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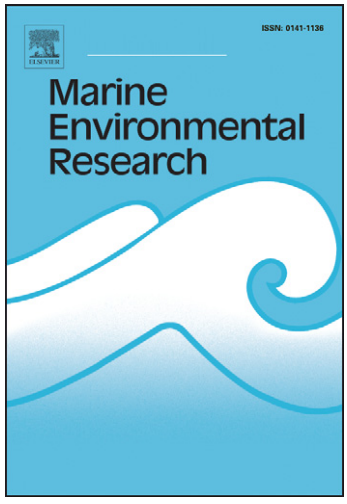

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Metallothioneins and cytosolic metals in *Neomysis integer* exposed to cadmium at different salinities

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Abstract

In the present study the induction of metallothioneins (MTs) and its relation to cytosolic metal concentrations (Zn, Cu and Cd) in the euryhaline crustacean *Neomysis integer* exposed to Cd at different salinities was studied. *N. integer* was exposed to the same free cadmium ion activity of 5.74×10^{-9} mol l^{-1} (i.e. 1/5 of the 96h LC_{50} value expressed as cadmium activity) in hypo-osmotic (5 psu), isosmotic (16 psu) and hyper-osmotic media (25 psu) for 7 days. In this way, the effect of salinity on cadmium speciation was eliminated and therefore the physiological effect of salinity on Cd accumulation and MT induction could be studied.

The accumulation of cytosolic Cd in *N. integer* changed with salinity from 1.11±0.05 µmol l^{-1} at 5 psu up to 1.43±0.17 µmol l^{-1} at 25 psu. This could indicate that the physiological response of euryhaline estuarine invertebrates like *N. integer* to salinity changes can influence the rate of trace metal uptake from solution. While the salinity changes did not cause significant differences in cytosolic Zn concentrations (mean value of all tested salinities: 34.4±2.8 µmol l^{-1}), an inverse relationship between salinity and cytosolic Cu concentration was observed. The highest concentration of 15.7±2.3 µmol Cu l^{-1} was determined at 5 psu and the lowest 10.9±1.4 µmol Cu l^{-1} at 25 psu. This could point to a possible relationship between the copper concentration and the hemocyanin metabolism in *N. integer*.

This is the first time that differential pulse voltammetry method was applied to MT assays with *N. integer*. Although the exposure to Cd resulted in a higher Cd cytosolic concentration, no subsequent MT increase was detected. The significant positive correlation between MT levels and cytosolic Cu concentrations (Spearman correlation coefficient r_s = 0.356, p = 0.009) implies a strong relationship between MT and Cu in *N. integer*.

Key words:

mysid, *Neomysis integer*, cadmium, salinity, metallothionein, cytosolic metals

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1. Introduction

Beside natural sources, human activities significantly contribute to the presence of cadmium in the environment (Hutton, 1983). Because of its toxicity, persistence and accumulation in the environment (Cole and Volpe, 1983; Herber, 2004) cadmium is identified as priority hazardous substance within the Water Framework Directive (WFD, 2000). Anthropogenic metal contamination influences both freshwater and coastal water bodies (Charlesworth and Service, 2000; Ibhadon et al., 2004). In this respect, molluscs, crustaceans and other marine invertebrates living in the littoral zone are known to accumulate high levels of metals in their tissues and yet survive in these polluted environments (Bryan et al., 1985; Rainbow, 2002; Fränzle, 2003). This tolerance depends on the ability of these animals to regulate metal in many of their tissues and to accumulate excess metal in non-toxic forms in other particular tissues (Viarengo and Nott, 1993). One of the proposed metal homeostasis and detoxification mechanisms in marine invertebrates is binding to specific soluble ligands, the most important of which are metallothioneins (MTs) (Viarengo and Nott, 1993, Amiard et al., 2006). Metals bound to metallothioneins, may be more available to predators than metals associated with insoluble cellular constituents (Wallace and Luoma, 2003; Zhang and Wang, 2006). Studies of the trophic transfer of metal contaminants have shown that the trophic transfer of certain metals in aquatic systems may be controlled by the internal distribution of metal within prey and that this distribution may be influenced by detoxification mechanisms (Wallace and Lopez, 1996; Fisher and Reinfelder, 1995). The ecological significance of these findings is that detoxification mechanisms in prey organisms may mediate the bioreduction or bioaccumulation of toxic metals along food chains by altering metal bioavailability (Wallace and Lopez, 1997).

Furthermore, an important factor governing the accumulation and toxicity of metals in aquatic animals is the physico-chemical form in which the metal is present in the medium (Rainbow,

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2002). Free cadmium species account for ca. 90% of the cadmium in the freshwater zone, whereas in marine systems chloro-complexes dominate the speciation distribution (Stumm and Morgan, 1996; Sadiq, 1992). It has been generally accepted that the toxicity of cadmium to aquatic animals changes as a function of ambient salinity with the metal generally being more toxic at low salinities. The effect of salinity on cadmium toxicity occurs primarily due to greater complexation of the free cadmium ion (Cd^{2+}) by the conservative ligand Cl^- (Sunda et al., 1978; De Lisle and Roberts, 1988). Study on the permeability of cadmium through lipid bilayer membranes suggested that cadmium transport and toxicity were protein mediated and correlated with Cd^{2+}, not $CdCl_2$, concentration (Gutknecht, 1983).

Next to physico-chemical factors, physiological factors modulate the response of organisms to metal challenge. For example, in a study with the euryhaline crustacean *Mysidopsis bahia*, the isosmotic point coincides with the salinity at which maximum tolerance to cadmium was observed (De Lisle and Roberts, 1988). For the euryhaline crustaceans *Orchestia gammarellus*, *Carcinus maenas* and *Necora puber* it is reported that decreased salinity is associated with reduced cadmium uptake (Rainbow et al., 1993; Rainbow and Black, 2005). This physiological response may include reductions in apparent water permeability with reduced salinities. Such physiological effects may be restricted to euryhaline organisms as opposed to aquatic invertebrates in general.

Neomysis integer used in this study is one of the most common mysids inhabiting estuaries along the European coasts and it has been shown to be sensitive to many toxicants at environmentally relevant concentrations (Roast et al., 2001; Verslycke et al., 2003, Wildgust and Jones, 1998). *N. integer* is a hyper- and hypo-osmoregulator with the isosmotic point at approximately 16 psu (De Lisle and Roberts, 1987). Thus, at low salinities it actively maintains its hemolymph hyperosmotic to the external environment and at high salinities the hemolymph is maintained in a hypo-osmotic state. As a result of the water fluxes associated

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with hypo- and hyperosmotic state of an organism, a number of physiological processes (e.g. uptake of major ions via ionic pumps, or excretion of unwanted salts) occur in order to keep the homeostasis of the organism (Rainbow, 1995; Roast et al., 2001). Consequently, the trace metal uptake can be facilitated due to increased activity of ionic pumps when an organism is hyper-osmoregulating. Uptake can also occur via the gut when the organism is hypo-osmoregulating.

This study is aimed at examining metallothionein induction in *N. integer* resulting from water-borne Cd exposure at different salinities. Concurrently, the Cd concentration in the cytosolic fraction was examined, as well as the constituent concentrations of the essential metals zinc and copper. Information on cytosolic metal concentrations enables the assessment of the relationship between cytosolic metals directly responsible for MT induction and the level of MT protein.

Expression of the exposure concentration in terms of Cd^{2+}, rather than total cadmium (Cd_T), reduces the apparent effect of salinity on cadmium toxicity (Engel and Fowler, 1979). In our study we used a similar approach. By using the same Cd^{2+} exposure concentration at different salinities, the effect of salinity on cadmium speciation was eliminated. Therefore, the true effect of salinity as an abiotic factor on Cd accumulation and the MT induction could be studied.

2. Materials and Methods

2.1. Animal collection and maintenance

N. integer was collected by hand net (about 2500 animals were sampled) from the dock B3 in the harbour of Antwerp (Belgium). Dock B3, situated on the right bank of the river Scheldt, is connected to the river through the Berendrecht and Zandvliet sluices. Salinity at the sampling

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location was 5 psu. The animals were transported to the laboratory in 15 L buckets containing ambient water within 2 hours after sampling.

In the laboratory the organisms were transferred to 200 L glass aquaria. Culture medium was artificial seawater (Instant Ocean®, Aquarium Systems, France) diluted with aerated deionized tap water to a final salinity of 5 psu. Water temperature was maintained at 15±1°C, and 12h light:12h dark photoperiod was used during culturing. Animals were fed *ad libitum* daily with 24 - 48h old *Artemia* nauplii. Hatching of the *Artemia* cysts was performed in 1 L conical vessels under vigorous aeration and continuous illumination at 25°C (Sorgeloos et al., 1986).

2.2 Exposure experiment

The experiments were performed at three different salinities: 5 psu (lower osmotic pressure than haemolymph of *N. integer*), 16 psu (isosmotic point) and 25 psu (higher osmotic pressure than haemolymph of *N. integer*). To avoid mortality during the experiment, the cadmium test concentration used was 5.74×10^{-9} mol l^{-1} expressed as free cadmium ion activity. This represents 1/5 of the cadmium activity of the reported 96h LC_{50}: 2.87×10^{-8} mol l^{-1} expressed as free cadmium ion activity at 5 psu or 45 µg l^{-1} in terms of the total dissolved cadmium (Verslycke et al., 2003). Free cadmium ion concentrations and activities were calculated using the visual MINTEQ software (geochemical speciation model; downloaded from http://www.lwr.kth.se/English/OurSoftware/vminteq/) and an average seawater composition (Sadiq, 1992). The content of DOC in Instant Ocean was low (0.2 mg l^{-1} at salinity 30 psu) and did not affect the cadmium speciation. Based on the vMINTEQ calculations, the same Cd^{2+} activity was used at each of the test salinities. The selected test concentrations in terms of total dissolved cadmium were 7.2, 23.0 and 38.1 µg l^{-1} at 5, 16 and 25 psu, respectively. The duration of the exposure experiment was 7 days and the animals were sampled on day 1, 4 and 7. The control and exposure media were renewed every 48h.

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Juveniles of approximately the same size (1 cm) were used for the experiment in order to minimise the differences in weight, sex and reproductive status. Animals (the average wet weight: 6.3±2.1 mg) were collected from the culture aquarium and randomly distributed into 10L solid glass experimental aquaria. For control and Cd-treatment at each salinity and time-point, one aquarium containing 120 individuals was set up. Test animals were allowed 24h to acclimate (osmotically) to different test salinities prior to the cadmium exposure. This acclimation period has been shown to be sufficient for *N. integer* to attain a new steady state, since mysids rapidly acclimate to salinity changes (De Lisle and Roberts, 1987).

Exposure experiments were performed in a temperature-controlled chamber (Liebher®, Laborimpex, Belgium) at 15±1°C and a 12h light:12h dark photoperiod was used during experiments. The required salinities (5, 16 and 25 psu) were obtained by dissolving appropriate amounts of Instant Ocean® (Aquarium Systems, France) in deionized tap water. The salinities were confirmed with a portable refractometer (Digit 032, CETI, Belgium). The total cadmium concentrations of the stock solutions were checked using atomic absorption spectrometry (flame-AAS, SpectrAA-100, Varian, Germany) and were within 10% of the nominal values. The animals were fed daily with 24 - 48h old *Artemia* nauplii *ad libitum* to prevent cannibalism.

2.3. Isolation and quantification of metallothioneins

Composite samples containing 20 test organisms were placed in pre-weighed Eppendorf tubes and weighed. Three samples were analysed for each salinity and time point for both control and Cd-exposed groups. Samples were stored at -80°C until analysis. Homogenization was performed in 20 mM Tris-HCl buffer (pH 8.6 at 4°C) containing 0.006 mM leupeptine, 0.5 mM phenylmethyl-sulphonylfluoride and 0.01% β-mercaptoethanol (tissue wet weight : buffer volume = 1:5) using a PTFE pestle and motorized homogenizer. The homogenate was

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centrifuged at 60,000 *g* for 60 minutes at 4°C. The resulting supernatant was the cytosolic fraction. Three aliquots of the supernatant were transferred to Eppendorf tubes, diluted 5× with 0.9% NaCl, and placed in a water bath at 85°C for 10 minutes. Subsequently, the samples were placed on ice for 30 minutes and then centrifuged at 10,000 *g* for 15 minutes at 4°C. The resulting supernatant, consisting of the purified MT fraction, was transferred to a new tube and stored at -80°C for MT quantification.

Determination of MT content was based on a modified Brdička procedure (Raspor, 2001; Raspor et al., 2001) using differential pulse voltammetry (DPV). This was the first time that DPV method was applied to MT quantification in mysid crustaceans. Measurements were performed using 797 VA Computrace (Metrohm, Switzerland) with a hanging mercury drop electrode as a working electrode, an Ag/AgCl/saturated KCl reference electrode and a platinum auxiliary electrode. The voltammetric response showed good correspondence between standard MT and MT curves from *N. integer*, allowing MT quantification. Concentrations of MTs in the samples were derived from the calibration curve, which was constructed – since a MT standard for mysids does not exist – by using purified rabbit liver MT standard material. MT standard used in this study was Ikzus Zinc Metallothionein (MT-95-L) solution (concentration 5.0 mgMT ml^{-1}; >95%-pure rabbit native zinc-metallothionein dissolved into 5 mM Tris-HCl pH 7.5). Calibration curve was constructed in the concentration range from 1.96×10^{-5} to 1.18×10^{-4} mg MT l^{-1}. Relation between MT concentration and peak height of MT signal was linear and can be expressed by equation $y = -4.230\times10^{-4}\ x - 7.663\times10^{-10}$; $R^2 = 0.998$.

2.4. Metal analysis

Concentrations of Zn, Cu and Cd in the cytosolic fraction of Cd-exposed and control *N. integer* were determined. Prior to metal analysis, the isolated cytosolic fraction (see 2.3) was

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diluted ten times with bi-distilled water. Metal analyses were performed using atomic absorption spectrometry (Varian, SpectrAA 220). Flame AAS (air/acetylene) was applied for the Zn (at 213.9 nm) and Cu (324.8 nm) measurements, while graphite furnace AAS with universal platforms (Varian GTA-100) was used for the Cd measurements (at 228.8 nm). A deuterium lamp was used for background correction. External calibration was performed for each metal using the appropriate dilutions of respective metal standard solutions (Merck) in diluted (10×) homogenizing buffer. Detection limits were 0.007 μg ml^{-1}, 0.002 μg ml^{-1} and 0.023 ng ml^{-1} for Zn, Cu and Cd, respectively.

2.5. Statistical analysis

All analyses were performed with the software package Statistica™ (Statsoft, Tulsa, OK, USA). The differences between control and Cd-exposed groups for all of the measured parameters were detected using the Mann-Whitney U test. The effects of salinity and exposure duration were tested for significance using analysis of variance (Tukey's Honestly Significant Difference Test; Bonferroni's Test). Correlations among variables (MT and metal concentrations) were assessed using the Spearman rank order correlation coefficient. All tests were performed at a probability level of 0.05.

3. Results

3.1. Cytosolic metal concentrations

Cadmium, zinc and copper concentrations in the whole body cytosolic fractions of control and cadmium exposed *N. integer* were determined (Fig. 1A-C). Molar concentrations of individual metals were used to evaluate their particular molar ratios.

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Measured cytosolic Cd concentrations in the control groups at all tested salinities (Fig. 1C) ranged from 0.014 to 0.022 μmol l^{-1}. In the control organisms, there were no significant differences in the cytosolic Cd concentrations in mysids exposed to the different salinities. Also no significant effect of the exposure time was observed (Tukey HSD test, $p>0.05$). Therefore, the mean value of all measured controls during the whole exposure period (0.019 ± 0.002 μmol l^{-1}) was used as the estimate of cytosolic Cd concentration in the control group on day 0 (Fig. 1C).

Cytosolic Cd concentrations in all Cd-exposed groups were significantly higher than those in the respective control groups (Mann-Whitney U test, $p<0.05$). The highest concentrations of cytosolic Cd (from 0.384 to 1.427 μmol l^{-1} on day 1 and day 7, respectively) in the Cd-exposed groups were noted in organisms exposed at 25 psu (Fig. 1C). These concentrations were significantly higher than those measured at 5 and 16 psu (Tukey HSD test, $p<0.05$). Differences in cytosolic Cd at salinities of 5 and 16 psu were not significant (Tukey HSD test, $p>0.05$).

If we take into consideration the total cadmium concentration, despite the fact that the exposure concentration of total Cd was 3 times higher at 16 psu (i.e. 23.0 μg l^{-1}) than at 5 psu (7.2 μg l^{-1}), it was not reflected in cytosolic concentrations of accumulated Cd. The cytosolic Cd concentrations in *N. integer* at these two salinities were practically the same (Fig. 1C).

In general, the differences in Zn and Cu concentrations between the control and Cd-exposed groups were not significant (Mann-Whitney U test, $p>0.05$). The Zn concentrations were higher than those observed for copper (Fig. 1A and B). Differences in cytosolic Zn in organisms exposed at different salinities were not significant (Tukey HSD test, $p>0.05$). The concentrations varied (at all tested salinities) in a narrow range, i.e. from 25.4 to 39.4 μmol l^{-1} (Fig. 1A).

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Cu concentrations, however, were significantly different between all tested salinities (Tukey HSD test, $p<0.05$). Measured values (Fig. 1B) ranged from 7.9 μmol l^{-1} at salinity 25 psu to 19.4 μmol l^{-1} at salinity 5 psu. On average, cytosolic Cu concentrations determined at 16 psu and 25 psu were 19% and 31% lower than at 5 psu, respectively.

3.2. Metallothionein contents

Metallothioneins were determined on the whole body heat-treated cytosolic fraction (Fig. 2). In this study we used the control group as a reference, thus eliminating potential stress effects that might be attributed to the sampling location. The differences in MT levels (μg per mg of wet weight) between the control and Cd-exposed groups were not significant (Mann-Whitney U test, $p<0.05$; results not shown) for any salinity – exposure duration combination. Therefore, the data for control and Cd-exposed groups were pooled for subsequent statistical analyses. The measured MT values at all tested salinities ranged from 1.2 to 2.2 μg mg^{-1} w.w. Two-way ANOVA was performed using salinity and exposure duration as independent variables. Significant differences were found between the different salinity treatments ($p<0.0001$). There was a statistically significant interaction between changes in salinity and the day of exposure ($p = 0.04$). To detect which groups differ from the others a multiple comparison procedure was used. *Post hoc* analysis revealed significant differences between 5 and 16 psu and between 5 and 25 psu (Bonferroni's Test, $p<0.05$) only on day 7. The largest difference (42%) was observed on day 7 between the 5 and 16 psu treatment. While the cadmium exposure did not influence the MT levels in *N. integer*, the salinity obviously did. Exposure duration did not significantly affect the MT levels ($p = 0.07$).

4. Discussion

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Although cadmium is a non-essential metal, it is taken up from water by all aquatic organisms that depend on water for the exchange of ions and gases (Rainbow, 1985). This study was designed to eliminate the effect of salinity – a dominant physico-chemical factor in marine environments – on cadmium speciation. Therefore, Cd exposure concentration expressed as Cd^{2+} was the same at each of the tested salinities.

Our study presents the first data on cytosolic metal concentrations in *N. integer*. Based on these concentrations it is clear that cadmium accumulation in *N. integer* exposed to the same concentration of bioavailable Cd is dependent on the salinity (Fig. 1C). The concentrations of cytosolic cadmium were significantly higher (up to 22 % at day 7) at 25 psu than at 5 and 16 psu. In general, the accumulated metal concentration depends on the net difference between the uptake and excretion rate (Luoma and Rainbow, 2005). How much metal can be retained by a tissue depends on the nature and extent of the metal detoxification processes available (Rainbow, 2002). All these processes are strongly affected by features of the biology of the organism i.e., the crustacean's morphology, its physiology, its mode of feeding and its ecophysiological adaptations to the physico-chemistry of the environment (Rainbow, 1998). *N. integer* being a strong osmoregulator keeps the ionic pressure of its hemolymph within a narrow range over a salinity range of 2-30 psu (De Lisle and Roberts, 1987). In a medium of higher osmotic pressure than their hemolymph, crustaceans lose water via osmosis, which is replaced by uptake of seawater via the mouth (i.e., drinking), the anus or both (Roast et al., 2001). Therefore, when hypo-regulating increased uptake of trace metals may occur through indiscriminate uptake as a results of e.g. increased drinking. The increased cadmium accumulation observed in our study at high salinity (25 psu) may be the result of this phenomenon.

If there was an additional uptake of Cd in some form other than Cd^{2+} at 25 psu due to higher total Cd concentration (38.1 $\mu g\ l^{-1}$), the same situation would be also expected to occur at

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salinity 16 psu with respect to 5 psu, which was not the case. This result was difficult to explain unequivocally. It is very likely that the specific physiological adaptations in *N. integer* are responsible for certain specificities in metal uptake and accumulation at different salinities.

At the isosmotic point, water exchange is minimal because osmotic pressures between the hemolymph and external medium are in equilibrium. The associated low ionic exchange has been suggested to result in reduced uptake (and toxicity) of cadmium in *Mysidopsis bahia* (De Lisle and Roberts, 1988). The reduction in cadmium toxicity at the isosmotic point compared to salinities below and above the isosmotic point, has also been reported for *N. integer* (Wildgust and Jones, 1998). In the present study, cadmium accumulation was 43% (day 1) to 22% (day 7) lower at the isosmotic point (16 psu) than at 25 psu (Fig. 1C), which confirms the observations and statements made by the above cited authors. It should be noted however, that we did not measure the uptake but net accumulation.

At 5 psu, the cytosolic Cd levels in Cd-exposed *N. integer* were comparable to the levels observed at isosmotic point. Such a relatively high Cd level at low salinity could be due to the metal being taken up non-specifically via ionic pumps for major ions (Rainbow, 1995). The cadmium ion can enter via calcium channels since it has an ionic radius of 0.9 Å, which is very similar to that of Ca^{2+} (0.99 Å) (Rainbow, 1997). On the other hand, a reduction in the rate of cadmium uptake from solution could be expected in a medium of low salinity if the organism makes a physiological response to low salinity (Rainbow and Black, 2002), such as the reduction in apparent water permeability observed in some euryhaline crustaceans (Rasmusen and Andersen, 1996). However, this was clearly not the case in our study.

Since Zn and Cu were not added into the tested media we assume that the observed cytosolic levels of these essential metals correspond to their constituent levels. We did not observe any significant differences in cytosolic Zn and Cu concentrations between the control and Cd-

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exposed groups (Fig. 1A and B). Also changes in salinity did not cause significant differences in cytosolic Zn levels (Fig 1A). It is known that decapod crustaceans (e.g. crabs, shrimps and prawns) possess mechanisms (active regulation, storage, or a combination of both mechanisms) to regulate their body concentrations of some essential metals, especially zinc and copper. Active regulators maintain stable body concentrations over a wide range of external metal availability conditions by excreting the metal at rates comparable to the intake rate (Rainbow, 1998). This observation is corroborated by our study on mysid species in which the measured concentrations of cytosolic Zn (Fig. 1A) were relatively constant over the range of tested salinities.

An interesting finding in this study was the inverse relationship between salinity and cytosolic Cu concentration in *N. integer* (Fig. 1B). In the hemolymph of mysids, oxygen is carried by hemocyanin, which contains copper in an active site. In shrimps, generally more than 40–50% of the body copper load is associated with the hemolymph (Depledge, 1989). Taylor et al. (1985) examined the effects of salinity acclimation on the oxygen-transporting properties of the hemolymph of the intertidal prawn *Palaemon elegans*. They observed an increase in the hemocyanin content of the hemolymph with a decrease in the salinity of the medium, i.e. an inverse relationship between hemocyanin content and salinity to which the animals were acclimated. This is in agreement with the inverse relationship between salinity and cytosolic Cu concentration in *N. integer* observed in our study.

We also observed that changes in Cd accumulation due to salinity were not reflected in the MT levels. Indeed, no significant differences in MT levels between the control and Cd-exposed groups were noted. Apparently, cadmium exposure concentration used in this study was too low to induce significant increases in MT levels (Fig. 2). However, changes in Cd accumulation may have been reflected in increased MT turnover, as suggested in a related malacostracan crustacean, *Orchestia gammarellus* (Mouneyrac et al., 2002). Furthermore,

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high molar ratios of essential metals vs. cadmium (Table 1) indicated an excess of metals other than cadmium that can bind to MTs (especially Cu and Zn). In such case, despite the high affinity of MT to bind Cd it is possible that Cd cannot successfully compete for the binding sites on the MTs. Instead, cadmium may be bound to other soluble cytosolic molecules, e.g. glutathione. As such, glutathione has been considered as the first line of defence against heavy metal cytotoxicity (Viarengo and Nott, 1993).

In order to determine the relationship between MT and cytosolic metal concentrations linear regression analysis was applied on all data, i.e. data obtained at all tested salinities irrespective of the exposure duration. A significant positive correlation was noted between MT levels and cytosolic Cu concentrations (Table 2). One of the characteristics of MTs is coexistence of different isoforms with different functions within organisms (Amiard et al., 2006). Recently, three MT encoding genes have been identified in the blue crab, *Callinectes sapidus*: MT-I, inducible by cadmium, zinc and copper; MT-II, inducible by cadmium and zinc; and MT-III, inducible by copper only (Syring et al., 2000; Brouwer et al., 2002). Their results support the hypothesis that the copper-specific metallothionein (CuMT-III) is involved in copper homeostasis associated with both the synthesis and degradation of hemocyanin. In our study, the significant positive correlation between MT levels and cytosolic Cu concentrations, and the inverse relationship between salinity and MT may point to the predominance of copper specific MT in *N. integer*.

5. Conclusions

This study presents the first results of MT quantification in the estuarine mysid *N. integer.* Although, an increase in cytosolic concentrations of Cd in *N. integer* was observed, no subsequent increase in MT level was detected. However, a significant positive correlation was

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found between MT levels and cytosolic Cu concentrations implying a strong relationship between MT and Cu in *N. integer*.

The accumulation of cytosolic Cd in *N. integer* was dependent on the salinity, which confirms the fact that physiological changes in this euryhaline organism can affect trace metal uptake from solution.

An inverse relationship between salinity and cytosolic Cu concentration was observed. The suggested possible relationship between copper concentrations and hemocyanin metabolism in *N. integer* needs further research.

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Figure captions

Figure 1.

Concentrations of zinc (A), copper (B) and cadmium (C) in cytosolic fractions of *Neomysis integer* (symbols and bars represent mean values and standard deviations, respectively; in Fig. 1C day 0 concentration was estimated as an mean value of all measured controls during the exposure period), ◆ - 5 psu, control; ◇ - 5 psu, Cd-exposed; ■ - 16 psu, control; □ - 16 psu, Cd-exposed; ▲ - 25 psu, control; △ - 25 psu, Cd-exposed

Figure 2.

Metallothionein concentration in *Neomysis integer* (data for control and Cd-exposed groups were pooled) at different salinities on day 1, day 4 and day 7 of the experiment. Graphs are showing median values, quartiles (boxes) and range (whiskers).

Tables

Table 1.

Molar ratios of cytosolic zinc and copper vs. cadmium in *Neomysis integer* exposed to cadmium for 1, 4 and 7 days and at three salinities (5, 16 and 25 psu).

Table 2.

Spearman correlation coefficients (r_s) and respective probability levels (p) between metallothionein and cytosolic metals in *Neomysis integer* for all tested salinities (a – control groups, b – exposed groups, * – significant correlation, $p<0.05$).

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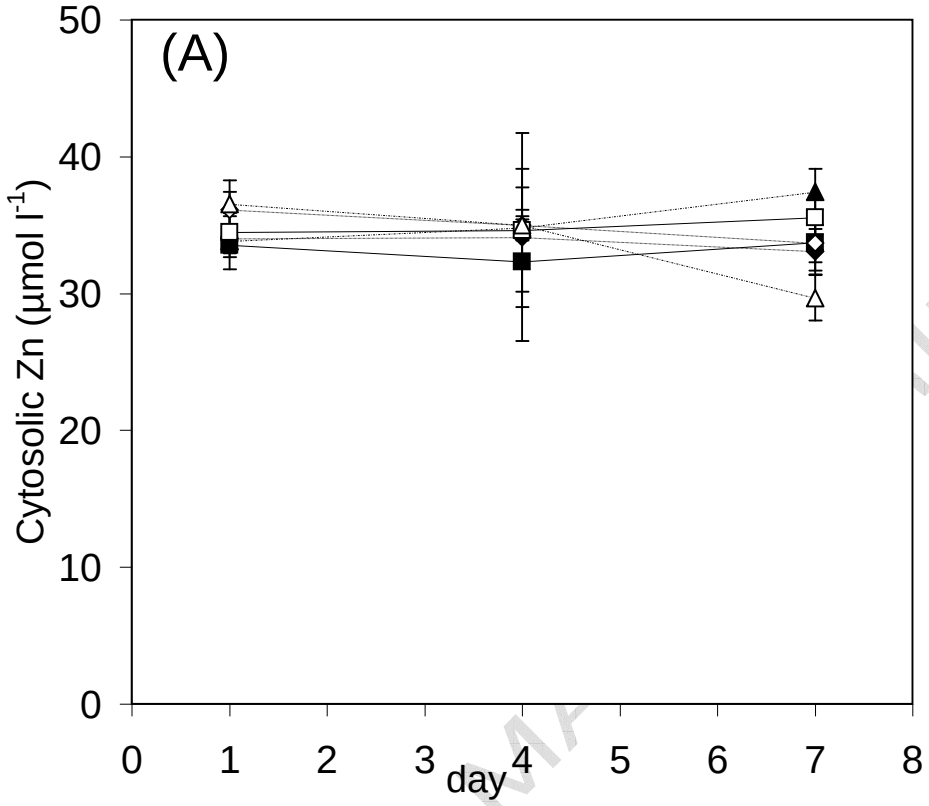

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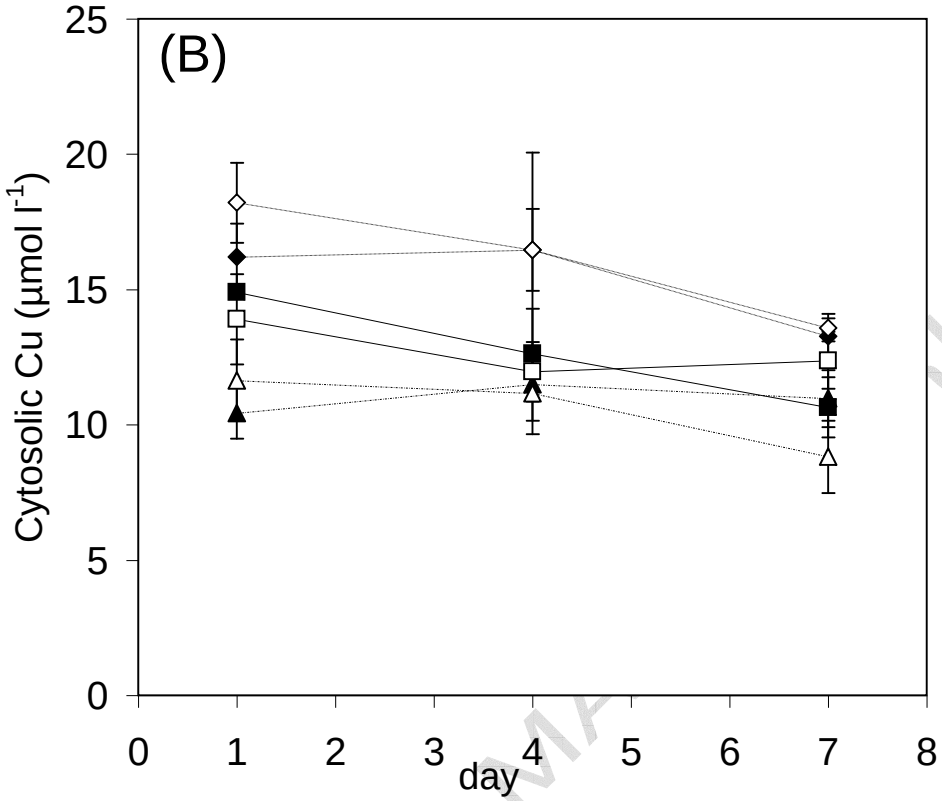

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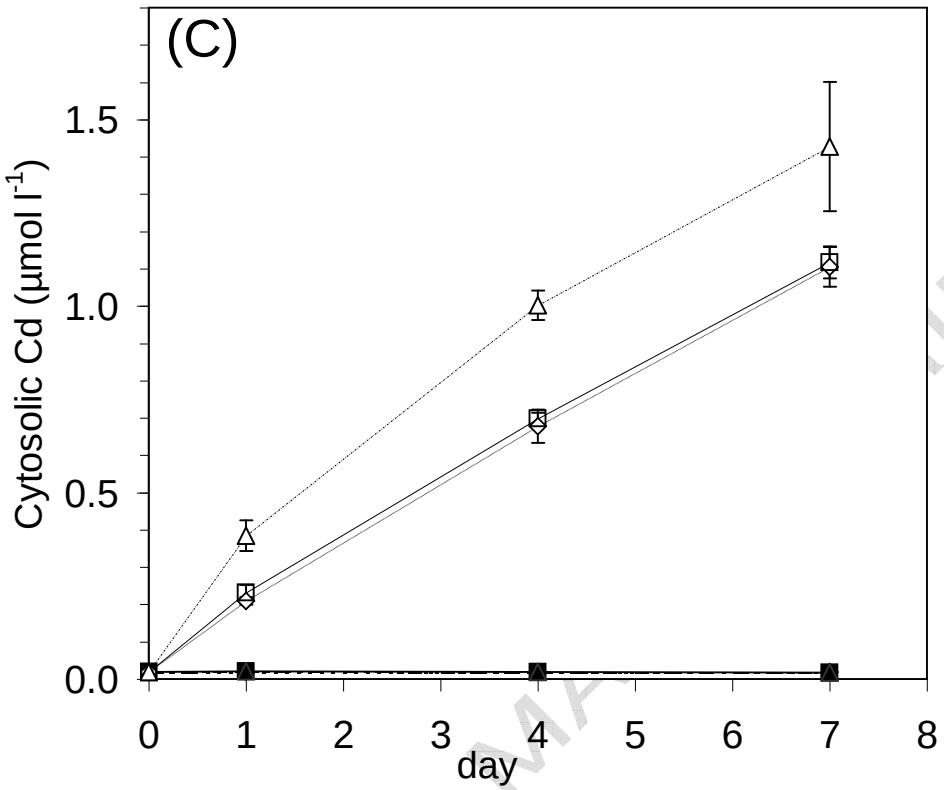

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Figure_2PowerPoint

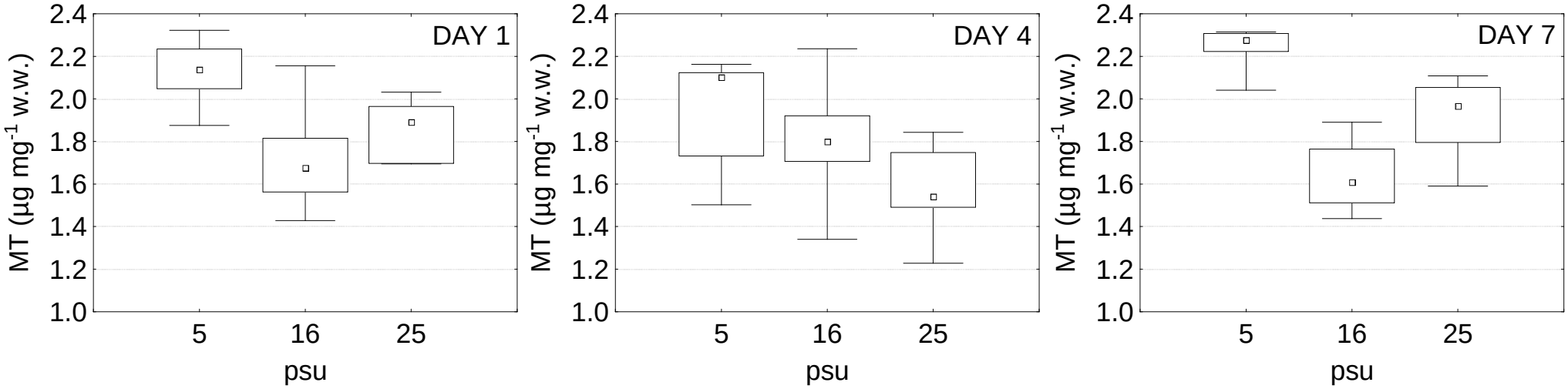

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Table 1.

Molar ratios of cytosolic zinc and copper vs. cadmium in *Neomysis integer* exposed to cadmium for 1, 4 and 7 days and at three salinities (5, 16 and 25 psu).

	5 psu			16 psu			25 psu		
	day 1	day 4	day 7	day 1	day 4	day 7	day 1	day 4	day 7
Zn:Cd	173	52	30	148	50	32	95	35	21
Cu:Cd	87	24	12	60	17	11	30	11	6

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Table 2.

Spearman correlation coefficients (r_s) and respective probability levels (p) between metallothionein and cytosolic metals in *Neomysis integer* for all tested salinities (a – control groups, b – exposed groups, * – significant correlation, $p<0.05$).

	R	p
Zn	0.117	0.403
Cu*	0.356	0.009
Cd[a]	-0.101	0.616
Cd[b]	-0.162	0.430

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