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

Marijana Erk^{a,b*}, Brita T.A. Muysen^b, An Ghekiere^c and Colin R. Janssen^b

^a Ruđer Bošković Institute, Division for Marine and Environmental Research, Bijenička c. 54, P.O.Box 180, HR-10002 Zagreb, Croatia

^b Laboratory of Environmental Toxicology and Aquatic Ecology, Ghent University, J. Plateaustraat 22, B-9000 Ghent, Belgium

^c EURAS, Kortrijksesteenweg 302, B-9000 Ghent, Belgium

* corresponding author; current address Ruđer Bošković Institute, Division for Marine and Environmental Research, Bijenička c. 54, P.O.Box 180, HR-10002 Zagreb, Croatia

phone: +385 1 4680216

fax: +385 1 4680242

e-mail: erk@irb.hr

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 $5.74 \times 10^{-9} \text{ mol l}^{-1}$ (i.e. 1/5 of the 96h LC₅₀ 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 $1.11 \pm 0.05 \text{ } \mu\text{mol l}^{-1}$ at 5 psu up to $1.43 \pm 0.17 \text{ } \mu\text{mol l}^{-1}$ 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: $34.4 \pm 2.8 \text{ } \mu\text{mol l}^{-1}$), an inverse relationship between salinity and cytosolic Cu concentration was observed. The highest concentration of $15.7 \pm 2.3 \text{ } \mu\text{mol Cu l}^{-1}$ was determined at 5 psu and the lowest $10.9 \pm 1.4 \text{ } \mu\text{mol Cu l}^{-1}$ 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

1 **1. Introduction**

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

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

1 2002). Free cadmium species account for ca. 90% of the cadmium in the freshwater zone,
2 whereas in marine systems chloro-complexes dominate the speciation distribution (Stumm
3 and Morgan, 1996; Sadiq, 1992). It has been generally accepted that the toxicity of cadmium
4 to aquatic animals changes as a function of ambient salinity with the metal generally being
5 more toxic at low salinities. The effect of salinity on cadmium toxicity occurs primarily due to
6 greater complexation of the free cadmium ion (Cd^{2+}) by the conservative ligand Cl^- (Sunda et
7 al., 1978; De Lisle and Roberts, 1988). Study on the permeability of cadmium through lipid
8 bilayer membranes suggested that cadmium transport and toxicity were protein mediated and
9 correlated with Cd^{2+} , not CdCl_2 , concentration (Gutknecht, 1983).

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

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

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

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

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

19

20

21 **2. Materials and Methods**

22 2.1. Animal collection and maintenance

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

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

3 In the laboratory the organisms were transferred to 200 L glass aquaria. Culture medium was
4 artificial seawater (Instant Ocean[®], Aquarium Systems, France) diluted with aerated deionized
5 tap water to a final salinity of 5 psu. Water temperature was maintained at $15\pm 1^\circ\text{C}$, and 12h
6 light:12h dark photoperiod was used during culturing. Animals were fed *ad libitum* daily with
7 24 - 48h old *Artemia* nauplii. Hatching of the *Artemia* cysts was performed in 1 L conical
8 vessels under vigorous aeration and continuous illumination at 25°C (Sorgeloos et al., 1986).

9

10 2.2 Exposure experiment

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

1 Juveniles of approximately the same size (1 cm) were used for the experiment in order to
2 minimise the differences in weight, sex and reproductive status. Animals (the average wet
3 weight: 6.3 ± 2.1 mg) were collected from the culture aquarium and randomly distributed into
4 10L solid glass experimental aquaria. For control and Cd-treatment at each salinity and time-
5 point, one aquarium containing 120 individuals was set up. Test animals were allowed 24h to
6 acclimate (osmotically) to different test salinities prior to the cadmium exposure. This
7 acclimation period has been shown to be sufficient for *N. integer* to attain a new steady state,
8 since mysids rapidly acclimate to salinity changes (De Lisle and Roberts, 1987).

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

18 19 2.3. Isolation and quantification of metallothioneins

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

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

7 Determination of MT content was based on a modified Brdička procedure (Raspor, 2001;
8 Raspor et al., 2001) using differential pulse voltammetry (DPV). This was the first time that
9 DPV method was applied to MT quantification in mysid crustaceans. Measurements were
10 performed using 797 VA Computrace (Metrohm, Switzerland) with a hanging mercury drop
11 electrode as a working electrode, an Ag/AgCl/saturated KCl reference electrode and a
12 platinum auxiliary electrode. The voltammetric response showed good correspondence
13 between standard MT and MT curves from *N. integer*, allowing MT quantification.
14 Concentrations of MTs in the samples were derived from the calibration curve, which was
15 constructed – since a MT standard for mysids does not exist – by using purified rabbit liver
16 MT standard material. MT standard used in this study was Izkus Zinc Metallothionein (MT-
17 95-L) solution (concentration 5.0 mgMT ml⁻¹; >95%-pure rabbit native zinc-metallothionein
18 dissolved into 5 mM Tris-HCl pH 7.5). Calibration curve was constructed in the concentration
19 range from 1.96×10⁻⁵ to 1.18×10⁻⁴ mg MT l⁻¹. Relation between MT concentration and peak
20 height of MT signal was linear and can be expressed by equation $y = -4.230 \times 10^{-4} x -$
21 7.663×10^{-10} ; $R^2 = 0.998$.

22

23 2.4. Metal analysis

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

1 diluted ten times with bi-distilled water. Metal analyses were performed using atomic
2 absorption spectrometry (Varian, SpectrAA 220). Flame AAS (air/acetylene) was applied for
3 the Zn (at 213.9 nm) and Cu (324.8 nm) measurements, while graphite furnace AAS with
4 universal platforms (Varian GTA-100) was used for the Cd measurements (at 228.8 nm). A
5 deuterium lamp was used for background correction. External calibration was performed for
6 each metal using the appropriate dilutions of respective metal standard solutions (Merck) in
7 diluted (10×) homogenizing buffer. Detection limits were $0.007 \mu\text{g ml}^{-1}$, $0.002 \mu\text{g ml}^{-1}$ and
8 0.023 ng ml^{-1} for Zn, Cu and Cd, respectively.

9

10 2.5. Statistical analysis

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

18

19

20 3. Results

21 3.1. Cytosolic metal concentrations

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

1 Measured cytosolic Cd concentrations in the control groups at all tested salinities (Fig. 1C)
2 ranged from 0.014 to 0.022 $\mu\text{mol l}^{-1}$. In the control organisms, there were no significant
3 differences in the cytosolic Cd concentrations in mysids exposed to the different salinities.
4 Also no significant effect of the exposure time was observed (Tukey HSD test, $p>0.05$).
5 Therefore, the mean value of all measured controls during the whole exposure period ($0.019 \pm$
6 $0.002 \mu\text{mol l}^{-1}$) was used as the estimate of cytosolic Cd concentration in the control group on
7 day 0 (Fig. 1C).

8 Cytosolic Cd concentrations in all Cd-exposed groups were significantly higher than those in
9 the respective control groups (Mann-Whitney U test, $p<0.05$). The highest concentrations of
10 cytosolic Cd (from 0.384 to 1.427 $\mu\text{mol l}^{-1}$ on day 1 and day 7, respectively) in the Cd-
11 exposed groups were noted in organisms exposed at 25 psu (Fig. 1C). These concentrations
12 were significantly higher than those measured at 5 and 16 psu (Tukey HSD test, $p<0.05$).
13 Differences in cytosolic Cd at salinities of 5 and 16 psu were not significant (Tukey HSD test,
14 $p>0.05$).

15 If we take into consideration the total cadmium concentration, despite the fact that the
16 exposure concentration of total Cd was 3 times higher at 16 psu (i.e. 23.0 $\mu\text{g l}^{-1}$) than at 5 psu
17 (7.2 $\mu\text{g l}^{-1}$), it was not reflected in cytosolic concentrations of accumulated Cd. The cytosolic
18 Cd concentrations in *N. integer* at these two salinities were practically the same (Fig. 1C).

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

1 Cu concentrations, however, were significantly different between all tested salinities (Tukey
2 HSD test, $p < 0.05$). Measured values (Fig. 1B) ranged from $7.9 \mu\text{mol l}^{-1}$ at salinity 25 psu to
3 $19.4 \mu\text{mol l}^{-1}$ at salinity 5 psu. On average, cytosolic Cu concentrations determined at 16 psu
4 and 25 psu were 19% and 31% lower than at 5 psu, respectively.

5

6 3.2. Metallothionein contents

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

23

24

25 4. Discussion

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

6 Our study presents the first data on cytosolic metal concentrations in *N. integer*. Based on
7 these concentrations it is clear that cadmium accumulation in *N. integer* exposed to the same
8 concentration of bioavailable Cd is dependent on the salinity (Fig. 1C). The concentrations of
9 cytosolic cadmium were significantly higher (up to 22 % at day 7) at 25 psu than at 5 and 16
10 psu. In general, the accumulated metal concentration depends on the net difference between
11 the uptake and excretion rate (Luoma and Rainbow, 2005). How much metal can be retained
12 by a tissue depends on the nature and extent of the metal detoxification processes available
13 (Rainbow, 2002). All these processes are strongly affected by features of the biology of the
14 organism i.e., the crustacean's morphology, its physiology, its mode of feeding and its
15 ecophysiological adaptations to the physico-chemistry of the environment (Rainbow, 1998).
16 *N. integer* being a strong osmoregulator keeps the ionic pressure of its hemolymph within a
17 narrow range over a salinity range of 2-30 psu (De Lisle and Roberts, 1987). In a medium of
18 higher osmotic pressure than their hemolymph, crustaceans lose water via osmosis, which is
19 replaced by uptake of seawater via the mouth (i.e., drinking), the anus or both (Roast et al.,
20 2001). Therefore, when hypo-regulating increased uptake of trace metals may occur through
21 indiscriminate uptake as a results of e.g. increased drinking. The increased cadmium
22 accumulation observed in our study at high salinity (25 psu) may be the result of this
23 phenomenon.

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

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

5 At the isosmotic point, water exchange is minimal because osmotic pressures between the
6 hemolymph and external medium are in equilibrium. The associated low ionic exchange has
7 been suggested to result in reduced uptake (and toxicity) of cadmium in *Mysidopsis bahia* (De
8 Lisle and Roberts, 1988). The reduction in cadmium toxicity at the isosmotic point compared
9 to salinities below and above the isosmotic point, has also been reported for *N. integer*
10 (Wildgust and Jones, 1998). In the present study, cadmium accumulation was 43% (day 1) to
11 22% (day 7) lower at the isosmotic point (16 psu) than at 25 psu (Fig. 1C), which confirms
12 the observations and statements made by the above cited authors. It should be noted however,
13 that we did not measure the uptake but net accumulation.

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

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

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

10 An interesting finding in this study was the inverse relationship between salinity and cytosolic
11 Cu concentration in *N. integer* (Fig. 1B). In the hemolymph of mysids, oxygen is carried by
12 hemocyanin, which contains copper in an active site. In shrimps, generally more than 40–50%
13 of the body copper load is associated with the hemolymph (Depledge, 1989). Taylor et al.
14 (1985) examined the effects of salinity acclimation on the oxygen-transporting properties of
15 the hemolymph of the intertidal prawn *Palaemon elegans*. They observed an increase in the
16 hemocyanin content of the hemolymph with a decrease in the salinity of the medium, i.e. an
17 inverse relationship between hemocyanin content and salinity to which the animals were
18 acclimated. This is in agreement with the inverse relationship between salinity and cytosolic
19 Cu concentration in *N. integer* observed in our study.

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

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

7 In order to determine the relationship between MT and cytosolic metal concentrations linear
8 regression analysis was applied on all data, i.e. data obtained at all tested salinities
9 irrespective of the exposure duration. A significant positive correlation was noted between
10 MT levels and cytosolic Cu concentrations (Table 2). One of the characteristics of MTs is
11 coexistence of different isoforms with different functions within organisms (Amiard et al.,
12 2006). Recently, three MT encoding genes have been identified in the blue crab, *Callinectes*
13 *sapidus*: MT-I, inducible by cadmium, zinc and copper; MT-II, inducible by cadmium and
14 zinc; and MT-III, inducible by copper only (Syring et al., 2000; Brouwer et al., 2002). Their
15 results support the hypothesis that the copper-specific metallothionein (CuMT-III) is involved
16 in copper homeostasis associated with both the synthesis and degradation of hemocyanin. In
17 our study, the significant positive correlation between MT levels and cytosolic Cu
18 concentrations, and the inverse relationship between salinity and MT may point to the
19 predominance of copper specific MT in *N. integer*.

20

21

22 **5. Conclusions**

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

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

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

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

9

10

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5

ACCEPTED MANUSCRIPT

1 **Figure captions**

2

3 Figure 1.

4 Concentrations of zinc (A), copper (B) and cadmium (C) in cytosolic fractions of *Neomysis*
5 *integer* (symbols and bars represent mean values and standard deviations, respectively; in Fig.
6 1C day 0 concentration was estimated as an mean value of all measured controls during the
7 exposure period), ◆ - 5 psu, control; ◇ - 5 psu, Cd-exposed; ■ - 16 psu, control; □ - 16 psu,
8 Cd-exposed; ▲ - 25 psu, control; △ - 25 psu, Cd-exposed

9

10 Figure 2.

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

14

15

16

17 **Tables**

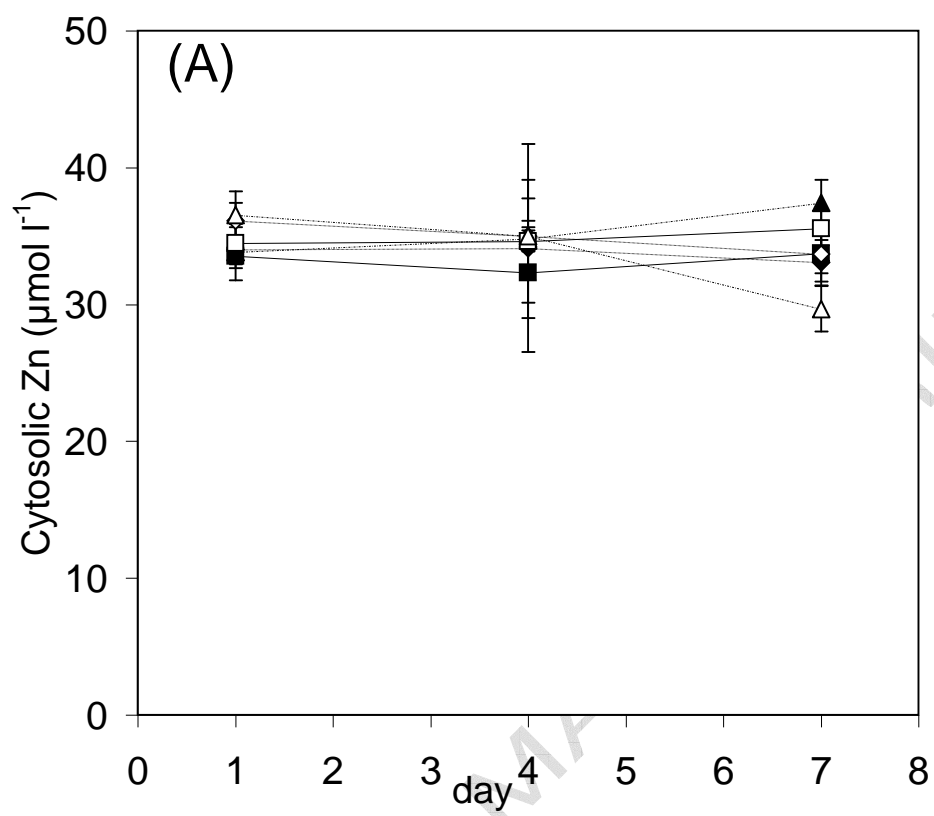
18 Table 1.

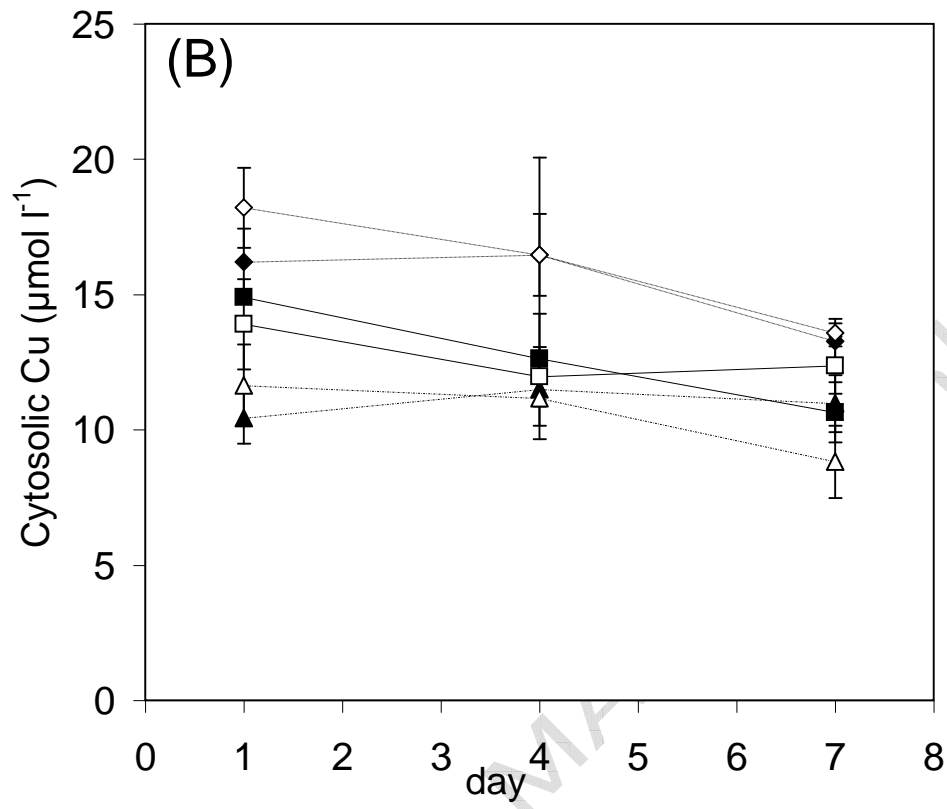
19 Molar ratios of cytosolic zinc and copper vs. cadmium in *Neomysis integer* exposed to
20 cadmium for 1, 4 and 7 days and at three salinities (5, 16 and 25 psu).

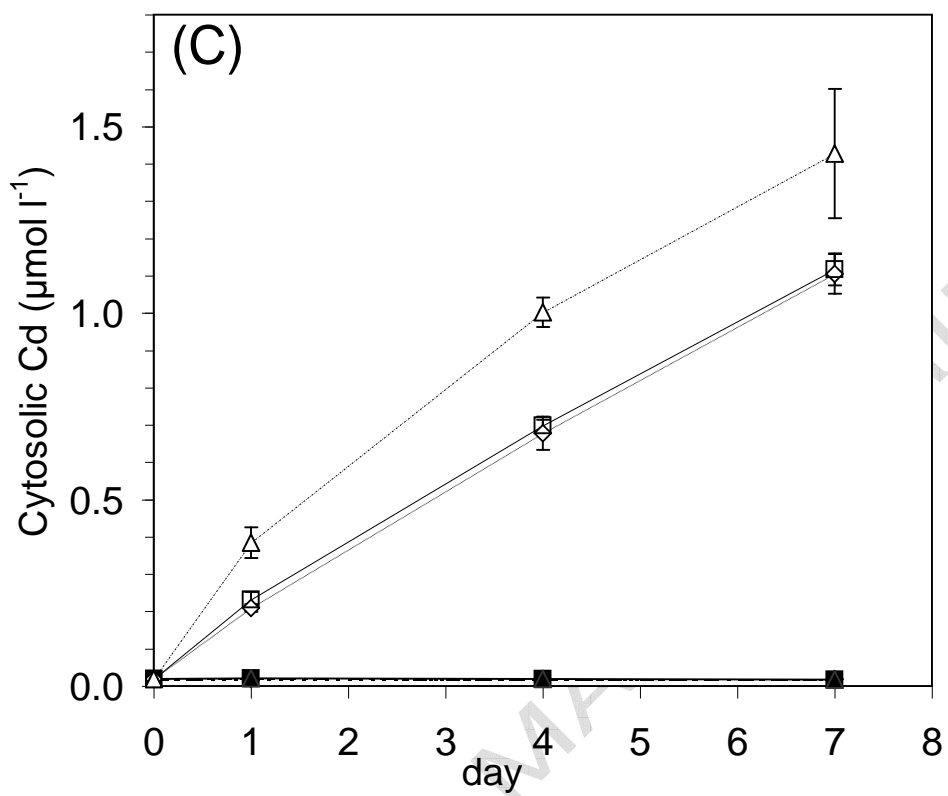
21

22 Table 2.

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







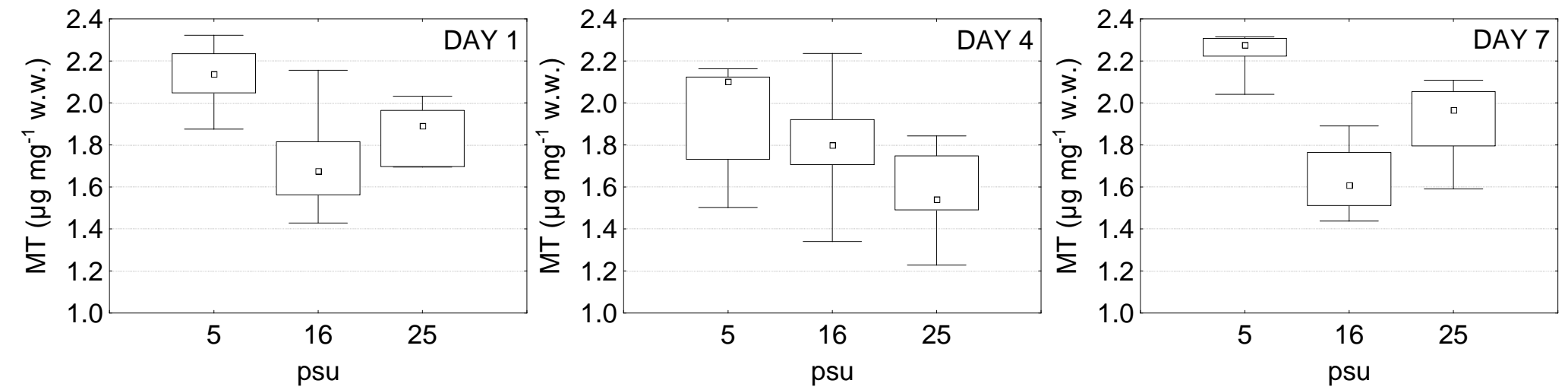


Table 1.

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

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