Marine Pollution Bulletin 112 (2016) 452–458

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Baseline

A baseline study of the metallothioneins induction and its reversibility in *Neohelice granulata* from the Bahía Blanca Estuary (Argentina)

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ARTICLE INFO

Article history:
Received 2 October 2015
Received in revised form 4 July 2016
Accepted 6 July 2016
Available online 18 July 2016

Keywords:
Heavy metals
Sediments
Metallothioneins
Neohelice granulata
Depuration

ABSTRACT

Contamination by heavy metals causes serious effects in marine systems. Nowadays, the combination of chemical and biological data is recommended in monitoring programs. Metallothioneins (MT) are early-warning signals of metal exposure and are widely used in biomonitoring. The present research evaluates the heavy metals levels in sediments and the MT synthesis in the crab *Neohelice granulata* from the Bahía Blanca Estuary (BBE). Then, the recovery capabilities of *N. granulata* followed by a depuration phase are assessed. Results demonstrate a slow decline in the level of metals in the study area. In relation to MT, female crabs showed elevated MT when compared to males. Furthermore, MT synthesis diminished after the depuration phase. These data constitute the first MT information in organisms from the BBE and may be considered as baseline for future studies in this matter. Although pollution level found was low, further biomonitoring considering both types of data is recommended.

Contamination by heavy metals has received much attention in the last decades, since these pollutants depending on their oxidation state, can be highly reactive and toxic to most organisms (Pinto et al., 2003). The main concerns exist in estuarine and harbor areas, where point and non-point metal sources are predominant, especially in intertidal zones, which receive chemical input from a variety of anthropogenic sources (Banni et al., 2005; Tlili et al., 2010). In this regard, evaluation strategies have been historically developed aiming at determining the presence, concentration and distribution of contaminants in abiotic and biotic compartments. Although the exclusive use of these traditional methods of assessment of metals is a useful guide to evaluate environmental contamination, it has a limited application. Since the main purpose of environmental monitoring is to protect the biological/ecological systems, it is necessary to study the biological effects of exposure to substances present in the environment (Langston et al., 2003). In this way, to improve the efficacy of monitoring programs, the combination of chemical data and biological responses is strongly recommended (Clements, 2000; van der Oost et al., 2003; Laffon et al., 2006). Among the wide range of biological responses, biochemical markers have played a singular role, representing early-warning signals whose detection can prevent adverse effects at hierarchical levels (van der Oost et al., 2003).

Metallothioneins (MT) are small non-enzymatic metalloproteins that bind to metals through cysteine bonds (Amiard et al., 2006). These proteins act in the cellular regulation of metabolically important metals, such as Cu and Zn, as well as in reducing the toxicity of non-essential metals, *i.e.* Cd and Pb (Livingstone, 1993; Viarengo et al., 1997). The induction of MT synthesis in response to metal contaminants in many marine species has been demonstrated. The relationship between metal levels in the environment and MT levels in animal tissues has led to the use of these proteins as biomarkers for monitoring the biological effects resulting from exposure to metals (Hylland et al., 1992; Livingstone, 1993). In aquatic invertebrates it has been demonstrated that MT also acts against cellular oxidative stress, being directly connected to the cellular antioxidant defense system (Viarengo et al., 2000; Cavaletto et al., 2002).

According to their toxicity, impacts by metals have been reported for various species of organisms. However, little is known about the recovery capabilities of aquatic organisms after exposure to trace metals (Baudrimont et al., 2003; Hédouin et al., 2007; Reichmuth et al., 2010; Mouchet et al., 2015). Moreover, there is little research concerning the reversibility or irreversibility of metal impacts in aquatic organisms at the biochemical, genetic and physiological scale after decrease or disappearance of metal contamination. Assessing the responses generated by metal exposure and the persistence of disturbances after contamination ends would provide a better understanding of the metal depuration

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http://dx.doi.org/10.1016/j.marpolbul.2016.07.007
0025-326X/

process in aquatic organisms since the way that different species respond to depuration is also poorly understood. In addition, this phase of decontamination/purification aims to study whether the altered biomarkers during exposure are resilient.

The Bahía Blanca Estuary (BBE) is located in the SW area of Buenos Aires province, Argentina (38°45′–39°25′ S and 61°45′–62°30′ W) (Fig. 1). This estuary extends over about 2300 km^2 and comprises several tidal channels, islands and extensive tidal flats with patches of low salt marshes and crab caves or "cangrejales" from populations of the crab *Neohelice granulata* (Piccolo et al., 2008). At the northern boundaries of the estuary various cities (Bahía Blanca, Punta Alta, Gral. Cerri) and industries (oil, chemical, and plastic factories) are located. The great majority of these industries discharge their effluents within the estuary. In this area is located the only deep water harbor system of Argentina, consisting of various harbors: Galván, Ing. White, Rosales and Naval Base Pto. Belgrano. Thus, the Main Channel is navigated consistently by fishing boats, cargo ships and fuel and cereal transports. For this reason the Main Channel is subjected to periodic dredging, affecting contaminant transport (Marcovecchio, 2000). At this site, there are good chances to introduce pollutants into the environment through man-activities. The main tributaries are the Sauce Chico River (drainage area of 1600 km^2) in the head of the estuary and the Napostá Grande Creak (drainage area of 920 km^2) upward Bahía Blanca city on the northern margin (Perillo et al., 2001).

Since 1978, studies related to the distribution and concentration of heavy metals in different compartments were carried out in this estuary; but so far nothing is known about the induction or expression of biomarkers in organisms from this area. In this context the main goal of the present study was to evaluate for the first time the levels of MTs in the hepatopancreas of the crab *Neohelice granulata* from the BBE, as well as, the potential reversibility of these sequestration proteins synthesis after the depuration phase. Additionally, we analyzed the possible relationships among MTs and heavy metals concentration in sediments.

Neohelice granulata is a benthic and abundant crab that lives intimately associated with sediments, and is considered of ecological importance as an structuring agent of the coastal ecosystem. It is an important member of the estuarine food chain due to its high abundance and its multiple roles as a scavenger, predator and prey. As a key species within this ecosystem, *N. granulata* could play a major role in the transference of pollutants to higher trophic levels. Given *N. granulata*'s ecological role and the extensive knowledge about their biology and maintenance conditions in captivity, it is an excellent model for ecotoxicological studies.

Specimens of *N. granulata* of both sexes were collected in April 2014 at Cuatreros Harbor (innermost area of the BBE) adjacent to the town of Gral. Cerri (Fig. 1). This site is characterized by a water salinity range of 20.8–31.9 psu, a water temperature range of 8.5–23.3 °C, and a sediment composition of mainly silt–clay (96.3%) (Botté, 2005; Angeletti et al., 2014). Sluggish crabs or those lacking one or more appendixes were discarded. Thirty eight mature crabs of each sex in the intermolt stage with a carapace width (maximum distance between the two prominent lateral spines) between 20 and 30 mm for females and between 25 and 35 mm for males were selected. Crabs were transported to the laboratory in thermally isolated boxes with *in situ* collected water.

Complementing sediment samples (approximately 10 to 15 cm thickness of the surface sediment) were also extracted for analysis of heavy metals. Sediment samples were immediately transported to the laboratory in refrigerated boxes and stored at 4 °C. Simultaneously, temperature, conductivity/salinity, pH, turbidity, and dissolved oxygen (DO) concentration were *in situ* measured using a Horiba U-10 multisensor device.

Once in the laboratory, one group of males and females crabs was anesthetized by freezing, weighed and their carapace width was

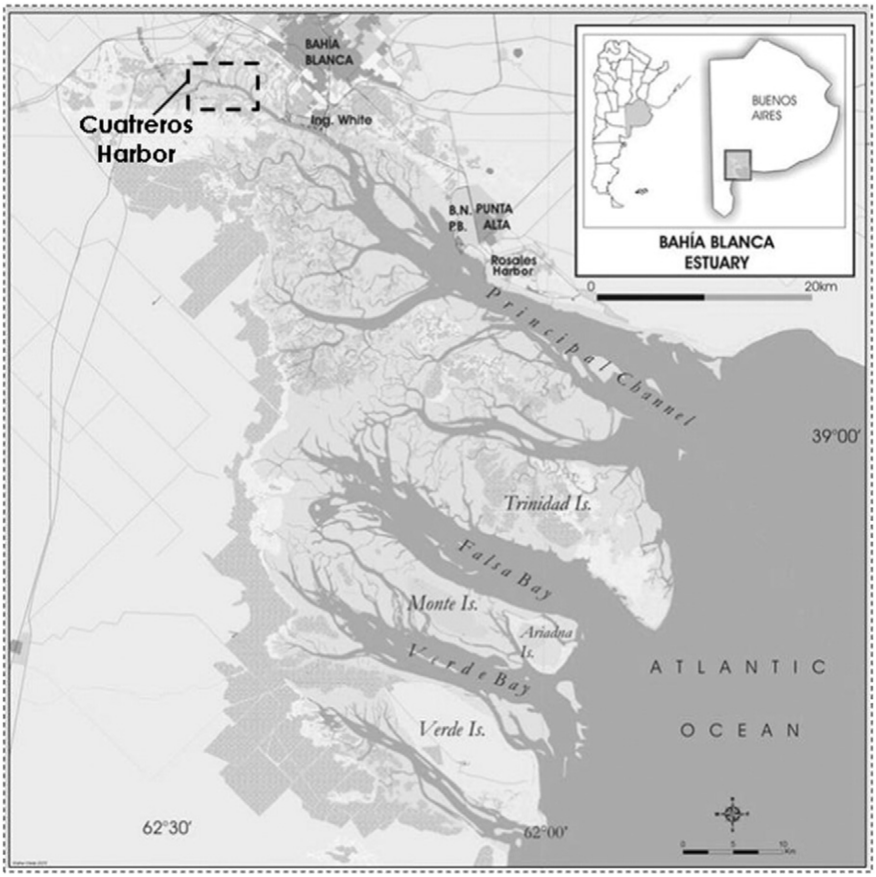

Fig. 1. Location of the study area in the Bahía Blanca Estuary.

measured with an electronic calliper (0.01 mm accuracy). Precautionary measures to prevent contamination during collection, dissection, and analysis were taken by cleaning all glassware and equipment in diluted nitric acid (5% *v*/v; APHA, 1998). Samples were carefully dissected out to collect the hepatopancreas, then it was weighed and stored at −80 °C. The condition index (CI) (calculated as the ratio between total body wet weight (g) and cephalothorax width (cm)) (Pereira et al., 2006) and the hepatosomatic index (HSI) (calculated as the percentage of hepatopancreas wet weight in relation to the total body wet weight) (Ferreira et al., 2006; Comoglio et al., 2008) of *N. granulata* were evaluated. Another group of males and females crabs was kept in separated aerated glass containers covered with artificial seawater (Marine Mix) in a culture chamber. The crabs were maintained during 20 days under the same conditions than those of the environment (temperature: 14 °C day – 11 °C night, photoperiod: 12 h light/12 h dark cycle, pH 8, salinity 28.0–29.0 psu). They were fed *ad libitum* with granulated fish food twice a week and the water of the aquariums was renewed after each feeding. After 20 days of purification we proceeded in the same way as with the other organisms mentioned above.

Hepatopancreas tissue from three crabs were pooled according to sex and sampling site and ice-cold homogenized (1:4) in 0.5 M sucrose, 20 mM Tris-HCl (pH 8.6), 0.5 mM phenylmethanesulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT) in a Potter-Elvehjem glass/Teflon homogenizer. Homogenates were centrifuged at 30,000 *g* for 30 min at 4 °C. Metallothioneins content was evaluated according the spectrophotometric method described by Viarengo et al. (1997). The absorbance was read at 412 nm, and MT concentration was quantified using reduced glutathione (GSH) as a reference standard. A Jenway 6500 UV/VIS spectrophotometer was used. The amount of MT was calculated based on cysteine content 18 cysteine/mol (Hamer, 1986; Serra-Batiste et al., 2010), assuming a similar SH group content in *N. granulata* MTs. MT assay was carried out in triplicate, blanks were performed and the MTs concentration was reported as nmol MT per gram of wet tissue (wt).

Sediment samples were oven dried at 50 ± 5 °C to a constant weight for 4 days. To evaluate heavy metal concentration, large debris and fragments of biota were removed from the dried sediments before grounding. All the sediment subsamples were then ground in a porcelain mortar and sieved through stainless steel meshes in order to separate sediment with grain size <62 μm. Metal concentrations (Cd, Cu, Cr, Ni, Pb, Zn, Mn and Fe) in the fine fraction (FF) were analyzed according to the methodology of Marcovecchio and Ferrer (2005). About 0.50 g of sediment samples were mineralized with a mixture $HNO_3/HClO_4$ (5:1) at 110 ± 10 °C. Then the extract (1 ml) was filled with 0.7% HNO_3 to a volume of 10 ml. Metals concentrations were measured by inductively coupled plasma-optical emission spectroscopy (ICP-OES Optima 2100 DV Perkin Elmer).

The detection limit of the method (MDL) was calculated as the standard deviation (SD) of 10 blank replicates (Federal Register, 1984). The MDL for Cd, Cu, Pb, Zn, Mn, Ni, Cr and Fe were 0.0030, 0.0623, 0.0937, 0.1063, 0.0450, 0.0113, 0.0101 and 0.6139 mg l^{-1}, respectively. Analytical precision expressed as coefficients of variance was <10% based on replicate analysis. For the analytical quality control, reagent blanks, certified reference materials (CRM; Pond Sediments, R.M. No. 2, NIES, Japan), and analytical grade reagents (Merck or Baker) were used. The recovery percentages for all trace metals in CRM were higher than 90%.

The different conditions analyzed (natural *vs.* depuration phase) and sex variations of MT responses, CI and HSI were tested by a two-way ANOVA with conditions, sex and interaction “conditions × sex” as variables. Post-hoc tests (Tukey) were used to discriminate between means of values. Prior to analysis, data were tested for normality and homogeneity of variance. Statistical analyses were carried out with appropriate software, following Zar (1996). The acceptable level of statistical significance was 5%. Error values represent ±1SE. Pearson correlations were used to study the influence of CI and HSI on MT induction.

Physical characteristics of Cuatreros Harbor are summarized in Table 1.

Table 2 shows the mean values of measured metal concentrations in sediments FF expressed in μg/g and in mg/g in the case of Fe (dry weight). This table shows that all the metals evaluated were above de detection limit. The average concentrations of heavy metals in sediments FF at Cuatreros Harbor revealed the following order:

Fe > Mn > Zn > Cu > Cr > Ni > Pb > Cd.

The analysis of the induction of MT in hepatopancreas of *N. granulata* showed that the effects of conditions and sex were highly significant ($p < 0.005$; $p < 0.0005$ respectively) and the two-factor interaction was no significant ($p > 0.05$) (Fig. 2). MT synthesis was higher in the crabs from Cuatreros Harbor (62.23 ± 7.93 nmol MT/g wt) than that after the depuration phase (44.34 ± 0.58 nmol MT/g wt). Sex-related changes in MT status were seen, the levels in adult females (67.82 ± 5.97 nmol MT/g wt) being 1.8 times higher than in adult males (38.75 ± 1.10 nmol MT/g wt) (Table 3). The difference in MT concentration between males and females crabs were more pronounced after 20 days of depuration. In this condition MT levels in females were 92% higher than in males while in natural conditions MT concentration in females crabs was 64% higher than in males. After 20 days of depuration, MT induction in female crabs decreased 24.6% and in male crabs 35.6% (Fig. 2).

The CI and the HSI of *N. granulata* did not show differences between conditions ($p > 0.05$) but highly significant differences were found between females and males crabs ($p < 0.05$). No interaction between conditions and sex was found for any of the indices evaluated ($p > 0.05$). The CI in males crabs was higher (0.68 ± 0.03) than in females ones (0.35 ± 0.02) showing highly significant differences (Tukey's test: $p < 0.005$). On the contrary, the HSI presented significant differences (Tukey's test: $p < 0.05$) being higher in females crabs (3.47 ± 0.36%) than in males (2.49 ± 0.08%).

The correlations between the morphological and biochemical responses in crabs were statistically tested considering genders either separately or jointly. Since no significant correlations were obtained, test results were not represented.

Sediments are an important sink for metals and other pollutants; they have been described as a non-point source for metals and sediment-bound metals which can be released into overlying waters and adversely affect aquatic organisms (Wang et al., 2004). The concentration of heavy metals depends on the sources, the chemical characteristics of the elements, and physicochemical conditions and complex reaction such as adsorption, flocculation and redox condition of the estuarine sediments (Establier et al., 1984). Benthic invertebrates are known to take up and accumulate heavy metals, both essential and non-essential, from water and sediment as well as from their food supply (Rainbow, 1997; Wang and Fisher, 1999). The concentrations of all the trace metals evaluated in the intertidal sediments FF in this study were similar to those found in 2010–2011 (Buzzi et al., 2012). Considering previous reports, the levels of Cd, Cr and Pb were lower (less than half) than that indicated by Botté et al. (2010). The mean concentrations of metals in sediments at Cuatreros Harbor could represent the influence of freshwater input and urban runoff (which also encompass lithospheric and atmospheric sources) as ones of the main sources of

Table 1
Physical parameters of the water from Cuatreros Harbor.

Physical parameters	
Temperature (°C)	14.20
Turbidity (ntu)	51.00
Conductivity (CD) (mS)	49.00
Salinity (psu)	29.00
pH	7.97
Diss. O_2 (OD) (mg/l)	9.30

Table 2
Summary of trace metal concentration (μg g^{-1} dw; Fe in mg g^{-1} dw) in the fine fraction sediments from Cuatreros Harbor (mean ± SE) and TEL and PEL-SQGs comparison. n.d.: no data.

	Metal concentration							
	Cd	Cu	Pb	Zn	Mn	Ni	Cr	Fe
Cuatreros Harbor	0.043 ± 0.011	11.132 ± 0.578	4.664 ± 0.905	28.085 ± 4.825	309.875 ± 21.375	5.800 ± 0.596	7.286 ± 0.829	21.205 ± 0.805
TEL-SQGs[a]	0.68	18.70	30.24	124.00	n.d	15.90	52.30	n.d
PEL-SQGs[a]	4.21	108.20	112.18	271.00	n.d	42.80	160.40	n.d

[a] Buchman, 1999.

contaminants to the system. In addition, we must emphasize that heavy metals content in sediments is related to extrinsic factors such as water salinity and temperature, sediment composition, and organic matter content among others (Förstner and Wittmann, 1983; Salomons and Förstner, 1984), and that these physicochemical characteristics show some variation from the head to the mouth of the estuary (Freije and Marcovecchio, 2004; Freije et al., 2008; Píccolo et al., 2008). According to the results provided by other authors for different estuaries or coastal areas (Villaescusa-Celaya et al., 2000; Buggy and Tobin, 2008; Yang et al., 2012) heavy metals contents herein found were below those levels recognized as characteristic of polluted environments. Moreover, according to the Sediment Quality Guidelines (SQGs) (Table 2), the concentrations of heavy metals in sediments from the BBE are below the marine TEL (Threshold Effects Level) and hence below the PEL (Probable Effects Level) so the heavy metals analyzed in this study are rarely associated with adverse biological effects. The TEL/PEL SQGs are applied to assess the degree to which the sediment-associated chemical status might adversely affect aquatic organisms and are designed to assist in the interpretation of sediment quality (Macdonald et al., 1996, 2000; Long et al., 1998). The TEL was interpreted to show chemical concentrations below which adverse biological effects rarely occur, and the PEL was intended to point out chemical concentrations above which adverse biological effects frequently occur (Macdonald et al., 2000).

The burrowing crab *N. granulata* dominates the tidal flats and salt marshes and has strong effects on the sediment dynamics, such as destabilization of cohesive sediments, which directly affects its porosity and permeability (Escapa et al., 2008). The hepatopancreas was chosen as a target organ for biochemical assessment according to its ability to uptake and concentrate the contaminants by 5–10 folds higher than other organs (Gomot de-Vaufleury and Pihan, 2000; Beeby and Richmond, 2003). Particularly, the hepatopancreas is also considered the major storage organ in decapods crustaceans, mainly accumulating lipids and to a lesser degree, glycogen (Sanchez Paz et al., 2007). Biomonitoring tests are frequently applied to assess the impact of pollution on a marine ecosystem. Detection of early warning responses at subcellular level, such as MT production, seems to be very useful to prevent long-term ecological damage (Kalpaxis et al., 2004). The diverse methodologies used to isolate and quantify MTs and the different units to express MT concentrations complicate the interpretation and comparison of the results obtained from different studies (Pedersen et al., 1997; Legras et al., 2000; Correia et al., 2004, Lavradas et al., 2014). These are the first results in marine organisms from the BBE in which MT induction is used in an attempt to characterize a biomarker of metal exposure that is commonly applied in pollution monitoring programs (Viarengo et al., 1999, 2007). Moreover, it is important to note that this is one of the few studies where the concentration of MT is analyzed after a depuration process. We found that MT levels were different between conditions; the higher induction of MT in crabs from Cuatreros Harbor could be influenced by the presence of heavy metals in the environment since the environmental parameters during the depuration phase were the same as in natural conditions. Baudrimont et al. (2003) and Arini et al. (2014) showed a MT decrease of 37% and 45% in the bivalve *Corbicula fluminea* whole bodies after long term of decontamination. Our results suggested a decrease of only 28% in the hepatopancreas of *N. granulata* after 20 days of depuration and this decrease was higher in male crabs than in females. Even though, according to the SQGs, the metal concentration in sediments from Cuatreros Harbor are rarely to cause adverse biological effects, we must note that in this study the effects of heavy metals in the induction of MT synthesis are evaluated as a whole and not each metal separately. Moreover, we must not forget, as the name implies, that MTs are "early warning indicators" of pollution. Although MTs are mainly involved in the adaptive cell mechanism of protection against metal toxicity, we must not discard that a wide range of other factors such as hormones, cytotoxic agents, physical and chemical stress could interfere with MT biosynthesis (Amiard et al., 2006). It has been reported that tissues directly involved in metal uptake, storage and excretion, such as liver has a high capacity for MT synthesis (Roesijadi and Robinson, 1994; Amiard et al., 2006). These small proteins have been studied in the hepatopancreas of some crustacean species (Legras et al., 2000; Chiodi Boudet et al., 2013; Lavradas et al., 2014); and has been described as the organ presenting the highest MT concentrations (Legras et al., 2000; Amiard et al., 2006; Lavradas et al., 2014). Unfortunately, not many reports regarding MT in crustaceans are available and even fewer are describing differences between males and females. The specific reason for MT levels being higher in females than adult males of *N. granulata* is unknown but may be related to aspects of development, such as moulting, and reproduction as in the case of a natural population of the bivalve mollusc *Ruditapes decussatus* and in the fishes *Pleuronectes platessa* and *Holocentrus adsenscionis*. In these species, the MT levels were higher in adult females than in adult males and this fact was correlated with gonad development (Overnell et al., 1987; Hamza-Chaffai

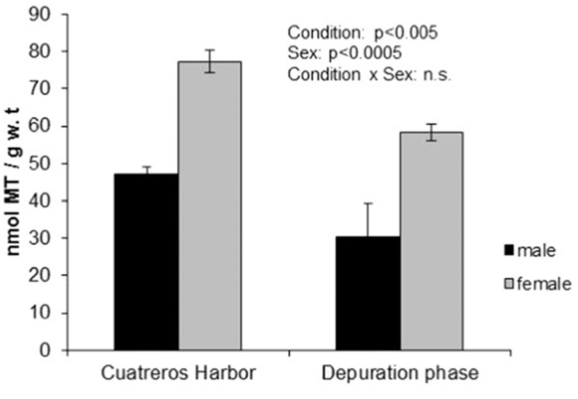

Fig. 2. Metallothioneins synthesis in hepatopancreas of *Neohelice granulata* from both studied conditions. The statistical significance was analyzed by two-way ANOVA test with condition and sex as factors. n.s. = not significant. Values are mean ± SE.

Table 3
Summary of Metallothioneins concentration in *Neohelice granulata* hepatopancreas (nmol MT/g wt) from Cuatreros Harbor, after the depuration phase and in all females' and males' crabs.

	Condition		Gender	
	Cuatreros Harbor	Depuration phase	Females crabs	Males crabs
Mean ± SE	62.23 ± 7.93	44.34 ± 0.58	67.82 ± 5.97	38.75 ± 1.10
Minimum	43.33	24.29	53.65	24.29
Maximum	88.23	61.15	88.23	49.21

et al., 1999; Thompson et al., 2002). MT levels were also found to be higher in adult females than in adult males of the anfphipod *Gammarus locusta* unexposed to heavy metals (Correia et al., 2004). In addition, as in this study, Lavradas et al. (2014) found higher MT concentrations in female *Callinectes* sp. crabs in comparison to males. These authors however, found that the levels of heavy metals in females' crabs were lower than in males, only Zn was significantly higher in females in viscera. Heavy metals in tissues crabs were not measured in this study. Nevertheless, previous studies in *N. granulata* from Cuatreros Harbor showed a tendency of a higher bioaccumulation of Cd and Cu in females crabs´ tissues than in males in autumn (Simonetti et al., 2012). In addition, the same authors (Simonetti et al., 2013) and in accordance with Lavradas et al. (2014), found that the levels of Zn were higher in soft tissue of females crabs than in males crabs. In the same way, the level of this metal was higher in *N. granulata* female's hepatopancreas than in males' crabs (Beltrame et al., 2011; Martinez et al., 2011). Probably the higher decline in MT induction in males crabs after 20 days of depuration is related to the differences in the uptake/elimination rate of some heavy metals between genders or even that the demand of this essential metal is different between both sexes (Beltrame et al., 2011). Some crustaceans, particularly decapods have a relatively constant total body concentration of essential metals, like Cu and Zn, over a wide range of environmental metal availabilities (Nugegoda and Rainbow, 1989; Rainbow, 1998; Pedersen and Lundebye, 1996); thus variability in Cu and Zn in the environment are considered not strong inducers of MTs in these organisms. Several studies (Engel, 1987; Brouwer et al., 1989; Couillard et al., 1995) have indicated that Cu and Zn MTs serve as storage forms for these metals and play a regulator role, especially as a metal donor for apohemocyanin (Cu) and in the synthesis of the carbonic anhydrase (Zn) (Couillard et al., 1995; Rainbow, 1997, 2007). Moreover, Zn is the most potent inducer of metallothionein transcription, regulation of MT genes by trace metals appear to be mediated by a zinc-sensitive inhibitor that interacts with a constitutively active transcription factor (Palmiter, 1994; Pourang et al., 2004). However, not all metals show similar patterns of accumulation in marine organisms, and the response to metals will vary between different animal groups. Therefore, MTs will only be effective indicators of heavy metal pollution where metal accumulation occurs in proportion to the environmental concentrations and where metals are effective inducers of MTs (Canli et al., 1997).

Several indices may be calculated in order to assess different and complementary information concerning the impairment caused by pollutants. In *N. granulata* changes in MT concentration with the CI and HSI were not clearly shown, since no statistical correlation was found contrary to the more marked influence evident in bivalves (Amiard-Triquet et al., 1998; Lemus et al., 2013). According to Pereira et al. (2006), organisms may exhibit reduced CI, as a result of environmental stress, but then these same authors did not find any differences in the CI of females shore crabs *Carcinus maenas* from a reference and a contaminated (metals and nutrients) sites (Pereira et al., 2008). In the same way, adult males of *C. maenas* exposed to fluoxetine were in similar health conditions at the end of the exposure (Mesquita et al., 2011). These results suggest no toxic effect at this level of biological organization (Ferreira et al., 2006). However, the significant differences in MT levels found in the hepatopancreas of *N. granulata* indicate alterations at molecular and biochemical levels in Cuatreros Harbor organisms. The presence of this early warning signal in crabs is an indication of possible toxicity effects (Moore, 1988). Further studies are necessary to clarify the relationship between CI and environmental stress due to pollutants. The HSI has been used to make inferences on the nutritional condition of crabs (*e.g.* Yamaguchi, 2001). In *N. granulata*, the HSI was higher in females crabs than in males, this could be suggesting a more intense reserve accumulation (Ituarte et al., 2006), probably because of the reduction in feeding activity of semiterrestrial crabs at low air temperatures (Wolcott, 1988) or due to a limited food supply during cold seasons. Added to that, females need to have more reserves to subsequently maintain oocyte development and provide maternal care. All these processes are simultaneous and demand a large amount of energy; the reserves stored in the hepatopancreas supply this energy, since feeding diminishes in ovigerous females (Fernández et al., 2000; Vazquez Boucard et al., 2004). Furthermore, the HSI is used as a morphological index which identifies possible organ diseases. This study showed that the HSI tends to decrease in crabs collected in Cuatreros Harbor, yet no statistical differences between both conditions were found. Sabatini et al. (2009) demonstrated a decrease in the HSI of *N. granulata* exposed to Cu but only at low salinities (2‰), they did not observed changes in this index at similar salinities to those in this study. A reduction of HSI was previously observed in *N. granulata* exposed to hexachlorobenzene (Chaufan et al., 2006) and in *C. maenas* exposed to different metals (Elumalai et al., 2005). This is interpreted as a negative impact of metals on the crabs' health. During chronic toxicity with heavy metals, liver damage in mammals occurs when the capacity of the liver to sequester free forms of metals is exceeded. Then metal–metallothionein is released into the blood and circulates to the kidneys where it is reabsorbed and accumulated in the renal epithelium (Barbier et al., 2005). A decrease of liver size as a consequence of a loss of hepatic lipid is a morphologic response of *N. granulata* liver to chronic toxicity (Sabatini et al., 2009) and this would explain how the amount of metal accumulated in liver is related with the HSI. We should not exclude that depuration time in this study was probably enough for the full recovery of the organisms and thus get a higher and statistically different HSI, or on the contrary, that the crab's defense system in which the MT are involved has still not been overwhelmed in organisms from Cuatreros Harbor. On the other hand, Mouneyrac et al. (2001) did not find any relation between MT synthesis and hepatopancreas weight in crabs.

The main novelty of this work is that MT levels have been studied for the first time in Bahía Blanca estuarine organisms. Considering previous studies that show the BBE as a moderately polluted ecosystem, these preliminary results allow the possibility to obtain a more complete and integrated ecotoxicological approach to the assessment of the biological impact and risk of heavy metals from industrial emissions, wastewater discharges, freshwater input and urban runoff in the estuary. Therefore, the use of the crab *N. granulata* may represent a relative easy process to improve the actual and classical monitoring techniques. In addition, the biological significance of the results presented here is important in terms of appearance of toxicological responses confirming that *N. granulata* is a good model for ecotoxicological studies. Anyway the inclusion of MT as a potential biomarker in biomonitoring programs with *N. granulata* requires some caution with respect to the sex of the organisms, as it was seen from the marked higher levels of MT in adult females compared to males. This information lays the groundwork for future studies related to heavy metals and future monitoring programs in this and other similar aquatic systems. Further research is required to evaluate the behavior of MT induction in relation to seasons in order to discriminate the seasonal factor from the interpretation of biomonitoring data. Then, the assessment of the recovery capacities of the crustacean *N. granulata* collected in the BBE shows interesting results, since MT synthesis in both sexes decreased after only 20 days of depuration. Although there are studies dealing with the effect of purification in organisms after exposure to heavy metals, most of them have been made in mollusks. Information on crustaceans is very scarce and further information related to the biochemical state of the organisms after this phase is required. Therefore, the analysis of the levels of MT, early-warning systems of exposure to heavy metals, in the crab *N. granulata* after the depuration phase is quite innovative and provides valuable information to be considered in future studies.

Acknowledgments

We thank to F. Biancalana, P. Simonetti, A. Berasategui and F. García for assistance in the laboratory and in the field. We also thank the IADO executive directors and Chemical Oceanography Area's staff. This research was supported by the National Agency for Promotion of Science and Technology (ANPCyT; PICT 2012-1383 and PICT 2012-2796), from Argentina.

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