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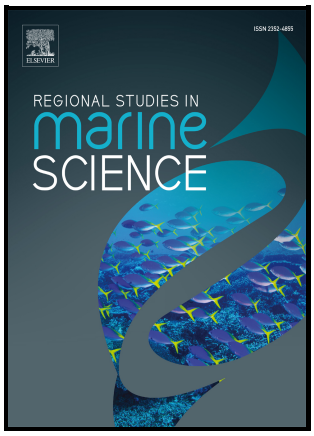

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Insights into metal depuration in different tissues of the burrowing crab *Neohelice granulata* (Dana, 1852)

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Abstract

In this article, we address the limited understanding of the recovery abilities of estuarine organisms following exposure to metals and the lack of research on the mechanisms involved in metal sequestration and detoxification. Specifically, our focus is on the burrowing crab, *Neohelice granulata,* found in the Bahía Blanca estuary. The objectives of this study were to a) evaluate the metal content in various tissues of *N. granulata* before and after the depuration process, b) identify metal-rich granules (MRG) in the crabs' hepatopancreas and c) examine the relationship between metal loads and the induction/activity or reversibility of biochemical biomarkers. Following the depuration

treatment, a noticeable decrease in metals was observed in the soft tissues compared to the hepatopancreas and the shell. Specifically, the depuration process effectively eliminated Mn and Fe in the form of MRG, but no significant differences were found for other metals, suggesting that MRG might not be the primary detoxification mechanism. Glutathione reductase (GR) and metallothionein (MT) levels significantly decreased after depuration. However, it is worth noting that the duration of the depuration period might not have been sufficient to fully counteract the oxidative stress and damage caused by field exposure.

Keywords: Metals; Depuration; Crabs; Metallothioneins; Biochemical biomarkers; IBR

1. Introduction

The toxicity of a specific metal is not solely determined by the total accumulated metal concentration, but rather by the threshold concentration of internally metabolically available metal (Rainbow, 2007). Toxicity arises when the rate of metal uptake from all sources exceeds the combined rates of detoxification and excretion (if present) of the specific metal in question (Rainbow, 2007). Consequently, elevated concentrations of metals pose a toxic risk to crustaceans, affecting various aspects of their biology, including growth, cellular damage, apoptosis, reproduction, the molt cycle, limb regeneration, biochemistry, oxidative stress, and physiology. These effects have been extensively documented in the literature (Capparelli et al., 2019; Luo et al., 2020; Frías-Espericueta et al., 2022).

Several studies have delved into the uptake and bioaccumulation of metals in aquatic animals, with notable contributions from Wang and Rainbow (2006) and Truchet et al. (2020). These investigations primarily focus on understanding the mechanisms underlying trace metal accumulation, the influence of geochemical and biological factors on trace metal

uptake and accumulation, and the transfer of metals along different aquatic food chains (Wang and Rainbow, 2006). Once metals enter an organism, they can take various routes, including binding to metallothionein, transport to mitochondria, accumulation by lysosomes, and transfer to the endoplasmic (Wallace et al., 2003; Arini et al., 2015).

Despite these advancements, there remains a notable gap in our understanding of the recovery capabilities of estuarine organisms following exposure to metals (Reichmuth et al., 2010; Anacleto et al., 2015; Khan et al., 2015; Buzzi and Marcovecchio, 2016). Additionally, there is a scarcity of research on the reversibility or irreversibility of metal impacts on aquatic organisms at the biochemical and physiological scales after a decrease or disappearance of metal contamination (Buzzi and Marcovecchio, 2016; Truchet et al., 2020). Furthermore, the mechanisms of sequestration and detoxification of metals have been poorly studied in highly human-impacted coastal and estuarine environments in the Southwest Atlantic Ocean (Truchet et al., 2020).

Depuration stands out as one of the most effective techniques for ensuring food safety by eliminating hazardous metals and microbes (Anacleto et al., 2015). Therefore, understanding the detoxification process in organisms is of paramount importance, as it represents a significant pathway for eliminating various toxic substances, especially in species consumed by humans (Anacleto et al., 2015; Birnstiel et al., 2019; Chinnandurai et al., 2022). Previous research has also reported that depuration and the activation of detoxifying enzymes play a pivotal role in limiting and reversing oxidative injury, as highlighted by Sabatini et al. (2015).

The burrowing crab, *Neohelice granulata*, has emerged as a model organism in developing countries due to its wide distribution along the coasts of the South Atlantic Ocean and the wealth of available information in various branches of biology and ecology. While species from the Northern Hemisphere have dominated research in different studies, *N*.

granulata has become the most extensively studied species globally, with a significant number of publications (Spivak, 2010). Nevertheless, the mechanisms underlying metal depuration in this species remain poorly documented in terms of metal concentrations (Simonetti et al., 2020), and biochemical parameters, specifically metallothioneins (Truchet et al., 2020).

Therefore, this study aims to achieve the following objectives: (a) evaluate the accumulation of metals (Cd, Cu, Pb, Zn, Mn, Ni, Cr, Fe) in the hepatopancreas, shell, and total soft tissue of *N. granulata* under natural conditions; (b) analyze the effectiveness of the depuration process in reducing the content of these metals; (c) investigate the presence of metal-rich granules (MRG) in the hepatopancreas of the crabs as a mechanism of metal detoxification; and (d) correlate this information with the induction/activity or reversibility of biochemical biomarkers (CAT, GST, GR, H_2O_2, TBARS, MT) resulting from depuration. These data will significantly contribute to expanding our understanding of the responses triggered by metal contamination and the persistence of disturbances even after the contamination has ceased. Additionally, this research aims to enhance our comprehension of the metal depuration process in *N. granulata*, thereby facilitating the development of comprehensive approaches for studying this emerging bioindicator species.

2. Materials and methods

2.1 Study area and field sampling

This study was carried out in the Bahía Blanca estuary (BBe) (Fig. 1a), which ranks as the second-largest estuarine system in Argentina, trailing only the La Plata estuary in the Southwestern Atlantic Ocean. The BBe stands out for its notable deep port, subject to continuous dredging activities, and is home to one of the largest petrochemical complexes in

South America. The BBe ecological significance is underscored by its provision of essential ecosystem services, including artisanal fishing, water regulation, and purification, air quality control, moderation of extreme events, biological control, preservation of cultural identity and heritage, scientific knowledge generation, and recreational opportunities, all of which are invaluable for human well-being (Speake et al., 2020). Nevertheless, the estuary has faced substantial challenges in recent decades, including concentrated coastal infrastructure development, wetland dredging and artificial filling, environmental contamination, and the introduction of exotic species such as the *Magallana gigas* oyster. These pressures have resulted in a decline in the delivery of these vital ecosystem services (Speake et al., 2020).

This research was conducted in Puerto Rosales (PR) (Fig. 1b), a pivotal port within the BBe. PR plays a crucial role as a hub for artisanal fishers and experiences substantial cargo traffic and naval vessel movement through the estuary main channel. Moreover, the vicinity of PR is affected by untreated sewage discharge from Punta Alta City, home to approximately 60,000 inhabitants. Consequently, this area bears a heavy burden of human activities and is characterized by pronounced eutrophication processes, primarily attributable to sewage contamination (Buzzi et al., 2021).

In February 2018, a total of 120 adult male *N. granulata* crabs were manually selected during the summer season in the southern hemisphere. These crabs were collected from a tidal flat near the sewage discharge point of Punta Alta City. Environmental parameters in the water column were measured in situ using a HANNA HI 9828 multisensor probe, yielding the following readings: pH 8.05, salinity 32 psu, and a temperature of 22.08 °C (data sourced from Truchet et al., 2020). Subsequently, the collected crabs were placed in a plastic container filled with seawater sourced from the collection site and then transported to the laboratory for further analysis.

2.2 Experimental design and depuration treatment

In the laboratory, we adhered to the experimental protocol detailed by Simonetti et al. (2020) and Truchet et al. (2020). The crabs underwent a 24-hour acclimation period in natural seawater. Following this, 60 individuals were anesthetized by freezing, and their morphological data, encompassing carapace width (CW) in millimeters and total wet weight (ww) in grams, were meticulously recorded. Subsequently, the crabs were dissected, and tissues from five individuals were combined to form a total of 12 pools: 8 pools of hepatopancreas, 4 pools of soft tissue (including hepatopancreas), and 4 pools of shells (derived from the soft tissue pools). These pools constituted the non-depurated (ND) samples. Out of the hepatopancreas pools, four were reserved for biochemical biomarker analysis and examination of metal-rich granules (MRGs), while the remaining pools of hepatopancreas, soft tissue, and shells were employed for metal content determination.

To assess their depuration capacity, the remaining crabs were housed in eight aerated glass tanks, each containing 7 to 8 individuals. These tanks were filled with artificial seawater, and the environmental conditions in the culture chambers were set to replicate the day/night temperatures, photoperiod, and humidity characteristics of the BBe (Fig. 1c) (Truchet et al., 2020). The crabs were fed at a rate equivalent to 1% of their total body weight every two days, using rabbit food pellets (GANAVE ®, 15% proteins). Additionally, tank cleaning took place the day after feeding to prevent water contamination from crab urine and feces. This feeding and cleaning regimen continued for a period of 25 days, concluding the experiment according to previous studies (Buzzi and Marcovecchio, 2016; Simonetti et al., 2020; Truchet et al., 2020).

Subsequently, 12 pools, each comprising five individuals (n = 12, with 4 organisms per pool), were randomly selected from the depurated (D) crabs. Similar to the ND crabs, the D crabs were rendered unconscious through exposure to cold temperatures at -20°C, and their morphological data were documented before dissection. Tissue pools were obtained and processed in the same manner as described previously for the ND crabs.

Metal and metallothionein (MT) data for the hepatopancreas of the ND and D crabs were obtained from Truchet et al. (2020). Meanwhile, the other biochemical biomarkers for the ND crabs were sourced from Truchet et al. (2023). In both cases, they involved crabs sampled simultaneously with those used by Truchet et al, (2020, 2023). The biomarkers for the D treatment, as well as the MRG analysis and metal concentrations in soft tissues and shells for both treatments, were specifically determined for this study. involved crabs This

2.3 Metals' and metal-rich granules (MRG) determinations

The homogenized pools of hepatopancreas and soft tissues were lyophilized, and carapaces were dried in an oven at 50°C ± 5°C until a constant dry weight was achieved. Subsequently, they were ground in a porcelain mortar for final homogenization. The acid digestion of crab tissues followed the protocol outlined in Buzzi et al. (2017). Duplicate samples of 0.5 g were digested with 5 ml of HNO_3 (65%, Merck) and 1 ml of $HClO_4$ (70-72%, Merck). The digestion process took place in a heated glycerin bath at 120 ± 10°C until complete digestion was achieved. The resulting extract was brought to a volume of 10 ml in centrifuge tubes by adding HNO_3 (0.7%). All equipment used for the dissection, digestion, and drying of samples was cleaned with 5% v/v nitric acid (APHA 1998).

The metal concentrations (Cd, Cu, Pb, Ni, Zn, Mn, Cr, and Fe) were determined using Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP OES Optima 2100 DV

Perkin Elmer). The method detection limit (MDL) was calculated based on the standard deviation of 10 blank replicates (Federal Register 1984). The MDL values (expressed as μg g^{-1} dw) were as follows: Cd 0.157, Cu 1.366, Pb 1.861, Zn 2.821, Mn 17.341, Ni 1.944, Cr 1.654, and Fe 31.102 (Simonetti et al., 2020). Analytical quality control measures involved using reagent blanks, certified reference materials and analytical grade reagents (Merck or Baker). The recovery percentages for all metals in the certified material exceeded 90%.

To analyze MRG, approximately 0.2 mg wet weight (ww) of hepatopancreas tissue was subjected to multiple centrifugations using NaOH solutions of 1 M and 0.5 M (Chiodi Boudet et al., 2019). The resulting fraction was then digested in a mixture of HNO_3 and $HClO_4$ in a 1:3 ratio to determine the metal content bound to this subcellular fraction. The samples were analyzed using ICP-OES and the results were expressed as μg g^{-1} ww.

2.4 Biochemical biomarkers

The determination of MTs followed the method outlined by Viarengo et al. (1997). After appropriate treatments, the samples were measured using a spectrophotometer at 412 nm. A reference standard solution of reduced glutathione (GSH, Sigma, 307.32 g/mol) was used, and the concentration of MTs was determined in triplicate, considering the cysteine content typically found in decapod crustaceans (18 residual cysteines per mole) (Buzzi and Marcovecchio, 2016). The results were reported as nmol of MT/g of wet weight (ww).

For the analysis of proteins, non-enzymatic, and enzymatic biomarkers associated with oxidative stress, approximately 25 mg of hepatopancreas tissue was homogenized in a cold phosphate buffer (0.1 M, pH 6.5) containing 1 mM EDTA, 14 mM DL-dithioerythritol (DTE), and 20% glycerol (Wiegand et al., 2000). The homogenates were then centrifuged at 10,000 g for 10 min at 4°C and the resulting supernatants were stored at -80°C until analysis.

Protein content was determined following the protocol described by Bradford (1976) and the absorbance was measured at 595 nm. Catalase (CAT) activity was measured at 240 nm, as described by Claiborne (1985); Glutathione-S-transferase (GST) activity at 340 following the method of Habig et al. (1974), Glutathione reductase (GR) activity was determined based on the method of Tanaka et al. (1994) and the activity was read at 340 nm. GST, CAT, and GR activities were expressed as U/mg of proteins. The H_2O_2 content was quantified at 560 nm by measuring the oxidation of Fe^{+2} by H_2O_2 in the presence of orange xylenol (Sigma), following the procedure described by Bellincampi et al. (2000). All these measurements were performed using a Biotek Synergy HTK microplate reader, and each assay was conducted in triplicate for each pool.

Lipid peroxidation levels (LPO) were determined by measuring the formation of thiobarbituric reactive substances (TBARS) in the homogenized samples described earlier, following the method outlined by Ale et al. (2018). The samples were read in a spectrophotometer at 535 nm, and the results were expressed as nmol MDA/mg of proteins.

2.5 Data analyses

2.5.1 Statistical analyses

The collected data underwent a comprehensive statistical analysis to evaluate several critical aspects. First, we assessed the normality of residuals using the Shapiro-Wilk test, checked for the homoscedasticity of variances using the Levene test, and ensured the independence of errors. In cases where it was necessary, data underwent log-transformation to meet the required statistical assumptions.

A factorial ANOVA test was applied to discern significant changes in metal concentrations between the tissues (ND and D), and metals in the tissues and biomarkers

before and after the treatments in the crab specimens. However, for the analysis of MRG data, the Mann-Whitney test was utilized due to the observed non-normality of residuals in the dataset.

All statistical analyses were conducted using the open-source software R, developed by the R Core Team in 2020. Additionally, we utilized specific R packages, including *dplyr*, *tidyverse*, and *ggradar*, to facilitate and enhance the statistical procedures.

2.5.2 Integrated Biomarker Response: IBR Index

The biomarkers analyzed in this study were combined to calculate a comprehensive stress index known as the integrated biomarker response index (IBR) (Beliaeff and Burgeo, 2002). To calculate the IBR, data standardization was performed, integrating 6 biomarkers (CAT, GST, GR, H_2O_2, LPO, and MT). These standardized biomarkers were then represented as vectors on a radial chart. The IBR was defined as the area enclosed by the polygon formed by connecting the ends of each vector (Broeg and Lehtonen, 2006). To account for the varying number of biomarkers in the set, the obtained IBR value was divided by the number of biomarkers used (n = 6) and referred to as IBR/n (Broeg and Lehtonen, 2006).

3. Results

3.1 Metals and MRG

The order of metals in the hepatopancreas differed between ND and D crabs. In ND crabs, the order was Fe > Cu > Zn > Mn > Ni > Cd (Cr and Pb were below the MDL), whereas, in D crabs, the order was Cu > Fe > Zn > Ni > Cd (Cr, Mn, and Pb were below the MDL). In the total soft tissue before the treatment, the order of metals was Fe > Cu > Mn >

Zn > Pb > Ni > Cr > Cd, and after the treatment, it was Fe > Cu > Zn > Mn > Ni > Pb > Cd (Cr was below the MDL). For the shell, the order of metals in ND crabs was Fe > Mn > Cu > Zn > Cd > Pb (Cr and Ni were below the MDL), and in D crabs, it was Fe > Mn > Cu > Zn > Cd (Cr, Pb, and Ni were below the MDL). In ND crabs, the total soft tissue had higher concentrations of Cd, Cr, Zn, Pb and Fe compared to shell tissue and hepatopancreas, while Mn tended to be higher in shell tissue and Ni in hepatopancreas (Fig. 2 and Table 1). The concentration of Cu tended to be similar in soft tissue and hepatopancreas but higher than in shell tissue (Fig. 2 and Table 1). After depuration, Cd, Cu, Zn and Ni were significantly higher in soft tissues and the hepatopancreas in comparison to the shell (Fig. 2 and Table 1).

Fig. 2 and Table 1 present the results of the depuration process of metals in *N. granulata* under experimental conditions, and Table 2 lists the percentages of reversibility and/or increases. The factorial ANOVA revealed that Cd, Pb, Mn, and Fe in the soft tissue showed a significant decrease after depuration ($p < 0.05$) (Fig. 2, Tables 1 and 2). However, in the shell, no significant differences were observed after depuration, except for Pb, which was below the MDL in D crabs (Fig. 2, Tables 1 and 2). In the hepatopancreas, significant decreases in Mn and Fe were observed ($p < 0.05$) (Fig. 2, Tables 1 and 2), while the other metals tended to decrease without significant differences.

Regarding the MRG, Mn and Fe showed a significant decrease after the assay ($p < 0.05$), while no significant differences were found between the treatments for the other metals, although they tended to decrease in D crabs and Cd and Pb were always below the MDL (Table 3).

3.2 Biochemical biomarkers and IBR

The biochemical biomarkers before and after the depuration process are shown in Fig. 4. As for the biochemical biomarkers, GR and MT significantly diminished after the depuration ($p < 0.05$) (Tables 1 and 2). At the same time, CAT and GST increased (Fig.3, Tables 1 and 2).

The radar plot of the IBR exhibited that ND crabs presented higher MT induction and GR activity than D crabs, where CAT and GST prevailed (Fig. 4). The IBR/n indicated that D crabs' values were lower than ND crabs (Fig. 4).

4. Discussion

4.1 Metals and MRG

4.1.1 Total soft tissues

After a 25-day period of depuration, a notable decrease in metals within the soft tissues was observed in comparison to the hepatopancreas and shell and this tissue also concentrated more metals than the others. Depuration research is marked by a scarcity of studies focused on total soft tissue metal detoxification in aquatic organisms, with the majority concentrating on bivalves (Freitas et al., 2012; Yin and Wang, 2018).

Notably, Simonetti et al. (2020) investigated the metal depuration process in decapod crustaceans, specifically *N. granulata*, albeit in a less disturbed location within the BBe. Their findings, in contrast to ours, did not detect Cr or Pb in the tissues, and only detected a decline in Ni, Cu and Fe in the soft tissues after the depuration, making direct comparisons challenging (Simonetti et al., 2020). A possible explanation for the lack of differences in Simonetti et al. (2020) study could stem from the fact that organisms from less contaminated sites may exhibit slower depuration rates compared to those collected from highly anthropized areas where depuration is more pronounced (Wang and Wang, 2014).

We observed a significant decrease in Cd levels post-depuration. As for this metal, McDonald et al. (2020) also observed a decrease in Cd levels in the entire body of the freshwater decapod crustacean *Paratya australiensis* following pulse exposure. They suggested that a portion of the metal bound to the gills was lost through desorption into the surrounding water. Conversely, Li et al. (2021) reported a pattern of Cd increase during the initial days of depuration in tissues such as muscles and gills of *Litopenaeus vannamei*, hinting at tissue-specific detoxification processes due to distinct tissue roles.

The higher levels detected of Cd in the total soft tissue compared to the hepatopancreas in ND crabs could be attributed to its accumulation in the gills. This phenomenon has been observed in other estuarine crabs, such as the mitten crab *Eriocheir sinensis* (Cheng et al., 2018) and *Ucides cordatus* (Duarte et al., 2019). However, for the other tissues, Cd did not exhibit significant depuration, underscoring the notion of tissue-specific depuration patterns (Wang and Wang, 2014).

Significant decreases in Mn and Fe were observed in the soft tissues of D crabs. Although Mn and Fe are essential metals, studies pertaining to their bioaccumulation and detoxification, whether in total tissue or specific tissue compartments in aquatic organisms, remain limited compared to those focused on other essential metals like Zn and Cu (Truchet et al., 2020). Previous research on oysters and clams has demonstrated the effective removal of metals, particularly essential ones such as Fe, from the soft tissues (El-Shenawy, 2004; Chinnadurai et al., 2022). The high depuration of these metals possibly reflects their weak binding within *N. granulata* tissues.

4.1.2 Shell

In relation to the shell, there is a dearth of studies examining the depuration of this hard tissue in estuarine organisms, with no significant differences noted in previous investigations (Simonetti et al., 2020). In our study, Pb was eliminated and found to be below the MDL after depuration. The previous work of Simonetti et al. (2020) did not detect Pb in shells, likely because their study was conducted in a less impacted location without the influence of untreated sewage discharges, as observed in our study area. Typically, Pb concentrations in crabs from other estuarine sites fall below the MDL (Truchet et al., 2023).

Even though all organisms in our study undergoing depuration were in the intermolt stage, and an increase in metal content in the shell was not observed, the reduction of some metals in tissues explained by their movement to the exoskeleton for expulsion in subsequent ecdysis periods cannot be ruled out. This was observed by Ramos et al. (2021) in the crab *Ucides cordatus*, where certain non-essential metals, including Pb, tended to be removed up to 60% during the ecdysis process. Similarly, Bergey and Weis (2007) provided evidence of metal transfer from soft tissues to discarded exoskeletons, specifically for Pb, in *Uca pugnax*.

4.1.3 Hepatopancreas

As highlighted by Truchet et al. (2020), the hepatopancreas exhibited a significant decrease in Mn and Fe levels after depuration, while Zn levels showed a significant increase, indicating differing behaviors among essential metals in this tissue. Similar to *Carcinus maenas*, it is plausible that Zn levels increased following depuration due to rapid expulsion and subsequent re-incorporation into the hepatopancreas (Reichmuth et al., 2010; Truchet et al., 2020). This notion suggests that crabs undergoing depuration in laboratory settings may re-absorb excreted metals through their gills, even with water changes in the tanks. Moreover, as proposed by McDonald et al. (2020) for *Paratya australiensis*, a transfer of this metal may

occur from the gills to the hepatopancreas during the depuration process, increasing the metal burden in this tissue.

4.1.4 MRG

In our study, except for Fe and Mn-MRG, most MRG did not exhibit significant statistical differences after the depuration process, although a slight decrease in metal levels was discernible. This trend was only noticeable for essential metals since Pb and Cd were both below the MDL in both ND and D crabs. Consequently, these metals were not stored or detoxified by MRG. Conversely, the reduced levels of Cu, Zn, Mn and Fe-MRG in depurated crabs can likely be attributed to the excretion of these granules from the hepatopancreas (Sánchez-Marín et al., 2023).

Several studies have pointed towards the formation of MRG as the primary mechanism for metal detoxification in metal-tolerant populations. In this regard, Chiodi Boudet et al. (2019) demonstrated the formation of Cd-MRG in the white shrimp *Palaemon argentinus*, which displayed tolerance to Cd. Additionally, Goto and Wallace (2010) observed that mummichogs (*Fundulus heteroclitus*) chronically exposed to high metal concentrations preferentially stored metals in insoluble MRG. Therefore, while further investigations into the distribution and subcellular partition of metals in *N. granulata* are warranted, our results suggest that MRG may serve as a defense mechanism, primarily against high Fe and Mn concentrations and to a lesser extent for Cu and Zn. This makes it a secondary detoxification mechanism for the latter metals. Notably, despite being essential metals, prior publications have highlighted their elevated concentrations in PR sediments, the location of crab collection (Truchet et al., 2020). Furthermore, Cu and Zn did not exhibit a reduction in their concentrations in the hepatopancreas of depurated crabs.

4.2 Biochemical biomarkers and IBR

Following the depuration period, a significant decrease in MTs was observed. Concerning these small proteins, when non-essential metals enter a cell, they inevitably compete with essential metals for intracellular ligands like MTs (Wang and Wang, 2014; Zhang et al., 2015). MTs may serve a role in metal detoxification and in combating intracellular ROS production by acting as scavengers (Bertrand et al., 2016; Giarratano et al., 2016; Truchet et al., 2023). 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 of metal exposure (Buzzi and Marcovecchio, 2016).

Buzzi and Marcovecchio (2016) examined the reversibility of MTs after 20 days of depuration in *N. granulata* and found that their induction in male crabs decreased to 35.6%. In contrast, in our study, this decrease amounted to 76.39% after the decontamination experiment. The reversibility of MTs coincides with the depuration of certain metals in the hepatopancreas, such as Mn and Fe. Thus, these proteins may indeed act as metal-binding proteins for these elements, even though this aspect has not been extensively studied (Truchet et al., 2020). Consequently, MTs may play a role in reversibility as metal-binding proteins and specific biomarkers of metals, as evidenced by their decrease following the experimental depuration (Amiard et al., 2006).

Regarding biochemical biomarkers associated with oxidative stress, CAT and GST exhibited a tendency to increase, while H_2O_2 and TBARS did not show significant differences. Furthermore, GR and MTs decreased significantly. In the case of *Ruditapes decussatus* and *R. philippinarum* clams, Freitas et al. (2012) noted a general decrease in LPO, CAT, and GST from environmental to depuration conditions. This suggests that depuration

mitigates the oxidative stress experienced by organisms in their environment. However, contrary to expectations based on previous depuration studies (Freitas et al., 2012; Nkoom et al., 2020; Solomando et al., 2021), *N. granulata* crabs exhibited increased CAT and GST activities in the hepatopancreas.

The elevated GST activity in the hepatopancreas of ND crabs likely indicates the stress caused by ROS resulting from metal exposure in the field. In contrast, D crabs displayed a decreasing trend, though not statistically significant, in lipid peroxidation, suggesting that the increase in GST activity is capable of detoxifying the stress caused by metals in the hepatopancreas tissue. Similar results were described by Wang et al. (2021) for Asian clams *Corbicula fluminea* after 7 days of depuration. Furthermore, the increase in CAT activity in D organisms could be attributed to the recovery of the enzyme, which regains its H_2O_2 removal capacity that was inhibited by its accumulation. Therefore, it can be inferred that the increased activities of CAT and GST in the hepatopancreas allow the organisms to manage and adapt to ROS-induced stress, thus maintaining homeostasis.

The observed increases in our study could be attributed to the relatively short duration of the depuration period, which might have been insufficient to effectively counteract the oxidative stress and damage caused by field exposure. This explanation aligns with the findings of the IBR radar plot, where CAT and GST emerged as the dominant biomarkers in D crabs, while MT was dominant in ND crabs. Additionally, the IBR/n plot suggests an overall reduction in the levels of biochemical biomarkers, consistent with other studies that have reported a general decrease in biochemical activity and induction (Freitas et al., 2012; Xu et al., 2021).

In the case of Cd depuration, Xu et al. (2021) observed in the crab *Sinopotamon henanense* that the recovery of antioxidant enzymes after metal depuration indicated the

restoration of redox equilibrium. They also found that glutathione reductase (GR) served as a good biomarker for depuration stages. However, the recovery process was faster in the gills than in the hepatopancreas, as dissolved metals are rapidly eliminated, promoting a more rapid recovery in antioxidant capacities (Xu et al., 2021).

5. Conclusions

Mn and Fe were found to be depurated as MRG during the experiment, suggesting that granules may not be the primary form of detoxification. The recovery of antioxidant enzymes after metal depuration suggests restoring redox equilibrium, with GR showing promise as a biomarker for depuration stages. However, the duration of depuration may not be enough to fully counteract the oxidative stress and damage caused by field exposure

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Authors contributions: Daniela M. Truchet: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Software; Writing - original draft; Writing - review & editing. Natali S. Buzzi: Investigation; Methodology; Supervision; Validation; Funding acquisition; Project administration; Writing - review & editing. C. Leandro Negro: Funding acquisition; Project administration; Investigation; Methodology; Supervision; Validation;

Writing - review & editing. M. Celeste Mora: Methodology. Jorge E. Marcovecchio: Funding acquisition; Supervision

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Figure captions

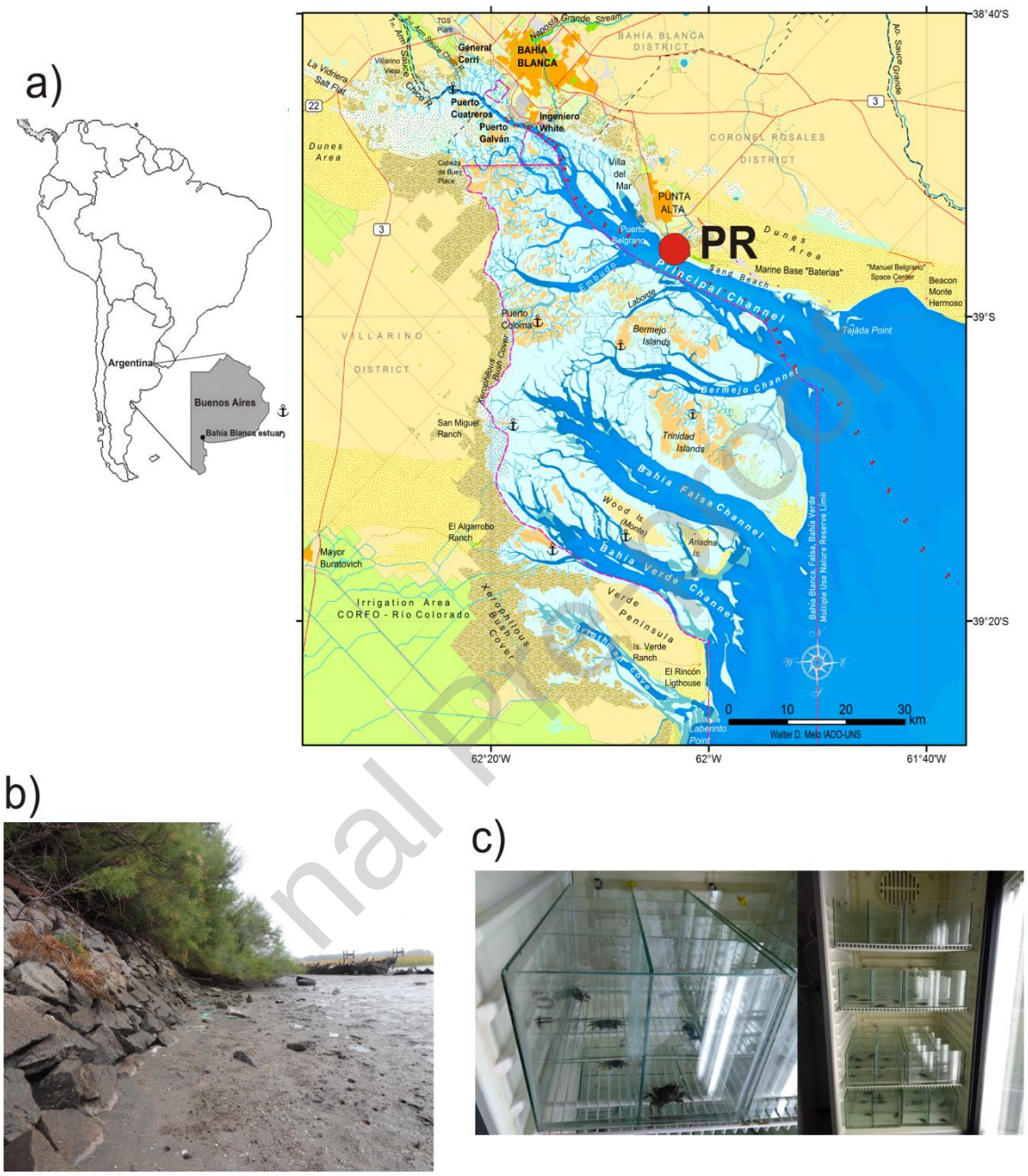

Figure 1: a) Location of the Bahía Blanca estuary and sampling site; b) PR mudflat; c) distribution of crabs in the culture chambers

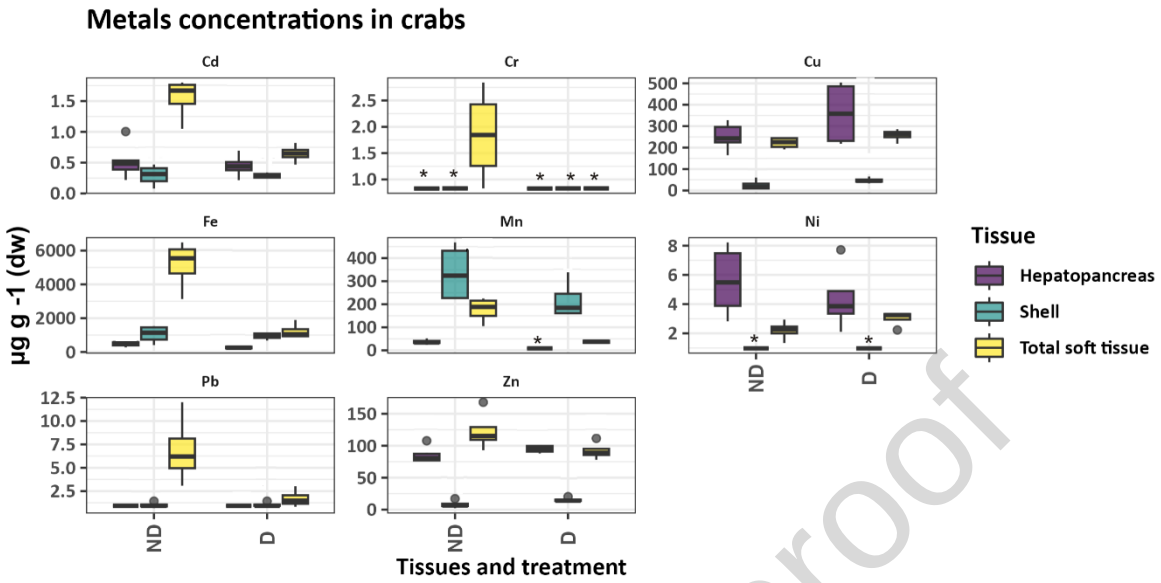

Figure 2: Metals before (ND) and after (D) the depuration experiment in the hepatopancreas, soft tissues (including hepatopancreas) and shell. * indicates values < MDL and dots/circles indicate outliers (data from ND and the hepatopancreas of D crabs taken from Truchet et al., 2020)

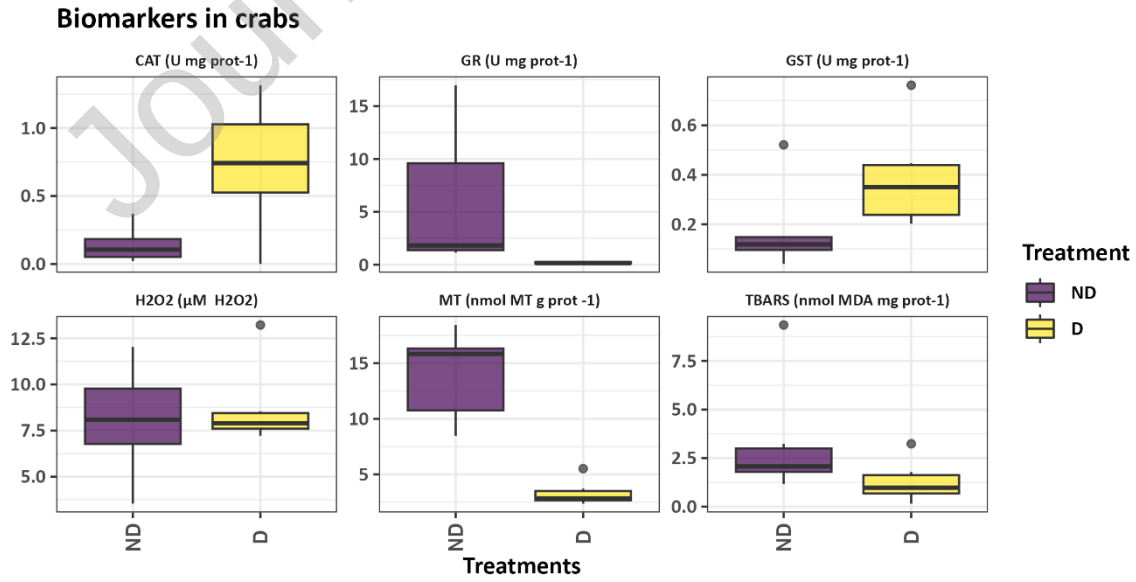

Figure 3: Biomarkers before (ND) and after (D) the depuration experiment. Abbreviations: CAT, catalase; GST, glutathione-s-transferase; GR, glutathione reductase; H_2O_2, hydrogen peroxide; TBARS, thiobarbituric acid reactive substances; MT, metallothioneins. The dots/circles indicate outliers (data from ND crabs taken from Truchet et al., 2020 and 2023)

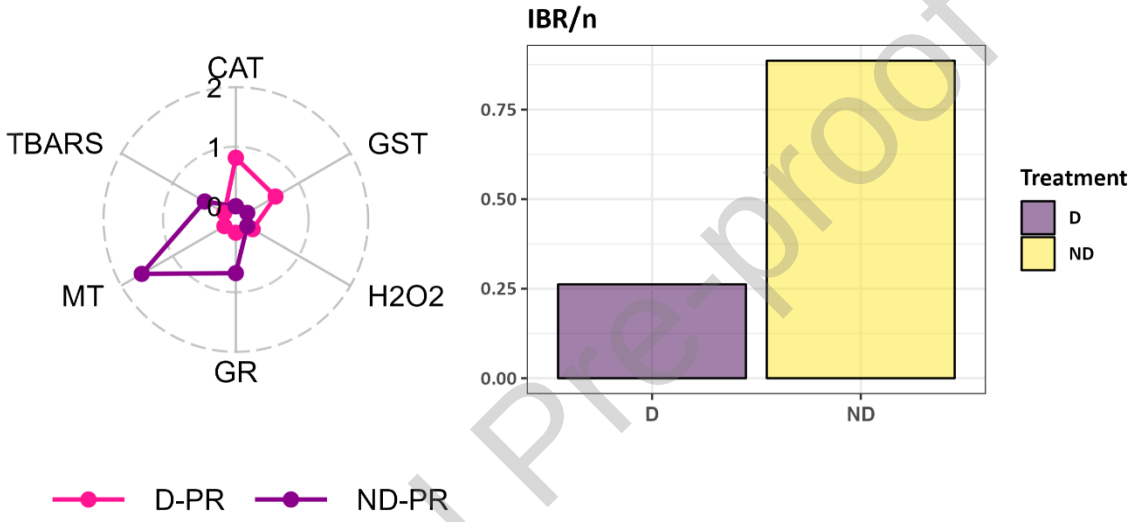

Figure 4: Integrated biomarkers response (IBR) showing the radar plot and IBR/n. Abbreviations: D-PR, depurated from PR and ND-PR non-depurated from PR; IBR/n: IBR value was divided by the number of biomarkers used (6); CAT, catalase; GST, glutathione-s-transferase; GR, glutathione reductase; H_2O_2, hydrogen peroxide; TBARS, thiobarbituric acid reactive substances; MT, metallothioneins.

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Table 1: Factorial ANOVA *p*-values after the depuration treatment for metals and biochemical biomarkers after depuration, and ND and D tissues. Values in bold are significant at $p < 0.05$

Metals / Tissue	Cd	Cu	Pb	Zn	Mn	Ni	Cr	Fe
Hepatopancreas	0.67	0.15		0.17	**0.0005**	0.43		**0.02**
Shell	0.96	0.16	0.75	0.09	0.2			0.7
Soft tissues	**0.002**	0.11	**0.03**	0.12	**0.002**	0.1	0.06	**0.002**
ND	**0.0001**	**<0.0001**	**0.0013**	**<0.0001**	**0.0002**	**0.0017**	**0.017**	**<0.0001**
D	**0.019**	**0.0026**	0.17	**<0.0001**	**0.0007**	**0.02**		**0.0035**
Biomarkers								
CAT	**0.002**							
GST	**0.02**							
GR	0.09							
H2O2	0.63							
TBARS	0.16							
MT	**0.0001**							

Values that are not shown are because of the given metal was < MDL

Table 2: Percentage of remotion (-) or increases (+) of metals and biomarkers after the experimental depuration in relation to non-depurated crabs (ND). In bold, differences are significant at $p < 0.05$

Metals / Tissue	Cd	Cu	Pb	Zn	Mn	Ni	Cr	Fe
Hepatopancreas	-13.46	+29.92		+10.34	**-76.31**	-21.64		**-45.21**
Soft tissues	**-58.06**	+13.96	**-75.11**	-25.25	**-78.88**	-27.15	-54.89	**-76.31**
Shell	-3.3	+46.33	**<MDL**	+46.41	-33.73			-10.72
Biomarkers	CAT	GST	H2O2	GR	MT	TBARS		
	+80.82	**+61.53**	-7.21	**-97.24**	**-76.39**	+39.63		

Table 3: Mean ± SD of metal-rich granules (µg g-1 ww) for *N. granulata* before (ND) and after (D) the depuration treatment. Values in bold indicate statistical differences between the treatments (p< 0.05; Mann-Whitney test)

Treatment	Cd	Cu	Pb	Zn	Mn	Ni	Fe

ND	<MDL	4.33±2.33	<MDL	4.45±1.87	3.42±0.33	0.17±0.4	24.46±3.97
D	<MDL	1.45±0.58	<MDL	2.74±0.2	**1.75±0.51**	0.13±0.01	**17.27±1.17**

Abbreviations: < MDL, below the method detection limit.

Credit author statement: Daniela M. Truchet: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Software; Writing - original draft; Writing - review & editing. Natali S. Buzzi: Investigation; Methodology; Supervision; Validation; Funding acquisition; Project administration; Writing - review & editing. C. Leandro Negro: Funding acquisition; Project administration; Investigation; Methodology; Supervision; Validation; Writing - review & editing. M. Celeste Mora: Methodology. Jorge E. Marcovecchio: Funding acquisition; Supervision

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.