Metallothionein and cellular energy allocation in the estuarine mysid shrimp Neomysis integer exposed to cadmium at different salinities

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Abstract

In the present study the induction of metallothioneins (MTs) and effects on the cellular energy allocation (CEA) in euryhaline crustacean Neomysis integer exposed to Cd at different salinities were studied. N. integer was exposed to the same sub-lethal concentration of free cadmium ion (1/5 of the cadmium activity of the reported 96h LC_{50} value) in hypo-osmotic (7.2 µg Cd/L at 5 psu), isosmotic (23.0 µg Cd/L at 16 psu) and hyper-osmotic media (38.1 µg Cd/L at 25 psu) for 7 days. By using the free Cd concentration as the basis for conducting the exposures, the effect of salinity on cadmium speciation was eliminated and therefore the true effect of salinity as an abiotic factor on the MT induction and CEA could be studied.

MT content was quantified by differential pulse voltammetry (DPV); this is the first time that this method is applied to assays with N. integer. No significant differences in MT levels between the control and Cd-exposed groups were observed. The measured MT levels (ranging from 32.3 to 75.7 $\mu g/mg_{(cytosolic\ protein)}$) probably represent the constitutive MT levels responsible for binding essential metals. The differences in MT levels observed at the different salinities indicate a possible relationship between some physiological process (possibly osmoregulation) other than detoxification and MT induction. A decrease in salinity caused an increase in MT level in N. integer, and this was significantly correlated to the CEA values (Spearman correlation coefficient $r = -0.56$, $p = 0.016$). Our results indicate that salinity can change the energy status and MT content of N. integer. These findings should be taken into account when using these biomarkers - and especially MTs - in field studies and environmental monitoring.

Key words: mysid, Neomysis integer, cadmium, salinity, bioavailability, metallothionein, cellular energy allocation

1. Introduction

Next to the natural sources, a large number of metals are present in the environment as a result of human activities. 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). In surface waters, cadmium can be – dependent on the abiotic factors – present in different physico-chemical forms (Stumm and Morgan, 1996). Free cadmium species account for ca. 90% of the cadmium in the freshwater zone, whereas in the marine area the chloro-complexes dominate the distribution (Stumm and Morgan, 1996; Sadiq, 1992). In this context, estuaries represent particularly variable environments characterised by large temporal and spatial fluctuations in abiotic factors such as salinity, temperature and dissolved organic matter (Baeyens et al., 1998; Uncles, 2002; Riba et al., 2003).

Biomarkers have been proposed and used to aid in the assessment of environmental risks posed by contaminants (Stagg, 1998; UNEP/RAMOGE, 1999). A biomarker can be broadly defined as a biochemical, cellular, physiological or behavioural alteration measured in tissue- or body fluid samples, or at the organismal level, that provides evidence of exposure to and/or effects of one or more chemical contaminants (Depledge, 1994). Use of metallothioneins (MTs) as potential molecular biomarkers of metal exposure is frequently reported (Amiard et al., 2006 and the references therein). However, variations in MT levels can also be caused by factors other than exposure to metals. Many biological functions of MTs have been reported; these include their primary role in maintaining cellular homeostasis of essential metals (e.g. cytosolic pool of Zn and Cu); non-essential metal detoxification (e.g., Cd, Hg and Ag) and their role in the inflammation process and free radical scavenging (Vallee and Maret, 1993). Other factors unrelated to metal exposure can also induce MT synthesis; these include organism handling, starvation, anoxia, freezing, and the presence of antibiotics, vitamins or herbicides (Amiard et

al., 2006). The influences of food abundance, reproductive cycle, and seasonal and spatial factors on the MT levels have been reported for marine bivalves (Cotou et al., 2001; Ivanković et al., 2005). From this it should be clear that the use of MTs as biomarkers of metal exposure in the field requires exact knowledge of these influencing factors. Without this information, differences in biomarker levels in organisms from distinct locations may erroneously be attributed to anthropogenic influences.

In addition to molecular biomarkers, physiological markers reflecting the energetics of an organism may contribute to the understanding of the mode of action of the toxicant (Lorenzon et al., 2000; Smith et al., 2001; Li et al., 2002; Nicholson and Lam, 2005). The differential allocation of energy to basal metabolism, growth and reproduction can change in response to changing environmental conditions and/or to contaminant exposure. Based on this concept, the cellular energy allocation (CEA) methodology has been developed (De Coen and Janssen, 1997) and successfully applied in both laboratory (Verslycke and Janssen, 2002; Verslycke et al., 2003a, 2003b) and field experiments (Verslycke et al., 2004; Smolders et al., 2004). Although the CEA assay has been used to evaluate effects of a number of organic substances on the energetic processes of N. integer in the laboratory (Verslycke, 2003), until now it has never been used to evaluate effects of metals on this invertebrate.

This study aimed at examining metallothionein induction (i.e. a molecular biomarker of metal exposure) and cellular energy allocation (i.e. physiological biomarker) in the euryhaline crustacean N. integer exposed to Cd at different salinities. To our knowledge, MT levels have never been determined in this species. N. integer was selected as a test organism, because it is one of the most common mysids inhabiting estuaries along the European coasts and it is sensitive to many toxicants at environmentally relevant concentrations (Roast et al., 2001; Verslycke et al., 2003a, Wildgust and Jones, 1998). The laboratory experiments were designed to answer the question on how N. integer reacts to a sub-lethal cadmium concentration at

different salinities (hypo-, iso-, and hyper-osmotic medium) when the concentrations of free cadmium ion were identical in all salinity treatments. By using the free Cd concentration as the basis for conducting the exposures, the effect of salinity on cadmium speciation was eliminated and therefore the true effect of salinity as an abiotic factor on the MT induction and CEA could be studied.

2. Material 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 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, and kept for seven days prior to the start of experiment. The 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 12-h light:12-h dark photoperiod was used during culturing. Animals were fed ad libitum daily with 24 to 48-h-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 Set-up and experimental conditions

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 in terms of free cadmium ion activity. It represents 1/5 of the cadmium activity of the reported 96h LC_{50}: 2.87×10^{-8} mol/L in terms of free cadmium ion activity at 5 psu or 45 μg Cd/L in terms of the dissolved cadmium (Verslycke et al., 2003a). 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). Based on the vMINTEQ calculations, the same Cd^{2+} activity was used at each of the tested salinities. The selected test concentrations in terms of the dissolved cadmium were 7.2, 23.0 and 38.1 μg/L 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. Cadmium was re-dosed during the experiment every 48h, i.e. the control- and exposure-media were renewed every 48h. Juveniles of approximately the same size (1 cm) were used for the experiment. By using this specific life-cycle stage, possible confounding factors in the lipid metabolism (high amounts of lipids and the lipid metabolism of ovigerous females being different from that of males) and thus in CEA measurements were avoided. Animals (the average wet weight of all animals used was 6.3±2.1 mg) were collected from the culture aquarium and randomly distributed into 10L solid glass experimental aquaria. For each 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 (De Lisle and Roberts, 1987).

Exposure experiments were performed in a temperature-controlled chamber (Liebher®, Laborimpex, Belgium) at 15±1°C and a 12-h light:12-h 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 dissolved 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 to 48-h-old Artemia nauplii; ad libitum to prevent cannibalism.

2.3. Metallothionein measurements

2.3.1. Isolation of the cytosolic fraction and purification of the MT fraction

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 the control and cadmium 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 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 then transferred to a new tube and stored at -80°C for MT quantification.

2.3.2. Quantification of MTs

Determination of MT content was based on modified Brdička procedure (Raspor, 2001; Raspor et al., 2001) using a differential pulse voltammetry (DPV). This is an electrochemical method for quantification of MTs, in which an electrochemical signal responds to thiol-groups (SH-groups) of cystein residues. Quantification of MT is based on the linear relationship between the

height of catalytic hydrogen peak (recorded at potential -1.46V) and the concentration of cystein residues. This was the first time that DPV method was applied to MT quantification in mysid crustaceans. The voltammetric response recorded for MT in *N. integer* showed a good correspondence with the standard MT material (Fig. 1A and B), allowing MT quantification. Voltammograms recorded in DP mode with the heat treated cytosolic fraction isolated from *N. integer* and with the standard MT both showed the MT peak at the potential -1.46V (Fig. 1A and B).

Measurements were performed using 797 VA Computrace (Metrohm, Switzerland) with hanging mercury drop electrode (HMDE, drop size 4) as a working electrode, an Ag/AgCl/saturated KCl reference electrode and a platinum auxiliary electrode. The analysis was performed in 10 mL of de-aerated Brdička supporting electrolyte (1M NH_4OH + 1M NH_4Cl and 0.6 mM $Co(NH_3)_6Cl_3$) to which 20 – 40μL of the sample was added. The instrumental working conditions were: negative potential scan from -0.9 to -1.65 V, pulse amplitude 0.02505 V, pulse time 0.057 s, voltage step 0.002594 V, voltage step time 0.2 s, sweep rate 0,0130 V/s. The peak used for MT quantification was recorded at potential -1.46±0.05V. All measurements were performed at constant temperature 20.0±0.5°C. 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-95L, Ikzus Proteomics, Italy). The MT concentrations were expressed as μg MT/mg cytosolic protein. Two aliquots of the cytosolic fraction were used for the quantification of the total cytosolic proteins (Bradford, 1976).

2.4. Cellular energy allocation measurements

Measurements of lipid, sugar and protein energy content and electron transport system (ETS) activity were performed in three composite samples for each salinity and time-point for both the

control and cadmium exposed groups. Each composite sample contained 5 test organisms. For wet weight measurements, mysids were blotted dry, placed in pre-weighed Eppendorf tubes and weighed on an analytical balance (± 0.1 mg). Samples were stored at -80°C until analysis.

CEA was measured according to Verslycke and Janssen (2002) with minor modifications. The different energy reserve fractions (lipid, protein, sugar = E_a, available energy) were determined spectrophotometrically and transformed into energetic equivalents using their respective energy of combustion (39500 mJ/mg lipid, 24000 mJ/mg protein, 17500 mJ/mg glycogen) (Gnaiger, 1983). The energy consumption (E_c) was estimated by measuring the activity of the mitochondrial electron transport system (ETS) according to Owens and King (1975). The quantity of oxygen consumed per mysid, as derived from the ETS data, was transformed into energetic equivalents using the oxyenthalpic equivalents for an average lipid, protein and sugar mixture (484 kJ/mol O_2) (Gnaiger, 1983).

The E_a, E_c and CEA value were calculated as follows:

E_a (available energy) = $E_{sugar} + E_{lipid} + E_{protein}$ (mJ/mg ww)

E_c (energy consumption) = ETS activity (mJ/mg ww/h)

CEA (cellular energy allocation) = E_a/E_c

From this, it can be evident that a decrease of CEA indicates either a reduction in available energy or higher energy expenditure, both resulting in a lower amount of energy available for growth or reproduction.

2.5. Statistical analysis

All analyses were done with the software package Statistica™ (Statsoft, Tulsa, OK, USA). Differences between control and Cd-exposed groups for all of the measured parameters (MT concentration, sugar, lipid and protein contents and ETS activity) were detected using the Mann-Whitney U test. For the calculated parameters E_a and CEA, the differences between the control

and Cd-exposed groups were evaluated using the Difference Test between two means. The effects of salinity and duration of the experiment were tested for significance separately, using one-way analysis of variance (Tukey's Honestly Significant Difference Test). All tests were performed at the probability level of 0.05.

3. Results

3.1. Metallothionein contents

The metallothioneins content of whole body homogenates were determined and expressed in μg per mg of cytosolic proteins (Fig. 2). The difference in MT levels between the control and Cd-exposed groups was not significant (Mann-Whitney U test, $p<0.05$) for any of the salinity – time-point combinations tested. The measured MT values ranged from 42.2 to 75.7, 34.7 to 63.4 and 32.3 to 56.1 $\mu g/mg_{(cytosolic\ protein)}$ at salinity 5, 16 and 25 psu, respectively. While the cadmium exposure did not influence the MT levels in N. integer, the salinity clearly did. On day 1, the MT level in control animals at salinity 5 psu was 30% and 42% higher (Tukey HSD test, $p<0.05$) than in control mysids exposed at 16 and 25 psu (Fig. 2).

Also, on day 7, MT levels at 5 psu in both control and Cd-exposed animals were significantly higher than those observed in the higher salinities (Tukey HSD test, $p<0.05$). MT levels in control animals were 56% and 41% higher at 5 psu compared to levels noted at 16 and 25 psu, respectively (Fig. 2). In Cd-exposed animals MT levels were 81% and 45% higher at 5 psu than that of organisms exposed to Cd at 16 and 25 psu, respectively (Fig. 2). Using the control group as a comparison in this study eliminates potential stress effects that might be attributed to the sampling location.

3.2. Energy budget

Sugar, protein and lipid contents of N. integer were determined (Table 1). To compare the relative contribution of these fractions to the organism's energy budget, these measurements were transformed into energetic equivalents using their respective energy of combustion. It can be observed that calculated energy equivalents of the lipid and protein fractions were quantitatively the most important energy sources with an average contribution of 56 and 41%, respectively (Table 1). In this paper, the respective energy contents will be referred to as sugar, protein and lipid content.

In general, there were no significant differences in the contents of the individual energy reserve fractions between control and Cd-exposed groups (Mann-Whitney U test, $p<0.05$). A few exceptions were found as indicated in Table 1. As observed for individual energy reserve fractions, there were no differences between control and Cd-exposed groups in total available energy (E_a), except at the 16 psu treatment on day 7 (Difference Test between two means, $p<0.05$). Hence, the data of the control and Cd-exposed groups were pooled to compare the salinities and time-points (Fig. 3). While the cadmium exposure did not influence the E_a values in N. integer, the salinity obviously did. E_a was significantly lower at 5 psu than at the other tested salinities, except on day 7 when E_a values at 5 and 25 psu were not significantly different (Difference Test between two means, $p>0.05$). As a function of time, an increase in E_a was observed at salinities 5 and 16 psu, while it decreased at 25 psu (Fig. 3).

The results showed that the differences in salinity did not significantly affect the energy consumption (E_c) in N. integer (Tukey HSD test, $p>0.05$) (Fig. 3). However, a significant increase in E_c as a function of time was observed (Fig. 3); i.e. from 38.46 mJ/mg ww/h on day 1 up to 62.06 mJ/mg ww/h on day 7 (Tukey HSD test, $p<0.001$).

Cellular energy allocation (CEA) of N. integer was calculated as the ratio of the amount of available energy (E_a) and the energy consumption (E_c) and is presented in Fig. 3. As already observed for all above mentioned parameters, there was no difference between the control and

Cd-exposed groups in CEA (except at 16 psu on day 7; Difference Test between two means, p = 0.0028). Again, data of the control and Cd-exposed groups were pooled to compare the salinities and time-points.

The CEA values in N. integer were affected by salinity: mysids kept at the lowest tested salinity allocated less energy to their reserves than those kept at higher salinities. On day 1 and 4, the CEA values in N. integer were significantly lower at 5 psu than at 16 and 25 psu (Difference Test between two means, $p<0.05$, Fig. 3). On day 7, the differences in the CEA values between 5 and 25 psu were not significant (Difference Test between two means, $p>0.05$, Fig. 3), while, at 16 psu, the CEA value was significantly higher than at 5 and 25 psu (Difference Test between two means, $p<0.05$, Fig. 3).

4. Discussion

This study was designed – by eliminating the effect of salinity on cadmium speciation - to assess the effect of cadmium on some aspects of the organism's physiological status at different salinities. In order to avoid mortality during the experiment, the mysids were exposed to only 1/5 of 96h LC_{50} value determined by Verslycke et al. (2003a). The cadmium test concentration used was 5.74×10^{-9} mol/L in terms of free cadmium ion activity. From the results it appears that this concentration was too low to induce significant increases in MT levels (Fig. 2). One possible explanation for this observation is that the constitutive MT levels, i.e. MTs that bind essential metals (e.g. Zn and Cu), were sufficient to detoxify Cd. Two fundamental characteristics of MTs are their high kinetic reactivity and their high affinity to bind metals (Stillman, 1995). Since the affinity of MT to Cd is higher than that for Zn, the exchange of Zn-ions with Cd-ions could occur, consequently complexing Cd and rendering it non-toxic.

The highest MT concentration of 75.7 μg/$mg_{(cytosolic\ protein)}$ determined in this study was found at the salinity of 5 psu. Since there were no significant differences between the control and Cd-exposed groups, and the bioavailability/speciation of Cd was the same at all studied salinities, some physiological mechanism other than detoxification could be responsible for the higher MT levels observed at low salinity. In field studies, salinity has been recognised as a natural factor affecting the MT concentrations in different estuarine invertebrates (Mouneyrac et al., 1998; Legras et al., 2000., Geffard et al., 2001; Leung et al., 2002). The higher MT level at lower salinities has usually been attributed to combined effect of physico-chemistry and physiology. Salinity plays a major role in the speciation of cadmium in seawater. The inverse relationship between salinity and free cadmium ion concentration occurs due to extensive complexation of Cd with chloride ions (Sadiq, 1992). On the other hand, considering the complexity of responsive elements and regulatory factors in MT gene expression and the evidence for MT induction by starvation, anoxia or freezing (Amiard et al., 2006), it is hypothesized that a change in osmotic pressure experienced by an organism under changing salinity affected the MT regulation mechanism of N. integer. The increased MT levels observed at low salinities - i.e. under environmental conditions favouring higher metal bioavailability - may be beneficial to the organism. Further research is required to address these questions.

Metabolic responses of crustaceans to changes in salinity are highly variable among species (Péqueux, 1995). One possible physiological response to low salinity is a reduction in apparent water permeability (AWP). AWP represents water fluxes in aquatic animals and is based on measurements of passive diffusion of isotope labelled water (Rasmussen and Andersen, 1996). The amphipod Gammarus duebeni lowers its AWP significantly when exposed to a low salinity medium, e.g. when the animal is transferred from 31 to 1 psu. The decapod Crangon crangon also reduces its AWP when transferred from 34 to 7 psu (Rasmussen and Andersen, 1996). Furthermore, it has been shown that in euryhaline species regulation of the intracellular

osmolarity is reflected in changes in the concentrations of small organic molecules, mostly free amino acids. For N. integer it has been reported that glycine concentrations, and to a lesser extent alanine and proline, increase with increasing salinity (Armitage and Morris, 1982).

Concerning the energy budget of N. integer, it appears that the cadmium concentration used was too low to produce observable changes. Salinity, on the other hand, did affect the energy budget of this mysid. Since the animals were fed during the experiment to avoid cannibalism, a significant decrease of the individual energy fractions was not expected. Indeed, in hypo-osmotic media an increase in all individual energy fractions of N. integer as a function of increasing exposure time was observed (Table 1). In the isosmotic medium there was no change in the protein contents during the experiment, while an increase in lipid and sugar energy fraction was noted (Table 1). However, in hyper-osmotic media both the lipid and protein energy fraction decreased and the sugar energy fraction remained the same (Table 1). The mysids were acclimated to a laboratory conditions at salinity of 5 psu and transferred into either isosmotic medium (16 psu) or hyper-osmotic medium (25 psu). Transfer to 25 psu requires the organism to shift from hyper-osmoregulation to hypo-osmoregulation. Some sugar was probably used by the organisms as a fast energy source to fuel these processes, as suggested by Verslycke and Janssen (2002).

These authors also noted large variations in lipid metabolism and suggested a sex-specific lipid metabolism, since the male and female organisms had been randomly used in their experiments. In our study we tried to avoid this problem by using juvenile organisms which did not have developed brood pouches with eggs. However, for practical reasons there was a delay in the start of the experiments at different salinities and the 25 psu experiment was carried out last. This might contributed to high lipid content. Considering all of the above issues, variation in lipid contents observed at the different salinities was difficult to explain univocally.

Regarding the protein energy fraction, since the exoskeleton of N. integer contains large amounts of protein it is suggested that the observed variation in protein contents may be affected by ecdysis. Indeed, some shed exoskeletons were observed in the exposure vessels during the experiment. Verslycke and Janssen (2002), however, suggested that their data supported the hypothesis that N. integer can actively use protein as an energy source under stress conditions.

Calculation of energy consumption was based on measurements of the electron transport system (ETS) activity in the mitochondria (Ovens and King, 1975). This can be considered as an estimate of the potential whole organism respiration rate. In the present study we did not find significant differences in ETS activity of N. integer in the salinity range 5 – 25 psu (Fig. 3). Similarly, no significant difference in the respiration rate of Palaemon peringueyi in the range from 15 to 35 psu was reported (Allan et al., 2006), implying that this shrimp species was well adapted to life in environments characterised by variations in salinity and temperature. N. integer is a hyper- and hypo-osmoregulator with its isosmotic point around 16 psu, and a tolerance range between 1 and 40 psu (Mauchline, 1971). In the Scheldt estuary, N. integer inhabits the water at salinities ranging from 8 to 25 psu with a maximal abundance at around 15 psu (Mees et al, 1994). While the salinity differences did not affect the ETS activity, the increased exposure time did increase the ETS activity (Fig. 3). This is in agreement with the observations of Verslycke and Janssen (2002) who reported that none of the tested abiotic parameters (temperature, salinity, dissolved oxygen) had a significant effect on the measured ETS activity. It was postulated that biotic variables (age, weight and sex) probably have a larger influence on respiration rates.

The calculated CEA – providing an integrated quantification of the organism's energy budget – was affected by salinity and the duration of the experiment. The observed relative decrease of the CEA values as a function of time at all studied salinities may be explained by the marked increase in the energy consumption (Fig. 3).

Since 16 psu is the isosomotic point and thus energetically favourable, organisms kept at lower and higher salinities should have lower CEA values. This was found to be true for the hypo-osmotic treatment on all observation days and at the end of the experiment for the hyper-osmotic exposures (Fig. 3). Lower variation in CEA values as a function of time was observed at 5 and 16 psu than at 25 psu. This could mean that the isosmotic and hypo-osmotic media are 'optimal' environments for N. integer, while the hyper-osmotic medium is a sub-optimal environment. This seems logical, since the test animals were collected at a site with a salinity of 5 psu, and the fact that N. integer has rarely been observed in waters of more than 18 psu (Mees et al., 1994).

When a crustacean is placed in a medium of higher or lower osmotic pressure, a number of physiological mechanisms are triggered which lead to marked changes in biochemical composition and different energetic requirements (Péqueux, 1995; Mantel and Farmer, 1983). Additionally, like protein synthesis in general, the synthesis of MT requires energy. Therefore, it was assumed that the decrease in salinity that caused an increase in MT level in N. integer, would also influence the biochemical composition of the animals and their energy metabolism. For that reason, the correlation between measured MT levels and CEA data was tested. To allow comparison with the CEA (calculated from the energy fractions expressed as mJ per mg of wet weight), MT values were expressed as μg per mg of wet weight (Fig. 4). A significant correlation was found between MT and CEA values (Spearman correlation coefficient, $r = -0.557563$, $p = 0.016210$, Fig. 4).

Further research is, however, needed (1) to explore the concentration-response relationship in MT level as a function of Cd exposure, and (2) to confirm the relationship between MT and CEA values.

5. Conclusions

This study presents the first results of metallothionein quantification in the estuarine mysid N. integer exposed to cadmium at different salinities. Since it was not possible to detect the differences in MT levels between the control and Cd-exposed groups, the measured MT levels probably represent the constitutive MT levels responsible for binding essential metals. However, the differences in MT levels at the different salinities indicate the relationship of some physiological process (probably osmoregulation), other than detoxification, with MT induction.

When N. integer was placed in the media of different osmotic pressure, marked changes in biochemical composition and energetic requirements were observed. Although, it was difficult to explain the variations in the individual energy reserve fractions, the calculated cellular energy allocation values showed a significant inverse relationship with the MT concentrations.

For this study it is concluded that the salinity affects the energy status and MT content of N. integer. This should be taken into account when using these biomarkers in field studies and environmental monitoring.

Acknowledgement

The research was supported by the Belgian Federal Science Policy Office ENDIS-RISK project. M. Erk is the beneficiary of a post-doc fellowship granted by the Belgian Federal Science Policy Office. B. Muyssen is the recipient of a grant from the Fund for Scientific Research F.W.O.-Vlaanderen, Belgium. We thank Dr. ir. Karel De Schamphelaere for his help in the application of the vMinteq software, Annick Verween for her assistance in collecting the test animals and Jill Van Reybrouck for the cadmium analysis. We acknowledge Professor Van Oostveldt for his support.

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Figure captions

Figure 1.

Voltammograms recorded in differential pulse mode (A) with the standard MT in the concentration range from 1.96×10^{-5} to 1.18×10^{-4} mg MT/mL and (B) with the heat treated cytosolic fraction isolated from *N. integer*.

Figure 2.

Metallothionein concentration (μg MT / mg cytosolic proteins) in N. integer exposed to $Cd^{2+}_{(aq)}$ 5.74×10^{-9} mol/L (+ control) at different salinities on day 1, 4 and 7 of the exposure. Median values, quartiles and range are presented; white boxes – control groups, pattern boxes – Cd-exposed groups; *significantly different from other tested salinities (Tukey HSD test, $p<0.05$)

Figure 3.

Total energy available (E_a), total energy consumption (E_c) and cellular energy allocation (CEA) in N. integer at different salinities on day 1, 4 and 7 of the exposure (data of control and Cd-exposed groups were pooled, mean values and standard deviations are presented).

Figure 4.

Correlation between metallothionein concentrations and cellular energy allocation values in N. integer.

Table

Table 1.

Energy reserve fractions in control and Cd-exposed N. integer at different salinities on day 1, 4 and 7 of the exposure (data are shown as mean ± standard deviation).

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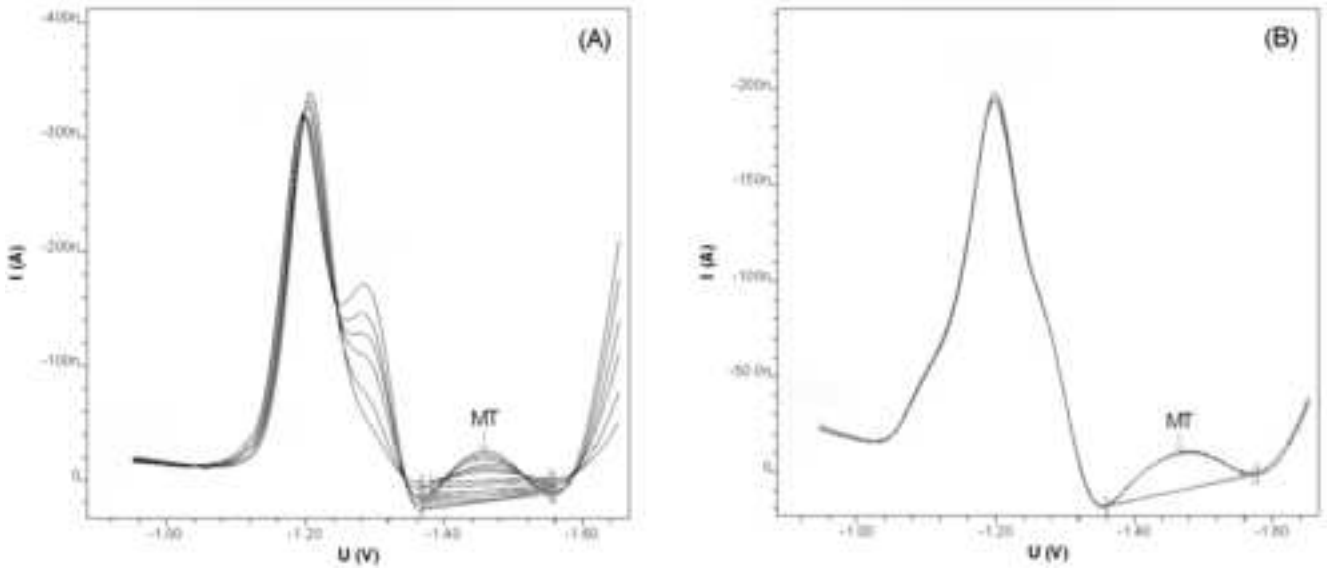

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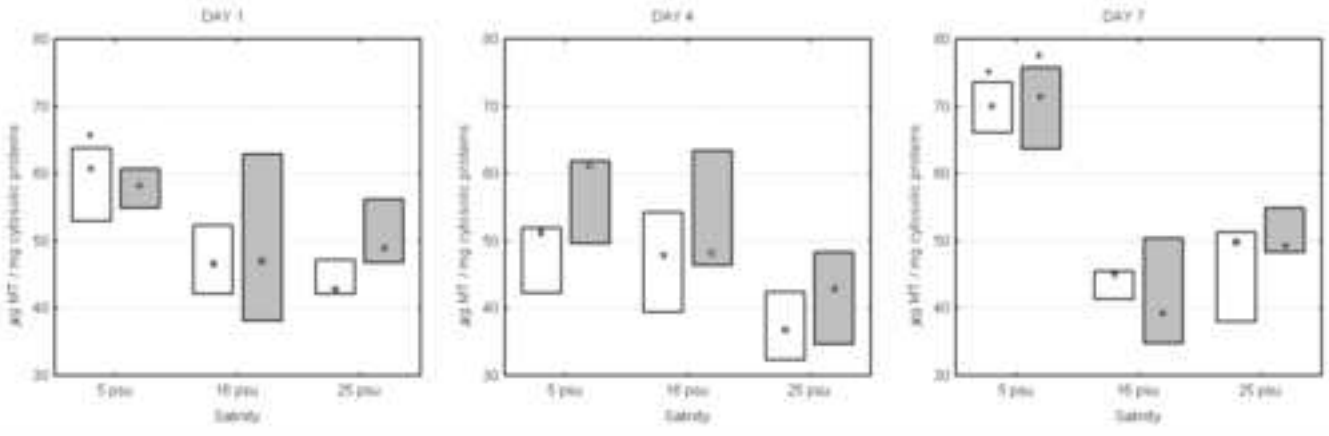

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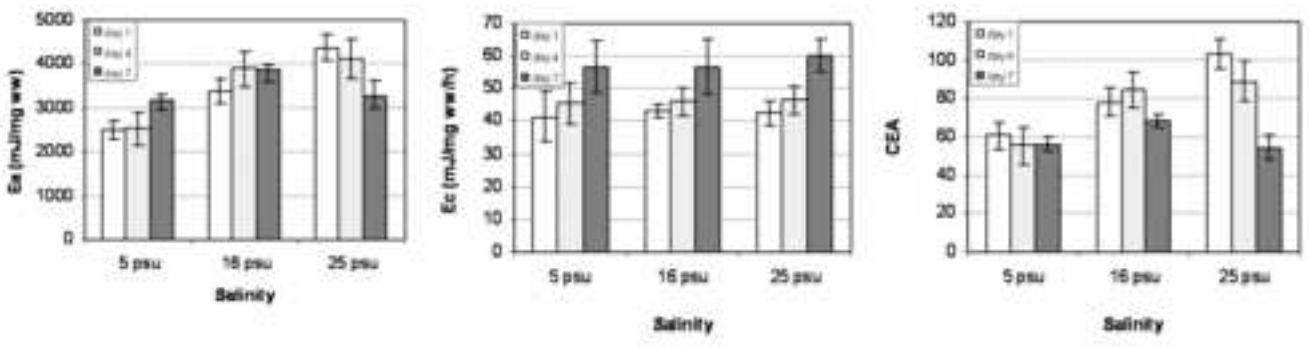

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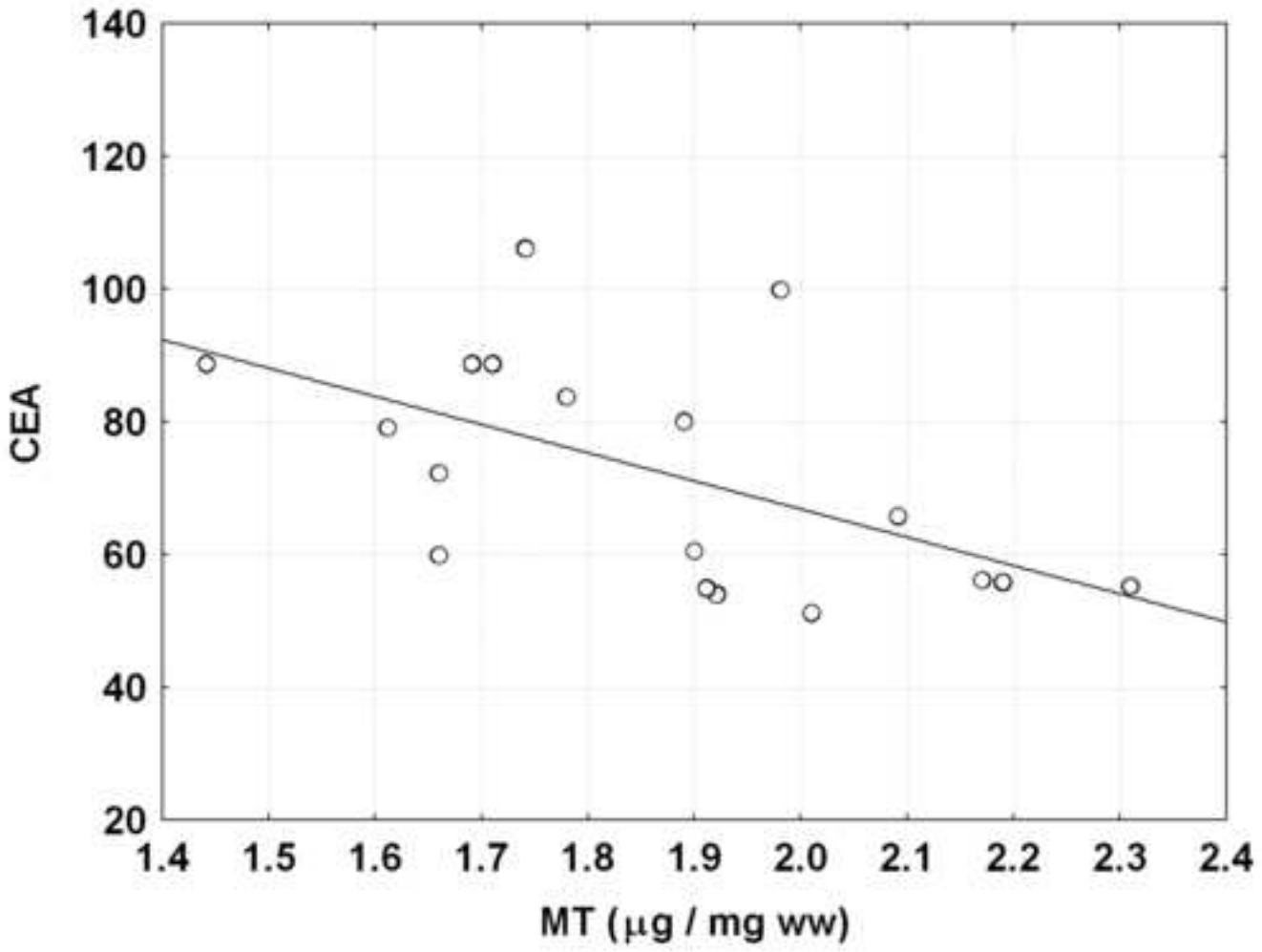

Table 1. Energy reserve fractions in control and Cd-exposed N. integer at different salinities on day 1, 4 and 7 of the exposure (data are shown as mean ± standard deviation).

Sample	Salinity (psu)	Day	E_{sugar} (mJ/mg ww)	%	$E_{protein}$ (mJ/mg ww)	%	E_{lipid} (mJ/mg ww)	%
Control	5	1	70.6 ± 10.3	2.8	986.1 ± 141.4	38.8	1482.0 ± 283.6	58.4
Cd-exposed	5	1	79.0 ± 4.1	3.2	1101.0 ± 52.4	44.5	1291.6 ± 89.9	52.3
Control	5	4	80.7 ± 9.1	3.2	1178.4 ± 137.3	47.3	1232.7 ± 322.8	49.5
Cd-exposed	5	4	115.4 ± 19.2*	4.5	1277.3 ± 96.8	50.1	1157.2 ± 404.4	45.4
Control	5	7	110.4 ± 26.9	3.5	1287.0 ± 112.0	40.3	1794.4 ± 44.9	56.2
Cd-exposed	5	7	127.2 ± 29.7	4.1	1169.0 ± 56.2	37.4	1826.1 ± 175.5	58.5
Control	16	1	81.8 ± 3.0	2.6	1409.0 ± 47.1	44.7	1664.2 ± 317.4	52.7
Cd-exposed	16	1	90.5 ± 12.8	2.5	1560.3 ± 160.1	43.2	1958.8 ± 203.5	54.3
Control	16	4	108.6 ± 13.6	2.7	1469.3 ± 111.2	36.1	2495.4 ± 328.6	61.3
Cd-exposed	16	4	104.8 ± 8.1	2.8	1621.0 ± 125.0	43.7	1985.9 ± 464.1	53.5
Control	16	7	124.4 ± 12.9	3.3	1518.3 ± 10.6	40.9	2072.7 ± 19.6	55.8
Cd-exposed	16	7	106.8 ± 16.0	2.6	1536.0 ± 144.4	37.9	2410.7 ± 124.5*	59.5
Control	25	1	79.4 ± 9.0	1.8	1565.8 ± 97.3	35.9	2718.4 ± 169.8	62.3
Cd-exposed	25	1	106.5 ± 23.2	2.4	1650.4 ± 133.6	37.5	2642.2 ± 349.2	60.1
Control	25	4	88.2 ± 12.2	2.1	1607.0 ± 50.6	37.6	2576.3 ± 623.8	60.3
Cd-exposed	25	4	95.8 ± 4.1	2.4	1562.3 ± 45.7	39.1	2338.6 ± 288.5	58.5
Control	25	7	101.0 ± 18.2	3.0	1504.5 ± 61.6	45.0	1737.1 ± 306.6	52.0
Cd-exposed	25	7	92.9 ± 18.8	2.9	1231.8 ± 124.5*	38.3	1890.1 ± 344.8	58.8

* significantly different from respective control ($p < 0.05$)