

1 **AMPHIPOD SUSCEPTIBILITY TO METALS: CAUTIONARY TALES**

2 M. Ramiro Pastorinho^{1*}, Trevor C. Telfer² and Amadeu M.V.M. Soares¹

3 *1- Centre for Environmental and Marine Studies (CESAM) and Department of Biology,*

4 *University of Aveiro, Portugal*

5 *2-Environmental Group, Institute of Aquaculture, University of Stirling, United Kingdom*

6 **ABSTRACT**

7 Heavy metals accumulated by aquatic crustaceans in environmental studies are
8 normally investigated using the whole body burden, with little regard paid to uptake in
9 different tissues, to potential gender of life stage differences, or to the influence of
10 nutrition on the test organism. This is likely to give erroneous conclusions for a dose-
11 response relationship within the toxicity test and potentially lead to wrong conclusions
12 for the ecological risks of metals where species may have higher sensitivities with
13 gender and life stage than indicated or that functionally metals may be sequestered
14 into parts of the body so are not bioavailable. This could lead to under-estimation or
15 over-estimation of the toxicity of metals, respectively, inaccuracy of metal budget
16 calculations and evaluation of trophic transfers of metals. This study evaluated the
17 influences of life stage, gender, and a priori nutritional state in the uptake of the
18 metals Zinc (an essential micro-nutrient; Zn) and Cadmium (a non-essential element;
19 Cd) in the amphipod *Echinogammarus marinus*. The study showed that life stage, and
20 nutritional stage did significantly influence the uptake and bioaccumulation for both

* Corresponding Author. Rua dos Santos Mártires, 8, Apartamento 32, 3810-171
Aveiro, Portugal. Fax: +351 234 372 587; e-mail: rpastorinho@ua.pt

metals, but only Cd showed differential uptake and bioaccumulation with gender. In addition, it was concluded that there was a significant uptake and accumulation of both metals within the exoskeleton of the amphipods, which though adding to the full body burden would add little to toxicity through lack of bioavailability. These results showed that care should be taken when interpreting results from tests normally performed on such test organisms.

Keywords: Gender; Life-Stage; Crustacean; Bioaccumulation; Diet; *Echinogammarus marinus*.

1. INTRODUCTION

A great deal of effort has been put into investigating effects of metals in the environment using amphipods. However, several aspects of currently used protocols have been disregarded due to a small number of studies whose conclusions, in our opinion, are seldom reflected in recent investigations. For example the use of routine evaluation of whole body bioaccumulation rates instead of considering the differential distribution of metals taken up between target organs (Rainbow, 2007; Weeks et al., 1992) or the amount of metal embedded on and coating exoskeletons (Viarengo and Nott, 1993) may give erroneous conclusions about dose-response relationships as it does not take into account the amount of sequestered metals which are not available for metabolic processes (Rainbow, 1993; Rainbow, 2007). In addition, only a limited number of studies using amphipods account for differences between different life stages (males/females/juveniles and neonates) (Marsden, 2002). Data regarding variations introduced by gender on metal levels is still patchy and inconsistent, with

results being frequently contradictory (e.g. Fialkowski et al., 2003; Marsden, 2002). Hence, for most studies only one life stage, adults, is used with no separation into gender (Fialkowski et al., 2003) leading Zhou et al. (2008) to state “There are overwhelming needs for the study of the gender-related differences in metal bioaccumulation”. Furthermore, studies evaluating the effect of the nutritional state of the test organisms prior to metal exposure are scarce despite this potentially leading to errors in metal budgeting in organisms/populations, evaluation of trophic transfers and ecodynamic extrapolation.

From an ecological point of view not taking these factors into account could lead to biased or erroneous data. This can have significant implications for ecological risk assessment calculations of “no-effect” metal levels in environmental compartments (e.g. water, sediment or tissue), which could potentially lead to population loss.

To test some of the sensitivity aspects of the issues raised, the ubiquitous inter-tidal amphipod *Echinogammarus marinus* Leach was chosen as test organism. It has a wide distribution, reported from Norway and Iceland to Portugal (Lincoln, 1979), commonly occurs in high abundances and plays an important trophic role (Dick et al., 2005; Maranhão and Marques, 2003), feeds as both as mesograzer and predator (Dick et al., 2005), is easy to culture and manipulate and reproduces rapidly (17 days at 20°C for production of a juvenile (Maranhão and Marques, 2003)).

The aim of this work was to investigate the influence of different life stages, of gender and of diet on uptake of metals by *Echinogammarus marinus* when in presence of available essential and non-essential metals. This was achieved in three experiments, 1) Exposing different life stages of *Echinogammarus* and genders to metal solutions, 2)

Subjecting adult *Echinogammarus* to different pre-exposure feeding regimes, and 3)
Evaluating the quantity of metals in the exoskeletons of *Echinogammarus*.

2. MATERIAL and METHODS

2.1. Experimental design

Echinogammarus marinus were collected from the Mondego estuary (40°08'N, 8°50'W), at the southernmost limits of its known distribution (Martins et al, 2002) along with *Fucus vesiculosus* L. In the laboratory, the organisms collected were placed in plastic tanks (40 x 20 cm) filled with 4 L of continuously aerated artificial seawater (SERA PREMIUM®) changed three times per week, under a 12 h dark/12 h light regime. The use of artificial seawater ensured that physic-chemical conditions affecting trace metal uptake were reproducible (Rainbow, 1997). *Echinogammarus* were maintained in these conditions for two weeks prior to the commencement of the experiments to allow acclimation to test conditions and allow depuration (Clason and Zauke, 2000).

All experiments consisted of 96h static exposures to 1 mg L⁻¹ Cadmium (CdCl₂·2H₂O, Sigma-Aldrich) or Zinc (ZnSO₄·7H₂O, Sigma-Aldrich) with five replicates (ten organisms per chamber) per experiment. No food was given to the organisms during the experiments. All materials (including experimental vessels) were acid washed and pre-soaked in the appropriate test medium for 24h to saturate all adsorption sites (after Rainbow et al., 2004).

To evaluate differences in metal bioaccumulation between development stage adults, juveniles and neonates were exposed in separate chambers to test solutions of the

two metals. Adults were separated by gender and exposed in separated chambers. Controls containing no metals were used for each life stage/gender.

The influence of pre-exposure diet on metal uptake was evaluated by feeding two types of foods to two groups of males, females and juveniles kept in separated chambers. *F. vesiculosus* collected at a site with oceanic conditions was fed to one of the groups and *Artemia salina* (brine shrimp) hatched from commercially obtained cysts (Sanders Brine Shrimp Co.) was fed to the other group. Each of these foods was the sole diet for one month prior to metal exposure. Metal levels present in *F. vesiculosus* and *A. salina* used as feeds were determined.

The amount of metals present in the exoskeleton (adsorbed and embedded) was assessed using equal numbers of males and female *Echinogammarus*. At the end of the exposure individuals were frozen at -80 °C then thawed on ice and the exoskeleton stripped from the remaining tissues under a dissecting microscope. Soft and exoskeletal tissues were separated for quantification of the respective metal burdens.

2.2. Metal analysis

Extraction of metals from amphipod tissues (500 mg ww for body and 150 mg ww exoskeleton), *F. vesiculosus* (2000 mg ww) and *A. salina* (2000 mg ww) was carried out using nitric acid – hydrogen peroxide digestion. The tissues were dried at 110 °C for 24 h and ground to a fine powder. A 500mg (*F. vesiculosus* and *A. Salina*), 50 mg (whole amphipod) or 30 mg (exoskeleton) sub-sample was added to a 20 mL Teflon screw top digestion vessel. Next 5 mL of concentrated nitric acid (69%, Aristar, BDH,106 U.K.) was added and the sample was heated to 110 °C for 24 h. Once cooled, 3 mL of hydrogen

peroxide (Aristar, BDH, U.K.) was added in 1 mL steps until the sample became totally clear and ceased effervescing. Samples were re-heated to 110 °C for a further 2 hours, allowed to cool and made up to 15 mL with distilled water and centrifuged at 2000 rpm for 15 minutes. A 1 mL sub-aliquot was analyzed using a THERMO™ ICP - Mass Spectrophotometer (Thermo Ltd, Huntingdon, UK). Calibration of the instrument was achieved using MERK CertiPUR standards and internal quality control performed using reference material (lobster hepatopancreas tissue TORT-2, NRC-CNRC). Certified values are 26.7±0.6 and 180±6 mg kg⁻¹ for Cd and Zn, respectively, whilst measured values were 25.3±0.9 and 176±7 mg kg⁻¹, respectively.

2.3. Statistical analysis

One-way Analysis of Variance (ANOVA) was performed on Log₁₀ transformed data to evaluate the significance of differences between life stages and different treatments. Normality of distribution was tested using the Kolmogorov-Smirnov test. Student-Newman-Keuls (SNK) Method was applied as pairwise multiple comparison procedure to further differentiate between groups/treatments.

All Statistical analysis was performed using SigmaStat (Version 3.5) statistical software.

3. RESULTS

3.1. Life stage metal accumulation

Figures 1 and 2 present the quantification of metal body burdens for females, males, juveniles and neonates in controls and test solutions of 1 mg L⁻¹ Cd and 1 mg L⁻¹ Zn, respectively.

These show that different life stages of *E. marinus* have differential bioaccumulation rates of cadmium and zinc. Post hoc results (SNK) for cadmium treatments (Figure 1) indicate that all exposed amphipods had significantly higher bioaccumulation than the controls and, while the adults, juveniles and neonates had significantly different bioaccumulation ($P < 0.001$), there was no difference between male and female adults ($P > 0.05$). No significant differences were seen between the controls ($P > 0.05$). Post hoc results (SNK) for zinc treatments (Figure 2) indicate that all exposed amphipods had significantly higher bioaccumulation than the controls. Adults, juveniles and neonates had significantly different bioaccumulation levels ($P < 0.001$). Significant differences were also shown between male and female adults ($P < 0.001$). No significant differences were seen between the controls ($P > 0.05$).

As a consequence, for cadmium bioaccumulation characteristics for *Echinogammarus* were:

Neonates >>Juveniles > Females = Males

For zinc bioaccumulation characteristics were:

Neonates >>Juveniles > Females > Males.

3.2. Pre - feeding influence on metal accumulation

Results for the analysis of metal content for *F. vesiculosus* and *A. salina* used in the pre-exposure feeding are presented in Table 1.

Figures 3 and 4 present the values for metal body burdens for organisms exposed to 1 mg L⁻¹ of cadmium or zinc, respectively, for 96 h after being previously fed different

diets. The results suggest that different pre-exposure diets did not affect water mediated cadmium uptake in *E. marinus*. The application of SNK method returned no significant difference ($P>0.05$) between the mean responses of similar life stage or genders offered different pre-exposure foods. On the contrary, the SNK analysis showed that when exposed to zinc, mean responses of the same life stage or gender given different pre-exposure feeds had significantly different metal uptake ($P<0.001$). This implies that different diets affect water mediated zinc uptake in *E. marinus*. For both metals there was significantly lower metal uptake shown by the controls than the treatments ($P<0.001$).

3.3. Exoskeleton metal quantification

Metals levels associated with the amphipod's exoskeletons as a percentage of the whole body burden are presented in Table 2. For both metals there was a statistically significant difference ($P<0.05$, ANOVA) in metal content within exoskeletons of exposed individuals and the exoskeleton of control organisms.

4. DISCUSSION

Amphipod crustaceans are net accumulators of trace metals both from solution and diet, with the accumulated metals levels in tissues being indicative of uptake over a period of time (Fialkowski et al., 2003; Rainbow, 1997). There is considerable potential, given the considerable number of internal binding sites, for metals to be accumulated in high concentrations (Rainbow, 1990). These high accumulated levels once bound are largely detoxified, mitigating the potential lethal effect (Hopkin 1990).

179 Aside from a few studies about metallothioneins (MT), which can have both metal-
180 detoxication and antioxidant functions, little is known about protective mechanisms in
181 amphipods against oxidative stress or about anti-oxidant defenses (Correia et al.,
182 2003). Antioxidant enzymes are an important protective mechanism and, like many
183 other biochemical systems, their effectiveness may vary with the stage of
184 development and other physiological aspects of the individual organism (Halliwell and
185 Gutteridge, 1999; Livingstone, 2001). The present study illustrated this to be true by
186 obtaining statistical evidence to show differences in cadmium and zinc uptake
187 between different life stages of *Echinogammarus*. Neonates accumulated higher
188 amounts of these metals than juveniles (threefold for Cd and approximately twofold
189 for Zn) or adults (fivefold for Cd and approximately 2.5 times for Zn). This observation
190 that smaller aquatic organisms bioaccumulate higher levels of toxicants has been well
191 documented for multitude of compounds (Rand, 1995). This can be explained by the
192 differing metabolic profiles which exist between early life and adult stages due to
193 progressively decreasing antioxidant enzyme activity (from 70% to 90%) during
194 development from neonates to adults (Correia et al., 2003). As higher levels of
195 enzymes are synonymous with higher detoxification capability, higher levels of metals
196 can be sequestered inside organisms without deleterious effects. In addition,
197 maintenance of an enlarged non-toxic zinc pool by early life stages (neonates and
198 juveniles) allow greater synthesis of metalloenzymes, which permit homeostasis of
199 many cellular processes (Amiard et al., 2006) related to cell division and proliferation
200 (McDonald, 2000), which are particularly important during these life stages (Sutcliffe,
201 1984). Similar results as found in this survey were obtained for the gammarid

amphipod *Gammarus locusta* (Correia et al. 2004) where juveniles were found to accumulate higher levels of zinc than adults. .

A similar process occurs with cadmium up take with the exception that all of the accumulated metal remains bound to metallothionein proteins whilst zinc remobilizes. In *Orchestia gammarellus*, zinc appeared in lysosomes after metallothionein degradation whilst cadmium remained metallothionein-bound in the cytosol of ventral caeca cells (Amiard et al., 2006). Furthermore as young stages molt more frequently than older individuals (Pöckl, 1995; Neuparth et al., 2002) and display an increased surface area to volume ratio and thinner body covering (Rand, 1995) there is the potential for even greater metal uptake in young stages. Thus for acute tests (96h) molting frequency has been highlighted as an important factor controlling differences in metal uptake and consequent susceptibility between life-stages (McGee et al., 1998).

Greater levels of polyunsaturated fatty acids (PUFA) adds to the decline in anti-oxidant activity, making the older animals more susceptible to higher lipid peroxidation and oxidative stress (Kawashima et al., 1999). PUFA account for a significant percentage of membrane phospholipids in amphipods and their peroxidation is promoted by metals (Correia et al., 2002; Roméo et al., 2000).

Saccharomyces cerevisiae yeast cells exposed to similar concentrations of cadmium and copper as in the present study, led to the observation of high levels of peroxidation in PUFA enriched membranes and a consequent loss of permeability (Howlett and Avery, 1997). In *Dicentrarchus labrax*, cadmium was shown to be less toxic than copper to the kidney lysosomal membrane, despite the higher uptake rate

of copper (Roméo et al., 2000). In adult specimens of *G. locusta* exposed to $4 \mu\text{g L}^{-1}$ water-borne copper over 10 days, lipid peroxidation increased after 1 day and peaked at day 4 before returning to control values by day 6. Simultaneously high concentrations of metallothionein were observed between days 6 and 10 concurrently with a decrease of lipid peroxidation (Correia et al., 2002). Due to higher contents in PUFA the peroxidative capability of metals, at high concentrations, causes an initial disruption of the adult's cellular membrane which could temporarily block metal uptake. This can help explain further discrepancies between early and adult life stages, but the need for further research is obvious. There is an indication in *G. locusta* that males possess higher levels of PUFA and lower levels of metallothionein (Correia et al., 2003, 2004). This could explain the differences found between genders in the present study. Marsden et al. (2003) found higher cadmium levels in females but the similar amounts of zinc between genders in talitrid amphipods.

Despite using the same concentrations (1 mg L^{-1}) in the present study, the results obtained for the uptake of the two metals were different. Zinc is an essential trace metal with the potential to vary in tissue concentration because of different physiological requirements of development stage, gender and reproductive state, as well as bioavailability in amphipods. Cadmium is a nonessential metal with accumulated concentrations expected to vary only in line with bioavailability differences (Marsden et al., 2003). Thus for a given molar concentration, the molar uptake of dissolved zinc by marine invertebrates is greater than that of cadmium (Rainbow and White, 1989).

The nutritional state of an individual is regarded as a non contaminant source of stress (Wolfe, 1992). Heugens et al. (2001) noted from literature that a negative correlation of approximately 80% existed between toxicity decrease and increasing values of nutritional state. The fact that the majority of experiments leading to such conclusions were performed by means of a comparison of sensitivity between fed and starved animals (e.g. Chandini, 1988, 1989; McGee et al., 1998) has raised criticism regarding the influence higher food levels cause upon metabolic rate, and consequent toxicokinetics. Information upon the effects of different diets is rare. In the present experiment uptake was affected differently by the two foods used. *Fucus vesiculosus* provides shelter and food for *E. marinus* creating good conditions for ecological fitness (Maranhão and Marques, 2003). *Artemia salina* when used as sole food has been known to cause low ecological fitness, low egg production rates and low survival rates (Cruz-Rivera and Hay, 2000).

For cadmium no statistical significant differences were found for uptake between individuals of the same development stage or same gender fed with *Artemia salina* or *Fucus vesiculosus*. Nevertheless, the amounts of metal present in the two foods have also to be considered. Cadmium content was approximately an order of magnitude different between *Fucus* ($0.28 \mu\text{g g}^{-1}$) and *Artemia* ($0.025 \mu\text{g g}^{-1}$). Under these conditions it is surprising that after the water exposure the amounts of cadmium taken up were approximately the same, especially as cadmium a non-essential, non-regulated metal (Marsden et al., 2003). A plausible explanation for this has two parts. Firstly, the high concentration in the water exposure precipitates mechanisms of peroxidation (see above). As herbivorous diets are lower in nutrients than the animal tissues they must build (Cruz-Rivera and Hay, 2000), lipid reserves stored are reduced.

The opposite happens with carnivorous diets where higher lipid stores will cause more oxidative stress. Carnivorous amphipods were shown to have decreased levels of antioxidant enzymes causing higher amount of peroxidation. Herbivorous amphipods had optimal levels of antioxidant enzymes resulting in no oxidative stress, which the authors attributed to the algal based diet (Obermüller et al., 2005). Secondly, the digestibility of the tissues where metaliferous compounds are stored in is the key to predicting their potential for food chain transfer (Amiard-Triquet et al. 1992) and it has been found that metals associated with algae are least assimilated primarily because of the thick cellulose wall that makes them less digestible (Wang & Fisher 1998).

Contrary to cadmium, there were significant statistical differences in zinc uptake between individuals fed different diets. Enhanced levels of zinc were taken up by *Echinogammarus* fed *Artemia*. This was probably due to the considerable difference in zinc levels within *Artemia* and *Fucus* ($179.4 \mu\text{g g}^{-1}$ versus $14.94 \mu\text{g g}^{-1}$) which means that zinc was accumulated through food exposure. In amphipods there is linearity between uptake in zinc and water concentrations. Nevertheless, the uptake rate was very small which causes the net effect to be close to that of regulation (Rainbow and White, 1989). Clason et al. (2003), working with *Chaetogammarus* (= *Echinogammarus*) *marinus* was unable to determine a toxicokinetic model for zinc accumulation, which inevitably led to the conclusion that the species was regulating zinc (a peculiar phenomenon for amphipods). The present data suggests otherwise as there is a significant difference in zinc uptake between the test groups (even adults) and control. However, Clason et al (2003) used concentrations which were two orders of magnitude smaller than the ones in the present study, implying that in the latter uptake rate

(which is a direct function of the concentration of aqueous metal) transcended the excretion rate and thus net accumulation occurred (Marsden and Rainbow, 2004).

The present results also provide an interesting insight into the gender dietary preferences of *E. marinus*. Dick et al. (2005) noted that gut contents from field collected females indicated they consumed 'animals only' significantly more frequently than males, the same pattern being verified in laboratory feeding preference assays. The explanation put forward was they would exploit different microhabitats more often than males, due to their smaller size, and therefore forage for high protein foodstuffs to invest in egg production. The present results suggest that females will possess higher metal body burdens, in accordance with their food preferences, which agrees with observations for the field collected amphipods (Marsden et al., 2003).

An important aspect when evaluating uptake of metals is the amount allocated to the exoskeleton matrix and adsorbed onto its surface. Literature references regarding metal content for exoskeletons of amphipods are rare, nevertheless, the present results returned values within the range found for other crustaceans. Table 3 presents a comparison of the calculated percentage of cadmium and zinc (either adsorbed, embedded or both, as is the case of this study) within exoskeletons of crustaceans. Unsurprisingly *E. marinus* exoskeleton showed percentages of adsorbed metal more similar to other Malacostraca (*Palaemon elegans* and *Penaeus indicus*) than to Maxillipoda (*Acartia spp.* and *Temora longicornis*). Despite the present study not being designed to discern the relative amount of metals embedded in the cuticle to that adsorbed to the surface, results appear to point to a different pattern for the two metals investigated. Cadmium showed a relative drop in exoskeleton concentration in

the treatment compared to the control. As can be seen in Table 3, cadmium possessed a lower affinity towards the exoskeleton compared to zinc. This suggests that storage of metal taken up was higher in soft tissues than in the exoskeleton (Amiard et al., 2006). Wright (1980) demonstrated using radiolabelling techniques that most cadmium uptake in amphipods may be internal rather than adsorption to or storage at the body surface. On the other hand, Nuñez-Nogueira and Rainbow (2005b) found that in *P. indicus* newly accumulated zinc was distributed to all organs but with the highest proportions being deposited in the exoskeleton. In addition, Wang and Fisher (1998) noted that compared to other metals, including cadmium, Zn binds in considerable quantities to the exoskeleton from the dissolved phase with Mouneyrac et al. (2002) calculating the adsorbed percentage onto *Orchestia gammarellus* exoskeleton as being $9\pm3\%$ of all zinc.

The results of the present study provided a clear demonstration of influences exerted on metal uptake by amphipods from parameters associated with the individual (sex, age) and external environment (types of foods offered). The need to include different stages of development and both genders of amphipods in acute exposure experiments (and chronic if pre-feeding is looked upon as exposure), as well as taking into consideration the amounts of metals related to the exoskeleton, was emphasized as an indispensable procedure if a clear evaluation of metal-biota issues is to be undertaken. Furthermore our study reinforces the high amenability and suitability of *E. marinus* as a test species.

Acknowledgements

FCT – Fundação para a Ciência e Tecnologia for funding this research through PhD Grant SFRH/BD/4778/2001.

REFERENCES

Amiard, J.C., Amiard-Triquet, C., Barka, S., Pellerin, J. Rainbow, P.S., 2006. Metallothioneins in aquatic invertebrates: their role in metal detoxification and their use as biomarkers. *Aquat. Toxicol.* 76, 160–202.

Amiard-Triquet, C., Martoja, R., Marcaillou, C., 1992. Alternative methodologies for predicting metal transfer in marine food webs including filter-feeders. *Water Environ. Technol.* 25, 197–204.

Chandini, T., 1988. Changes in food [*Chlorella*] levels and the acute toxicity of cadmium to *Daphnia carinata* (Daphnidae) and *Echinisca triserialis* (Macrothricidae)[Crustacea: Cladocera]. *B. Environ. Contam. Tox.* 41, 398–403.

Chandini, T., 1989. Survival, growth and reproduction of *Daphnia carinata* (Crustacea: Cladocera) exposed to chronic cadmium stress at different food (*Chlorella*) levels. *Environ. Pollut.* 60, 29–45.

Clason, B., Zauke, G.-P., 2000. Bioaccumulation of trace metals in marine and estuarine amphipods: Evaluation and verification of toxicokinetic models. *Can. J. Fish. Aquat. Sci.* 57, 1410-1422.

Clason, B., Langston, W. J., Zauke G.-P., 2003. Bioaccumulation of trace metals in the amphipod *Chaetogammarus marinus* (Leach, 1815) from the Avon and Tamar

361 estuaries (UK): Comparison of two compartment and hyperbolic toxicokinetic
362 models. Mar. Environ. Res. 57, 171-195.

363 Correia, A. D., Costa, M. H., Luis, O. J., Livingstone, D. R., 2003. Age-related changes in
364 antioxidant enzyme activities, fatty acid composition and lipid peroxidation in
365 whole body *Gammarus locusta* (Crustacea: Amphipoda). J. Exp. Mar. Biol. Ecol.
366 289, 83– 101.

367 Correia, A. D., Lima, G., Costa, M. H., Livingstone, D. R., 2002. Studies on biomarkers of
368 copper exposure and toxicity in the marine amphipod *Gammarus locusta*
369 (Crustacea) I: induction of metallothionein and lipid peroxidation. Biomarkers
370 7(5), 422–437.

371 Correia, A.D., Sousa, A., Costa, M.H., Moura, I., Livingstone, D. R., 2004. Quantification
372 of metallothionein in whole body *Gammarus locusta* (crustacea: amphipoda)
373 using differential pulse polarography. Toxicol. Environ. Chem. 86(1), 23–36.

374 Cruz-Rivera, E., Hay, M.E., 2000. The effects of diet mixing on consumer fitness:
375 macroalgae, epiphytes, and animal matter as food for marine amphipods.
376 Oecologia 123, 252–264.

377 Dick, J.T.A., Johnson, M.P., McCambridge, S., Johnson, J., Carson, V.E.E., Kelly, D.W.,
378 MacNeil, C., 2005. Predatory nature of the littoral amphipod *Echinogammarus*
379 *marinus*: gut content analysis and effects of alternative food and substrate
380 heterogeneity. Mar. Ecol-Prog. Ser. 291, 151–158.

381 Fialkowski, W., Fialkowska, E., Smith, B.D., Rainbow, P.S., 2003. Biomonitoring survey
382 of trace metal pollution in streams of a catchment draining a zinc and lead
383 mining area of upper silesia, Poland Using the Amphipod *Gammarus fossarum*.
384 Int. Rev. Hydrobiol. 88 (2), 187-200.

385 Halliwell, B., Gutteridge, J.M.C., 1999. Free radicals in biology and medicine. Oxford
386 University Press, Oxford.

387 Heugens, E.H.W., Hendriks, J.A., Dekker, T., Van Straalen, N.M., Admiraal, W., 2001. A
388 review of the effects of multiple stressors on aquatic organisms and analysis of
389 uncertainty factors for use in risk assessment. Crit. Rev. Toxicol. 31(3), 247–284.

390 Hopkin, S.P., 1990. Species-specific differences in the net assimilation of zinc,
391 cadmium, lead, copper, and iron by the terrestrial isopod *Oniscus asellus* and
392 *Porcellio scaber*. J. Appl. Ecol. 27, 260-474.

393 Howlett, N.G., Avery, S.V., 1997. Induction of lipid peroxidation during heavy metal
394 stress in *Saccharomyces cerevisiae* and influence of plasma membrane fatty acid
395 unsaturation. Appl. Environ. Microb. 63(8), 2971–2976.

396 Kawashima, H., Takeuchi, I., Ohnishi, M., 1999. Fatty acid compositions in four caprellid
397 amphipod species (Crustacea) from Otsuchi and Mutsu Bays in Northern Japan.
398 Journal of the Japanese Oil Chemists' Society 48, 595-599.

399 Lincoln, R.J., 1979. British marine Amphipoda: *Gammaridea*. British Museum (Natural
400 History), London.

401 Livingstone, D.R., 2001. Contaminated-stimulated reactive oxygen species production
402 and oxidative damage in aquatic organisms. Mar. Pollut. Bull. 42, 656– 666.

403 MacDonald, R.S., 2000. The role of zinc in growth and cell proliferation. J. Nutr.
404 130(5S), 1500S-1508S.

405 Maranhão, P., Marques, J.C., 2003. The influence of temperature and salinity on the
406 duration of embryonic development, fecundity and growth of the amphipod
407 *Echinogammarus marinus* Leach (Gammaridae). Acta Oecol. 24, 5–13.

408 Marsden, I.D., 2002. Life-history traits of a tube-dwelling corophioid amphipod,
409 *Paracorophium excavatum*, exposed to sediment copper. J. Exp. Mar. Biol. Ecol.
410 270, 57–72.

411 Marsden, I.D., Rainbow, P.S., Smith, B.D., 2003. Trace metal concentrations in two New
412 Zealand talitrid amphipods: effects of gender and reproductive state and
413 implications for biomonitoring. J. Exp. Mar. Biol. Ecol. 289, 93–113.

414 Martins, I., Maranhão, P., and Marques J-C. (2002) Modelling the effects of salinity
415 variation on *Echinogammarus marinus* Leach (Amphipoda, Gammaridae)
416 density and biomass in the Mondego Estuary (Western Portugal). Ecol. Model.,
417 152, 247–260

418 McGee, B.L., Wright, D.A., Fisher, D.J., 1998. Biotic factors modifying acute toxicity of
419 aqueous cadmium to estuarine amphipod *Leptocheirus plumulosus*. Arch.
420 Environ. Con. Tox. 34, 34–40.

421 Mouneyrac, C., Amiard, J.C., Amiard-Triquet, C., Cottier, A., Rainbow, P.S., Smith, B.D.,
422 2002. Partitioning of accumulated trace metals in the talitrid amphipod
423 crustacean *Orchestia gammarellus*: a cautionary tale on the use of
424 metallothionein-like proteins as biomarkers. Aquat. Toxicol. 57, 225–242.

425 Neuparth, T., Costa, F.O., Costa, M.H., 2002. Effects of temperature and salinity on life
426 history of the marine amphipod *Gammarus locusta*. Implications for
427 ecotoxicological testing. Ecotoxicology 11, 55–67.

428 Nuñez-Nogueira, G., Rainbow, P.S., 2005a. Cadmium uptake and accumulation by the
429 decapod crustacean *Penaeus indicus*. Mar. Environ. Res. 60(3), 339–354.

430 Nuñez-Nogueira, G., Rainbow, P.S., 2005b. Kinetics of zinc uptake from solution,
 431 accumulation and excretion by the decapod crustacean *Penaeus indicus*. Mar.
 432 Biol. 147(1), 93-103.

433 Obermüller, B. Karsten, U., Abele, D., 2005. Response of oxidative stress parameters
 434 and sunscreens compounds in Arctic amphipods during experimental exposure
 435 to maximal natural UVB radiation. J. Exp. Mar. Biol. Ecol. 323: 100–117.

436 Pöckl, M., 1995. Laboratory studies on growth, feeding, moulting and mortality in the
 437 freshwater amphipods *Gammarus fossarum* and *G. roeseli*. Arch. Hydrobiol.
 438 134(2): 223– 253.

439 Rainbow, P.S., 1990. Heavy metal levels in marine invertebrates. In: Furness, R. W.,
 440 Rainbow, P. S. (Eds.). Heavy metals in the Marine Environment. CRC Press, Boca
 441 Raton, FL, USA, pp. 67–80.

442 Rainbow, P.S., 1993. The significance of trace metal concentrations in marine
 443 invertebrates. In: Dallinger, R., Rainbow, P.S. (Eds.). Ecotoxicology of Metals in
 444 Invertebrates. Lewis Publishers, Chelsea, USA, pp. 3–23.

445 Rainbow, P.S., 1997. Ecophysiology of trace metal uptake in crustaceans. Estuar. Coast.
 446 Shelf S. 44, 169–175.

447 Rainbow, P.S., 2007. Trace metal bioaccumulation: Models, metabolic availability and
 448 toxicity. Environ. Int. 33, 576–582.

449 Rainbow, P.S., Moore, P.G., 1986. Comparative metal analysis in amphipod
 450 crustaceans. Hydrobiologia 141, 273–289.

451 Rainbow, P.S., White, S.L., 1989. Comparative strategies of heavy metal accumulation
 452 by crustaceans: zinc, copper and cadmium in a decapod, an amphipod and a
 453 barnacle. Hydrobiologia 174, 245–262.

454 Rainbow, P.S., Ng, T.Y.-T., Shi, D., Wang, W.-X., 2004. Acute dietary pre-exposure and
455 trace metal bioavailability to the barnacle *Balanus amphitrite*. J. Exp. Mar. Biol.
456 Ecol. 311, 315–337.

457 Rand, G.M., 1995. Fundamentals of aquatic toxicology (2nd ed.). Taylor and Francis,
458 Washington, DC.

459 Reinfelder, J.R., Fisher, N.S., 1994. Retention of elements absorbed by juvenile fish
460 (*Menidia menidia*, *Menidia beryllina*) from zooplankton prey. Limnol. Oceanogr.
461 39, 1783–1789.

462 Roméo, M., Bennani, N., Gnassia-Barelli, M., Lafaurie, M., Girard, J.P., 2000. Cadmium
463 and copper display different responses towards oxidative stress in the kidney of
464 the sea bass *Dicentrarchus labrax*. Aquat. Toxicol. 48, 185–194.

465 Sutcliffe, D.W., 1984. Quantitative aspects of oxygen uptake by *Gammarus* (Crustacea,
466 Amphipoda): a critical review. Freshwater Biol. 14, 443-489.

467 Viarengo, A., Nott, J.A., 1993. Mechanisms of heavy metal cation homeostasis in
468 marine invertebrates. Comp. Biochem. Physiol. 104C, 355–72.

469 Wang, W.-X., Fisher, N.S., 1998. Accumulation of trace elements in a marine copepod.
470 Limnol. Oceanogr. 43, 273–283.

471 Weeks, J.M., Rainbow, P.S., Moore, P.G., 1992. The loss, uptake and tissue distribution
472 of copper and zinc during the moult cycle in an ecological series of talitrid
473 amphipods (Crustacea: Amphipoda). Hydrobiologia 245, 15-25.

474 White, S.L., Rainbow, P.S., 1986. Accumulation of cadmium by *Palaemon elegans*
475 (Crustacea: Decapoda. Mar. Ecol.-Prog. Ser. 32, 17-25.

476 Wolfe, D. A., 1992. Selection of bioindicators of pollution for marine monitoring
477 programmes. Chem. Ecol. 6(1-4), 149–167.

Wright DA., 1980. Cadmium and calcium interactions in the freshwater amphipod *Gammarus pulex*. Freshwater Biol. 10, 123-133.

Zhou, Q., Zhang, J., Fu, J., Shi, J., Jiang, G., 2008. Biomonitoring: an appealing tool for assessment of metal pollution in the aquatic ecosystem. Anal. Chim. Acta 606(2), 135-150.

FIGURE CAPTIONS

Figure 1 – Mean whole body burdens ($\mu\text{g g}^{-1}$) after a 96h exposure to water-borne cadmium (nominal $1 \text{ mg L}^{-1} \text{ Cd}$) for *E. marinus*. Error bars are +St Dev. Treatments with the same letter were not significantly different; $p > 0.05$, ANOVA, post hoc SNK).

Figure 2 – Mean whole body burdens ($\mu\text{g g}^{-1}$) after a 96h exposure to water borne zinc (nominal $1 \text{ mg L}^{-1} \text{ Zn}$) for *E. marinus*. Error bars are + St Dev. Treatments with the same letter were not significantly different; $p > 0.05$, ANOVA, post hoc SNK).

Figure 3 – Mean whole body burdens ($\mu\text{g g}^{-1}$) of diet for *E. marinus* males, females and juveniles exposed for 96h to water borne cadmium (nominal $1 \text{ mg L}^{-1} \text{ Cd}$). Error bars are + St Dev.

Figure 4 - Mean whole body burdens ($\mu\text{g g}^{-1}$) of diet for *E. marinus* males, females and juveniles exposed for 96h to water borne zinc (nominal $1 \text{ mg L}^{-1} \text{ Zn}$). Error bars are + St Dev.

TABLE CAPTIONS

Table 1 - Mean metal content ($\mu\text{g g}^{-1} \pm \text{St Dev}$) for *F. vesiculosus* and *A. salina* used for pre-exposure feeding to two experimental groups of *E. marinus*.

502 **Table 2** – Metal levels, as percentage of whole body burden (\pm St Dev), within the
503 exoskeleton of *E. marinus*. Quantifications were performed after exposure to 1 mg L⁻¹
504 Cd (nominal) or 1 mg L⁻¹ Zn (nominal) for 96h.

505 **Table 3** - Levels of zinc and cadmium (as % + Std Dev) bound to crustacean
506 exoskeletons in different studies. (^a) – the authors did not present standard deviation
507 values; np – quantification of the metal was not performed in the study; Mat – Metal
508 embedded in the exoskeleton matrix; Ad – Metal adsorbed to the exoskeleton surface.

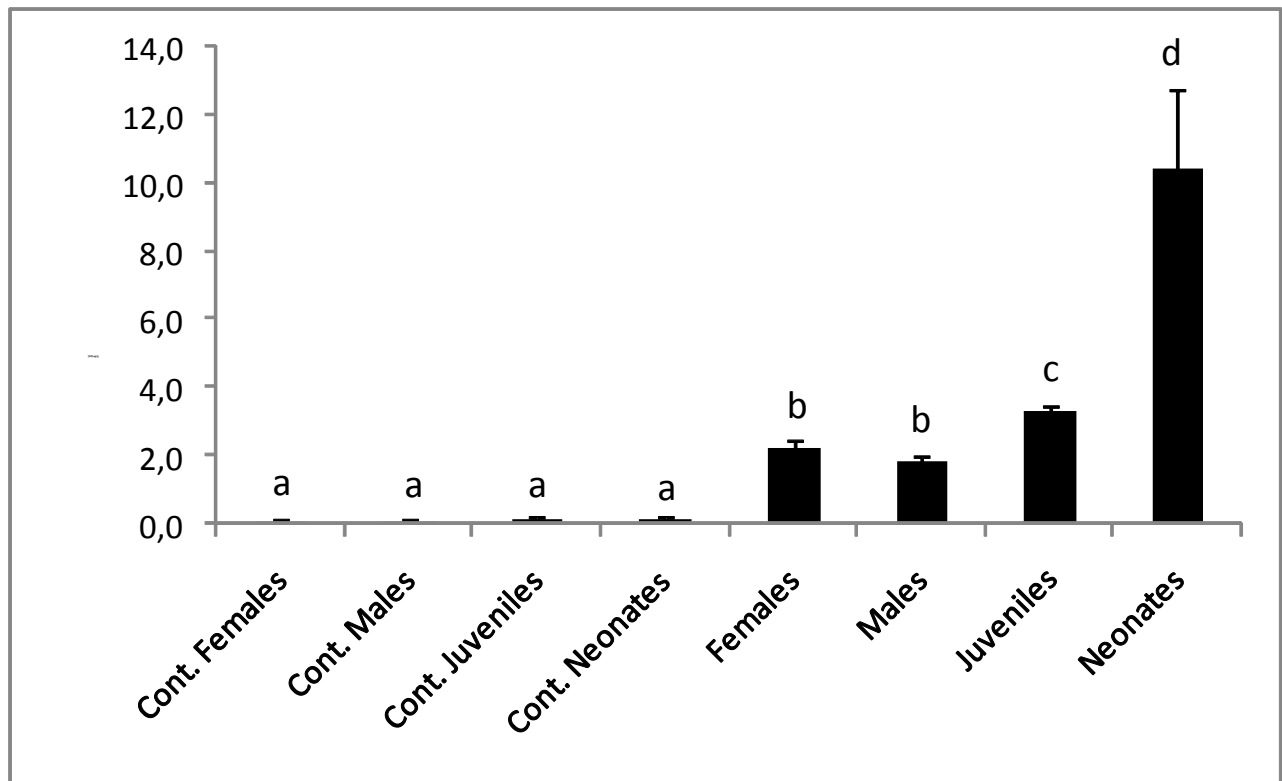


Figure 1 – Mean whole body burdens ($\mu\text{g g}^{-1}$) after a 96 h exposure to water-borne cadmium (nominal $1 \text{ mg L}^{-1} \text{ Cd}$) for *E. marinus*. Error bars are +St Dev. Treatments with the same letter were not significantly different; $p > 0.05$, ANOVA, post hoc SNK).

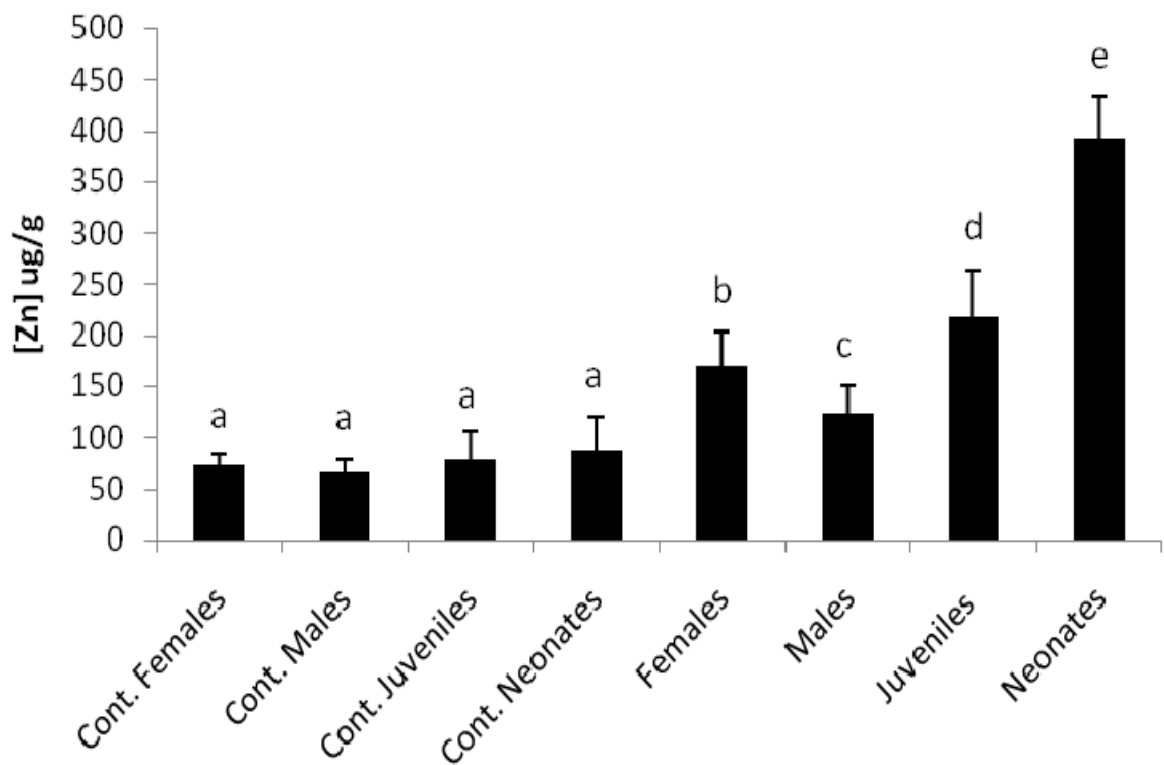


Figure 2 – Mean whole body burdens ($\mu\text{g g}^{-1}$) after a 96 h exposure to water borne zinc (nominal $1 \text{ mg L}^{-1} \text{ Zn}$) for *E. marinus*. Error bars are + St Dev. Treatments with the same letter were not significantly different; $p > 0.05$, ANOVA, post hoc SNK).

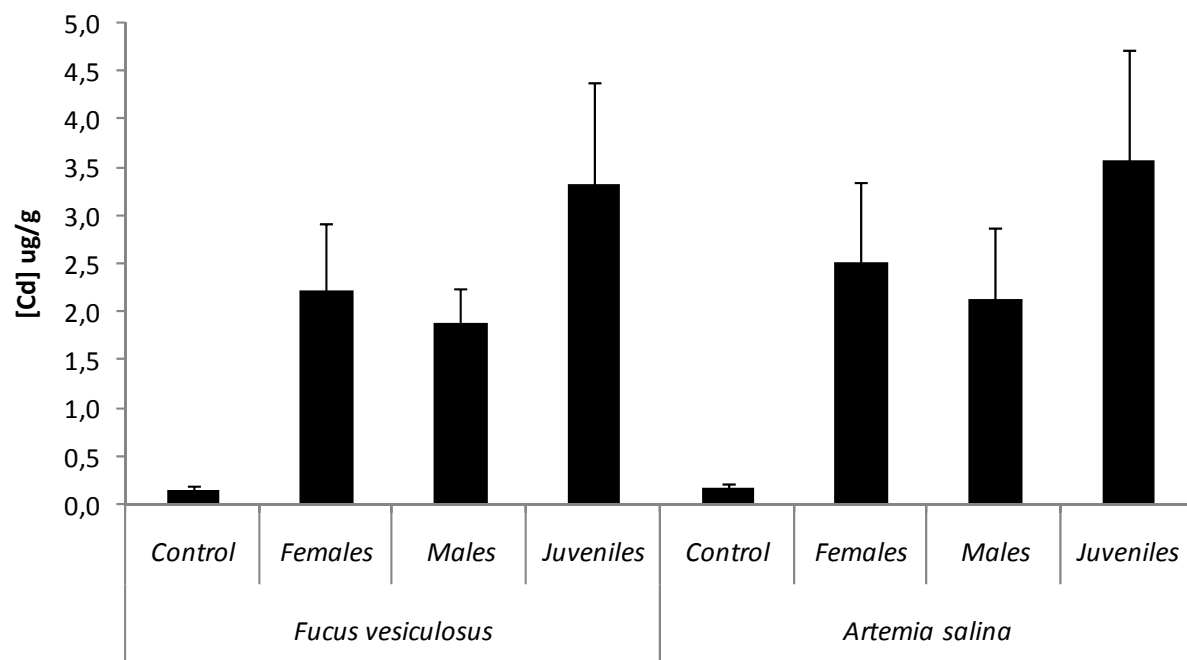


Figure 3 – Mean whole body burdens ($\mu\text{g g}^{-1}$) of diet for *E. marinus* males, females and juveniles exposed for 96 h to water borne cadmium (nominal $1 \text{ mg L}^{-1} \text{ Cd}$). Error bars are + St Dev.

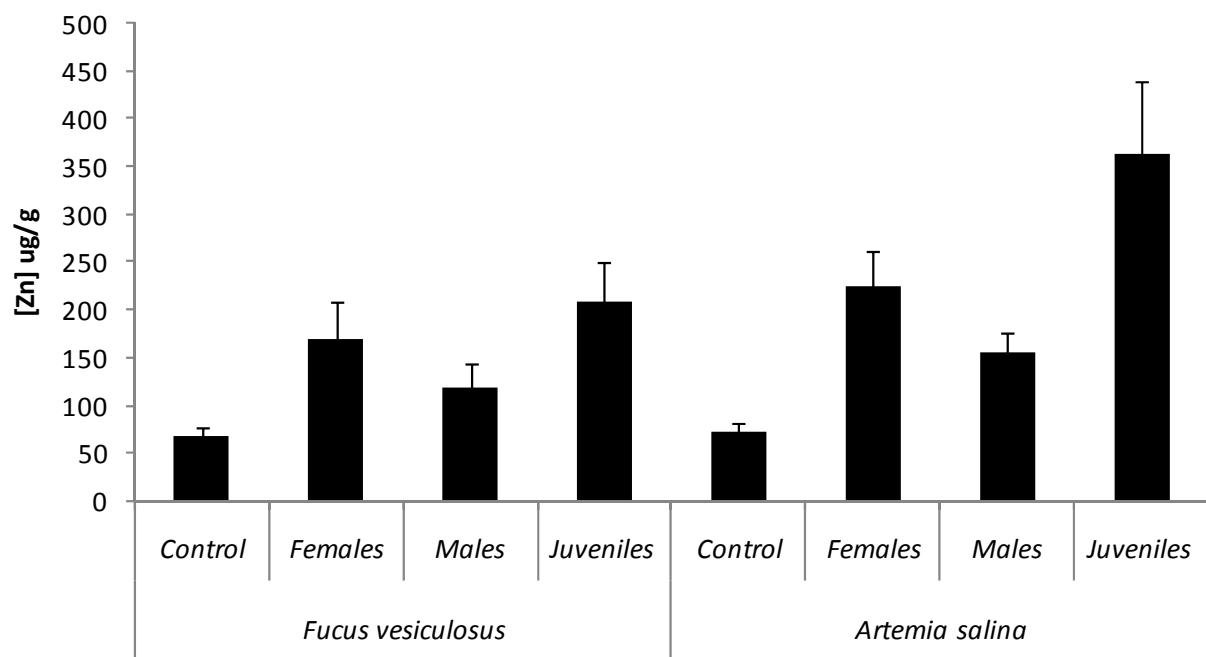


Figure 4 - Mean whole body burdens ($\mu\text{g g}^{-1}$) of diet for *E. marinus* males, females and juveniles exposed for 96 h to water borne zinc (nominal 1 mg L⁻¹ Zn). Error bars are + St Dev.

Table 1 - Mean metal content ($\mu\text{g g}^{-1} \pm \text{St Dev}$) for *F. vesiculosus* and *A. salina* used for pre-exposure feeding to two experimental groups of *E. marinus*.

	Cadmium	Zinc
<i>A. salina</i>	0.025 ± 0.0061	179.35 ± 21.35
<i>F. vesiculosus</i>	0.28 ± 0.047	14.94 ± 1.12

Table 2 – Metal levels, as percentage of whole body burden ($\pm \text{St Dev}$), within the exoskeleton of *E. marinus*. Quantifications were performed after exposure to 1 mg L^{-1} Cd (nominal) or 1 mg L^{-1} Zn (nominal) for 96 h.

Cadmium		Zinc	
Control	Exposed	Control	Exposed
28.68 ± 1.5	26.21 ± 1.75	23.57 ± 1.16	34.05 ± 1.47

Table 3 - Levels of zinc and cadmium (as % + Std Dev) bound to crustacean exoskeletons in different studies. (^a) – the authors did not present standard deviation values; np – quantification of the metal was not performed in the study; Mat – Metal embedded in the exoskeleton matrix; Ad – Metal adsorbed to the exoskeleton surface.

Test organism	% Zn	% Cd	Fraction	Reference
<i>Acartia spp.</i>	98.5	97.4 ^a	Mat + Ad	Reinfelder and Fisher, 1994
<i>Temora longicornis</i>	65.0 (2.0)	17.0 (1.0)	Ad	Wang and Fisher, 1998
<i>Palaemon elegans</i>	Np	46.0 (4.8)	Mat + Ad	White and Rainbow, 1986
<i>Penaeus indicus</i>	Np	27.5 (11.0)	Mat + Ad	Nunes-Norueira and Rainbow, 2005a
<i>Penaeus indicus</i>	41.0 (8.0)	Np	Mat + Ad	Nunes-Norueira and Rainbow, 2005b
<i>Orchestia gammarellus</i>	9.0 (3.0)	Np	Ad	Mouneyrac et al., 2002
<i>Echinogammarus marinus</i>	34.1 (1.5)	26.2 (1.8)	Mat + Ad	This study