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Research paper

Integrative assessment of the ecological risk of heavy metals in a South American estuary under human pressures

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ABSTRACT

Biomonitoring of heavy metal pollution through the use of biomarkers could be a difficult task since the organisms' physiological changes could shift regarding natural factors (i.e., the season of the year) and due to the anthropogenic pressures of the environment. In the Southwest Atlantic Ocean, where most industrial and developing countries are settled, it is essential to address these concerns to generate information for the stakeholders and monitoring programs that aim to use biochemical biomarkers as early warning signals to detect heavy metal pollution. The present study intended to determinate the heavy metal concentrations in sediments and the hepatopancreas of the crab species *Neohelice granulata* as well as the ecological risk through the use of biomarkers and geochemical indices in sites with different anthropogenic pressures of the Bahía Blanca estuary (SW Atlantic Ocean) during the warm and cold season. The results showed low to moderate heavy metal pollution in the sediments by Cu with possible effects on the biota in a site with sewage waters' discharges. Except for GST that was explained by Cd, the biomarkers employed were not useful to assess spatial heavy metal pollution, and they might be ruled out by physiological seasonal variations rather than anthropogenic constraints, or another type of pollutants in the area.

1. Introduction

Coastal and estuarine areas are essential places for human inhabitants since they are economic-important areas for fisheries and provide other ecosystem services, such as climate regulation via gases and ocean thermal regulation (Martinetto et al., 2019); still, they are often the ultimate receptacles of anthropogenic pollutants. In large marine ecosystems, such as the Southwest Atlantic Ocean, the inputs of toxic substances in coastal and estuarine areas were increased by the dominant productive economic model with extensive areas destined for agricultural and livestock activities, the increased human populations, along with portuary and industrial activities, and the untreated sewage discharges (Häder et al., 2020). Some studies have demonstrated that coastal and estuarine ecosystem restoration due to human pressures would take almost a hundred years in comparison to freshwater systems (Borja et al., 2010). In a long-term period, this problem and the effects of climate change would be challenging to overcome, and it would have effects on human health and small blue economies (Allison et al., 2009).

For assessing in situ heavy metal pollution in coastal and estuarine environments, the use of multiple indicators and biomarkers as early warning signals of pollution is thoroughly recommended for the monitoring, conservation, and recovery of these systems (Martín-Díaz et al., 2008). Some biochemical parameters known as biomarkers provide information on the effects of heavy metals in the environment and play a unique role since its detection can prevent adverse effects at higher hierarchical levels (Van der Oost et al., 2003). The exposure of marine

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animals to heavy metals in the field and under laboratory conditions has proved to induce oxidative stress throughout the formation of reactive oxygen species (ROS), which have deleterious effects and cell damage. Organisms have developed antioxidant defense mechanisms through the enzymatic activity that can intercept and inactive ROS to protect molecular targets against oxidative damage. Some of these enzymes are superoxide dismutase (SOD), catalase (CAT), peroxidases (like glutathione peroxidase, GPx), and glutathione S-transferases (GST). As an example, SOD converts superoxide anion radical (O_2^-) to hydrogen peroxide (H_2O_2), while CAT and GPx detoxify H_2O_2, and GST conjugates pollutants with reduced glutathione (GSH) to facilitate the excretion, which implies a biotransformation ability (Regoli et al., 2002).

On the other hand, metallothioneins (MT) are specific metal biomarkers because they are cysteine-rich proteins with the ability to sequestrate and detoxify excesses of essential and non-essential heavy metals, protecting the organism against possible damages caused by the excess of metals in the organs and tissues (Viarengo et al., 1985). However, biomarkers not only increase its activity or are induced by anthropogenic pollution, but they are also influenced by the seasonal factor and its effect on the organisms' physiology, as well as by the natural biological processes (Giarratano et al., 2011; Capparelli et al., 2019). Therefore, to generate tools for monitoring the environmental stress due to heavy metal pollution, it is fundamental to build approaches that include a possible effect of seasonal parameters on the organisms' physiology.

Special attention has been paid to benthic invertebrates as biomonitors, since they are in the bottom of food webs, triggering a "bottom-up" in top predators. These animals are sessile or with limited movements, which allow them to indicate local pollution. They are easy to sample and manipulate in laboratory conditions, and some of them are easy to identify and are adapted to environmental parameters changes (Rainbow, 2002). In the SW Atlantic Ocean, the information regarding biomonitors and early warning signals in coastal and estuarine environments is scarcer than in other regions of the world, especially in the Argentine Sea, one of the most productive seas worldwide (Martinetto et al., 2019). Among benthic invertebrates in these ecosystems, mostly mussels and few crustaceans have been studied in terms of the different biochemical responses in association with environmental heavy metal pollution (Comoglio et al., 2011; Giarratano et al., 2011, 2013, 2016; Di Salvatore et al., 2013).

Estuarine environments in the Argentine Sea have been even less studied than other coastal areas. The Bahía Blanca estuary (hereafter, BBE) is part of El Rincón Area, one of the few marine protected areas globally, and therefore is critical to understand its health status. In this estuary, the dominant macrobenthic species is *Neohelice granulata*, a varunid widespread crab known as the burrowing crab because of its ability to remove sediments (bioturbation) to burrow their depth caves that provide shelter against environmental conditions and predatory attacks, and are a useful area for sexual encounters and protection during molting stages and breeding (Luppi et al., 2004, 2013; Nuñez et al., 2015). Recent studies on heavy metal pollution have considered the hepatopancreas of this species as the main organ to accumulate and detoxify pollutants in comparison to other tissues such as gills, and therefore it is an essential organ for ecotoxicological studies (Buzzi and Marcovecchio, 2016; Truchet et al., 2020).

Hence, the seasonal biomonitoring of this estuarine system through *N. granulata*, its heavy metal bioaccumulation pattern, and biochemical early warning signals would provide stakeholders invaluable information to manage and protect this estuary. Therefore, the main objectives of this study were a) to assess the seasonal heavy metal pollution status (Cd, Cu, Zn, Pb, Ni, Mn, Cr, Fe) in the fine sediments and the potential ecological risk through geochemical indices in two sites with different human pressures in the BBE; b) to evaluate the seasonal accumulation of these heavy metals in the crab species *N. granulata;* and c) to determinate the biochemical responses (MT, GST, CAT, H_2O_2) to heavy metals and/or environmental parameters. This information would allow decision-makers to get a global picture of the heavy metal pollution process and dynamics and if seasonality affects the biomarkers' response instead of just the anthropogenic pressures as it is expected.

2. Materials and methods

2.1. Study area

The BBE (38°44′–39°27′ S and 61°45′–62°30′ W) is a mesotidal Pampean−Patagonian estuarine system located on the south of Buenos Aires province (Argentina) on the Southwest of the Atlantic Ocean (Fig. 1a, b). It is the second-largest estuary in Argentina after La Plata with an extension of 2300 km^2, including large mudflats and salt marshes with *Spartina alterniflora* as the dominant plant species (Parodi, 2004). It is a semi-enclosed estuary with limited exchange with the continental shelf, and therefore it is considered a vertical homogenous and hypersaline estuary from the mouth to the inner part (Freije et al., 2008). It is also considered a natural high eutrophic system, and the magnitudes of the nutrients are incredibly high in some areas, like in the discharges of untreated domestic waters (Spetter et al., 2015b). The nutrients supply is detected miles away from the estuary in El Rincón Area (Perillo, 1994), a recently protected maritime area that represents 10% of the Argentinian continental shelf (Fig. 1a) and is a productive area for fisheries since it is the nursery and mating zone of many fishes of commercial interest (Colautti et al., 2010).

The entire system is at serious risk since large petrochemical refineries, reservoirs, synthetic compounds, pesticide and fertilizer factories, cold-storage plants, and cereal silos are settled along the estuary. Therefore, this system receives natural and anthropogenic discharges of heavy metals, urban and agricultural pesticides, oil and its hydrocarbons, petrochemical residues, microplastics, and organic materials with high chemical and biochemical oxygen demands (Freije et al., 2008; Marcovecchio et al., 2008; Arias et al., 2010; Forero López et al., 2020). Besides, the Principal Channel (Canal Principal) is continuously under dredging activities to allow the circulation of great cargo ships removing and re-suspending pollutants and biota from the bottom sediments (López-Abbate et al., 2017).

This study was conducted in two sites of the BBE: Villa del Mar (VM) and Puerto Rosales (PR) (Fig. 1b). VM is a small coastal village in the middle-external part of the estuary, and most of its inhabitants (~500) are families of small-scale artisanal fishers that use the coasts as an alternative port (Truchet et al., 2019). People that inhabit this place are also dedicated to the rescue of endangered animals and to the use of the extensive salt marshes of *S. alterniflora, S. densiflora,* and *Sarcocornia perennis* for education, conservation, and recreation. The main dominant community is composed of *S. alterniflora* and the burrowing crab *N. granulata.* PR is located in the outer part of the estuary and is part of Coronel Rosales district, where the city of Punta Alta (~60,000 inhabitants) is located, and it is also the second biggest town of the estuary after Bahía Blanca city (~301,501 inhabitants). PR is a small-scale artisanal and commercial port that also receives the discharges from an oil plant located in the district and from untreated sewage waters with heavy metals and other pollutants from the city of Punta Alta (Spetter et al., 2015a). From a bio-ecological perspective, the area has small patches of salt marshes of *S. alterniflora* and extensive mudflats inhabited by microbial mats and *N. granulata*.

2.2. Field sampling and laboratory measures

In the intertidal zone of each wetland, 120 adult females and 60 adult males' inter-molt crabs were collected by hand from the mudflat of PR and the salt marsh with *S. alterniflora* of VM. Samplings were performed during the cold (winter, August 2018) and warm (spring, October 2018) season for the southern hemisphere. The carapace width was >15 mm, and those injured or with missing appendixes were discharged. Crabs were stored in plastic containers with in situ estuarine water and were

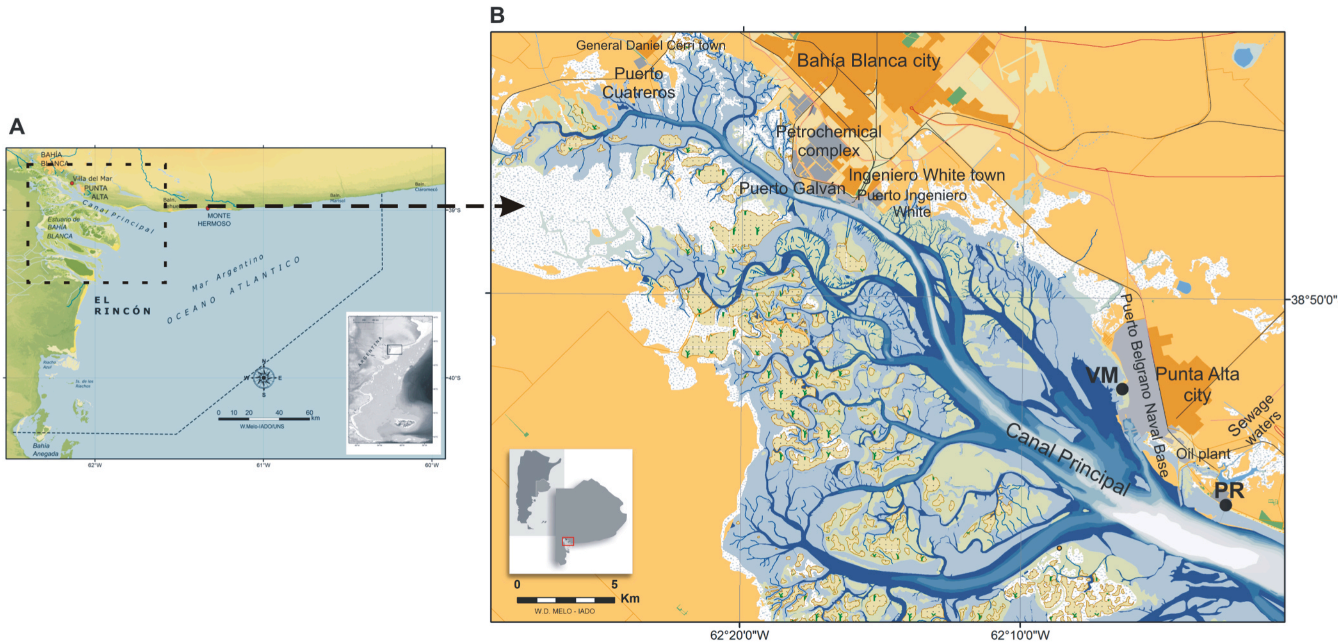

Fig. 1. Study area, the Bahía Blanca estuary (BBE). (a) The BBE as part of the marine protected area El Rincón (Argentine Sea) (b) Sampling sites in the BBE. The dots indicate the different sites. Abbreviations: VM: Villa del Mar, PR: Puerto Rosales.

transported to the laboratory. Also, 3 replicates of sediments were taken from each zone and were stored in plastic bags and refrigerated. The physicochemical parameters were measured in situ for each season and site with a HANNA® HI 9828 multisensor probe.

In the laboratory, females (F) and males (M) were sized and weight to obtain the biometric data, like carapace width (mm) and total weight (g) using an electronic caliper ±0.01 mm accuracy and a precision analytical balance OHAUSS® ± 0.001 g accuracy, for each respective measure. For the heavy metal determination, crabs were anesthetized by freezing at −20 °C and then were rapidly dissected out in the cold to obtain the hepatopancreas and avoid possible metal movements throughout the tissues due to the sudden temperature changes (Wiech et al., 2017). After dissection, the hepatopancreas was weighed (g). A total of 10 pools of 6 hepatopancreas of M and 10 pools of 12 F were obtained in each site and season (M: $n = 10$, 6 each; F: $n = 10$, 12 each), 5 pools were used for heavy metals determination, while the other ones were saved at −80 °C for the biomarkers and biochemical analysis. For heavy metals analyses, the tissue pools were lyophilized and then were mortared to homogenize the samples. The sediment samples were dried in an oven at 50 ± 5 °C until constant weight and then mortared. Large debris and fragments of animals and plants were removed before grinding and sieving to obtain the finest fraction of <63 µm ($n = 3$ pools of sediments' fine fraction). All the dry material (crabs and sediments) was saved in a desiccator at room temperature until analysis.

2.3. Heavy metals determination in sediments and crabs and cleaning procedure

Following the protocols proposed by Marcovecchio and Ferrer (2005), with modifications by Buzzi et al. (2017) for macroinvertebrates, subsamples of 0.5 g of dry material were digested with a mixture of HNO_3 and $HClO_4$ (5:1) (Merck® 69% and 70–72% pure, respectively) at 110 ± 10 °C, until the digestion was completed. The final extract (1 mL) was poured into a centrifuge tube, and it was filled up to 10 mL with diluted 0.7% HNO_3. Heavy metal concentrations were measured by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES AVIO® 500 DV, Perkin Elmer, Argentina).

Reagent blanks, certified reference materials (CRM), and analytical grade reagents were used for analytical quality control. The recovery percentages of all metals in CRM (n°6, NIE Tsukuba, Japan, mussel tissue flour, and n° 2 NIES, Japan, Pond Sediments) were higher than 90%. The method detection limit (MDL) was calculated for each metal by multiplying the standard deviation of 20 blank replicates by the Student *t* value at the 99% confidence level (n − 1° of freedom) (Federal Register, 1984; EPA, Environmental Protection Agency's, 2016). The MDL presented the following values (µg g^{-1}): Cd: 0.157, Cu: 1.366, Pb: 1.861, Cr: 1.654, Zn: 2.821, Mn: 17.341, Ni: 1.944, Fe: 31.102. In the cases where metal concentrations did not exceed the MDL, they were replaced with half of its value (MDL/2) for statistical analyses. To avoid heavy metal cross-contamination, prevention was taken during the whole process, and all the materials employed were washed with non-ionic soap, HNO_3 (5%) and distilled water following the recommendations of APHA-AWWA-WEF (1998).

2.4. Biochemical determinations in the hepatopancreas of crabs

2.4.1. Metallothioneins

Metallothioneins (MT) were determined following the method of Viarengo et al. (1997). Pools of hepatopancreas tissue of F and M crabs were ice-cold homogenized (1:4) in Tris-HCl buffer 0.02 M (pH 8.6), saccharose 0.5 M, Dithiothreitol (DTT) 1 mM, and Phenylmethylsulfonyl fluoride (PMSF) 0.5 mM using a Potter–Elvehjem tissue homogenizer. Then, samples were centrifuged at 30,000 g for 45 min at 4 °C and they were purified by two successive extractions with pure cold ethanol and chloroform. The pellets were dried under a soft N_2 flux and dissolved with NaCl 250 mM and Ethylenediaminetetraacetic (EDTA) 4 mM. Finally, they were reacted with 5,5′-Dithiobis 2-nitrobenzoic (DTNB, Sigma) 0.43 mM in phosphate buffer 0.2 M and read with a spectrophotometer at 412 nm. MT concentrations were quantified using a solution of reduced glutathione (GSH Sigma, 307.32 g mol^{-1}) as a reference standard based on the cysteine content of 18-cysteine residues per mol (Serra-Batiste et al., 2010) -assuming a similar tiol SH group content in *N. granulata.* The values were reported as nmol MT per gram (g) of wet tissue (wt) (Buzzi and Marcovecchio, 2016; Truchet et al., 2020).

2.4.2. Catalase (CAT), glutathione-s-transferase (GST), hydrogen peroxide (H_2O_2) and proteins content

For the measurements of the enzymatic activity, hydrogen peroxide and proteins in the hepatopancreas, the extracts were obtained in ice-cold phosphate buffer 0.1 M (pH 6.5), EDTA 1 mM, DL-ditioeritritol (DTE) 14 mM, and glycerol 20% in a Potter–Elvehjem tissue homogenizer, following the methodology proposed by Wiegand et al. (2000). Homogenates were centrifuged at 10,000 g for 10 min at 4° C, and the supernatants of each pool were stored at −80 °C until analysis. All the determinations of the enzymatic and non-enzymatic activities were done by using a microplate photometer Biotek Synergy HTK, and each enzymatic assay was carried out in triplicate for every pool. GST activity was determined at 340 nm, according to Habig et al. (1974), using 1-chloro-2, 4-dinitrobenzene (CDNB, Sigma) as a substrate. CAT activity was determined at 240 nm based on the decomposition of H_2O_2, according to Claiborne (1985). These enzymatic activities were expressed as nanokatals per mg protein. Protein content was determined following Bradford (1976) using serum bovine albumin as standard at 595 nm. Whereas H_2O_2 content (μM H_2O_2) was quantified at 560 nm, according to Bellincampi et al. (2000), based on the oxidation of Fe^{+2} by H_2O_2, followed by the reaction of Fe^{+3} with xylenol orange (Sigma) (Negro et al., 2019).

2.5. Data analyses

2.5.1. Statistical analysis

A two-way ANOVA was carried out to test the possible differences between sites and seasons for each metal in the sediments ($p < 0.05$), while, in the case of heavy metals in crabs and biomarkers' response, a three-way ANOVA was carried out to test the differences between sites, seasons and genders ($p < 0.05$). In the three cases, analyses were conducted after the normality of the distribution, samples' independence, and homoscedasticity were tested. A Post Hoc LSD Fisher was employed for the three analyses. A Principal Component Analysis (PCA) biplot was employed for describing the study sites, possible associations with heavy metals between sediments (MeS), and those accumulated in crabs' tissues (MeC), biomarkers, and environmental. This analysis was carried out with the Software Infostat® (Student free version, FCA, Universidad de Córdoba), and the values of heavy metals below the MDL were not employed for the PCA.

2.5.2. Methods of heavy metal pollution assessment

Several geochemical indices were calculated to establish sediments' pollution for the sampling sites and seasons. The geo-accumulation index (Igeo) (Müller, 1981) allowed us to determine and define metal contamination in sediments:

$$Igeo = log_2\left(\frac{C_n}{1.5 \times B_n}\right)$$

This index was distinguished into seven classes by Müller and Buccolieri et al. (1981, 2006): Igeo≤ 0, class 0, unpolluted; $0 < \text{Igeo} \leq 1$, class 1, unpolluted to moderately polluted; $1 < \text{Igeo} \leq 2$, class 2, moderately polluted; $2 < \text{Igeo} \leq 3$, class 3, from moderately to strongly polluted; $3 < \text{Igeo} \leq 4$, class 4, strongly polluted;$4 < \text{Igeo} \leq 5$, class 5, from strongly to extremely polluted; and $\text{Igeo} > 5$, class 6, extremely polluted.

The enrichment factor (EF) (Szefer et al., 1998) was calculated by comparing current metal concentrations with pre-industrial and/or natural levels:

$$EF = \frac{\left(\frac{M}{X}\right) sample}{\left(\frac{M}{X}\right) background}$$

Where M is the concentration of the metal of interest and X the normalization element (in this case, the values of Fe were employed) for the samples and geochemical background using the reference baseline international levels. The pollution level based on the EF was evaluated using the classification proposed by Sutherland (2000), considering the following five categories: 1) EF <2, depletion to minimal enrichment, suggests no or minimal pollution; 2) EF 2–5, moderate enrichment, suggests moderate pollution; 3) EF 5–20, significant enrichment, suggestive of a significant pollution signal; 4) EF 20–40, very highly enriched, indicating a reliable pollution signal; 5) EF >40, extremely enriched, indicating extreme pollution (Muniz et al., 2019).

Potential ecological risk index (PER, or ER for other authors) was also introduced to assess the pollution degree of heavy metals in the present sediments, and the equation was proposed by Guo et al. (2010) as the following:

$$PER = \sum E;\ \ E = T \times C;\ \ C = C_a \big/ C_b$$

Where C is the single element pollution factor, C_a is the content of the element in samples, and C_b is the reference value of the element. The sum of C for all the heavy metals examined represents the integrated pollution degree (C) of the environment. E is the potential ecological risk index of an individual element, while T is the toxic biological factor of an individual element determined for Zn = 1, Cu=6, Ni=Pb = 5, Cd = 30, Cr = 2 (Hakanson, 1980; Guo et al., 2010). Following Hakanson (1980), the ecological hazards associated with the PER are distinguished in four levels: PER ≤ 150 low-grade, 150 ≤ PER ≤ 300 moderate, 300 ≤ PER ≤ 600 severe, and 600 ≤ PER serious ecological risk.

Since many pollutants are in the environment, the mean ERM quotient (mERM-Q) method was applied to determine the adverse biological effects of coexisting contaminants (Long et al., 1998) as follows:

$$mERM - Q = \sum \left(C_i \big/ ERM_i\right) \big/ n$$

C_i is the concentration of contaminant or element *i*, ERM_i is the ERM value for contaminant *i,* and *n* is the number of contaminants. Values of mERM-Q a) ≤0.10 show no adverse biological effects; b) between 0.10 and 0.50 indicate potential adverse effects; c) between 0.50 and 1.5 indicate moderate adverse effects; and d) >1.5 reveal significant adverse effects (Long, 2000).

For the biota, the following indices were estimated: condition factor (CF), Hepatosomatic index (HSI), humidity content of the hepatopancreas (H) and bio-sediment accumulation factor (BSAF).

$$CF = \frac{body\ wet\ weight \quad (g)}{carapace\ width \quad (cm)}$$

$$HSI = \frac{hepatopancreas\ wet\ weight \quad (g)}{body\ wet\ weight \quad (g)}\ x\ 100\%$$

$$H = \frac{hepatopancreas\ dry\ weight \quad (g)}{hepatopancreas\ wet\ weight \quad (g)}\ x\ 100\%$$

To estimate the proportion in which metal occurs in crabs and the associated sediment, BSAF was performed following Szefer et al. (1998) as

$$BSAF = \frac{metal\ concen\ tration\ in\ the\ hepatopancreas \quad (\mu g g^{-1})}{metal\ concentration\ in\ the\ sediments \quad (\mu g g^{-1})}$$

3. Results

3.1. Oceanographic physicochemical parameters of the water column

The physicochemical parameters of the water column are shown in Table 1. The temperature was the main factor that decreased by half in winter and the conductivity in spring at VM. The salinity tended to gently decrease in winter, as well as the values of dissolved oxygen (DO).

Table 1
Seasonal and spatial oceanographic physicochemical parameters of the water column in the BBE, and carapace length (mm) and weight (g) of males (M) and females (F) of *N. granulata.*

Season	Site	Temperature (°C)	Salinity (psu)	pH	DO (mg L^{-1})	Conductivity (mS cm^{-1})	F length (mm)	F weight (g)	M length (mm)	M weight (g)
Spring	VM	18.05	**35**	8.2	12	23.4	20.13 ± 2.15	4.50 ± 1.52	26.24 ± 2.29	10.87 ± 2.66
	PR	**21.07**	34	8.3	11	45.2	23.84 ± 2.92	7.95 ± 2.56	**31.81 ± 2.11**	**20.32 ± 4.53**
Winter	VM	12.3	32	8.1	9.5	54.3	21.08 ± 2.61	4.64 ± 1.62	**25.38 ± 1.94**	**9.92 ± 2.29**
	PR	10.04	33	8.2	8.5	57.2	20.20 ± 3.68	4.83 ± 2.57	21.94 ± 4.49	6.59 ± 5.03

The highest values are shown in bold.

3.2. Morphological indices in N. Granulata crabs

Morphological measures are also shown in Table 1: in spring, M exhibited a total length and weight higher in PR than in VM, while in winter, it was observed otherwise. F presented similar values of weight and length in each site and season. Table 2 shows the spatial and seasonal morphological indices for females and males crabs: overall, the CF did not show statistical differences, except for M in PR, where the highest value of this index was recorded. The HSI showed the highest statistical value in F during spring in VM, followed by F from the same winter site. As for the hepatopancreas' humidity content, the highest value was recorded in F spring in PR.

3.3. Seasonal and spatial heavy metal concentrations in sediments and N. Granulata crabs

Heavy metal concentration in the sediments' fine fraction showed the following pattern Fe > Mn > Cu > Zn > Cr > Pb, while Cd was below the MDL. In males and females' crabs was Fe ≥ Cu > Zn > Mn > Cd, while Pb (except in F from PR in spring) and Cr were below the MDL, and Ni was <MDL in F in spring and M in winter both from PR. Seasonal and spatial metal distribution in sediments and comparisons with TEL and PEL values are shown in Table 3, where PR in spring registered the highest values of heavy metals, except Mn that was statistically higher in spring in VM. An important thing to point out is that Cu in spring in PR was above the TEL value recommended by the Sediment Quality Guidelines (SQGs) of the NOAA, and consequently, its concentration was higher than the value for unpolluted sediments.

Table 3 also shows the accumulation patterns in crabs: Cd and Zn did not show any statistical differences between seasons, sites and genders. Mn was statistically lower in F and M from PR in spring, while Cu was higher in F from VM in winter and M from PR in spring. Ni did not show any statistical difference but was below the MDL in F crabs in spring and M in winter from PR. Finally, the highest value of Fe was detected in M from PR during spring, which was also similar to F in the same season in VM.

Table 2
Mean (±SD) seasonal and spatial morphological indices of females (F) and males (M) crabs of *N. granulata.*

Index	Season	Site	Sex	Values
CF	Spring	VM	F	0.22 ± 0.04a
			M	0.05 ± 0.04c
		PR	F	0.33 ± 0.03b
			M	**0.63 ± 0.07d**
	Winter	VM	F	0.22 ± 0.02a
			M	0.39 ± 0.03b,c
		PR	F	0.26 ± 0.05a
			M	0.25 ± 0.05a
HSI (%)	Spring	VM	F	**6.17 ± 1.34d**
			M	3.38 ± 0.17b,c
		PR	F	3.10 ± 1.36b,c
			M	2.08 ± 0.21a
	Winter	VM	F	3.92 ± 0.7c
			M	3.01 ± 0.23b
		PR	F	2.54 ± 0.57a,b
			M	2.76 ± 0.44a,b
Humidity (%)	Spring	VM	F	29.22 ± 83a,b
			M	33.18 ± 4.97b
		PR	F	**82.89 ± 2.58c**
			M	20.4 ± 2.56a
	Winter	VM	F	32.02 ± 14.14b
			M	27.20 ± 2.41a,b
		PR	F	25.49 ± 2.22a,b
			M	23.76 ± 1.47a

Similar letters indicate no statistical differences (Fisher LSD Test, n = 5 pools, p>0.05). The highest values are shown in bold.

3.4. Geochemical indices, ecological risk and bioaccumulation patterns in N. Granulata

Table 4 shows geochemical indices, the employment of ecological risk assessment (PER) for sediments, and the bioaccumulation patterns in crabs using the BSAF index. The Igeo and EF values for Cu and Zn were higher in PR during spring, but only the EF showed a metal enrichment by Cu in the sediments, which suggests moderate pollution by this metal. In the case of Mn, both indices tended to be higher in the sediments from VM during both seasons but just like for all the other heavy metals in both sites and seasons, the Igeo ranged between 0 and 1, belonging to class 1: from unpolluted to moderately polluted, and the EF was <2 suggesting no or minimal pollution. Overall, mERM-Q indicated potential adverse effects to the biota in the sediments of PR, and PER indicated all sediments with low ecological risk. Meanwhile, as is evidenced by the BSAF values, crabs tended to accumulate more Cu from sediments followed by Zn, Ni, Mn and Fe in lower proportions, with the highest BSAF for Cu in F from VM during winter.

3.5. Seasonal and spatial biochemical biomarkers in N. Granulata in response to heavy metals and physicochemical parameters

Concerning biomarkers, MT showed a statistical difference between seasons, but not in the case of sites and genders. The highest values of MT were found during winter in F from VM and PR and the lowest value in F from PR during spring (Fig. 2a). GST showed the highest activity in F from VM during winter, followed by F from VM and PR in spring, and then the rest of the samples expressed significantly lower activity (Fig. 2b). CAT activity was also significantly higher in F from VM during winter, and in comparison with this value, M and F from PR exhibited significantly lower values of this biomarker (Fig. 2c). Meanwhile, H_2O_2 exhibited the highest concentration in winter in M from VM and the lowest in F from PR during spring, the rest of the measures were statistically similar to these values (Fig. 2d).

The PCA analysis (Fig. 3) revealed that two principal components explained 85.5% of the total variance. The first axis PC1 accounted for 56.3% of the variance and was represented negatively by MnS, CdC, MnC, NiC, the biomarkers and DO; and was positively correlated most of the heavy metals in the sediments, CuC, ZnC, and FeC, CF, the humidity of the hepatopancreas and all the other physicochemical parameters. PC2 accounted for 29.2% of the total variance and was represented mainly by the oceanographic physicochemical parameters (except conductivity), GST, HSI, and most heavy metals in crabs (except for Zn and Fe). The CuS was the variable that made the greatest contribution to the

Table 3
Summary of mean (± SD) seasonal and spatial distribution of heavy metals concentrations (μg g^{-1} dw) in the hepatopancreas of *N. granulata* and in fine sediments (<63 μm) and comparisons with TEL-Q and PEL-Q values.

Season	Site	Matrix		Cd	Cu	Pb	Zn	Mn	Ni	Cr	Fe
Spring	VM	Sediments		<MDL	11.79 ±1.26^{a}	3.86 ±0.23^{a}	38.31 ±1.58^{a}	**496.35 ±14.4**^{c}	7.96 ±0.4^{a,b}	5.7 ± 0.35^{a}	16,781.91 ±392.78^{a,b}
		Crabs	F	1.33±0.27^{a}	212.38±30.66^{a,b}	<MDL	53.78±16.84^{a}	**29.2±10.33**^{a}	2.36±2.17^{a}	<MDL	247.37±53.68^{a,b}
			M	1.1±0.1^{a}	159.11±15.16^{a,b}	<MDL	51.23±7.19^{a}	21.35±1.15^{a}	**4.04±3.57**^{a}	<MDL	210.95±60.78^{a}
	PR	Sediments		<MDL	**43 ±22.87**^{b}	4.31 ±0.68^{a,b}	**56.0 8±4.44**^{c}	238.24 ±14.32^{a}	**9.29 ±0.43**^{c}	6.52 ±0.35^{a}	**19,415.98 ±770.04**^{c}
		Crabs	F	1.19 ± 0.79^{a}	223.5±35.75^{a,b}	**2.94 ±4.02**	55.52±6.3^{a}	9.65±2.94^{b}	<MDL	<MDL	292.85±133.71^{a,b}
			M	0.66±0.08^{a}	293.31±67.72^{c,d}	<MDL	62.76±5.23^{a}	10.02±1.85^{b}	1.34±0.84^{a}	<MDL	**511.75±98.31**^{c}
Winter	VM	Sediments		<MDL	10.79 ±0.49^{a}	3.75 ±0.24^{a}	34.96 ±2.31^{a}	387.62 ±17.29^{b}	7.31 ±0.37^{a}	5.54 ±0.51^{a}	15.729.35 ±775.31^{a}
		Crabs	F	**1.73 ±0.87**^{a}	**304.85 ±103.48d**	<MDL	**69±30.27**^{a}	19.45±6.52^{a}	1.85±1.22^{a}	<MDL	336.95±124.31^{b},c
			M	1.2±0.49^{a}	177.72±50.65^{a,b}	<MDL	48.39±8.16^{a}	24.6±7.18^{a}	2.97 ±1.67^{a}	<MDL	347.31± 131.94^{a,b}, c
	PR	Sediments		<MDL	18.44 ±2.74^{a}	**4.7 ±0.38**^{b}	46.07 ±5.41^{b}	237.91 ±13.04^{a}	8.45±0.78^{b}, c	5.97 ±0.77^{a}	18,005.22 ±1,515.49^{b},c
		Crabs	F	0.38±0.06^{a}	160.96±20.44^{a}	<MDL	66±4.51^{a}	<MDL	1.07±0.22^{a}	<MDL	305.72±43.03^{a,b}
			M	0.52±0.09^{a}	231.9±54.85^{b},c	<MDL	54.31±2.81^{a}	<MDL	<MDL	<MDL	286.9±14.65^{a}
TEL-SQGS[a]				0.68	18.7	30.24	124		15.90	52.3	
PEL-SQGS[a]				4.21	108.2	112.18	271		42.8	160.4	
Unpolluted sediments[b]				0.01–0.2	5–25	5–25	20–100		10–20	30	

Abbreviations: <MDL, values below the Method Detection Limit; TEL-Q: threshold effect level quotients; PEL-Q: probable effect level quotients (PEL-Q) of the sediments quality guidelines (SQGs). F: females, M: males crabs. Similar letters indicate no statistical differences (Fisher LSD test, n = 3, p>0.05 for sediments; Fisher LSD test, n = 5 pools, p>0.05 for crabs)

[a] Buchman (1999).

[b] Bryan and Langston (1992).

Table 4
Metal pollution indices in sediments and crabs.

Season	Site	Index	Cd	Cu	Pb	Zn	Mn	Ni	Cr	Fe
Spring	VM	EF	NC	0.736	0.54	1.13	1.64	0.33	0.18	1
		Igeo	NC	0.05	0.039	0.08	0.12	0.02	0.013	0.07
		BSAF F	NC	18.01	NC	1.4	0.06	0.29	NC	0.02
		BSAF M	NC	13.49	NC	1.34	0.04	0.51	NC	0.01
		mERM-Q	0.09	No adverse effects						
		PER	3.51	Sediments with low ecological risk						
	PR	EF	NC	**2.32**	0.52	1.43	0.68	0.33	0.18	1
		Igeo	NC	**0.19**	0.04	0.12	0.06	0.03	0.01	0.08
		BSAF F	NC	5.19	0.68	0.1	0.04	NC	NC	0.02
		BSAF M	NC	6.82	NC	0.42	0.04	0.14	NC	0.03
		mERM-Q	**0.18**	Potential adverse effects						
		PER	**7.27**	Sediments with low ecological risk						
Winter	VM	EF	NC	0.72	0.56	1.1	1.37	0.32	0.18	1
		Igeo	NC	0.05	0.04	0.07	0.09	0.02	0.01	0.07
		BSAF F	NC	**28.25**	NC	1.97	0.05	0.25	NC	0.02
		BSAF M	NC	16.47	NC	1.38	0.06	0.41	NC	0.02
		mERM-Q	0.09	No adverse effects						
		PER	3.27	Sediments with low ecological risk						
	PR	EF	NC	1.07	0.62	1.27	0.73	0.33	0.17	1
		Igeo	NC	0.08	0.05	0.1	0.06	0.02	0.01	0.08
		BSAF F	NC	8.73	NC	1.43	NC	0.13	NC	0.02
		BSAF M	NC	12.26	NC	1.17	NC	NC	NC	0.02
		mERM-Q	**0.12**	Potential adverse effects						
		PER	4.46		Sediments with low ecological risk					

Abbreviations: NC: not calculated; EF: enrichment factor; Igeo: geo-accumulation index; BSAF: biosediment accumulation factor in females (F) and males (M); mERM-Q: mean effects-range-median quotient; PER: potential ecological risk. The highest values are shown in bold

main component (0.95), while the variables of DO, temperature and salinity represented the more remarkable contributions in the conformation of component 2 (1, 0.89 and 0.82, respectively). The different cases were separated more by sites than by seasons, differentiating VM from PR in both seasons through this analysis. On the right side of the plot are observed WPR and SPR; both cases are characterized by high heavy metal concentrations in sediments but differ in oceanographic parameters and MT content. On the left side of the plot are presented SVM and WVM: these cases are characterized by high CAT and GST activity, H_2O_2 content and Ni, Cd and Mn concentration in crabs and Mn in sediments. Similarly to PR, SVM and WVM differ in the oceanographic parameters and MT content. None of the heavy metals in crabs and sediments were correlated, except for CuC-CuS in SPR and GST was better explained by CdC in SVM.

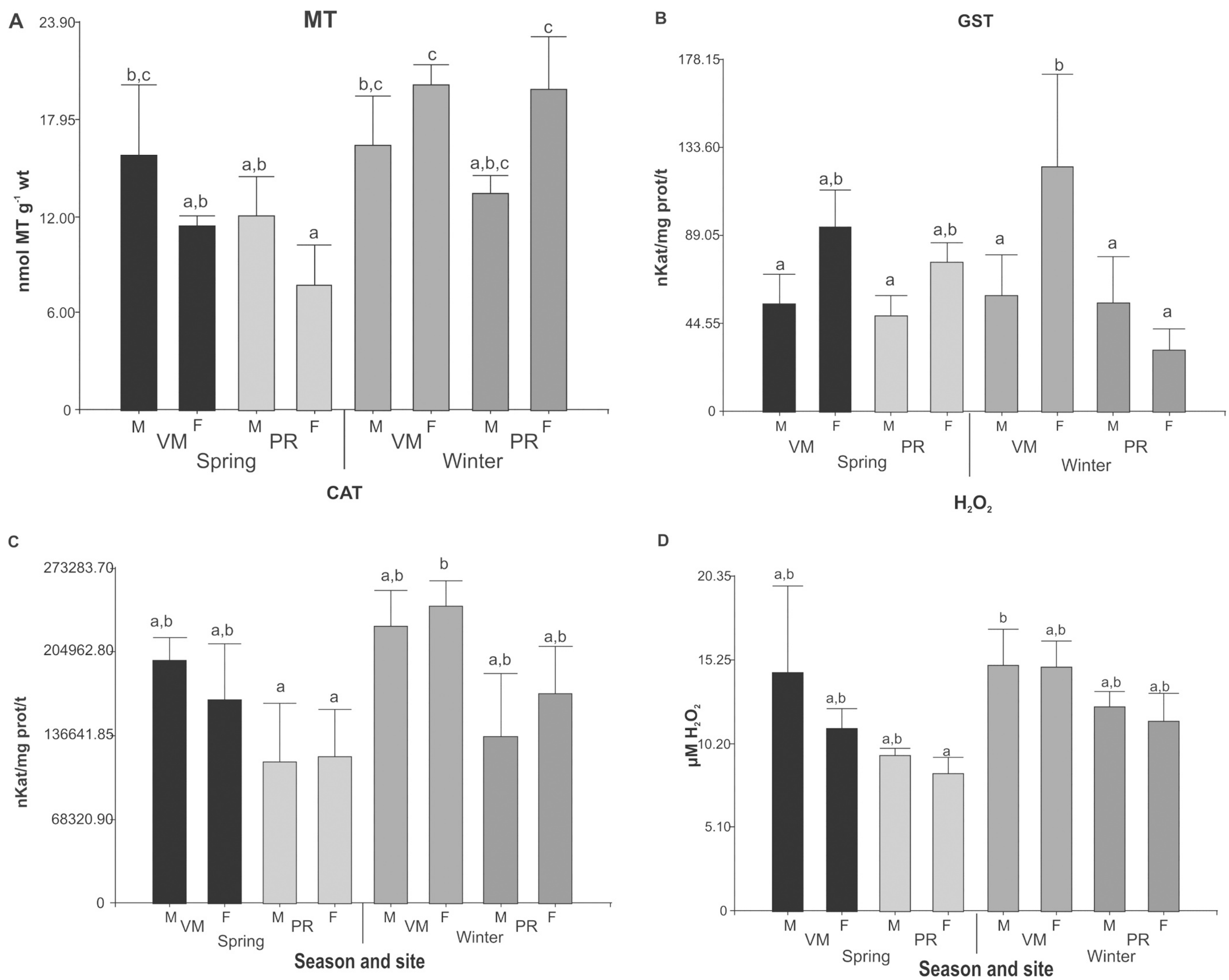

Fig. 2. Seasonal and spatial mean concentrations and standard error of (a) MT, (b) GST, (c) CAT, and (d) H_2O_2 in the hepatopancreas of males (M) and females (F) of *N. granulata.* Similar letters indicate no statistical differences (Fisher LSD test, n = 5 pools, p˃0.05).

4. Discussion

4.1. Heavy metals in sediments and crabs, sediment quality indices and ecological risk

Studies that assessed the potential ecological risk of heavy metals in estuarine sediments have been well recorded globally, but in those studies, the geochemical properties have been evaluated separately from the use of biomarkers (i.e., Muniz et al., 2019; Williams and Antoine, 2020), or otherwise (Capparelli et al., 2019). Few studies have achieved the same goal of evaluating the ecological risk of heavy metals in sediments using geochemical indices, biomarkers, and bioaccumulation patterns of heavy metals in resident species (Pereira et al., 2009; Aljahdali and Alhassan, 2020). This research is a first integrative approach conducted in the BBE to assess the metal pollution in sediments and their biological implications on a native benthic invertebrate, the burrowing crab *N. granulata.*

For fine sediments, the detected values of Cu indicated pollution and enrichment (EF) by this metal in PR during spring, since it exceeded the recommended values of this metal according to Bryan and Langston (1992) for unpolluted sediments and also it is above the TEL values proposed by Buchman (1999), indicating occasional adverse biological effects. Overall, the geochemical indices exhibited that the BBE is low to moderately polluted by heavy metals, but allowed to get different pictures of the same area. As an example, PER indicated that both sites' sediments were rarely associated with adverse biological effects and presented a low ecological risk. mERM-Q indicated potential adverse effects to the biota in the sediments of PR that suggests moderate pollution due to industrial, port and urban activities in the area (where a sewage water discharge and an oil plant are settled). Whereas, the Igeo showed unpolluted to moderately polluted sediments (class 1).

In the sediments, the PCA showed that the heavy metals loading is more influenced by sites than by seasons, allowing to observe two groups, PR and VM, being PR the site explained by most heavy metals loads. This could also point out that untreated sewage waters from Punta Alta city and the portuary activities developed there (i.e., antifouling for small ships and vessels containing Pb and Cu) were significant input of heavy metals. The same pattern of higher heavy metals values in this site was observed by other authors (Spetter et al., 2015a; Simonetti et al., 2017; Truchet et al., 2020). Heavy metal loads for the two sites were higher in spring, probably influenced by the higher activity of burrowing species in the intertidal that might affect organic matter and metals bioavailability (Mendez Casariego et al., 2011; Andrade et al., 2019). Mn was the only metal that was higher in VM and Hempel et al. (2008) reported in this salt marsh higher levels of this metal in the sediments associated with the roots of *S. alterniflora* in comparison with Mn values

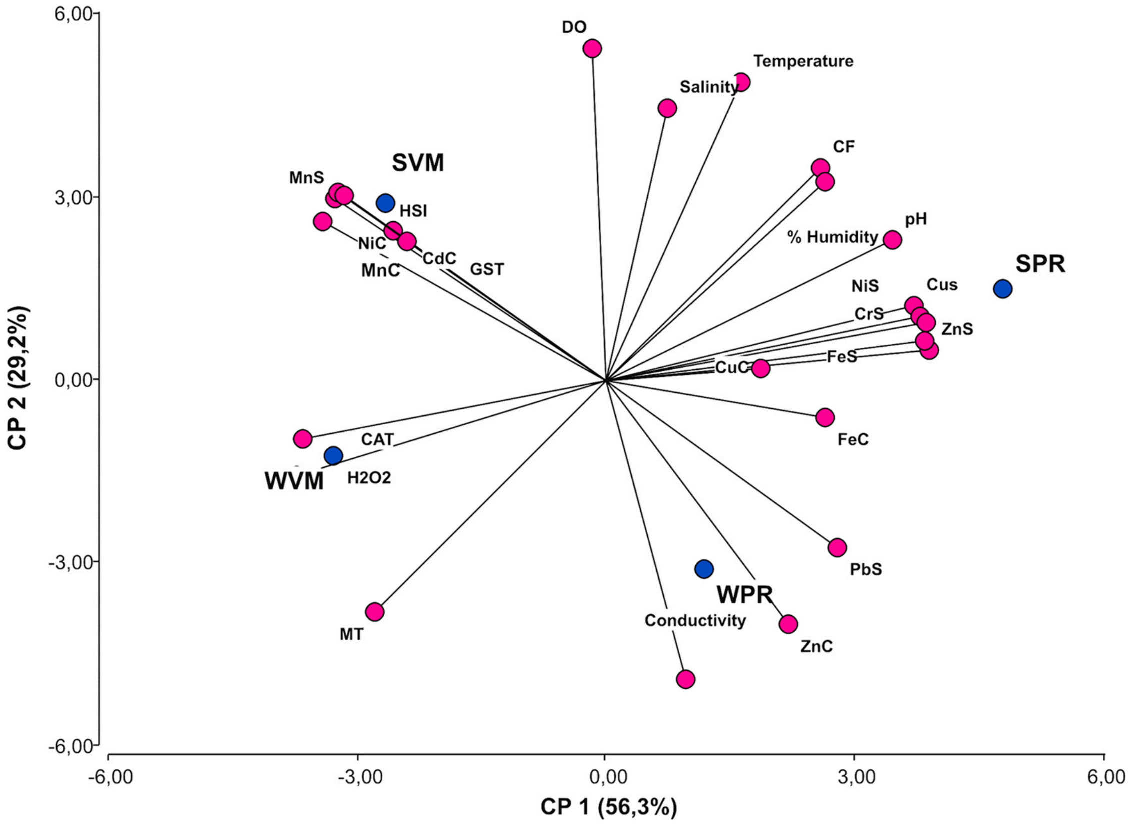

Fig. 3. Principal component analysis (PCA) biplot. With seasonal (W, winter; S, spring) and spatial (VM, PR) concentrations of metals (Cd, Cu, Zn, Pb, Ni, Mn, Cr, Fe) in crabs (C) and sediments (S), biochemical responses (MT, GST, CAT, H_2O_2) and physicochemical parameters (salinity, pH, conductivity, DO, temperature).

in mudflat sediments. These authors considered that the sediments' environmental characteristics, most notably the pH and redox potential, must be affecting the dynamic of heavy metals. Thus, the higher Mn concentration found in VM might be related to this halophyte and different sediments' conditions absent in PR. Also, values in VM could be lower since it is a minor port with few small ships, and heavy metals in the sediments could be uptaken and sequestrated by the extensive salt marshes of *S. alterniflora* in the area (Hempel et al., 2008; Negrin et al., 2019).

Regarding the influence of physicochemical parameters on the heavy metals in fine sediments, none of the measures (temperature, salinity, DO, conductivity) explained the analyzed metals except the pH with Zn, Mn and Fe. For the BBE, Simonetti et al. (2017) also determined that the pH correlates with metals in sediments, along with the organic matter in the case of Fe. Other authors explained that salinity might affect heavy metals behavior and mobilization and, besides, salinity enhances the pH of the water along estuaries, influencing metal dynamics. Hence, pH could have a stronger influence on metals at lower salinities in the estuaries' inner parts (Souza Machado et al., 2016).

As we stated before, in the sediments, all metals were present except Cd, and the dominant ones were essential metals like Fe, Cu, Zn and Mn. The same pattern was observed in other studies performed at the BBE (Table S1, supplementary material), whereas Cd was only detected by Buzzi and Marcovecchio (2018) in small concentrations in inner sites of the estuary that receives riverine runoffs associated with agriculture. The absence of large agricultural settlements near the sampling sites could be the main reason why Cd (mostly related to organophosphates) was not detected in the sediments in the present study. In comparison with other estuarine and coastal systems of the Argentine Sea (Table S1, supplementary material), the BBE has lower ranges of metals in the sediments, except in the case of Cu, and sometimes the excess of it is associated with industrial and port (antifouling paints) activities and urban settlements (Comoglio et al., 2011). The values of heavy metals in our study are even lower than in other worldwide marine environments, especially those conducted in some developing countries and the industrial areas of China. These systems are severely affected by human activities such as industries, mining and landfills, which are common in industrialized countries, and could be potential sources of metals in coastal areas and estuaries (Souza Machado et al., 2016). Therefore, it is essential to continue monitoring the heavy metal pollution in emerging countries with developing economies and high demographic rates, as the anthropogenic pressures might affect these ecosystems' health status.

Regarding heavy metal bioaccumulation in *N. granulata,* our results showed that seasonality has no effect on metal accumulation patterns in crabs. Unlike previous studies by Saher and Siddiqui (2019) in the crab *Macrophthalmus depressus*, we found that environmental endpoints of water did not explain heavy metal accumulation in crabs. Also, metals in the sediments did not explain crabs' concentrations, suggesting that the uptake of most metals by *N. granulata* might not be strictly through the sedimentary route (Simonetti et al., 2020). The BSAF indicated higher bioaccumulation of Cu in F from VM during winter and could be due to the high loads of this metal in the sediments and the spring reserve for future reproductive females (Capparelli et al., 2019). But in general terms, this metal was the most bioaccumulated one and is an essential metal for the normal development of benthic organisms like bivalves and decapods, as it is a principal constituent of the hemolymph. However, a long-term exposition to high concentrations of this metal might have adverse effects on the organisms, as it was registered in some biochemical biomarkers (GST, GPx) of the estuarine Ocypodidae crab, *MinUca rapax* (Capparelli et al., 2020) that also feeds on metal-polluted sediments.

In comparison to other benthic *taxa* from the BBE, like bivalves*, N. granulata* accumulates higher loads of metals, and therefore, it would be a suitable heavy metal biomonitor for metal pollution for the Argentine

Sea (Table S2, Supplementary material). Compared to the heavy metals concentrations in other estuarine and coastal crabs from other areas of developing countries (i.e., Brazil, Pakistan, Iraq and Taiwan), Cd and essential metals like Cu and Zn were still higher in our study in *N. granulata*. In our case, the uptake of Cd might come from the dissolved and particulate phase of water (Fernández Severini et al., 2013; La Colla et al., 2015; Villagran et al., 2019), or the dietary route of the main trophic item (*S. alterniflora, S. densiflora*) of this species (Hempel et al., 2008; Negrin et al., 2018 personal communication). Also, Liu and Wang (2013) demonstrated that the interaction Zn-Cd facilitates the uptake of Cd from estuarine waters and sediments by *Magallana (=Crassotrea) hongkongensis*. Thus a similar process might occur in *N. granulata* in the BBE (Simonetti et al., 2020).

The morphological measures and patterns of heavy metal accumulation in the hepatopancreas of *N. granulata* were also assessed in the present study. Though the values of CF were higher in PR, the use of HSI to assess the health status of the crabs' populations described a better condition in VM, and the results of the PCA exhibited that this morphological biomarker could be better to assess metal accumulation in the crabs. Some studies in *Brachidontes rodriguezii* in the BBE demonstrated differences in the CF according to the seasons but not by sites (Buzzi et al., 2017; Buzzi and Marcovecchio, 2018). These authors explained that higher levels of Cd, Ni and Cr showed worse body CFs than those with lower values of these metals. However, in other coastal areas of the Argentine sea with the same crab species, these indices were not employed to establish further comparisons to evaluate heavy metal pollution.

4.2. Biochemical biomarkers as early warning signals for heavy metal pollution

In general terms, none of the biomarkers employed showed spatial differences, but while comparing the data with seasonal responses, MT, CAT, GST and H_2O_2 showed higher values in winter, especially in VM, and this might indicate that crabs suffered pro-oxidant pressures during the cold season. The same seasonal pattern of responses in winter was observed in benthic crabs *Carcinus maenas*, *MinUca rapax* and the bivalve *Mytilus edulis chilensis* (Pereira et al., 2009; Duarte et al., 2011; Capparelli et al., 2016, 2019). These authors explained that invertebrates' biochemical responses could be higher in winter since it indicates the beginning of the reserve accumulation period. So it could be considered a high exposure period, with higher food uptake and the rates of some pollutants that are not easily eliminated, as it might have happened in our study in VM.

Our results exhibited that the levels of metals in the fine sediments did not explain any of the employed biomarkers (except MnS with GST in SVM), indicating that the battery of biomarkers was not useful to evaluate heavy metal pollution in the sediments. Previous studies by Martin-Díaz et al. (2008) for Spanish ports determined that increased levels of ROS activity in invertebrates might be induced by a synergic effect of both organic (PAHs and PCBs) and metallic pollution. Concerning heavy metals in crabs, only GST was explained by CdC, suggesting its induction as a protective response to Cd-initiated oxidative injury. However, though some authors demonstrated that indeed Cd pollution increased the levels of GST in benthic organisms (Figueira et al., 2012), others found better correlations with other metals like Fe or Cr (Giarratano et al., 2016) or even sometimes no correlations have been found between this biomarker and metals (Giarratano et al., 2011, 2013). Despite this, Cd tends to induce ROS due to inhibition in the mitochondria electron transport (Stohs et al., 2000), and other studies suggest that the increase in GST activity display a particular GPx activity, which may play a protective role against oxidative stress induced by metals or be related to their detoxification (Pereira et al., 2009). However, the GST mechanism as a biomarker for metal pollution in estuarine invertebrates is not yet well understood. CAT levels strongly explained H_2O_2 levels, and this would indicate that the activities are complementary and that CAT activity is related to the protective response to peroxide concentrations increase. To study the levels of hydrogen peroxide as a biomarker is thoroughly recommended since its increase could lead to further oxidative damage in the hepatopancreas of crabs (Negro et al., 2019).

For some authors (i.e., Gagné et al., 2008, among others), MT represents the first line of defense against reactive oxygen species since an increase in MT activity tends to reduce ROS activity. In our study, MT activity tended to be higher in winter, like the ROS-related biochemical markers. Contrary to expected, MT showed no higher affinity for metals in the hepatopancreas of crabs, but with CAT and H_2O_2 they explained WVM. This might suggest that MT activity is indeed related to the oxidative stress defense. These results coincide with the findings of Giarratano et al. (2016) that determinate no relationship between MT and heavy metals in tissues of *N. granulata*, suggesting that the levels of these metal-binding proteins could be a result of increased production of ROS to protect the cells against the oxidative stress acting as an oxyradical scavenger, as it was also suggested by Viarengo et al. (2000).

Therefore, the induction of the biochemical activities (except for GST) might not be strictly related to heavy metal pollution. A recent study by Villagran et al. (2020) demonstrated the evidence of high microplastics accumulation in *N. granulata* during a cold season in VM in comparison with PR, probably due to the inputs of plastics in the case of fishers' gears and nets, and the human litter left in these coasts through the recreational activities, like tourism. Therefore, a possible explanation by the ROS induction could be the presence of these pollutants in the environment, or even a mixture of several pollutants inducing a synergic effect on the biota, such as PAHs and TBTs that were recorded in the sediments of the area (Oliva et al., 2015; Quintas et al., 2016).

5. Conclusions

Our results indicated a low to moderate heavy metal pollution in sediments of the middle – outer area from BBE. Punctually, it was detected a moderate enrichment of Cu and, consequently, potential effects on the biota in PR (a site profoundly impacted by untreated sewage waters). Several indices to assess metal pollution in coastal systems are highly recommended since they reflect different information that needs to be complemented. In the sediments, heavy metals exhibited a spatial pattern rather than a seasonal one, and the highest values were recorded in PR. Therefore, it is necessary to keep on studying this area with high human pressures, and decision-makers should take into account that sewage waters are probably a significant input of metals to this estuarine environment that might have future biological effects on the biota.

The battery of morphological indices employed in *N. granulata* demonstrated that HSI index is better to assess the health status of crabs and was lower in PR, suggesting that though the crabs have higher CF values in this site, the hepatopancreas presents a better status in VM. Except for GST, the set of biochemical biomarkers evaluated in *N. granulata* as early warning signals for heavy metal pollution showed that they responded more to a seasonal variability than a spatial one, since they were higher during winter, especially in VM. GST was explained by Cd in crabs, indicating an induction as a protective response to Cd-initiated oxidative injury. Therefore, the biomarkers only suggest seasonal variations and not heavy metal pollution in the sediments and crabs in the studied area.

We conclude that *N. granulata* has a key ecological role in processes of estuarine systems, and in terms of accumulation, compared with other coastal systems of the Argentine Sea, this species was a better biomonitor to assess heavy metal pollution than other benthic organisms, especially in the case of Cu. But as for biochemical responses, this species was ruled out by seasonality rather than anthropogenic impacts. These results should be taken into account for stakeholders to discuss and explore the best ways to assess heavy metal pollution to create integrative monitoring programs of pollution assessment.

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CRediT authorship contribution statement

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

Declaration of Competing Interest

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2020.111498.

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