AMPHIPOD SUSCEPTIBILITY TO METALS: CAUTIONARY TALES

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ABSTRACT

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

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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 preformed 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

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

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

56 To test some of the sensitivity aspects of the issues raised, the ubiquitous inter-tidal
57 amphipod *Echinogammarus marinus* Leach was chosen as test organism. It has a wide
58 distribution, reported from Norway and Iceland to Portugal (Lincoln, 1979), commonly
59 occurs in high abundances and plays an important trophic role (Dick et al., 2005;
60 Maranhão and Marques, 2003), feeds as both as mesograzer and predator (Dick et al.,
61 2005), is easy to culture and manipulate and reproduces rapidly (17 days at 20^{o}C for
62 production of a juvenile (Maranhão and Marques, 2003)).

63 The aim of this work was to investigate the influence of different life stages, of gender
64 and of diet on uptake of metals by *Echinogammarus marinus* when in presence of
65 available essential and non-essential metals. This was achieved in three experiments,
66 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 ($CdCl_2 \cdot 2H_2O$, Sigma-Aldrich) or Zinc ($ZnSO_4 \cdot 7H_2O$, 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

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

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

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

103

104 **2.2. Metal analysis**

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

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

121 **2.3. Statistical analysis**

122 One-way Analysis of Variance (ANOVA) was performed on Log_{10} transformed data to
123 evaluate the significance of differences between life stages and different treatments.
124 Normality of distribution was tested using the Kolmogorov-Smirnov test. Student-
125 Newman-Keuls (SNK) Method was applied as pairwise multiple comparison procedure
126 to further differentiate between groups/treatments.

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

128

129 **3. RESULTS**

130 **3.1. Life stage metal accumulation**

131 Figures 1 and 2 present the quantification of metal body burdens for females, males,
132 juveniles and neonates in controls and test solutions of 1 mg L^{-1} Cd and 1 mg L^{-1} Zn,
133 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

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

165

166 **3.3. Exoskeleton metal quantification**

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

171

172 **4. DISCUSSION**

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

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

225 of copper (Roméo et al., 2000). In adult specimens of *G. locusta* exposed to 4 µg L^{-1}
226 water-borne copper over 10 days, lipid peroxidation increased after 1 day and peaked
227 at day 4 before returning to control values by day 6. Simultaneously high
228 concentrations of metallothionein were observed between days 6 and 10 concurrently
229 with a decrease of lipid peroxidation (Correia et al., 2002). Due to higher contents in
230 PUFA the peroxidative capability of metals, at high concentrations, causes an initial
231 disruption of the adult's cellular membrane which could temporarily block metal
232 uptake. This can help explain further discrepancies between early and adult life stages,
233 but the need for further research is obvious. There is an indication in *G. locusta* that
234 males possess higher levels of PUFA and lower levels of metallothionein (Correia et al.,
235 2003, 2004). This could explain the differences found between genders in the present
236 study. Marsden et al. (2003) found higher cadmium levels in females but the similar
237 amounts of zinc between genders in talitrid amphipods.

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

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

260 For cadmium no statistical significant differences were found for uptake between
261 individuals of the same development stage or same gender fed with *Artemia salina* or
262 *Fucus vesiculosus*. Nevertheless, the amounts of metal present in the two foods have
263 also to be considered. Cadmium content was approximately an order of magnitude
264 different between *Fucus* (0.28 μg g^{-1}) and *Artemia* (0.025 μg g^{-1}). Under these
265 conditions it is surprising that after the water exposure the amounts of cadmium taken
266 up were approximately the same, especially as cadmium a non-essential, non-
267 regulated metal (Marsden et al., 2003). A plausible explanation for this has two parts.
268 Firstly, the high concentration in the water exposure precipitates mechanisms of
269 peroxidation (see above). As herbivorous diets are lower in nutrients than the animal
270 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 were 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 µg g^{-1} versus 14.94 µg 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

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

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

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

317 the treatment compared to the control. As can be seen in Table 3, cadmium possessed
318 a lower affinity towards the exoskeleton compared to zinc. This suggests that storage
319 of metal taken up was higher in soft tissues than in the exoskeleton (Amiard et al.,
320 2006). Wright (1980) demonstrated using radiolabelling techniques that most
321 cadmium uptake in amphipods may be internal rather than adsorption to or storage at
322 the body surface. On the other hand, Nuñes-Nogueira and Rainbow (2005b) found that
323 in *P. indicus* newly accumulated zinc was distributed to all organs but with the highest
324 proportions being deposited in the exoskeleton. In addition, Wang and Fisher (1998)
325 noted that compared to other metals, including cadmium, Zn binds in considerable
326 quantities to the exoskeleton from the dissolved phase with Mouneyrac et al. (2002)
327 calculating the adsorbed percentage onto *Orchestia gammarellus* exoskeleton as being
328 9±3% of all zinc.

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

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FIGURE CAPTIONS

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

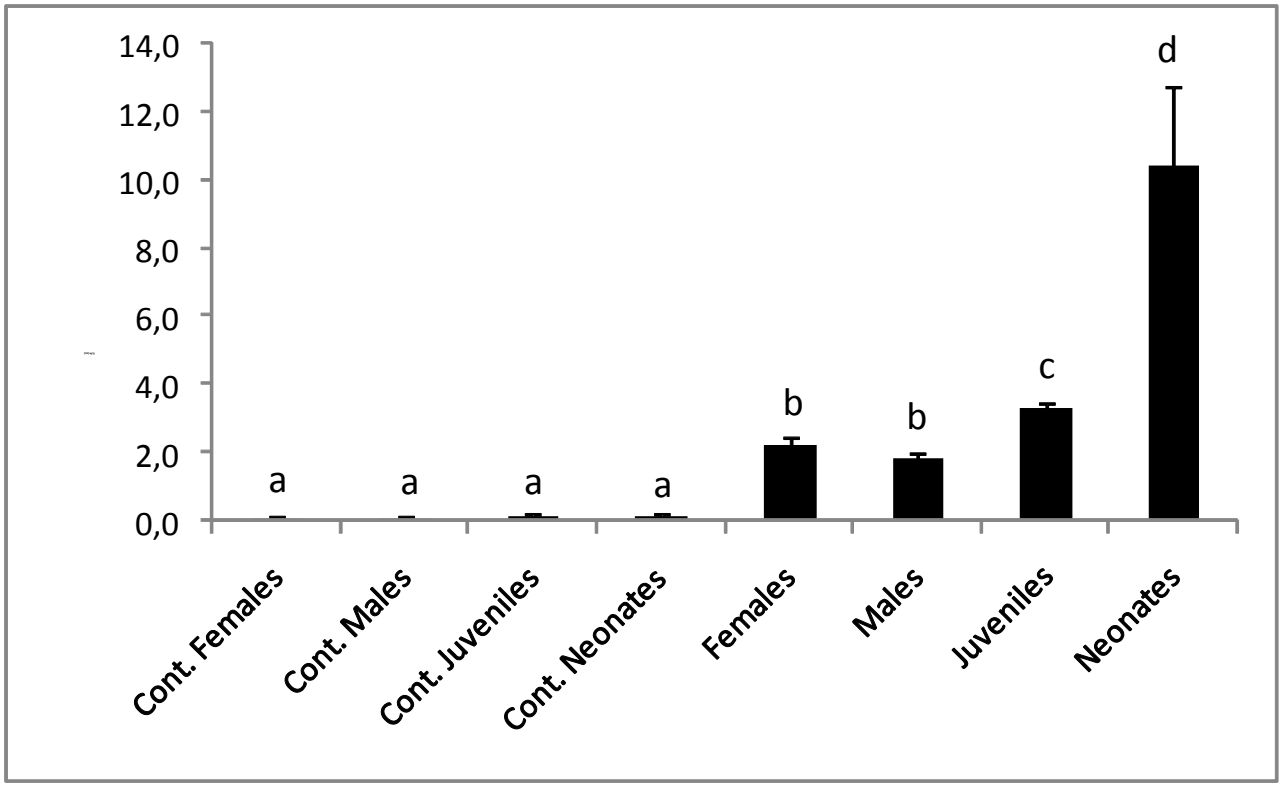
Figure 4 - Mean whole body burdens (µg g^{-1}) of diet for *E. marinus* males, females and juveniles exposed for 96h to water borne zinc (nominal 1 mg L^{-1} Zn). Error bars are + St Dev.

TABLE CAPTIONS

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

Table 2 – Metal levels, as percentage of whole body burden (± 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 96h.

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.



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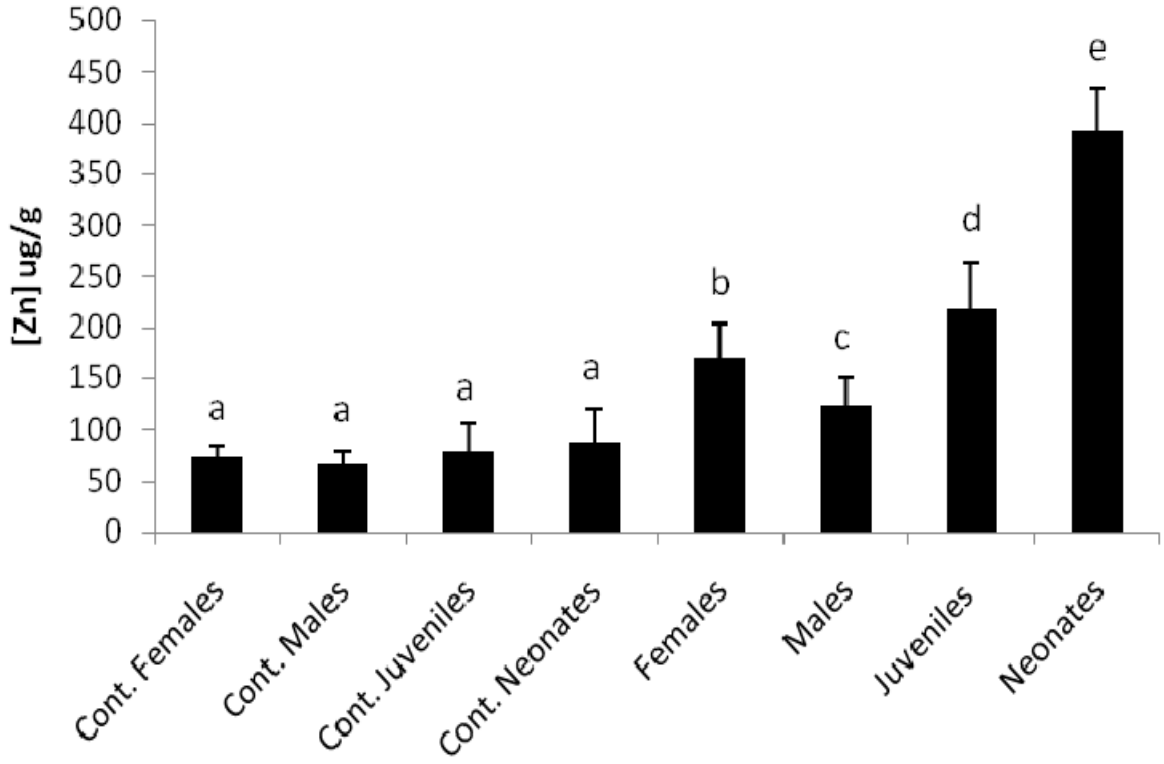
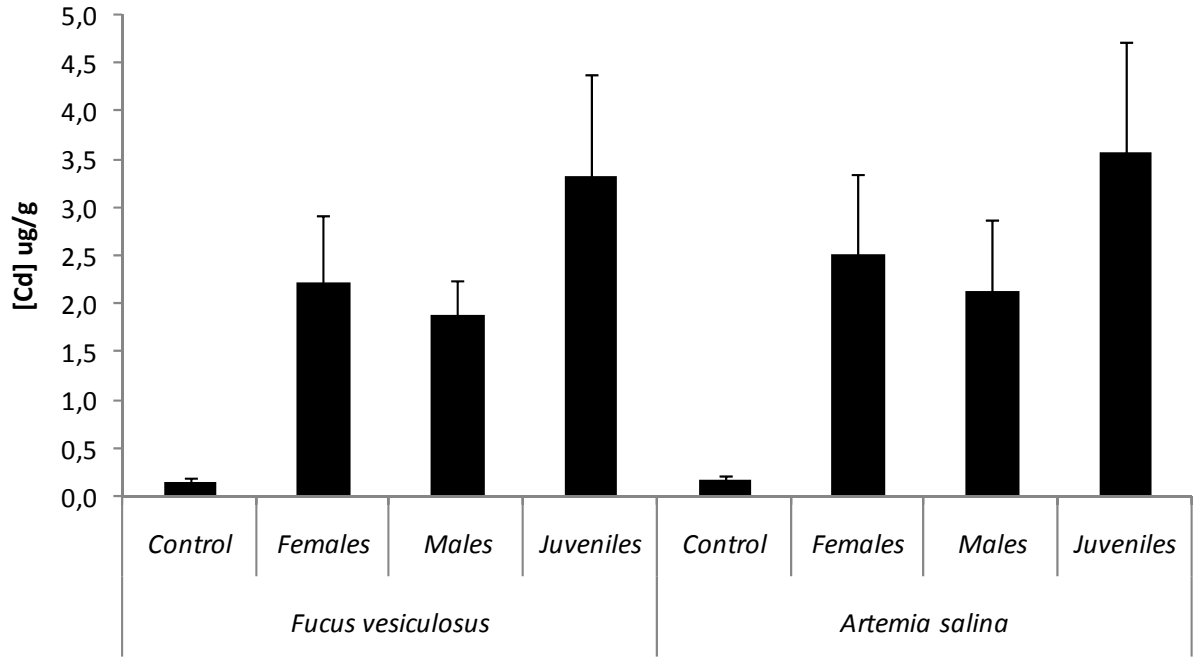


Figure 2 – Mean whole body burdens (µg g^{-1}) after a 96 h exposure to water borne zinc (nominal 1 mg L^{-1} Zn) for *E. marinus*. Error bars are + St Dev. Treatments with the same letter were not significantly different; $p > 0.05$, ANOVA, post hoc SNK).



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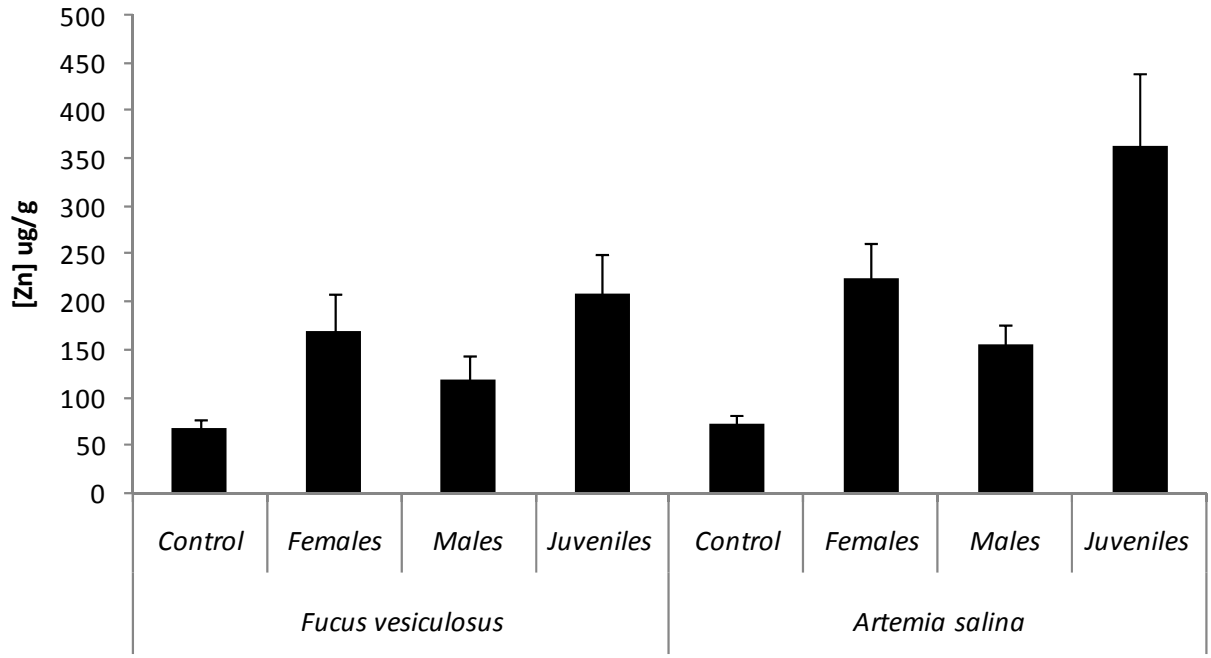

Figure 4 - Mean whole body burdens (µg g^{-1}) of diet for *E. marinus* males, females and juveniles exposed for 96 h to water borne zinc (nominal 1 mg L^{-1} Zn). Error bars are + St Dev.

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

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

Table 2 – Metal levels, as percentage of whole body burden (±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
Acartia spp.	98.5	97.4[a]	Mat + Ad	Reinfelder and Fisher, 1994
Temora longicornis	65.0 (2.0)	17.0 (1.0)	Ad	Wang and Fisher, 1998
Palaemon elegans	Np	46.0 (4.8)	Mat + Ad	White and Rainbow, 1986
Penaeus indicus	Np	27.5 (11.0)	Mat + Ad	Nunes-Norueira and Rainbow, 2005a
Penaeus indicus	41.0 (8.0)	Np	Mat + Ad	Nunes-Norueira and Rainbow, 2005b
Orchestia gammarellus	9.0 (3.0)	Np	Ad	Mouneyrac et al., 2002
Echinogammarus marinus	34.1 (1.5)	26.2 (1.8)	Mat + Ad	This study