Studies on biomarkers of copper exposure and toxicity in the marine amphipod *Gammarus locusta* (Crustacea): I. Copper-containing granules within the midgut gland

A.D. Correia*‡, M.H. Costa*, K.P. Ryan† and J.A. Nott†

*IMAR—Institute of Marine Research, Department of Environmental Sciences and Engineering, College of Sciences and Technology, Lisbon New University, 2829-516 Caparica, Portugal. †Marine Biological Association, The Laboratory, Citadel Hill, Plymouth, Devon, PL1 2PB. ‡Corresponding author, e-mail: adc@mail.fct.unl.pt [IMAR-Instituto do Mar, DCEA, Faculdade de Ciências e Tecnologia, Universidade Nova Lisboa, 2829-516 Caparica, Portugal.]

Sub-lethal exposure of the marine amphipod *Gammarus locusta* to a range of copper concentrations (Cu) in water and spiked sediments were performed, and the resulting bioaccumulation of Cu into intracellular granules investigated. The presence of Cu-granules was demonstrated histochemically by the rubeanic acid method. The granules were quantified by automated image analyses (expressed as volume fraction, V_V). The metal composition of the granules was characterized by X-ray microprobe analysis. The results showed that granules, rich in copper and sulphur, were formed in response to Cu exposure in water and sediment. These granules appeared in the B-cells of the hepatopancreas. V_V values increased over the dose-range of Cu compared with control in water ($P<0.001$) and sediment ($P<0.01$) exposures. The abundance of the granules also increased with increasing whole-body Cu content, suggesting that at least part of the increasing Cu level was incorporated into the granules, as a strategy for Cu detoxification along with normal storage of Cu during the moult cycle. The presence of sulphur within the granules is thought to represent an organic detoxification mechanism for Cu. The formation of Cu-granules as a cellular response is a useful biomarker of Cu-exposure in ecotoxicological studies with amphipods. The rubeanic acid method is a useful screening tool for this copper.

INTRODUCTION

Amphipods merit special attention for ecological and ecotoxicological studies for a number of reasons, including ecological relevance, sensitivity to environmental disturbance, short life cycle and amenability to experimental investigation (Conlan, 1994; Costa & Costa, 2000). Standardized methodologies for sediment toxicity tests are available for a series of freshwater species (ASTM, 2001a) and for marine and estuarine amphipods (ASTM, 2001b). The marine amphipod *Gammarus locusta* L. has been proposed for acute sediment toxicity testing in Europe (Costa et al., 1998). Also, a chronic sediment toxicity test using this species is currently under development (Correia et al., 2001).

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 & 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 and to better understand pathways of contaminant metabolism, detoxification and toxic action, e.g. for metals, see reviews by Livingstone (1993) and Langston & Bebianno

(1998). With respect to the latter aspect, the mechanisms of metal sequestration and detoxification merit special attention. Once their capability is exceeded, metal ions can become free to interact with a number of subcellular systems and induce several manifestations of toxicity (Livingstone, 2001). Various combinations of intracellular chemical binding and compartmentalization serve as detoxification systems (Nott, 1991). This is the case of the sequestration of metals by soluble cytoplasmic ligands, such as metallothionein (MT) (Roesijadi, 1994) or to insoluble metal-containing granules, which are generally thought to be residual lysosomes (Brown, 1982; Taylor & Anstiss, 1999). Nevertheless, the significance of these mechanisms is poorly understood in crustaceans, especially in amphipods. Interest has increased in the subcellular sequestration of metals to elucidate the biology of metals, including uptake, storage and excretion in these organisms (Icely & Nott, 1980; Weeks, 1992; Nassiri et al., 2000).

The authors performed a study that in the first instance included research on biochemical responses (including induction of MT) of *Gammarus locusta* to copper in water and sediments (Correia et al., 2002b). The work presented here describes the concomitant histological examination of intra-cellular, copper-containing granules (CuCG) in *G. locusta* in order to investigate the intracellular responses induced by this metal and its subsequent potential use as a biomarker of Cu-exposure. Copper was chosen as a contaminant because it is one of the major sediment metallic

contaminants in Sado estuary (western coast of Portugal), where ecotoxicological studies with this species have been performed. The water and sediment are likely major routes of contaminant uptake by *G. locusta* and Cu dose is an important determinant of Cu toxicity (Costa et al., 1998; Correia & Costa, 2000). The hepatopancreas is the primary organ of interest due to its ability to store metals (Icely & Nott, 1980; Weeks, 1992). The general structure and function of this organ has been described in detail for many amphipod species (see a review by Schmitz, 1992), including *G. locusta* (Moritz et al., 1973; Correia et al., 2002a).

MATERIALS AND METHODS

Animals, chemicals and sediment

Adult (~10 mm length) and juvenile (2–4 mm length class) *Gammarus locusta* were obtained from laboratory cultures. Chemicals were obtained from Sigma, Portugal. DORM-1 and DORM-2 dogfish muscle and liver reference material (Canadian National Research Council Standards) were obtained from the National Research Council of Canada. The sediment used was a sand with 0.9% fine fraction (<0.063 mm) and 0.9% total volatile solids, collected from a clean site at Sado estuary, (38°27′N 08°43′W) that 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 hours.

Laboratory experiments

Water exposure experiment

The protocol used followed the general procedure outlined in Costa et al. (1996) with some modifications. Temperature was 23°C ±0.5 and salinity 33–34 psu. Before the commencement of the experiment, animals were acclimated for three days to laboratory test conditions, with unlimited fresh food, macroalgae *Ulva* sp. The macroalgae was collected in the *G. locusta* natural population site. Adult specimens, both male and female were placed in test chambers containing 100 ml of seawater (control condition) or of seawater containing $CuCl_2$, with one amphipod in each chamber. Animals were exposed to 3, 5 and 10 $\mu g\, Cu\, L^{-1}$ for 96 hours. The concentrations and exposure period were selected after preliminary lethality tests. During exposures, seawater or seawater containing Cu were renewed every two days, and the organisms were fed with fresh macroalgae *Ulva* sp. on an *ad libitum* basis.

Sediment exposure experiment

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 wt sediment. Five replicates were conducted for the control (no Cu added) and each Cu concentration. A 28-d static assay was carried out at 20°C ±0.5 and 33–34 psu salinity with juveniles that had previously been acclimated for three days to the assay conditions. 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 next day exactly 70 juveniles were placed in each tank. Seawater renewal took place at ten day intervals. Aeration was provided with plastic tips placed at 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-d exposure period, the contents of each chamber were gently sieved through 1500-μm mesh sieves to collect the adult males from the original cohort.

Histochemistry

Control and exposed males in intermoult (a total of eight animals per treatment) were sampled at the end of the experiments and the entire pair of tubules of the hepatopancreas (hepatopancreatic caeca) were dissected out after cutting the ventral nerve cord of each animal (Correia et al., 2002a). Copper was stained with rubeanic acid (Pearse, 1980). Fresh hepatopancreas tissues of animals from controls and Cu-treatments (four animals per treatment) were fixed in an alcoholic solution of 0.1% dithiooxamide (rubeanic acid) for 10 min after adding sodium acetate 15 mM for 48 hours. The staining solution was poured off and the tissues washed in 70% alcohol for 60 min before being dehydrated with absolute alcohol for 24 hours. Subsequently they were cleared in chloroform for 30 min, mounted whole on a slide in DPX and examined under a light microscope. The same slides were used for semi-quantitative analysis of CuCG as described later. It should be noted that soluble copper in the cytoplasm will be removed by this treatment.

The hepatopancreas of animals from controls and Cu-treatments (four animals per treatment) were also stained with dithiooxamide, dehydrated in alcohols, rinsed in benzene and embedded in paraffin. Sections 5–7 μm thick were examined under a light microscope without counter-staining the tissue background. The same slides were used for X-ray microanalysis as described later. Copper salts or copper protein complexes stained as dark-black precipitates.

Semi-quantification of CuCG by automated image analysis

Slides of stained fresh hepatopancreas of *G. locusta* (inter-moult males) from water and sediment experiments were used for quantifying the CuCG. The number of tubules analysed per hepatopancreas was based on the integrity of the organ after the staining procedure, but at least one tubule was selected for the analysis. This was made using an Olympus computer-assisted stereological toolbox CAST-Grid system (Software version 1.5) on live images. The volume fraction (V_V) of the CuCG in relation to the hepatopancreas tubules was estimated by the method of point counting (Howard & Read, 1998). This method used a software-generated grid which had two systems of points with a relationship of 1:15. The first system was used for the reference space (tubule) and the latter for the granules. The V_V was estimated by dividing the sum of all points hitting the CuCG by the sum of all points hitting the
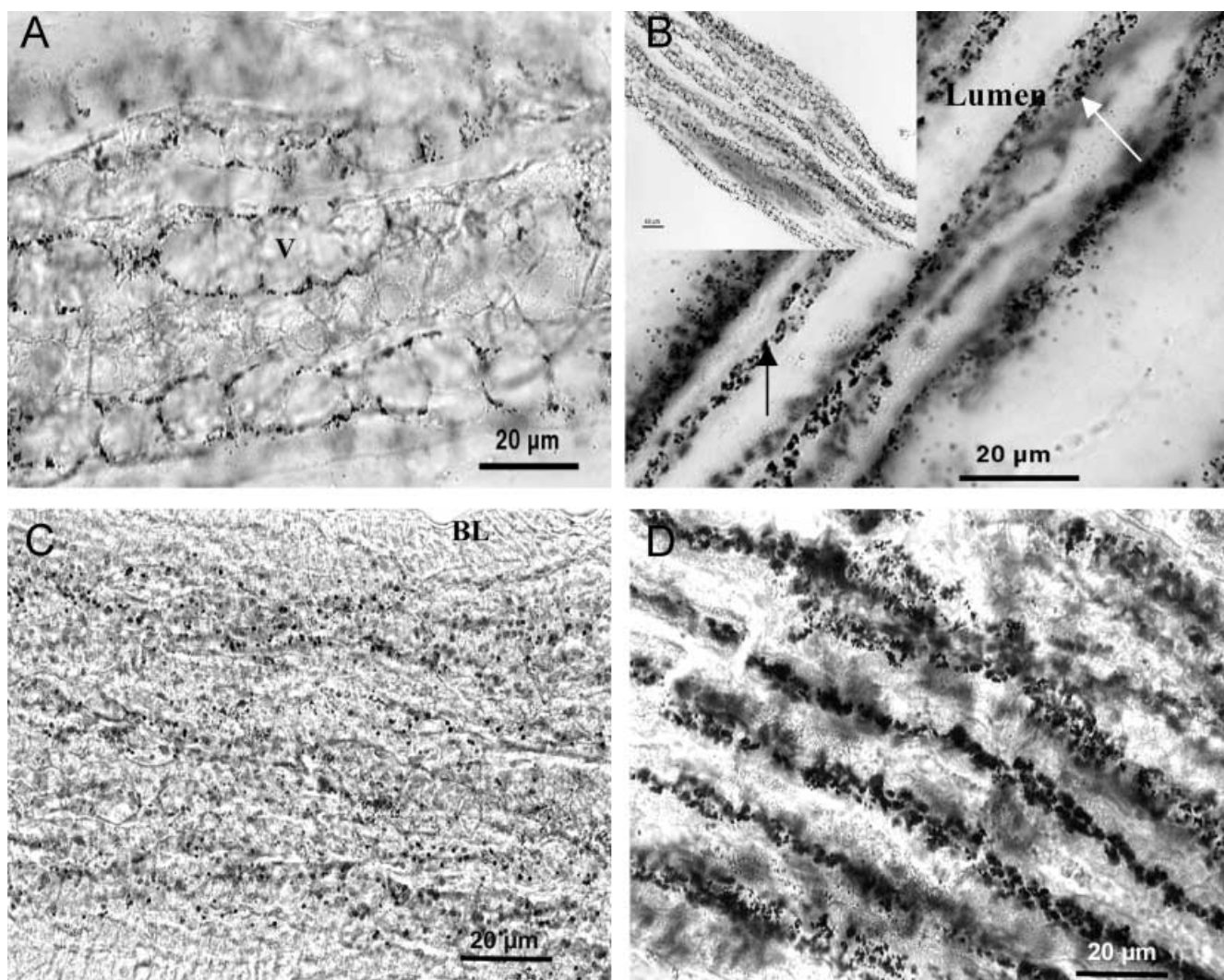
Figure 1. Exposure to water-borne Cu over 96 hours (A and B) and copper-spiked sediments over 28-days (C and D): showing variation of copper content in hepatopancreas whole-mounts from *Gammarus locusta* intermoult males. Copper-containing granules— CuCG (arrow) were stained with dithiooxamide (rubeanic acid). (A) Control—a few small copper granules. (B) Animal exposed to $10\,\mu g\,Cu\,L^{-1}$ showing a denser population of granules. Micrograph, in the top left corner, showing an overview of the mid-region of the hepatopancreas displaying CuCG in longitudinal lines. (C) Control—hepatopancreas exhibiting few CuCG. (D) Animal exposed to $3\,mg\,Cu\,kg^{-1}$ dry wt. A dense population of CuCG. Arrows, CuCG in the epithelium of the hepatopancreas; V, Vacuoles; BL, Basal lamina.

tubules over all fields analysed (results are presented as a percentage).

Point counting was carried out on images captured under a ×100 oil immersion lens (NA 1.35) and matching condenser. This greatly reduces the depth of focus (Howard & Read, 1998) and produced on the screen a thin ($\sim 0.5\,\mu m$) focused optical slice within the tubule fresh mounts (thickness $\sim 50\,\mu m$). Systematic sampling was used for field selection, using the motorized stage controlled by the software. The outline of the tubule to be studied was traced, manually, on the computer screen, being the image acquired with the ×10 objective. (The distal zone of each caecum was not taken into account as reference space as it was free of Cu deposits.) A mean of 25 fields was sampled per tubule. The auto-focus of the motorized stage was used to define within each sampled field which optical section in the tubule would be evaluated.

Qualitative X-ray microprobe analysis

X-ray microanalysis was used to investigate the specificity of rubeanic acid for Cu staining. Paraffin sections of hepatopancreas ($5–7\,\mu m$ thick) stained with dithiooxamide were used to detect the presence of Cu in dense inclusions. Those sections, mounted on glass slides, had their cover-glass removed by soaking in xylene and were fixed onto aluminium stubs, after cutting the slides bearing the sections into suitably sized pieces using a diamond. The section sometimes remained attached to the cover-glass, then this was used as a support. The sections were then coated with a conductive coating of carbon by evaporation in an Edwards's 12E6 coating unit. Specimens were examined in a JEOL JSM-35C scanning electron microscope, and the granules visualized using a Robinson back-scattered electron detector. Analyses were carried out
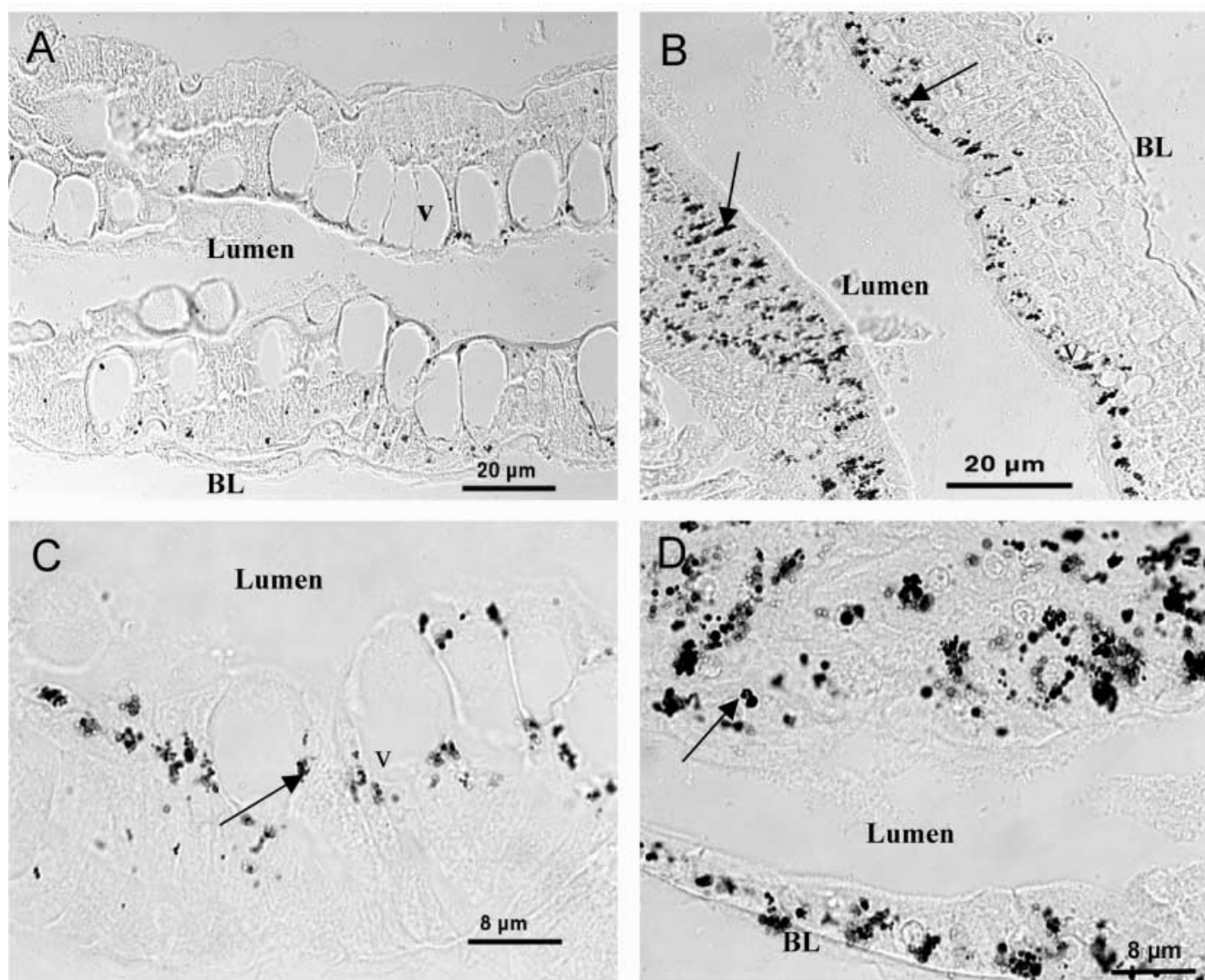
Figure 2. Longitudinal sections of hepatopancreas tubules of male *Gammarus locusta* exposed to sediments (A and B) and water (C and D). (A) From a control, showing the lumen of the tubule and the vacuoles of B-cells are free of CuCG. (B) Animal exposed to $1 \, mg \, Cu \, kg^{-1}$ dry wt CuCG are located throughout the cytoplasm of the R/F cells and are concentrated apically in those cells. (C) Animal exposed to $3 \, \mu g \, Cu \, L^{-1}$. CuCG delimiting the vacuoles of B-cells. (D) Animal exposed to $10 \, \mu g \, Cu \, L^{-1}$. Hepatopancreas displaying a pronounced increase in the number of CuCG with a general tendency for fusing together. Arrows, CuCG in the epithelium of hepatopancreas; V, Vacuoles; BL, Basal lamina.

with a Link Systems 860 energy dispersive X-ray micro-analyser.

Quantitative analyses of whole body Cu content

Quantification of tissue Cu was made on three to four pools of whole animal (pool wet wt $\sim 0.05 \, g$) from controls and metal treatments sampled at the end of water and sediment experiments. 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. Cu concentrations were expressed as $\mu g \, Cu \, g^{-1}$ dry wt of whole body.

In whole body analyses of animals for quantification of Cu uptake, the stage of the moult cycle is irrelevant because negligible concentrations of copper are lost during moulting in amphipods (Weeks et al., 1992). In the other

sections of this work, i.e. histochemistry and X-ray microanalysis, it was important to use only intermoult animals (as determined by carapace hardness). It must be borne in mind that Cu is cycled during the moult cycle to produce haemocyanin as the animals grow after moulting, so the stores of copper in granules then become depleted.

Statistical analyses

Analysis of variance (one-way ANOVA) was used to determine treatment and dependent effects on V_V of CuCG. Significant differences were established at $P < 0.05$ level using the least significant difference test (LSD test) for multiple range comparisons between pairs of the means. Linear regression analysis was made to study the relationship between treatment and whole body Cu content. The goodness of fit was assessed by r^2 determination. All statistical analyses were performed with the Statistica/W®5.0 (StatSoft™) package using a PC computer.
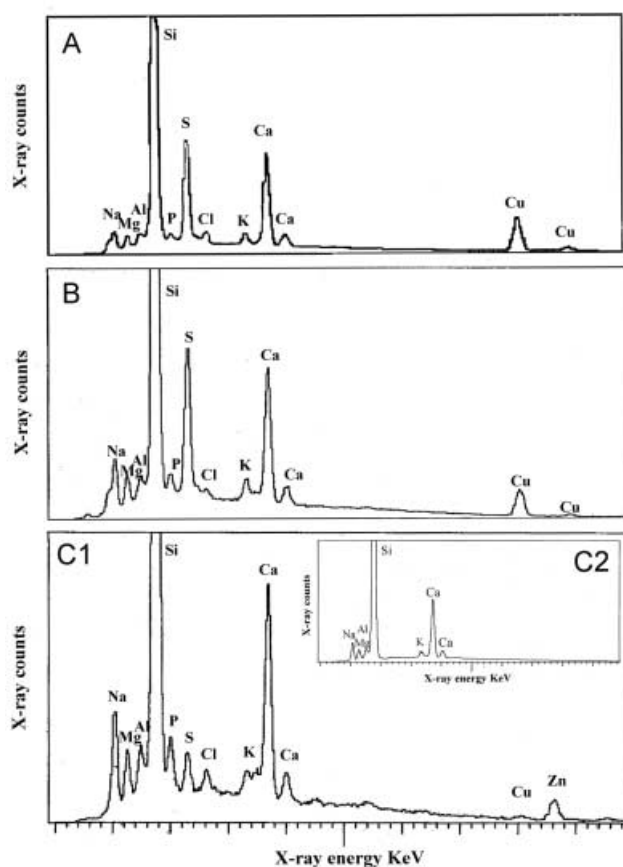
Figure 3. X-ray spectra derived from analyses of hepatopancreas sections of *Gammarus locusta* exposed to copper. (A) Animal exposed to $5\,\mu g\,Cu\,L^{-1}$ in water. The spectrum was obtained from a dense inclusion and shows major peaks for Cu and S and minor peaks for P and Cl. (B) Animal exposed to $1\,mg\,Cu\,kg^{-1}$ dry wt in sediment. Analyses of a dense inclusion give a comparable spectrum. (C1) Control. The spectrum obtained from a dense granule as detected by the back-scattered electron detector shows almost no Cu and minor peaks for S, P and Cl. In this example the support was cover-glass, which produces a peak for Zn as well as Na, Mg, Al, and Si. (C2) The peaks for Na, Mg, Al, Si, K and Ca are derived from the specimen support, in this case glass-slide. It should be noted that fresh tissue would show a high peak for K, this and other diffusible elements were lost during the staining method. The copper is thought to be associated in copper sulphide, so these peaks tend to correlate together, note the small S peak in the control where there is minimal Cu.

RESULTS

Histological observations of CuCG

Figure 1 shows the general pattern of CuCG accumulation along the entire hepatopancreas of *Gammarus locusta* after exposures to Cu in water and sediments. The cellular arrangement of granules in longitudinal sections through the hepatopancreas is shown in Figure 2.

In unexposed animals there were either no positive staining granules in the hepatopancreas or very few (Figure 1A & C). Where granules were present, they tended to be arranged in longitudinal lines along each hepatopancreatic tubule (Figure 1). A general view of those lines
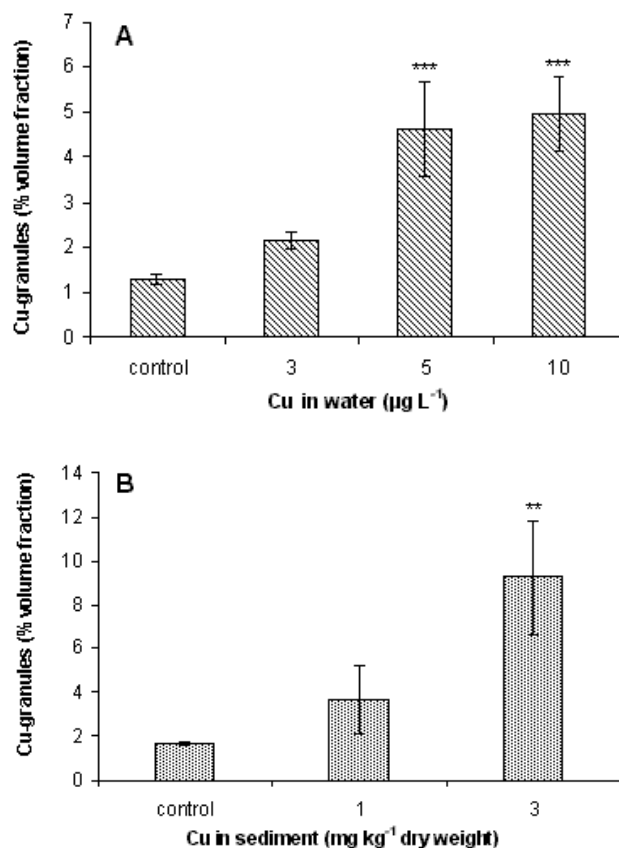

Figure 4. Effects of copper exposures in the volume fraction of copper granules (V_V) (expressed as percentages) in hepatopancreas of *Gammarus locusta*. (A) 3, 5 and $10\,\mu g\,Cu\,L^{-1}$. (B) 1 and $3\,mg\,kg^{-1}$ dry wt. All results are expressed as mean ±SD. **, Indicates significant differences from control ($P<0.01$); ***, indicates significant differences from control ($P<0.001$).

is shown in Figure 1B. The presence of CuCG appeared to be more pronounced on the mid-region of the tubules and the degree of Cu accumulation decreased from anterior to posterior (i.e. proximal to distal) zones of hepatopancreas. The embryonic zone was completely free of CuCG.

Exposed animals revealed great numbers of densely staining deposits of CuCG increasing with the dose of metal in the medium in both experiments (Figures 1 & 2). Animals exposed to low doses of Cu contained more granules with a spherical shape, ranging from $0.8–1.0\,\mu m$ in diameter. At the higher treatments, they had a tendency to fuse together, appearing in large agglomerates. Animals exposed to $10\,\mu g\,Cu\,L^{-1}$ and $3\,mg\,Cu\,kg^{-1}$ dry wt formed plenty of CuCG in the entire hepatopancreas and the colour and profusion of such granules were so pronounced that often this organ appeared 'black' (Figure 1B & D). In longitudinal sections of exposed tubules, the granules tend to be confined to the epithelium of hepatopancreas and mostly located in the apical region of the cells (Figure 2B). Evidence of extrusion of CuCG from the cells was not confirmed by histological observations. Granules appeared concentrated basally in B-cells (Figure 2C). Their presence was also noticed in R/F cells (Figure 2B & D). There was no indication of Cu-positive staining in the hepatopancreas without rubeanic acid treatment.
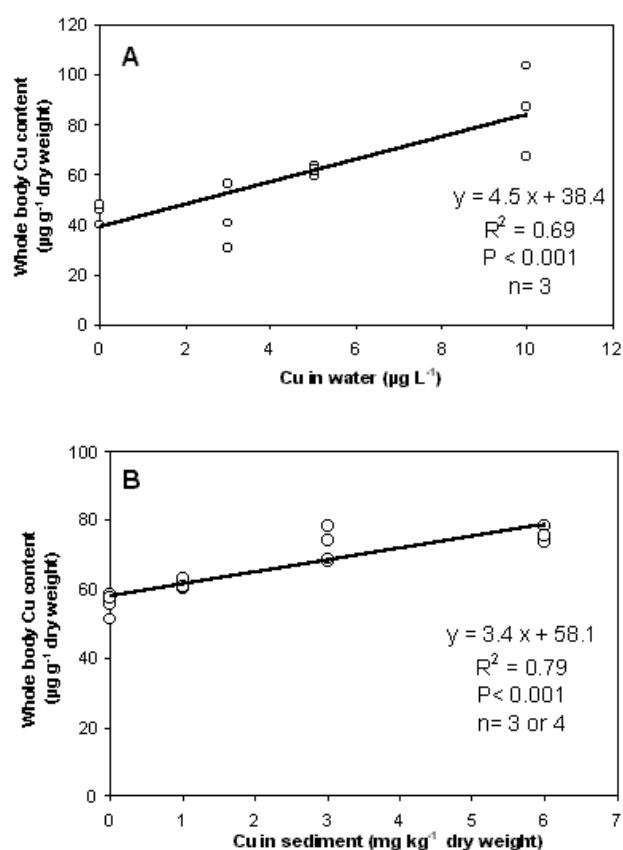
Figure 5. Patterns of whole body accumulation of copper (Cu) by *Gammarus locusta* in water, after 96 h of exposure (A), and sediment, after a 28-d of exposure (B). The regression analyses were performed using combined data from control and exposed individuals. The equations describing each relationship and the associated coefficients of determination (r^2) are indicated on the graphs. n, number of pools per treatment.

Qualitative X-ray microprobe analysis

X-ray microprobe analysis indicated the presence of Cu peaks in intracellular granules of exposed hepatopancreas (Figure 3A,B). Sulphur was the major element associated with these granules that also contained traces of phosphorus and chlorine. Analyses of granules from control animals revealed very little or no Cu but traces of sulphur, phosphorus and chlorine (Figure 3Cl). Copper was not seen as a background element in the microscope in analyses of the graphite specimen stage but peaks for Si, Na, Al, Mg, K and Ca were generated by the glass supports to which the sections were attached (Figure 3C2). The cover-glass also contained Zn (Figure 3Cl).

Semi-quantification of CuCG

The results for CuCG accumulation under dose-dependent exposures (water and sediment) are presented in Figure 4. The volume fraction of CuCG-V_V (%), increased in a dose-dependent manner under exposure to Cu in the water. The values were about two, three, and four-fold higher ($P < 0.001$) than in the control (1.3%) for, respectively the 3, 5 and $10 \mu g \, Cu \, L^{-1}$ conditions (Figure 4A). The V_V values for the two higher exposure

conditions were also significantly different from the $3 \mu g \, Cu \, L^{-1}$ condition ($P < 0.01$).

V_V values increased in response to Cu-spiked sediments (Figure 4B). In the treatments 1 and $3 \, mg \, Cu \, kg^{-1}$ dry wt they were two- ($P > 0.05$) and six-fold ($P < 0.01$) higher than in control (1.6%), respectively. In these treatments the values differed significantly from each other ($P < 0.01$). V_V values for the highest exposure condition ($6 \, mg \, Cu \, kg^{-1}$ dry wt) were not obtained due to high mortality at this concentration from a marked increase in cannibalism.

Quantitative analyses of whole-body Cu content

Figure 5A shows typical patterns of short-term accumulation of Cu by *G. locusta* from water-borne exposures. A significant correlation was found between whole body Cu content and levels of Cu in the medium ($r^2 = 0.69$, $P < 0.001$), using combined data from control and exposed individuals. A similar pattern for long-term accumulation ($r^2 = 0.79$, $P < 0.001$) of Cu by *G. locusta* from sediment was observed in this study (Figure 5B).

DISCUSSION

Gammarus locusta responds to copper concentrations from seawater and from sediment–water systems by sequestering this metal in inclusions in the hepatopancreactic cells. These inclusions (or precipitates) contain granular Cu and sulphur and they are commonly referred to as Cu–S containing granules (Brown, 1982; Viarengo & Nott, 1993). These granules are related to the normal metabolism of haemocyanin and the moult cycle in crustaceans (Al-Mohanna & Nott, 1989). Also, the accumulation of Cu into granules appears to be a generalized response by diverse organisms to sequester Cu within a cell, thus protecting cytological components from the injurious effects of copper ions (Viarengo & Nott, 1993; Langston et al., 1998).

We observed that at the highest Cu concentrations tested in water and sediment, the volume fraction of CuCG (V_V) in the *G. locusta* hepatopancreas was about four-fold ($P < 0.001$) and six-fold ($P < 0.01$) higher than in the control, respectively. The abundance of the granules also increased with increasing *G. locusta* whole-body Cu content, suggesting that at least part of the increasing Cu level was incorporated into the granules, as a strategy for Cu detoxification along with normal storage of Cu during the moult cycle. Dietary copper loading has resulted in the formation of CuCG in the hepatopancreas of some amphipod species (Weeks, 1992; Nassiri et al., 2000). In some of these organisms, high concentration of Cu, achieving 50% of the levels in whole animals, were found in the hepatopancreas (Weeks, 1992), the major site for metal detoxification (Icely & Nott, 1980; Rainbow, 1998). This underlines the importance of these granules regarding the detoxifying role they have in regulating Cu in these animals.

The biology of the hepatopancreas seems to regulate the processes of storage–excretion of metals since Cu-granules are incorporated preferentially in the epithelial cells of this organ (Nott, 1991). Cells are replaced by mitotic division of embryonic cells at the distal end, migrating towards the proximal end where they eventually break down and are shed into the lumen, along with their contents (Schmitz, 1992). The profusion of CuCG in the proximal zones of

the hepatopancreas of *G. locusta* suggested, therefore, the release of granules into the gut lumen when the hepatopancreactic cells break down. The release of granules in this way was also observed in other amphipod species (Icely & Nott, 1980; Weeks, 1992), although the degree of cell turnover and, therefore, the potential excretion of granules are poorly known in these organisms (Rainbow, 1998). Differences between species in rates of turnover of each cell type or indeed of the whole hepatopancreatic epithelium will cause differences in the patterns of metal accumulation (Rainbow, 1998). This seems particularly evident when comparisons are made between amphipod species. For instance, Cu seems to be regulated by *Hyalella azteca* at low environmental concentrations (Borgmann & Norwood, 1995). However, an inverse tendency was noticed in *G. locusta* that showed, in this study, a continued increase of Cu body burdens, in particular, at low doses of Cu in the medium. This behaviour was probably due to either the low turnover of hepatopancreactic cells or the storage capacity of these animals that was never exceeded. Limited granular excretion is evident in talitrid amphipods since these organisms accumulate high levels of copper from environments heavily contaminated by this metal (Weeks, 1992).

R/F-cells are more abundant than B-cells in the mid-region of the amphipod hepatopancreas and are possibly the main cells involved in metal release due to the preferential site for Cu, Fe and Ca accumulation (Icely & Nott, 1980, 1985). The metals are released or excreted, into the gut lumen when those cells break down. A comparable role seems to be played by the B-cells of *G. locusta*, probably due to their capacity in taking macromolecular substances from the tubule lumen (Moritz et al., 1973). Some authors demonstrated that the Cu-granules are preferentially located in the lysosomes of the hepatopancreatic R and F-cells of amphipods (Icely & Nott, 1980; Nassiri et al., 2000). A similar pathway is indicated in bivalve molluscs, where elimination of metals involves the accumulation in lysosomes (Viarengo et al., 1987). Metabolic processing of the ingested metal ions occurs during their passage through the cell interior involving a particular group of cytosolic metal-binding proteins (MT), commonly present in the hepatopancreas of crustaceans (Pedersen et al., 1998). The presence of Cu–S granules in the hepatopancreatic cells of many crustaceans and molluscs suggests these granules are perhaps the product of lysosomal breakdown of metallo-sulphur proteins (Weeks, 1992; Viarengo & Nott, 1993; Langston et al., 1998; Nassiri et al., 2000). The biochemical mechanisms which underlie the incorporation of heavy metals into intracellular granules have not been completely elucidated.

Sulphur was also present in the Cu-granules of *G. locusta*, even the intensity of the sulphur peak in the X-ray spectrum may be partially due to the sulphur in the molecule of dithiooxamide (rubeanic acid). The suspicion that these granules are composed of residues of MT is reinforced by the biochemical analysis performed in a complementary study (Correia et al., 2002b). The results revealed the induction of MT by *G. locusta* in response to exposures of 3, 5 and 10 μg Cu L^{-1}. Information on the contribution and specific role of these metal binding proteins in metal detoxification in amphipods is very limited. However, the increase in the levels of MT being directly correlated with

the increase of CuCG in these animals may indicate that at least two mechanisms of Cu sequestration (MT-binding and CuCG) exist in *G. locusta*. Indeed, both mechanisms may have contributed to reduce the potential toxicity of Cu by the fact that no effects on survival were observed in this experiment.

The histochemical test presented here showed conclusively the presence of CuCG in the hepatopancreas of Cu exposed *G. locusta*. Similar results have been reported by some authors using the rubeanic acid method in other crustacean species (Weeks, 1992; Vogt & Quinitio, 1994). The results from the present semi-quantitative analyses of CuCG quantified alterations in the levels of Cu in tissues of *G. locusta*, suggesting that this histochemical method is applicable to the semi-quantitative evaluation of tissue Cu. In early studies, Irons et al. (1977) also obtained consistent results using semi-quantitative analysis for identification of abnormal Cu levels in the liver of humans.

In summary, the results indicate that the tissue responses related to the formation of Cu-granules have potential for use as biomarkers of Cu-exposure in ecotoxicological studies with *G. locusta*. Information gained from the use of this biomarker would contribute to our understanding of the mechanisms of Cu sequestration and detoxification, which seems to be effected by either MT or by CuCG. From a complementary study, MT has shown promise as a biomarker of exposure in *G. locusta* in controlled toxicological studies (Correia et al., 2002b). This marker also offers potential for application to assess the toxicity of field sediments when coupled to tissue Cu levels. The rubeanic acid method described here for staining granules works alongside the MT approach and provides a simple alternative, reliable screening method for the identification of Cu, it also offers advantages over chemical analysis, in particular when low traces of metals are present in tissues.

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