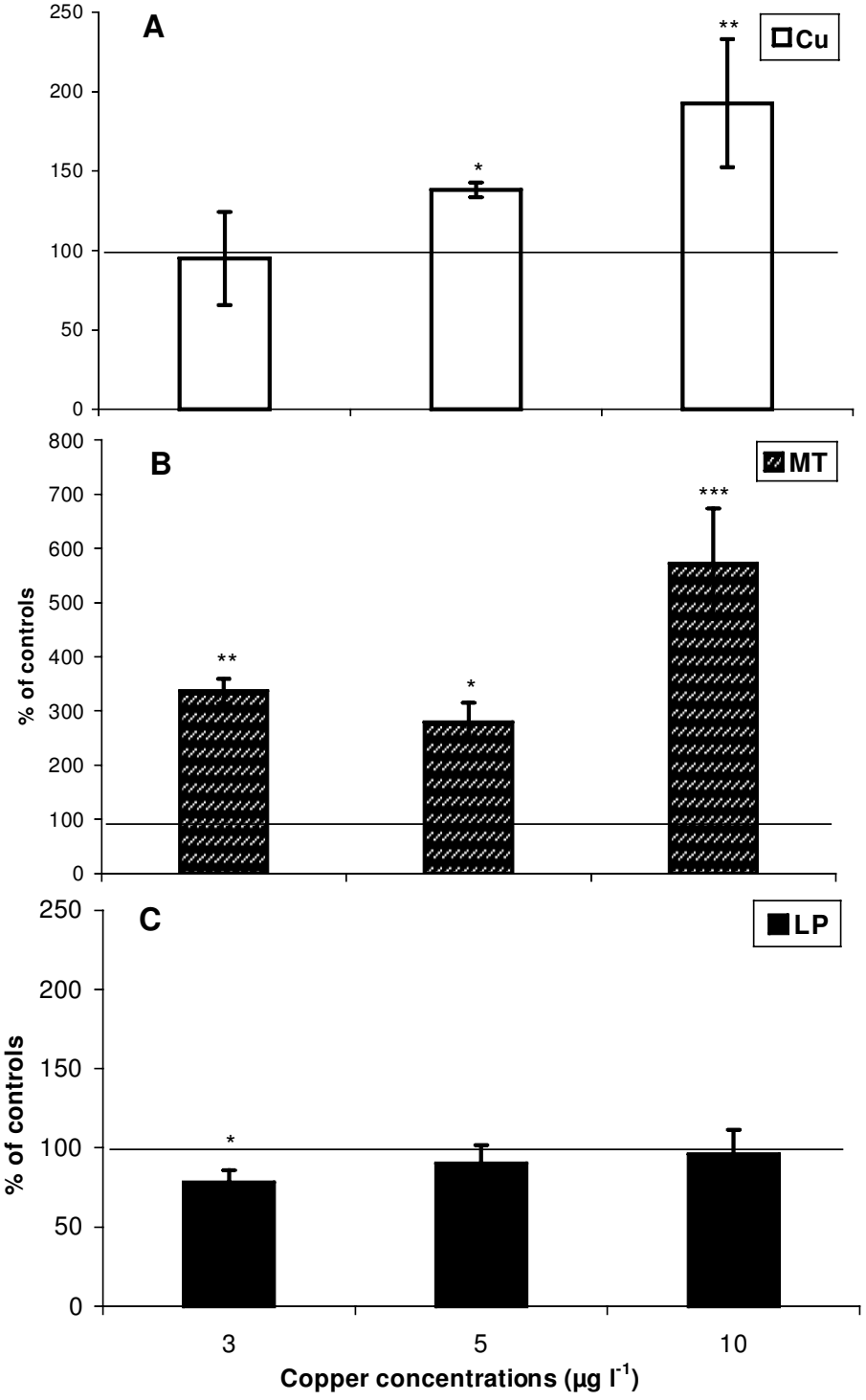

Figure 1. Effects of water-borne Cu (3, 5 and 10 µg Cu l^{-1}) on levels of (a) Cu body burden (Cu), (b) metallothionein (MT) and (c) lipid peroxidation (LP) in *Gammarus locusta* after 4 days of exposure. All results are expressed as percentage of control (mean ± SD). Significant differences from control: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

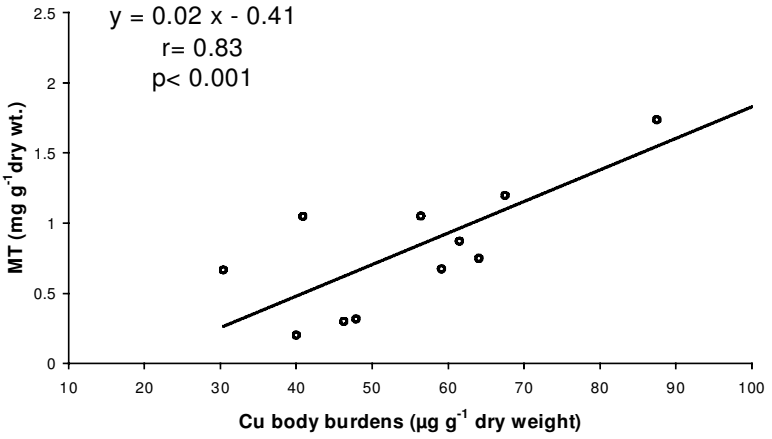

Figure 2. Relationship between levels of MT and Cu body burdens (data from controls and treatments, $n = 3$) of *Gammarus locusta* in the dose–response experiment to water-borne Cu.

$p < 0.001$) (figure 2). The relationship between the two variables is given by the linear equation:

$$\text{MT (mg g}^{-1}\text{ dry weight)} = 0.02\,\text{Cu (µg g}^{-1}\text{ dry weight)} - 0.41$$

In contrast to Cu accumulation and increased MT levels, no marked effects on LP were observed with exposure to Cu, as evidenced by the lower (at 3 µg Cu l^{-1}, $p < 0.05$), or similar levels of MDA in exposed animals compared with controls (12.6 nmol MDA g^{-1} wet weight) (figure 1c and table 1). Despite the reduced levels of LP in exposed animals, a trend of slightly increasing MDA concentration with increasing Cu dose was indicated (figure 1c).

Time-course experiment. A preliminary report of these data was presented in Correia *et al*. (2002). Total body Cu content of exposed animals was significantly higher than controls ($p < 0.05$) at every sampling time, except after 10 days (figure 3a). The reason for the latter was that the body Cu content of control animals at day 10 was higher (*ca*. 25%) than at any other control sampling time. The mean of body Cu content in controls was 61.5 ± 9.8 µg Cu g^{-1} dry weight) (table 1).

The levels of MT in exposed animals followed a similar trend to that exhibited by Cu body-burden. MT levels increased by about 36% compared with controls by day 2 ($p < 0.001$), and were still higher in relation to controls at days 6 and 10 (55% and 38%, respectively) ($p < 0.001$) (figure 3b and table 1). MT concentrations in control amphipods over time varied between 0.62 and 1.04 mg MT g^{-1} dry weight. The maximum levels of MT and Cu body content in exposed animals (both *ca*. 55% > control) were observed at day 6, although overall no significant correlation could be demonstrated between these two variables ($r = 0.025$) as was found for the dose-dependent experiment.

LP changed with exposure to Cu, but showed a different time-course of events compared with MT. Levels of MDA were indicated to increase after 1 and 2 days of exposure, subsequently peaking at day 4 at 68% greater than control ($p < 0.001$) (figure 3c). MDA levels returned to control values at both days 6 and 10. The mean of MDA concentrations in controls was 16.9 ± 1.9 nmol MDA g^{-1} wet weight (table 1). The decrease in LP in exposed amphipods at days 6 and 10 coincided with maximum levels of MT (figures 3b, c).

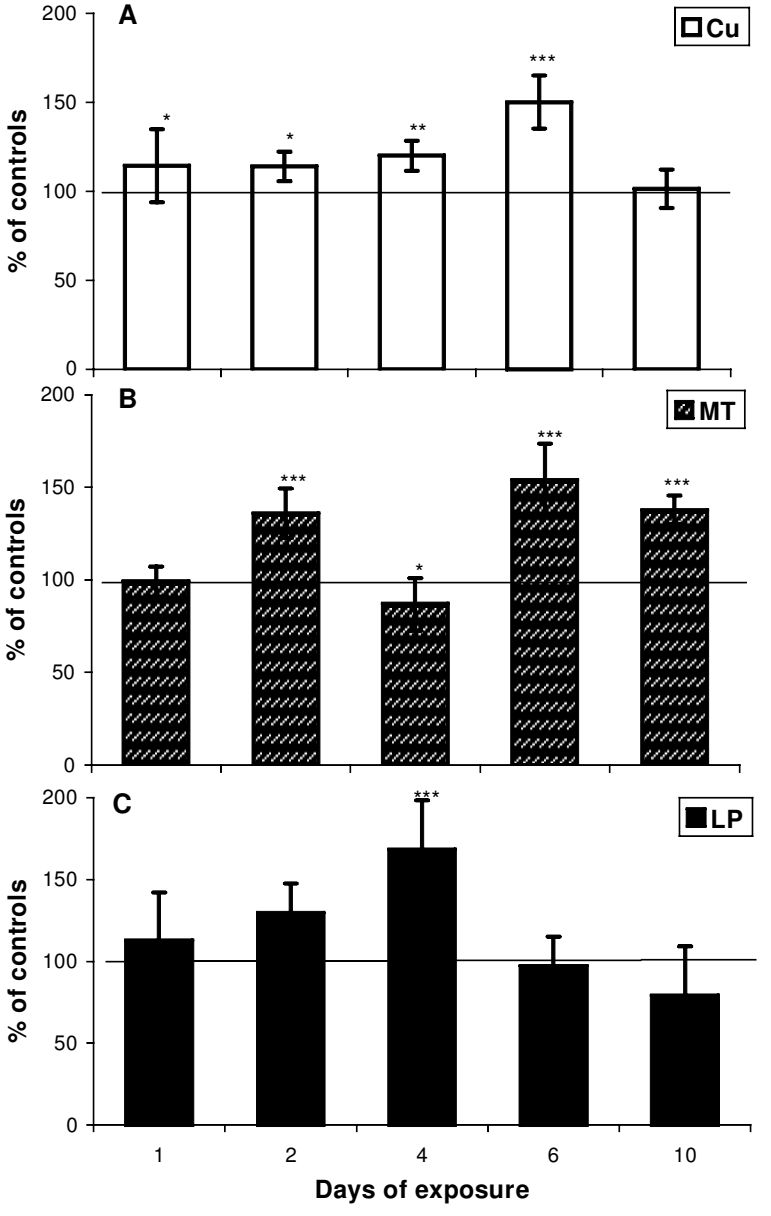

Figure 3. Effects of water-borne Cu ($4\,\mu g\,Cu\,l^{-1}$) on levels of (a) Cu body burden (Cu), (b) metallothionein (MT) and (c) lipid peroxidation (LP) in *Gammarus locusta* after 1, 2, 4, 6 and 10 days exposure. All results are expressed as percentage of control (mean $\pm$ SD). Significant differences from control: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Sediment exposure to Cu

Survival of animals in the treatments 1 and $3\,mg\,Cu\,kg^{-1}$ dry weight did not differ from the control ($p > 0.05$) condition, but the average of values at higher treatment ($6\,mg\,Cu\,kg^{-1}$ dry weight) was about sixfold lower than the control ($p < 0.001$). The average survival in the control condition after 28 days was 46%.

The accumulation of Cu increased in a dose-dependent manner under chronic exposure to Cu-spiked sediments, the levels being higher than controls by 10, 29 and 36%, respectively, for the 1 ($p < 0.05$), 3 and 6 ($p < 0.001$) mg Cu kg^{-1} dry weight conditions (figure 4a and table 1). The tissue Cu levels for the two higher

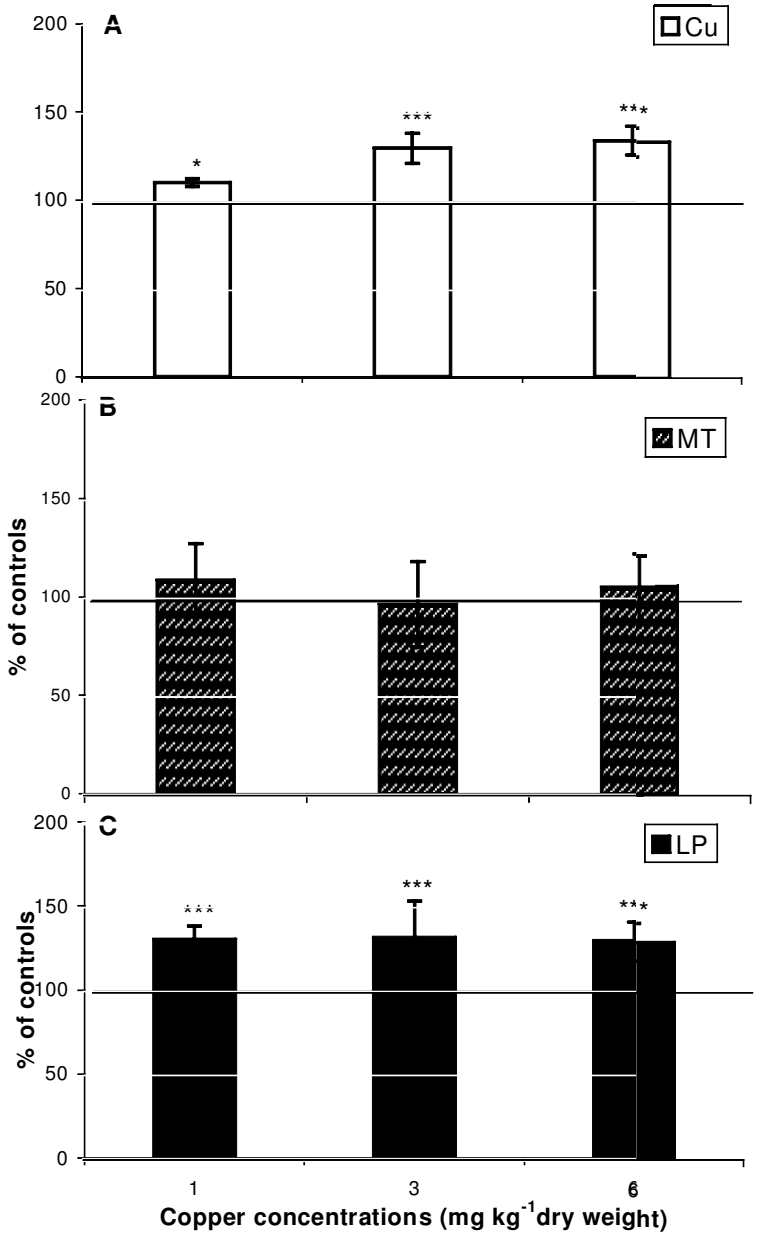

Figure 4. Effects of Cu-spiked sediments (1, 3 and 6 mg Cu kg^{-1} dry weight) on levels of (a) Cu body burden (Cu), (b) metallothionein (MT) and (c) lipid peroxidation (LP) in *Gammarus locusta* after 28 days exposure. All results are expressed as percentage of control (mean ± SD). Significant differences from control: * $p < 0.05$; *** $p < 0.001$.

exposure conditions were also significantly different from the 1 mg Cu kg^{-1} dry weight condition ($p < 0.001$). The level of Cu in control animals was 56.0 ± 3.2 µg Cu g^{-1} dry weight. No increase in MT levels in animals was observed in any of the Cu-treated conditions relative to the control level of 1.23 ± 0.1 mg MT g^{-1} dry weight (figure 4b and table 1), whereas, in contrast, about 20% higher LP was seen in all Cu-treated conditions ($p < 0.001$) compared with control levels of 15.3 ± 1.0 nmol MDA g^{-1} wet weight (figure 4c and table 1).

Discussion

The main aim of the experiments was the investigation of the responses of MT and LP in *G. locusta* with exposure to Cu via the water column or sediment, and their potential for use as biomarkers of, respectively, Cu exposure and toxicity in integrated ecotoxicological studies. Copper was taken up by *G. locusta* from free seawater and from a seawater/sediment system, with maximal increases in Cu body-burden for the different exposure concentrations and exposure times ranging from 1.36 to 1.93-fold compared with controls (see table 1). The marked increase in Cu body burden of *G. locusta* with increasing water-column concentrations of Cu is indicative of a limited capacity for regulation of total tissue Cu concentrations, at least in the short-term, consistent with the findings of Clason and Zauke (2000). Similar results were found with the marine amphipod *Allorchestes compressa*, where Cu body-burden increased twofold for a water column exposure concentration of 10 µg Cu l^{-1} (Ahsanullah and Williams 1991). Freshwater *Hyalella azteca* also accumulated Cu in short-term experiments, whereas in long-term experiments the animals were able to regulate this metal (Borgmann and Norwood 1995). Elevated tissue Cu concentrations (90–360 µg g^{-1}) were found in the amphipods *Orchestia gammarellus* (Icely and Nott 1980) and *Corophium volutator* (Weeks 1992) from environments strongly contaminated by this metal. Overall the ability of these species to survive with elevated body burdens of Cu is consistent with the existence of mechanisms of Cu detoxication in amphipods.

Copper is known to be both an inducer of MT and a pro-oxidant producing LP in many types of animals (Heuchel *et al.* 1995, Halliwell and Gutteridge 1999), including aquatic invertebrates (Viarengo 1989, Ringwood *et al.* 1998, Langston and Bebianno 1998, Livingstone 2001). In spite of Cu being a less efficient inducer of MT than certain other metals in some crustaceans (Lundebye and Depledge 1998, Pedersen *et al.* 1998), elevated MT concentrations have been seen following exposure to this metal in a number of crustacean groups, including crabs (Schlenk and Brouwer 1991, Pedersen *et al.* 1997, Engel *et al.* 2001), lobsters (Brouwer *et al.* 1989, Canli *et al.* 1997) and copepods (Barka *et al.* 2001). The existence of a Cu-inducible MT(s) is also clearly indicated in the amphipod *G. locusta*, increasing whole-body levels of putative MT being seen with increasing bioaccumulation of Cu from the water-column ($p < 0.001$), over periods of up to at least 6 days exposure. The pronounced increase of MT (up to fivefold) in these animals after 4 days of exposure to 10 µg l^{-1} is comparable with the levels observed in whole body of the copepod *Tigriopus brevicornis* following the same period of exposure to a water-column concentration of 9 µg Cu l^{-1} (Barka *et al.* 2001). The reason for the lack of observed elevation of whole-body MT in *G. locusta* with 28 days exposure to Cu in sediment is unknown, but may be related to various factors, including increased stress resulting from the longer period of exposure (higher mortality and

higher LP occurred in the sediment-exposure experiments compared with the water-exposure experiments); the participation of MT in metal-granule formation; and the older age of the animals at the end of the sediment (45 days old) compared with the water column exposure (30 to 35 days old). These different aspects are discussed below. The assay procedure employed for MT in the studies with *G. locusta* depends upon the heating procedure removing all, or the large majority, of the other non-MT thiol-containing proteins (see Materials and Methods). Although some non-MT higher molecular weight proteins were found not to be removed by heat treatment in molluscan species, they were also seen not to be inducible by metal exposure (Bebianno *et al.* 1992). Recently, we have shown that 92% of post-30 000 *g* supernatant total protein in whole-body *G. locusta* was removed by the heat treatment, leaving principally a protein fraction of 23 ± 0.6 kDa apparent molecular weight (SDS-PAGE), consistent with the average size of dimeric MTs found in several aquatic invertebrate species (Viarengo *et al.* 1997, Engel 1999).

Information on the contribution and specific role of these MT-like metal-binding proteins in metal detoxication and toxication in amphipods is limited. However, the incidence of fast induction of MT in the amphipods *Echinogammarus echinosetosus* (Martínez *et al.* 1996) and *Gammarus pulex* (Stuhlbacker and Maltby 1992) following cadmium (Cd) exposure suggests the possibility of prompt cellular responses of these animals to metal contamination. With respect to *G. locusta*, the related patterns of change of MT and Cu body-burden levels in the water column experiments are consistent with detoxication of at least part of the increasing Cu level by binding into the MT pool. This presumed detoxication also matches the inverse relationship observed between MT and LP levels, the latter being one possible toxic consequence of increased levels of 'free Cu' (Halliwell and Gutteridge 1999). The capability of MT to store metals in non-toxic form, so allowing protection against increased metal uptake, have been proposed for other invertebrates such as molluscs (i.e. mussels and oysters) (Viarengo *et al.* 1987, Bebianno *et al.* 1992, Langston and Bebianno 1998) and other crustaceans, including *Carcinus maenas* (Pedersen *et al.* 1997, 1998), *Penaeus vannamei* (Moksnes *et al.* 1995), *Tigriopus brevicornis* (Barka *et al.* 2001), *Procambarus clarkii* and *Artemia* sp. (Del Ramo *et al.* 1995) exposed to Cu and other metals. In bivalve molluscs, elimination of metals involves accumulation in lysosomes (Viarengo *et al.* 1985, 1987) in which the metals are bound to lipid peroxidation products or to polymerized MT (Viarengo *et al.* 1985, George 1990). A similar pathway is indicated in crustaceans, residual bodies of Cu (termed Cu-containing granules) having been seen in the lysosomal system of amphipods (Icely and Nott 1980, Weeks 1992, Nassiri *et al.* 2000), as well as in decapods (Vogt and Quinitio 1994) and interpreted as breakdown products of MT. Thus, the accumulation of such granules in target organs of these species has been argued as a temporary mechanism of Cu detoxication.

Histological examination of *G. locusta* from the current studies revealed the presence of Cu-containing granules in the hepatopancreas of animals from both water-borne and sediment exposures (A. D. Correia, K. Kyan and M. H. Costa, unpublished data). The volume fraction of the granules increased with increasing whole-body Cu body-burden, similar to earlier findings for talitrid amphipods, in which it was concluded that tolerance to metal contamination was related to the ability of these granules to prevent metal toxicity (Weeks 1992). Thus, at least two

mechanisms of Cu sequestration (MT-binding and granules) appear to be present in *G. locusta*. Indeed, the lack of observed elevation of MT levels in the long-term sediment compared with the short-term water column Cu exposures may be related to temporal differences in MT production, Cu binding, and incorporation of MT-products into the granules. MT is also known to play an important endogenous role in Cu homeostasis in crustaceans, mainly during the moulting cycle where cyclic changes in Cu levels occur due to the breakdown of haemocyanin (Engel and Brouwer 1991, Engel *et al.* 2001). Thus, some of the variability in MT levels in control and exposed *G. locusta* observed over the time-course experiment may be linked to the use of pooled whole-body samples and the animals being at different stages of the moulting cycle.

Copper can act as a pro-oxidant both as a redox cycling catalyst promoting initiation and subsequent propagation reactions of LP, and through inhibition of antioxidant enzyme activities (Viarengo *et al.* 1990, Halliwell and Gutteridge 1999). Although MDA may react with other biological molecules (Janero 1990), its accumulation *in vivo* has been successively used as a measure of LP in aquatic organisms exposed to contaminants (Livingstone 2001). Exposures to sublethal concentrations of Cu have thus resulted in increased LP in tissues of a range of invertebrates, including bivalve molluscs (Viarengo *et al.* 1990, 1991, Ringwood *et al.* 1998), crustaceans (Reddy and Bhagyalakshmi 1994, Livingstone 2001) and polychaetes (Nusetti *et al.* 2001). The changes in LP levels in these studies showed varying time courses and, in some cases, correlated with decreased antioxidant protection. Similar results were obtained for *G. locusta*, increased LP being seen with both water column (time-course experiment) and sediment exposures to Cu, and differences in MDA levels being evident over time with exposure to Cu. The greatest increase in whole-body LP of about 70% after 4 days exposure to $4\,\mu g\,Cu\,l^{-1}$ compares with a maximum of about 100% in the hepatopancreas of the freshwater crab *Oziotelphusa senex* after 7 days exposure to $100\,\mu g\,Cu\,l^{-1}$ (Reddy and Bhagyalakshmi 1994). The indicated increases in LP in *G. locusta* and *O. senex* were paralleled by marked decreases in tissue levels of MT and total glutathione, respectively. Variations of LP were also seen for *G. locusta* between similar studies, i.e. increased LP was seen with exposure to $4\,\mu g\,Cu\,l^{-1}$ for 4 days, but not to 3 or $5\,\mu g\,Cu\,l^{-1}$ for 4 days, which presumably may be related to differences in the physiological condition of different animal batches (Dick 1995). A similar situation of varying toxic response under identical testing conditions has also been seen for other amphipod species (Borgmann *et al.* 1993).

The inverse relationship seen between MT and LP levels in *G. locusta* exposed to Cu indicates a protective antioxidant role for MT, either by binding free Cu, or by scavenging ROS. Evidence for the latter function has been indicated in bivalve molluscs exposed to Cu and Cd (Viarengo *et al.* 1987, Roesijadi *et al.* 1997, Ringwood *et al.* 1998, Viarengo *et al.* 2000). In the studies on bivalves, as was also observed for the *G. locusta* water column time-course study, an initial increase in LP was followed by an increase in MT level and a subsequent decrease in LP. Thus, the MT induction was considered part of a recovery and repair process following initial cellular injury caused by the LP (Viarengo *et al.* 1987, Ringwood *et al.* 1998). The lack of MT induction, but increased LP, in *G. locusta* in the chronic sediment-exposure experiment may be related to the older age of these animals and a lack of ability to compensate for decreasing levels of antioxidant protection that occurs with increasing age, as has been proposed for bivalve

molluscs (Viarengo *et al.* 1991, Kirchin *et al.* 1992, Hole *et al.* 1993). Reduced antioxidant protection may also have contributed to the much lower survival levels (about sixfold lower than in control) at the highest sediment dose (6 mg Cu kg^{-1} dry weight) compared with controls. However, equally well, the reduced health status and observed greater lethality may have occurred at any stage of growth of the *G. locusta* through the phenomenon of cannibalism (see Dick 1995), which in this species is directed most intensely towards smaller or less healthy animals (Costa and Costa 2000, Christie and Kraufvelin 2001). Thus, animals sublethally affected by sediment Cu, which otherwise would have survived until the end of the tests, may well have been cannibalized by stronger conspecifics. Because improved diet leads to accelerated growth (Costa *et al.* 1996, Christie and Kraufvelin 2001), the cannibalism may also explain the increased body length of survivors at the highest Cu treatment compared with controls (data not shown). Copper has also been shown to induce whole animal sublethal effects, including decreased growth and somatic indices in aquatic animals, including vertebrate (Baker *et al.* 1998, Schlenk *et al.* 1999) and invertebrate species (Hummel *et al.* 1997, Conradi and Depledge 1998); in some cases the effects were associated with altered MT expression (Farag *et al.* 1995, Perkins *et al.* 1997).

Overall, the results indicate that MT and LP have potential for use as biomarkers of, respectively, Cu exposure and Cu toxicity in *G. locusta* in controlled laboratory ecotoxicological studies. Furthermore, although MTs can be induced by metals other than Cu (Livingstone 1993, Langston and Bebianno 1998, Halliwell and Gutteridge, 1999) and LP can be produced by other pro-oxidants (Livingstone 2001), they also offer the potential for application to assess the toxicity of field sediments when coupled with metal-body burden levels and more detailed MT analysis, i.e. measurement of metals in the MT pool.

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References

Ahsanullah, M. and Williams, A. R. 1991, Sublethal effects and bioaccumulation of cadmium, chromium, copper and zinc in the marine amphipod *Allorchestes compressa*. *Marine Biology*, **108**, 59–65.

Baker, R. T. M., Handy, R. D., Davies, S. J. and Snook, J. C. 1998, Chronic dietary exposure to copper affects growth, tissue lipid peroxidation, and metal composition of the Grey Mullet, *Chelon labrosus*. *Marine Environmental Research*, **45**, 357–365.

Barka, S., Pavillon, J. F. and Amiard, J. C. 2001, Influence of different essential and non-essential metals on MTLP levels in the copepod *Tigriopus brevicornis*. *Comparative Biochemistry and Physiology*, **128C**, 479–493.

Bebianno, M. J. and Langston, W. J. 1989, Quantification of metallothioneins in marine invertebrates using different pulse polarography. *Potugaliae Electrochimica Acta*, **7**, 59–64.

Bebianno, M. J., Langston, W. J. and Simkiss, K. 1992, Metallothionein induction in *Littorina littorea* (Mollusca: Prosobranchia) on exposure to cadmium. *Journal of the Marine Biological Association of the United Kingdom*, **72**, 329–342.

Borgmann, U. and Norwood, W. P. 1995, Kinetics of excess (above background) copper and zinc in *Hyalella azteca* and their relationship to chronic toxicity. *Canadian Journal of Fisheries and Aquatic Science*, **52**, 864–874.

Borgmann, U., Norwood, W. P. and Clarke, C. 1993, Accumulation, regulation and toxicity of copper, zinc, lead and mercury in *Hyalella azteca*. *Hydrobiologia*, **259**, 79-89.

Brouwer, M., Winge, D. R. and Gray, W. R. 1989, Structural and functional diversity of copper-metallothioneins from the American lobster *Homarus americanus*. *Journal of Inorganic Biochemistry,* **35**, 289–303.

Canli, M., Stagg, R. M. and Rodger, G. 1997, The induction of metallothionein in tissues of the Norway lobster *Nephrops norvegicus* following exposure to cadmium, copper and zinc: the relationships between metallothionein and the metals. *Environmental Pollution*, **96**, 343–350.

Christie, H. and Kraufvelin, P. 2001, Mechanisms regulating amphipod population density within macroalgal communities with restricted predator impact. Proceedings of 36th European Marine Biology Symposium (Maó, Menorca, Spain), p. 22.

Clason, B. and Zauke, G. P. 2000, Bioaccumulation of trace metals in marine and estuarine amphipods: evaluation and verification of toxicokinetic models. *Canadian Journal of Fisheries and Aquatic Science,* **57**, 1410–1422.

Conlan, K. E. 1994, Amphipod crustaceans and environmental disturbance: a review. *Journal of Natural History*, London, **28**, 519–554.

Conradi, M. and Depledge, M. H. 1998, Population responses of the marine amphipod *Corophium volutator* (Pallas, 1766) to copper. *Aquatic Toxicology*, **44**, 31–45.

Correia, A. D. and Costa, M. H. 2000, Effects of sediment geochemical properties on the toxicity of copper-spiked sediments to the marine amphipod *Gammarus locusta*. *Science of the Total Environment*, **247**, 99–106.

Correia, A. D., Costa, F. O., Neuparth, T., Diniz, M. E. and Costa, M. H. 2001, Sub-lethal effects of copper-spiked sediments on the marine amphipod *Gammarus locusta*: evidence of hormesis? *Ecotoxicology and Environmental Restoration*, **4**, in press.

Correia, A. D., Livingstone, D. R. and Costa, M. H. 2002, Effects of water-borne copper on metallothionein and lipid peroxidation in the marine amphipod *Gammarus locusta*. *Marine Environmental Research*, in press.

Costa, F. O. and Costa, M. H. 2000, Review of the ecology of *Gammarus locusta* (L.). *Polskie Archiwum Hydrobiologii*, **47**, 541–559.

Costa, F. O., Correia, A. D. and Costa, M. H. 1996, Sensitivity of a marine amphipod to non-contaminant variables and to copper in the sediment. *Ecology*, **27**, 269–276.

Costa, F. O., Correia, A. D. and Costa, M. H. 1998, Acute marine sediment toxicity: a potential new test with the marine amphipod *Gammarus locusta*. *Ecotoxicology and Environmental Safety*, **40**, 81–87.

Costa, F. O., Neuparth, T., Costa, M. H., Theodorakis, C. W. and Shugart, L. R. 2002, Detection of DNA strand-breakage in a marine amphipod by agarose gel electrophoresis. *Biomarkers*, in press.

Del Ramo, J., Torreblanca, A., Martinez, M., Pastor, A. and Mayans, J. D. 1995, Quantification of cadmium-induced metallothionein in crustaceans by the silver-saturation method. *Marine Environmental Research*, **39**, 121–125.

Dick, J. T. A. 1995, The cannibalistic behaviour of two *Gammarus* species (Crustacea: Amphipoda). *Journal of Zoology*, **236**, 697–706.

Di Giulio, R. T., Benson, W. H., Sanders, B. M. and Van Veld, P. A. 1995, Biochemical mechanisms: metabolism, adaptation, and toxicity. In *Fundamentals of Aquatic Toxicology, Effects, Environmental Fate, and Risk Assessment*, edited by G. Rand (London: Taylor & Francis), pp. 523–561.

Engel, D. W. 1999, Accumulation and cytosolic partitioning of metals in the American oyster *Crassostrea virginica*. *Marine Environmental Research*, **47**, 89–102.

Engel, D. W. and Brouwer, M. 1991, Short-term metallothionein and copper changes in blue crabs at ecdysis. *Biological Bulletin*, **180**, 447–452.

Engel, D. W., Brouwer, M. and Mercaldo-Allen, R. 2001, Effects of molting and environmental factors on trace metal body-burdens and hemocyanin concentrations in the American lobster, *Homarus americanus*. *Marine Environmental Research*, **52**, 257–269.

Farag, A. M., Stansbury, M. A., Hogstrand, C., MacConnell, E. and Bergman, H. L. 1995, The physiological impairment of free-ranging brown trout exposed to metals in the Clarck Fork River, Ontana. *Canadian Journal of Fish and Aquatic Science*, **52**, 2038–2050.

Francesconi, K. A., Pederson, K. L. and Højrup, P. 1998, Sex-specific accumulation of Cd-metallothionein in the abdominal muscle of the coral prawn *Metapenaeopsis crassissima* from a natural population. *Marine Environmental Research*, **46**, 541–544.

Garnacho, E., Peck, L. S. and Tyler, P. A. 2000, Variations between winter and summer in the toxicity of copper to a population of the mysid *Praunus flexuosus*. *Marine Biology*, **137**, 631–636.

George, S. G. 1990, Biochemical and cytological assessments of metal toxicity in marine animals. In *Heavy Metals in the Marine Environment*, edited by R. W. Furness and P. S. Rainbow (Boca Raton, FL, USA: CRC Press), pp. 123–142.

Halliwell, B. and Gutteridge, J. M. C. (editors) 1999, *Free Radicals in Biology and Medicine* (Oxford: Oxford University Press).

Heuchel, R., Radtke, F. and Schaffner, W. 1995, Transcriptional regulation by heavy metals, exemplified at the metallothionein genes. In *Inducible Gene Expression*, Vol. 1, *Environmental Stresses and Nutrients, Progress in Gene Expression*, edited by P. A. Baeuerle (Boston: Birkhäuser), pp. 206–240.

Hole, L. M., Moore, M. N. and Bellany, D. 1993, Age-related cellular reactions to copper in the marine mussel *Mytilus edulis*. *Marine Ecology Progress Series*, **94**, 175–179.

Hummel, H., Modderman, R., Amiard-Triquet, C., Rainglet, F., van Duijn, Y., Herssevoort, M., De Jong, J., Bogaards, R., Bachelet, G., Desprez, M., Marchand, J., Sylvand, B., Amiard, J. C., Rybarczyk, H. and De Wolf, L. 1997, A comparative study on the relation between copper and condition in marine bivalves and the relation with copper in sediment. *Aquatic Toxicology*, **38**, 165–181.

Icely, J. D. and Nott, J. A. 1980, Accumulation of copper within the 'hepatopancreatic' caeca of *Corophium volutator* (Crustacea: Amphipoda). *Marine Biology*, **57**, 193–199.

Janero, D. R. 1990, Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radical Biology and Medicine*, **9**, 515–540.

Kirchin, M. A., Moore, M. N., Dean, R. T. and Winston, G. W. 1992, The role of oxyradicals in intracellular proteolysis and toxicity in mussels. *Marine Environmental Research*, **34**, 315–320.

Langston, W. J. and Bebianno, M. J. (editors) 1998, *Metal Metabolism in Aquatic Environments* (London: Chapman & Hall).

Livingstone, D. R. 1993, Biotechnology and pollution monitoring: use of molecular biomarkers in the aquatic environment. *Journal of Chemical Technology and Biotechnology*, **57**, 195–211.

Livingstone, D. R. 2001, Contaminated-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. *Marine Pollution Bulletin*, **42**, 656–666.

Livingstone, D. R. and Goldfarb, P. S. 1998, Aquatic environmental biomonitoring: use of cytochrome P450 1A and other molecular biomarkers in fish and mussels. In *Environmental Biomonitoring: The Biotechnology Ecotoxicology Interface, Biotechnology Research Series*, Vol. 6, edited by J. Lynch and A. Wiseman (Cambridge University Press), pp. 101–129.

Livingstone, D. R., Chipman, J. K., Lowe, D. M., Minier, C., Mitchelmore, C. L., Moore, M. N., Peters, L. D. and Pipe, R. K. 2000, Development of biomarkers to detect the effects of organic pollution on aquatic invertebrates: recent molecular, genotoxic, cellular and immunological studies on the common mussel (*Mytilus edulis* L.) and other mytilids. *International Journal of Pollution*, **13**, 56–91.

Lundebye, A. K. and Depledge, M. H. 1998, Automated interpulse duration assessment (AIDA) in the shore crab *Carcinus maenas* in response to copper exposure. *Marine Biology*, **130**, 613–620.

Martínez, M., Del Ramo, J., Torreblanca A., Pastor, A. and Díaz-Mayans, J. 1991, Presence of Cd-binding proteins in pre-exposed and not pre-exposed cadmium brine shrimp *Artemia*. *Toxicology and Environmental Chemistry*, **31**, 417–424.

Martínez, M., Del Ramo, J., Torreblanca A., Pastor A. and Díaz-Mayans, J. 1996, Cadmium toxicity, accumulation and metallothionein induction in *Echinogammarus echinosetosus*. *Journal of Environmental Science and Health*, **A31**, 1605–1617.

Martínez, M., Del Ramo, J., Torreblanca A. and Díaz-Mayans, J. 1999, Effect of cadmium exposure on zinc levels in the brine shrimp *Artemia parthenogenetica*. *Aquaculture*, **172**, 315–325.

McCarthy, J. F. and Shugart, L. R. (editors) 1990, *Biomarkers of Environmental Contamination* (Boca Raton, FL, USA: Lewis Publishers).

McPherson, C. and Chapman, P. M. 2000, Copper effects on potential sediment test organisms: the importance of appropriate sensitivity. *Marine Pollution Bulletin*, **40**, 656–665.

Moksnes, P. O., Lindahl, U. and Haux, C. 1995, Metallothionein as a bioindicator of heavy metal exposure in the tropical shrimp, *Penaeus vannamei:* A study of dose-dependent induction. *Marine Environmental Research*, **39**, 143–146.

Nassiri, Y., Rainbow, P. S., Amiard-Triquet, C., Rainglet F. and Smith, B. D. 2000, Trace-metal detoxification in the ventral caeca of *Orchestia gammarellus* (Crustacea: Amphipoda). *Marine Biology*, **136**, 477–484.

Nusetti, O., Esclapes M., Salazar, G., Nusetti, S. and Pulido, S. 2001, Biomarkers of oxidative stress in the polychaete *Eurythoe complanata* (Amphinomidae) under short-term copper exposure. *Bulletin of Environmental Contamination and Toxicology*, **66**, 576–581.

Ohkawa, H., Ohishi, N. and Yagi, K. 1979, Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry*, **95**, 351–358.

Palecek, E. and Pechan, Z. 1971, Estimation of nanogram quantities of proteins by pulse polographic techniques. *Analytical Biochemistry*, **42**, 59–71.

Pedersen, S. N., Lundebye, A. K. and Depledge, M. H. 1997, Field application of metallothionein and stress protein biomarkers in the shore crab (*Carcinus maenas*) exposed to trace metals. *Aquatic Toxicology*, **37**, 183–200.

Pedersen, S. N., Pedersen, K. L., Højrup P., Knudsen J. and Depledge, M. H. 1998, Induction and identification of cadmium-, zinc- and copper-metallothioneins in the shore crab *Carcinus maenas* (L.). *Comparative Biochemical and Physiology*, **120C**, 251–259.

Perkins, E. J., Griffin, B., Hobbs, M., Gollon, J., Wolford, L. and Schlenk, D. 1997, Sexual differences in mortality and sublethal stress in channel catfish following a 10 week exposure to copper sulphate. *Aquatic Toxicology*, **37**, 327–339.

Reddy, P. S. and Bhagyalakshmi, A. 1994, Lipid peroxidation in the gill and hepatopancreas of *Oziotelphusa senex* Fabricius during cadmium and copper exposure. *Bulletin of Environmental Contamination and Toxicology*, **53**, 704–710.

Ringwood, A. H., Conners, D. E. and Di Novo, A. 1998, The effects of copper exposures on cellular responses in oysters. *Marine Environmental Research*, **46**, 591–595.

Ringwood, A. H., Hammeedi, M. J., Lee, R. E., Brouwer, M., Peters, E. C., Scott, G. I., Luoma, S. N. and DiGiulio, R. T. 1999, Bivalve biomarker workshop: overview and discussion group summaries. *Biomarkers*, **4**, 391–399.

Ritterhoff, J., Zauke, G. P. and Dallinger, R. 1996, Calibration of the estuarine amphipods, *Gammarus zaddachi* Sexton (1912), as biomonitors: toxicokinetics of cadmium and possible role of inducible metal-binding proteins in Cd detoxification. *Aquatic Toxicology*, **34**, 351–369.

Roesijadi, G. 1992, Metallothionein in metal regulation and toxicity in aquatic animals. *Aquatic Toxicology*, **22**, 81–114.

Roesijadi, G., Brubacher, L. L., Unger, M. E. and Anderson, R. S. 1997, Metallothionein mRNA induction and generation of reactive oxygen species in molluscan hemocytes exposed to cadmium *in vitro*. *Comparative Biochemistry and Physiology*, **118C**, 171–176.

Sato, M. and Bremner, I. 1993, Oxygen free radicals and metallothionein. *Free Radical Biology and Medicine*, **14**, 325–337.

Schlenk, D. 1996, The role of biomarkers in risk assessment. *Human and Ecological Risk Assessment*, **2**, 251–256.

Schlenk, D. and Brouwer, M. 1991, Isolation of three copper metallothionein isoforms from the blue crab (*Callinectes sapidus*). *Aquatic Toxicology*, **20**, 25–34.

Schlenk, D., Davis, K.B. and Griffin, B. R. 1999, Relationship between expression of hepatic metallothionein and sublethal stress in channel catfish following acute exposure to copper sulphate. *Aquaculture*, **177**, 367–379.

Stuhlbacker, A. and Maltby, L. 1992, Cadmium resistence in *Gammarus pulex* (L.) *Archives of Environmental Contamination and Toxicology*, **22**, 319–324.

Thomas, J. D. 1993, Biological monitoring and tropical diversity in marine environments: a critique with recommendations and comments on the use of amphipods as bioindicators. *Journal of Natural History*, **27**, 795–806.

Viarengo, A. 1989, Heavy metals in marine invertebrates: mechanisms of regulation and toxicity at the cellular level. *CRC Critical Reviews in Aquatic Sciences*, **1**, 295–317.

Viarengo, A., Moore, M. N., Pertica, M., Mancinelli, G., Zanicchi, G. and Pipe, R. K. 1985, Detoxification of copper in the cells of the digestive gland of mussel: the role of lysosomes and thioneins. *Science of the Total Environment*, **44**, 135–145.

Viarengo, A., Moore, M. N., Mancinelli, G., Mazzucotelli, A., Pipe, R. K. and Farrar, S. V. 1987, Metallothioneins and lysosomes in metal toxicity and accumulation in marine mussels: the effect of cadmium in the presence and absence of phenanthrene. *Marine Biology*, **94**, 251–257.

Viarengo, A., Canesi, L., Pertica, M., Poli, G., Moore, M. N. and Orunesu, M. 1990, Heavy metal effects on lipid peroxidation in the tissues of *Mytilus galloprovincialis* Lam. *Comparative Biochemistry and Physiology*, **97C**, 37–42.

Viarengo, A., Canesi, L., Pertica, M., Livingstone, D. R. and Orunesu, M. 1991, Age-related lipid peroxidation in the digestive gland of mussels: The role of the antioxidant defence systems. *Experientia*, **47**, 454–457.

Viarengo, A., Ponzano, E., Dondero, F. and Fabbri, R. 1997, A simple spectrophotometric method for metallothionein evaluation in marine organims: an application to mediterranean and antartic molluscs. *Marine Environmental Research*, **44**, 69–84.

Viarengo, A., Burlando B., Ceratto, N. and Panfoli, I. 2000, Antioxidant role of metallothionein: A comparative overview. *Cellular and Molecular Biology*, **46**, 407–417.

Vogt, G. and Quinitio, E. 1994, Accumulation and excretion of metal granules in the prawn *Penaeus monodon*, exposed to water-borne copper, lead, iron and calcium. *Aquatic Toxicology*, **28**, 223–241.

Walker, C. H., Hopkin, S. P., Sibly, R. M. and Peakall, D. B. 1996, *Principles of Ecotoxicology* (London: Taylor & Francis), 321 pp.

Weeks, J. M. 1992, Copper-rich granules in the ventral caeca of talitrid amphipods (Crustacea; Amphipoda: Talitridae). *Ophelia*, **36**, 119–133.

Wlostowski, T. 1993, Involvement of metallothionein and copper in cell proliferation. *BioMetals*, **6**, 71–76.