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Metals from mine waste as potential cause of oxidative stress in burrowing crab *Neohelice granulata* from San Antonio bay

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ABSTRACT

The Natural Protected Area San Antonio bay is of particular importance for its congregation of migratory shorebirds and it has been declared one of the Western Hemisphere Shorebird Reserve Network International site (WHSRN). Present study represents the first assessment of variation on oxidative stress biomarkers in male crab *Neohelice granulata* from San Antonio bay (Río Negro, Argentina) under field conditions, associated mainly to metal contamination coming from passive mining wastes. Three sites were sampled once every three months from November 2012 to August 2013 within this sea inlet (Pile, Fishery and Port) and a control site at the southeast of the bay (Punta Perdices). Accumulation of Ni, Zn, Cr and Al varied only with seasons although without a constant trend, meanwhile Cd, Cu and Pb also varied among sites being highest in Pile and Port. Biochemical results indicated that variations in catalase activity was only site specific being maximum in Pile; meanwhile lipid radical, α-tocopherol and metallothioneins were only seasonal specific being higher in autumn and winter. Seasonal variation was also found for total thioles, being the content higher in summer and autumn than in winter. Correlation analysis revealed that malondialdehyde and α-tocopherol have a positive association with Al and negative with Ni, meanwhile GST has a positive association with Fe. Crabs from the closest area to the waste pile did not exhibit a differentiated oxidative pressure despite the higher accumulation of metals. It is possible that crabs from contaminated areas have developed a tolerance to metals, indicating a strong ecotoxicological selective pressure. More studies are needed to assess whether there is a transfer of metals through the food chain.

1. Introduction

Since a comprehensive analysis and assessment of the whole chemical universe appears to be impossible, approaches are required to reduce the complexity of possible or actual environmental contamination while limiting the chance of overlooking significant contributors to risks and effects (Brack et al., 2016). There is general agreement that the most appropriate way to assess the quality of aquatic environments with respect to hazardous substances requires the use of chemical and biological measurements at several levels of biological organisation in an integrated way (OSPAR, 2011). At present, it is widely accepted that new ecological perspectives require holistic and multidisciplinary approaches that use multiple lines of evidence such as studies on the presence of contaminants, their availability for aquatic organisms and their adverse effects (De Jonge et al., 2012). In the framework of the EU-funded BEEP project (Biological Effects of Environmental Pollution in marine coastal ecosystems), one of the most promising and world-wide applied approaches to quantify the impact of xenobiotics on organisms is biological-effect monitoring with the use of biomarkers.

The Natural Protected Area San Antonio bay is of particular importance for its congregation of migratory shorebirds and it has been declared one of the Western Hemisphere Shorebird Reserve Network International site (WHSRN). In the Patagonian coastal area there are virtually no mining activities releasing metals into the environment, except for San Antonio Bay in the northwest of San Matías Gulf (Río Negro Province) until 1980. Waste tailings generated between 1960 and 1980 by the electrolysis of minerals Pb, Zn, Ag and V from Gonzalito mine (located 120 km from the city of San Antonio Oeste) in an electrochemical plant within the town of San Antonio Oeste, have produced a dispersion of various

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metals over time. Solid waste from the process (pile), enriched in some metals, were laid in the open air around the city of San Antonio Oeste and on the edge of the bay. There are also deposits within the urban area where even houses have been built on top of them. High levels of Pb (14,500 ppm), Cu (4750 ppm), Zn (7300 ppm) and Cd (7.5 ppm) have been found in sediments (Gil et al., 1999), mussels (Vázquez et al., 2007) and crabs (Gil et al., 2006) evidencing that waste piles from the abandoned mine are still leaching various metals into the environment.

Essential and non-essential metals possess the ability to produce reactive oxygen species (ROS), resulting in DNA damage, lipid peroxidation, depletion of protein sulfhydryls (Valko et al., 2005), enzyme inhibition (Chen et al., 2002), cell signalling impairment (Shimada et al., 1998), disruption of calcium homeostasis (Ahearn et al., 2004) and changes in gene regulation (Kim et al., 2014). In order to protect themselves against the effect of toxic metals, organisms developed a variety of detoxification mechanisms. The first line of defence consists of low molecular mass antioxidants that include water-soluble compounds, which usually function as free radical scavengers (Lushchak, 2011). Another defensive mechanism comprises high molecular mass antioxidant group such as enzymes, and proteins that prevent ROS-induced damage by binding to transition metal ions such as metallothioneins and ferritin (Lushchak, 2011). An induction of the antioxidant system can be considered as an adaptation of the organisms to overcome an unsafe environment and to prevent toxicity. A deficiency in the enzyme activities suggests a precarious state characterized by a higher susceptibility to environmental stress, and potential adverse effects can be expected. The use of biomarkers has been proposed as sensitive tools for biological effects monitoring, playing an important role in the risk assessment of complex ecosystems (Monserrat et al., 2007). The use of toxicant-specific biomarkers such as metallothionein has been widely employed to indicate the presence of trace metals. Since several pollutants can modify directly or indirectly the balance between the concentration of pro-oxidants and antioxidants, the determination of several oxidative stress and/or antioxidant responses in aquatic species is commonly and successfully employed as a non-specific biomarker (Bainy et al., 1996).

San Antonio bay is a very dry region with no freshwater input, except scarce rains (mean rainfall = 240 mm/year), and with strong eastward winds. Salinity and temperature are usually higher in San Antonio Bay than in the open ocean, due to low freshwater input and excessive evaporation. The low organic matter content in the sediment implies that food is not available inside or around burrows and it could be a factor forcing crabs to use living plants of *Spartina alterniflora* as food, and move intensely between mudflat and salt marsh (Luppi et al., 2013).

The semiterrestrial estuarine crab *N. granulata* (Brachyura, Varunidae) is widely distributed along the Atlantic coast of South America from southern Brazil (23°S) to the northern Argentinean Patagonia (41°S). Crabs are herbivorous–detritivorous when associated with salt marshes and deposit feeders when living in mud flats (Iribarne et al., 1997). *N. granulata* represents an important link in the trophic web considering that all stages in the crab's life cycle make them a relevant food component for other species, such as fishes, shellfishes and birds. For this reason, they could play a major role in the transference of pollutants to higher trophic levels (Beltrame et al., 2011; Simonetti et al., 2013).

The San Antonio inlet is located at the northwest of the bay and receives the pollution produced by the San Antonio Oeste city and the remaining wastes of the Gonzalito mine closed thirty five years ago. Although some field studies of environmental monitoring were performed in Argentina by evaluating *N. granulata* as bioindicator of trace metals (Beltrame et al., 2011; Simonetti et al., 2013) only one with crabs from San Antonio bay (Gil et al., 2006), but none in regard to the application of oxidative stress biomarkers for field monitoring.

Due the deposition of solid wastes enriched in metals from mining activity in the open air around the city of San Antonio Oeste and on the edge of the bay, it can be hypothesized that the coastal waters and sediment near these piles contain concentrations of metals which can generate oxidative stress in the organisms that live in the surrounding area. The evaluation of oxidative stress parameters using crabs from a mining impacted area is of high relevance since, as far as we know, it is the first time that has been made in Argentina. The main goal of the present work is elucidate if selected biomarkers give relevant information for the assessment of the oxidative stress status of crabs affected by passive mining wastes. The possible associations between biochemical responses and metals were also evaluated.

2. Material and methods

2.1. Study sites and sampling

San Antonio bay is located at the northeast of the Argentinean Patagonia within the San Matías gulf (Fig. 1) and is characterized by a large tidal range (up to 9 m) that produces large watermass exchange. Three sites were sampled within this sea inlet: in the upper part, close to the waste pile (Pile); in the middle, in front of

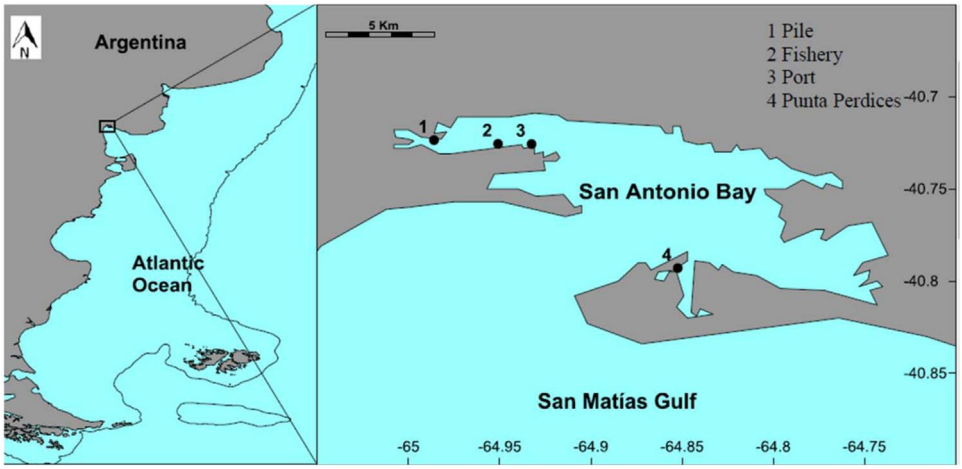

Fig. 1. Map showing the sampling sites.

a fish plant (Fishery) and in the port area (Port). Punta Perdices located at the southeast of the bay was considered as the control site, due to low levels of metals that have been reported in sediments and in the mussel *Brachidontes rodriguezi* (Vázquez et al., 2007).

N. granulata is a species sexually dimorphic with males identified by enlarged chelae and triangular shape of abdominal perimeter. Forty male specimens were hand-picked during low tide at the crab beds on mudflats once every three months, from November 2012 to August 2013 at each site. Crabs were transported to laboratory in boxes with *in situ* collected sea water and maintained in aerated aquaria shielded by a plastic cover to reduce disturbance for 24 h to depurate. The means of width and length of cephalothoraxes were 30.25 ± 2.82 mm and 25.45 ± 2.22 mm, respectively. After cleaning the bodies with distilled water, all crabs were anaesthetized before dissection by chilling on ice for 60 min for subsequent removal of whole soft tissues (WST) contained in the cephalothoraxes. Ten WST were weighed (1.12 ± 0.05 g), washed with distilled water and quickly kept at -80 °C for subsequent biochemical analyses. The other 30 WTS (1.39 ± 0.09 g) were separated into 3 composite samples of 10 WTS each and frozen at -20 °C for analysis of trace metals.

2.2. Metal analysis

All material used for handling of samples was previously cleaned with diluted nitric acid to prevent contamination and special care was maintained to avoid contact with metal surfaces. The 3 pooled samples of 10 crabs each were oven-dried at 60 °C and then grounded in an agate mortar. The 3 composite homogenates (two replicates of 500 mg each) were extracted for metal analysis by digestion with hot concentrated nitric acid (Merck) according to Boletín Oficial del Estado (1991). Metal concentrations (Cd, Cu, Pb, Zn, Ni, Fe, Al and Cr) were determined using an IL-457 atomic absorption spectrophotometer with air-acetylene flame, except for Al and Cr that were measured using a nitrous oxide-acetylene flame. All concentrations are expressed in parts per million (μg/g) on a dry weight (dw) basis. Reference material of oyster tissue NIST-SRM 1566a was used for the quality control of trace metal analysis. Recovery of all metals varied between 84% and 115%. Atomic absorption spectrometry standard solutions for all elements (Merck) were used to build up calibration curves. They were prepared from a stock solution of 1000 mg/L for each metal by successive dilutions. Aqueous solutions of reagents and standards were prepared using deionized water.

2.3. Biochemical analysis

Lipid radical content (LR) was measured by electron paramagnetic resonance (EPR)-spin trapping on homogenates from tissues prepared in a fresh stock solution containing 40 mM N-t-butyl-α-phenyl nitrone (PBN) and pure dimethyl sulfoxide (DMSO) (1:10) (Lai et al., 1986). EPR spectra were obtained at room temperature using a Bruker spectrometer ECS 106, operating at 9.8 GHz with a modulation frequency of 50 kHz. EPR instrument settings for the spin trapping experiments were: microwave power 19.85 mW, time constant 81.92 ms; scans number 2; center field 3515 G; modulation amplitude 1.194 G, receiver gain 2×105; sweep with 100 G and conversion time 82 ms (Jurkiewicz and Buettner, 1994). Spin adduct was calibrated using an aqueous solution of 2,2,5,5-tetramethyl piperidine 1-oxyl (TEMPO). The concentration of spin adduct was calculated according to Kotake et al. (1996) and expressed per mg of wet tissue.

Lipid peroxidation products analyzed as malondialdehyde (MDA) content were measured according to Shaw et al. (2004). Samples were homogenized in Tris–HCl buffer 20 mM pH 7.4 (1:3 w/v) and centrifuged at $3000 \times g$ for 20 min (4 °C). An aliquot of supernatant was mixed with 650 μl of 1-methyl-2-phenylindole in acetonitrile: methanol (1:3) and 150 μl of 37% HCl. This mixture was incubated at 45 °C for 40 min; the reaction was stopped in ice and further centrifuged at $15{,}000 \times g$ for 10 min (4 °C) to precipitate proteins. Absorbance was read at 586 nm versus a standard solution of 1,1,3,3-tetramethoxypropane treated similarly. MDA content was expressed as nmol MDA/g wet tissue.

The content of α-tocopherol (α-T) in crab tissue was quantified by reverse-phase HPLC with electrochemical detection using a Bioanalytical Systems LC-4C amperometric detector with a glass carbon working electrode at an applied oxidation potential of 0.6 V. Samples (0.5 g) were extracted with 1 ml of ethanol and 4 ml of hexane. After centrifugation at $600 \times g$ for 10 min, the hexane phase was removed and evaporated to dryness under N_2. Extracts were dissolved in methanol:ethanol (1:1) and injected for HPLC analysis (Desai, 1984). D,L-α-Tocopherol (Sigma, St Louis, MO) was used as standard and results were expressed as nmol of α-T/mg wet tissue.

For the biochemical measurements of catalase (CAT), glutathione-S-transferases (GST) and protein content, tissues were homogenized (1:3 w/v) in ice-cold buffer (20 mM Tris-base, 1 mM EDTA, 1 mM DL-dithiothreitol, 500 mM sucrose and 150 mM KCl) with pH adjusted to 7.60 (Bainy et al., 1996). Homogenates were centrifuged at $9000 \times g$ for 30 min (4 °C), the supernatants were collected and enzymatic activities were measured. CAT activity was assayed in tissues following the method of Beutler (1982). Appropriate amount of sample was added to 0.44 M H_2O_2 in 50 mM phosphate buffer (pH 7.0) at 30 °C. The decrease in absorbance was followed at 240 nm every 5 s for 1 min. CAT activity was calculated from the extinction coefficient of H_2O_2 as 0.04 $\mu M^{-1}\,cm^{-1}$. One unit of CAT activity was defined as 1 μmol of H_2O_2 decomposed per minute. The activity of GST was assayed by the method of Habig et al. (1974). The reaction mixture (1.0 ml) consisted of 0.1 M potassium phosphate buffer (pH 7.0), 100 mM GSH, 50 mM 1-chloro-2,4-dinitrobenzene (CDNB) and an aliquot of sample to be tested. The enzyme activity was calculated by using a molar extinction coefficient of 2,4-dinitrophenyl-S-glutathione as 9.6 $nM^{-1}\,cm^{-1}$ at 340 nm and 25 °C. One unit of GST was defined as the amount of enzyme catalyzing the formation of 1 μmol of 2,4 dinitrophenyl-S-glutathione per min. Specific activities of each enzyme were defined as the unit of enzyme activity per mg of protein measured by the method of Lowry et al. (1951), with bovine serum albumin as standard.

Total thiol groups were quantified according to the method of Ellman as modified by Sedlak and Lindsay (1968). Briefly, reaction mixture containing 0.2 M Tris–HCl and 0.02 M EDTA buffer (pH 8.2), sample and 0.01 M DTNB (in methanol) was incubated for 15 min at room temperature then was centrifuged at $3000 \times g$ for 15 min (4 °C). The supernatant was collected and the absorbance was read at 412 nm. Results were calculated from the standard curve of reduced glutathione (2–100 nM) and expressed as nmol of sulfhydryl content per mg protein.

Metallothioneins (MT) were analyzed by the spectrophotometric assay as described in Viarengo et al. (1997). Tissues were homogenized in 1:3 (w/v) Tris–HCl buffer 20 mM pH 8.6, 0.5 M sucrose, 0.006 mM leupeptin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 0.01% β-mercaptoethanol and centrifuged at $30{,}000 \times g$ for 45 min (4 °C). After acidic ethanol/chloroform fractionation of the tissue homogenate, MT was quantified using reduced glutathione (GSH) as standard to build a calibration curve. Results were expressed as nmol sulfhydryl content per g of wet tissue.

Biochemical analyses were performed according to protocols that are widely applied by the international scientific

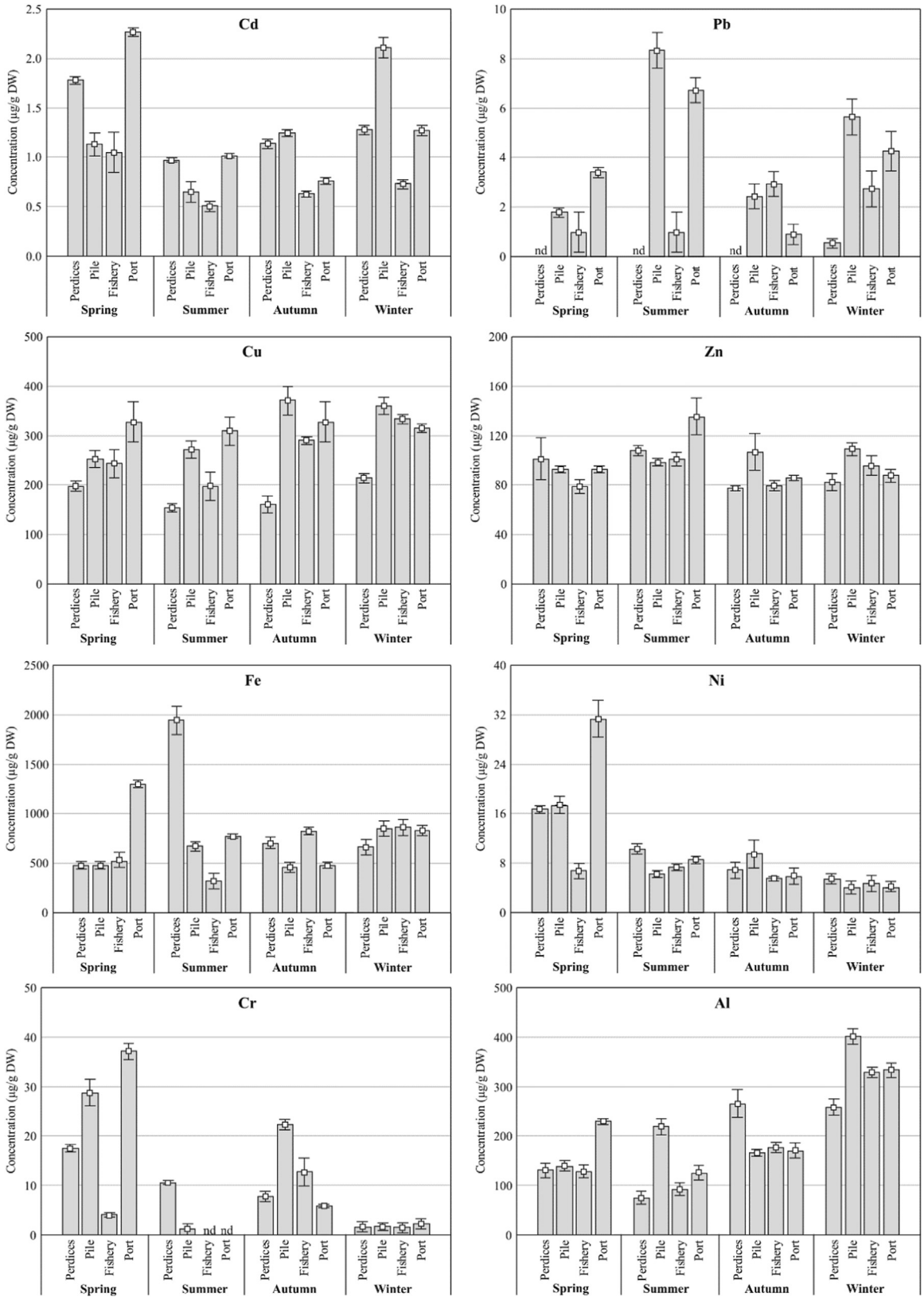

Fig. 2. Trace metal concentrations (µg/g dw) measured in soft tissues of male *N. granulata* from San Antonio bay. Data are presented as the mean ± S.E.M. nd = non detectable.

community. Calibration curves were prepared by spiking of the Sigma-Aldrich analytical standards in the corresponding buffer. Each curve included at least 4 points (3 replicates each), besides a blank. For each regression fit, we obtained determination coefficients above 0.99. Precision was calculated based on results obtained from one sample that was processed 5 times. Variation coefficients were below 10% for all biochemical assays.

2.4. Statistical analysis

Significant changes in concentrations of metals and in oxidative stress responses in tissue crabs were tested by analysis of variance (factorial ANOVA). Following all significant ANOVA, Tukey's HSD multiple comparisons were performed between groups for the main effects or between interacting factors where interactions were significant ($\alpha = 0.05$). In the case of metal concentrations that were lower than the detection limits of the applied analytical method, a value of one-half the detection limit was assigned for statistical treatment. Data were checked to meet the assumptions of normality and homogeneity of variances prior to analysis. Principal components analysis (PCA) was performed in order to visualize in an integral way the behaviour of the oxidative stress responses due to combinations of factors and their relationship with metal accumulation. Associations between endpoints (oxidative stress parameters and metal bioaccumulation) were examined using Pearson Product Moment correlation analysis.

3. Results

In the present study cephalothoraxes' length of *N. granulata* ranged from 20 to 31 mm and the width from 22 to 36 mm. Significant differences in size among seasons were not recorded in our study. This result implies that size could not be responsible for the observed seasonal bioaccumulation patterns of the studied metals in male crabs from San Antonio bay.

Mean seasonal concentrations of metals in soft tissues of burrowing male crab *N. granulata* from San Antonio bay are presented in Fig. 2 and the statistical results of Factorial ANOVA on Table 1. Fe was the most bioaccumulated metal, with levels (321–1943 μg/g dw) several times as high as those for the other metals measured. Significant differences among sites were not detected for concentrations of Zn, Fe, Ni, Cr and Al. The lowest concentration of Cd was measured in crabs from Fishery ($P < 0.005$). Pb was significantly higher in crabs from Pile than in those from Perdices ($P < 0.001$). Concentrations of Cu in crabs from Pile and Port were the highest, followed by values measured in crabs from Fishery and the lowest one in crabs from Perdices ($P < 0.001$). All metals, except Fe, showed significant variations throughout the year ($P < 0.05$), but without a clear tendency. Cd and Cu concentrations were minimum in summer, whilst Cd was maximum in spring and Cu in winter ($P < 0.001$). Concentrations of Pb and Zn measured in summer were significantly higher than in spring an autumn ($P < 0.05$). Levels of Ni and Al were significantly higher in spring and winter respectively, than in other seasons ($P < 0.001$). Cr was maximum in spring and minimum in winter ($P < 0.001$).

Table 1
Factorial ANOVA results for interactions between factors of independent variables.

Metal	**Cd**	**Pb**	**Cu**	**Zn**	**Fe**	**Ni**	**Cr**	**Al**
	P	*P*	*P*	*P*	*P*	*P*	*P*	*P*
Factor								
Site	**0.004**	**0.000**	**0.000**	0.185	0.246	0.062	0.131	0.134
Season	**0.000**	**0.011**	**0.000**	**0.010**	0.396	**0.000**	**0.000**	**0.000**
Site*season	**0.000**	**0.034**	**0.000**	0.207	0.101	0.079	**0.027**	**0.000**

Biomarker	**LR**	**MDA**	**α-TOC**	**CAT**	**GST**	**Thiol**	**MT**
	P	*P*	*P*	*P*	*P*	*P*	*P*
Factor							
Site	**0.000**	**0.000**	0.228	**0.008**	**0.002**	0.132	**0.046**
Season	**0.000**	**0.000**	**0.000**	0.605	0.170	**0.009**	**0.000**
Site*season	**0.000**	**0.000**	0.063	**0.000**	**0.000**	**0.044**	**0.000**

Bold values are significant at $P < 0.05$.

Oxidative stress biomarkers are presented in Figs. 3 and 4 and results of Factorial ANOVA are in Table 1. LR content was the highest in crabs from Perdices and Fishery (Fig. 3(A)), while the highest MDA level was in organisms from Pile and Perdices and the lowest in those from Port (Fig. 3(B)). LR and α-T contents in crabs varied significantly among seasons being the highest in winter and higher in autumn than in other seasons (Fig. 3(A) and (C)), meanwhile MDA content was the highest in winter and the lowest in spring (Fig. 3(B)). CAT and GST activities showed significant differences only among sites. CAT activity was higher in crabs from Perdices than in the other sites (Fig. 4(A)), while GST activity in Perdices and Port was higher than in Fishery and Pile (Fig. 4(B)). Regarding total thioles, differences were only found among seasons being the content higher in summer and autumn than in winter (Fig. 4(C)). Finally, significant differences in MT were found among sites and seasons. Higher values of MT were registered in crabs from Perdices and Fishery than in those from Port. From the seasonal point of view, the highest value of MT was measured in autumn, followed by that of winter and the lowest ones in spring and summer (Fig. 4(D)).

In order to evaluate the combinations of variables, a Principal Component Analysis (PCA) was applied employing all oxidative stress responses besides metal concentrations measured in male crab tissues in spring, summer, autumn and winter. A graphical representation of the estimated factor values corresponding to each case (studied site) is presented in order to confirm the descriptions of these new factors (Fig. 5). The first principal factor accounting for 31% of the variance represents LR, MDA, α-T and MT on positive axis in crabs from all sites in winter and from Perdices and Fishery in autumn; total thiols and concentrations of Ni and Cr on negative axis in crabs from Port in spring and from Perdices and Port in summer. The second factor accounting for 17% of the variance shows the enzymatic activity of CAT on positive axis and the metal contents of Cu and Pb on negative one. Positive values were found for crabs from Perdices in all seasons; meanwhile negative values were found in crabs from Pile in summer, autumn and winter and in those from Port in summer. Bivariate correlation analyses of biomarker crab responses denoted significant positive Pearson's correlation between Al and MDA (0.68, $P < 0.05$), Al and α-T (0.75, $P < 0.05$), Fe and GST (0.69, $P < 0.05$) and negative values for Ni with both MDA and α-T (-0.55, $P < 0.05$).

4. Discussion

Males and females can show different patterns of metal accumulation and biochemical responses, since females dispend great amount of energy in breeding strategy, resulting in important physiological alterations. In order to minimize variability, only males were used for the present study.

In the present study, no consistent general trend emerged from the reported seasonal metal concentrations. Cd, Ni and Cr concentrations were the highest in spring, Pb in summer and Al in winter. These results highlight the significance of a simultaneous sampling (the same period within all the studied sites) to correctly develop biomonitoring programs.

Results showed that crabs from Pile have higher levels of Pb than those from Perdices and Fishery. Accumulation of Cd and Cu were significantly higher in crabs from San Antonio inlet than in those from control site Perdices. This fact suggests that the corresponding bioavailabilities of these two pollutants within both areas are different. Concentrations of Cd (0.5–2.2 μg/g dw), Cu (154–372 μg/g dw) and Zn (77–135 μg/g dw) determined in tissue of *N. granulata* from San Antonio Bay were relatively high compared to reports of Gil et al. (2006) for identical species within the same ecosystem (Cd: < 0.08, Cu:85–101, Zn:68–86 μg/g dw).

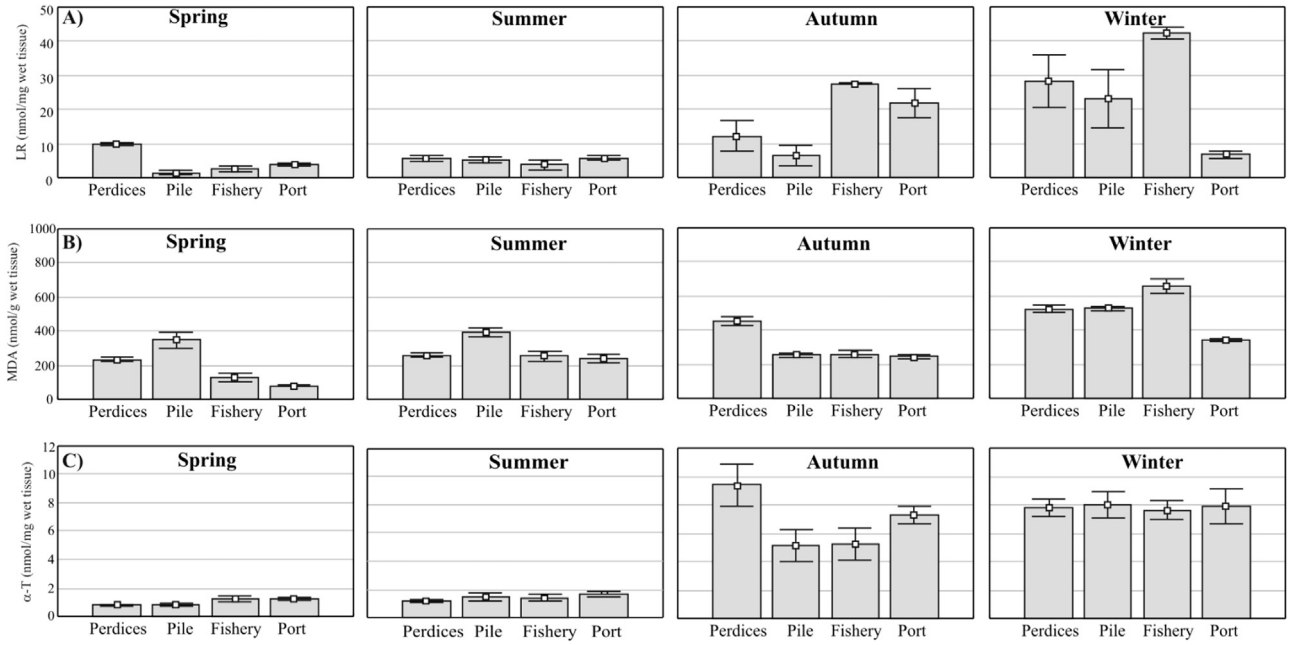

Fig. 3. Contents of lipid radical (A), malondialdehyde (B) and α-tocopherol (C) in soft tissues of male *N. granulata* from San Antonio bay. Data are presented as the mean ± S.E.M.

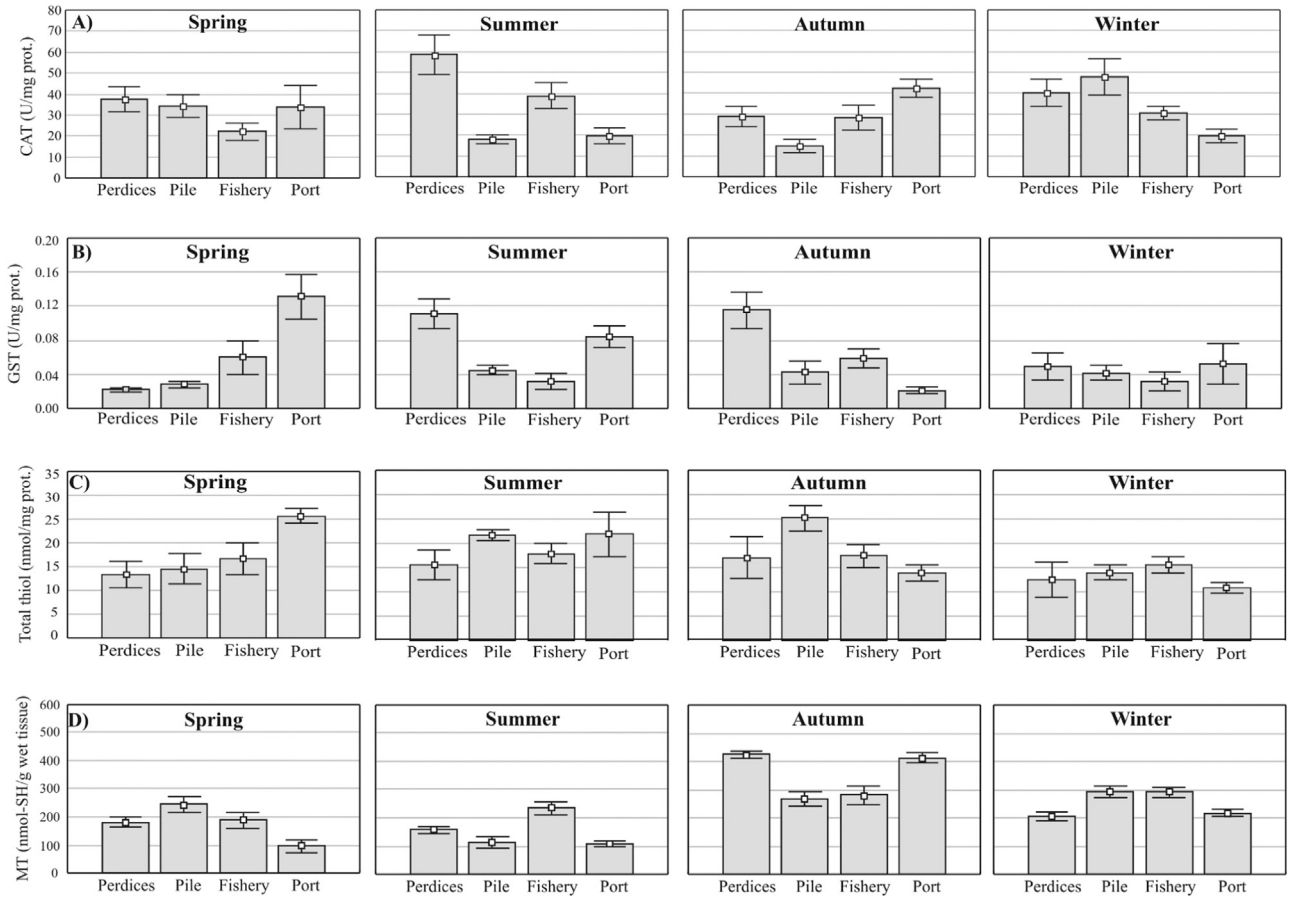

Fig. 4. Catalase activity (A), glutathione-S-transferases activity (B), total thiols (C) and metallothionein content (D) in soft tissues of male *N. granulata* from San Antonio bay. Data are presented as the mean ± S.E.M.

Bonuccelli and Malan (2004) have demonstrated migration of metals from waste pile towards the channel by lixiviation process. The lowest concentrations of Pb were measured in crabs from Perdices during all seasons (< 0.2 – 0.5 μg/g dw), meanwhile in San Antonio channel concentrations ranged from < 0.9 to 9.1 μg/g dw, being lower than the range of 10–13 μg/g dw reported by Gil et al. (2006). Despite bioavailability of Pb seems to be diminishing over time; values are high even when they are compared with

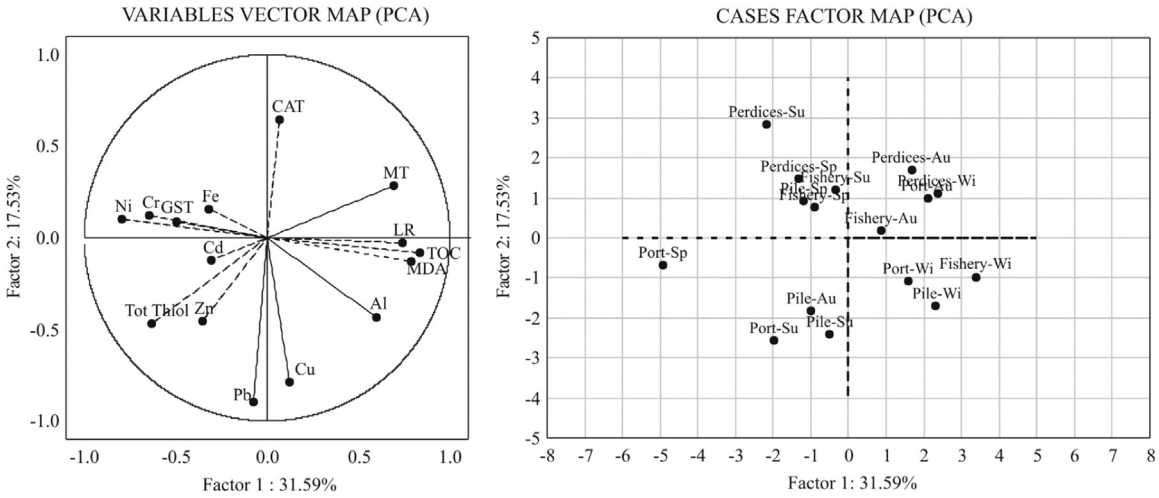

Fig. 5. Representation of Factors 1 and 2 scores estimation for each of the 16 cases evaluated employing biomarker responses and metal concentrations after multivariate analysis. Sp: spring, Su: summer, Au: autumn, Wi: winter.

values from an urban and industrial impacted area such as the estuary of Bahía Blanca, where Ferrer (2001) reported non-detectable values of this metal for the same crab species. Ferrer (2001) reported values of Cd and Zn similar to those of the present study, but lower for Cu (155 ± 10 μg/g dw), Fe (464 ± 58 μg/g dw) and Cr (< 0.03 μg/g dw). Existing data of Al for *N. granulata* could not be found. Comparing with other crab species, present results (74–401 μg/g dw) were lower than those reported by Sparling and Lowe (1996) for *Sesarma cinereum* (1847 μg/g dw) from Chesapeake Bay and *Pachygrapsus* sp. (1965 μg/g dw) from San Francisco Bay.

A distinction has to be made between two different components of accumulated metals, specifically the accumulated metal that has been detoxified and the metal that is metabolically available to play an essential role and in extreme circumstances have a toxic role in biochemistry (Rainbow, 2002). Metals can produce effects through their interaction with cellular sulfhydryl groups in proteins. Thiols participate in several processes ranging from simple electron donation (GSH) and antioxidant catalysis to complex biochemical sensing and antioxidant responses (Winyard et al., 2005). Protein thiol groups may scavenge oxidants, thus sparing antioxidants and/or preventing cellular constituents from attack. When the availability of free thiol group is low, enhanced expression of toxicity in the form of oxidative stress could occur (Rao et al., 2006). The interaction of Ni with thiol-containing cellular constituents and related free radical generation from molecular oxygen, especially in the presence of lipid hydroperoxides, may be involved in the mechanisms of Ni toxicity and carcinogenicity (Shi et al., 1993).

An induction of GST due to Fe was found. The prooxidant potential of free intracellular Fe resides in its potential to catalyze formation of partially reduced oxygen intermediates including hydroxyl radicals via the Fenton reaction which initiate lipid peroxidation. Evidence suggests that induction of GST is a protective response to iron-initiated oxidative injury, though the biochemical mechanism of this induction is not well understood (Tjalkens et al., 1998). Nitric oxide, a strong chelator for Fe, could be bound to Fe and endogenous thiols generating different nitrosyl–Fe complexes. GST binds these nitrosyl-iron complexes with extraordinary high affinity (Pedersen et al., 2007).

Significant positive correlation was also found between Al and both MDA and α-T. This finding is in accordance with previous works reporting that lipid peroxidation is induced by Al at sublethal levels (Yousef, 2004). Alpha-tocopherol is an important antioxidant in biological systems. It inhibits peroxidation of membrane lipids by scavenging lipid peroxyl radicals, and is converted into a tocopheroxyl radical as a consequence (Arita et al., 1998). Despite several authors have reported a greater vitamin E concentration associated to lower lipid peroxidation (Malanga et al., 2007; Huang and Huang, 2004), enhanced α-tocopherol seemed to be unable to interrupt lipid peroxidation chain reaction in *N. granulata*. Tocopherols are synthesized only by photosynthetic organisms, so animals must obtain them from plant sources (Vismara et al., 2003). Tocopherols are then passed through the aquatic animal food chain in herbivorous species, such as *N. granulata* that has been described as mainly herbivorous/detritivorous in the mudflat (Iribarne et al., 1997). Salt marshes of San Antonio are better characterized by the presence of the halophytic genera Spartina and Sarcocornia. Despite the offer of plant species from the salt marsh, as well as the diverse species of micro and macroalgae present in mudflat that could represent sources of α-tocopherol, *N. granulata* feeds almost exclusively on cordgrass species and sediments from burrows (Bas et al., 2005). In that sense, herbivory on *Spartina densiflora* (Alberti et al., 2011) as well as on *S. alterniflora* (Luppi et al., 2013) has been documented. Our results also suggest that intense herbivory in autumn and winter would explain the high content of α-tocopherol found in crabs from all sites. In this sense, *N. granulata* from Mar Chiquita (Argentina) showed seasonal herbivory on *Spartina densiflora* leaves, being most annual consumption occurring in fall (Alberti et al., 2011). This behaviour did not reveal the same pattern that the seasonal variation in N-content in leaves (highest % nitrogen in winter). These results suggest that even though crabs prefer N-rich leaves, other factors are driving the seasonal variation in herbivory. This seasonal pattern could result from higher plant exposure to the herbivory in fall due to longer tidal submersion intervals, ontogeny of the crab and associated nutritional requirements, which may vary among seasons, and availability of more palatable food sources than in other seasons (Persson and Brönmark, 2002).

The increased levels of MT in whole tissue of the burrowing crab could be a result of an increased production of ROS since no relationship was found between MT and metals. This hypothesis is based on the fact that MT can protect cells against the oxidative stress not only through the essential metal binding/release dynamics, but also acting as an oxyradical scavenger (Viarengo et al., 2000).

In general, long-term exposure of living organisms to contaminants results primarily in their accumulation in organs and tissues and secondly, in irreversible molecular alterations due to their continuous deleterious action (Barata et al., 2005). Of particular interest are transition metals like Fe, Cu, Cr and V that through

the Fenton reaction are able to facilitate the conversion of superoxide anion to the highly reactive hydroxyl radical. Other metals including Cd, Ni, Pb and Hg may also produce oxidative stress indirectly, depleting glutathione levels or via metal-induced displacement of redox metal ions (Stohs and Bagchi, 1995). Oxidative damage can occur when antioxidant and detoxifying systems are deficient and not able to neutralize the active intermediates produced by toxics and their metabolites (Frenzilli et al., 2001).

Considering the above discussion, additive effects of metals, seasons, and other abiotic and biological variables, as well as their interactions, can play a crucial role on the induction of the biochemical effects measured in crabs (Pereira et al., 2009). It has been reported that temperature, pH changes during tidal cycles, dissolved oxygen and air exposure affect the metabolism of intertidal species (Freire et al., 2011), but only changes in temperature were indirectly considered in present study through seasonal samplings. Salinity is not considered a significant variable in San Antonio bay since it is high and constant. Biological differences between sites can arise from many environmental and ecological factors independent of pollutant stress. In the context of ecological risk assessment, a role for biomarkers has been identified both in site-specific assessments as well as in regional biomonitoring programs. In general, current results demonstrate the rational of the biomarker selection, giving support to the idea presented by Monserrat et al. (2007) that when aquatic species are facing complex environmental changes the determination of non-specific biomarker is recommended.

5. Conclusions

The current study represents the first assessment of variation in oxidative stress biomarkers in crab *N. granulata* from San Antonio bay under field conditions, associated mainly to metal contamination coming from passive mining wastes. Despite the described variation of the antioxidant responses, male crabs did not cope efficiently with LR production and oxidation of polyunsaturated fatty acids occurred, leading to lipid peroxidation measured as MDA content. This highlights the environment degradation state at the studied area.

It is concluded that the inheritance of historical mining in San Antonio bay is the ongoing presence of sediments rich in bioavailable metals to local deposit feeders. The area is exposed to a semidiurnal macrotidal regime, making possible that deposited material to be moved and be redeposited. The low organic matter content in the sediment implies that food is not available inside or around burrows and it could be a factor forcing crabs to use living plants of *Spartina alterniflora* and *densiflora* as food, and move intensely between mudflat and salt marsh (Luppi et al., 2013).

Crabs from the closest area to the waste pile zone did not exhibit a differentiated oxidative pressure. An explanation to this matter is that the toxicity of metals to the accumulating organism is not necessary directly related to the total accumulated concentration, simply because accumulated trace metals are often stored in detoxified form and are therefore unavailable to interact with sites in metabolism where they might exert a toxic effect. It is also possible that crabs from contaminated areas have developed a genetically based tolerance to metals, indicating a strong ecotoxicological selective pressure (Luoma and Rainbow, 2008). More importantly, from these metal-rich sediments deposited long ago, deposit-feeding invertebrates still accumulate high concentrations of metals that have the potential to affect them. More studies are needed to evaluate if metals can be passed up through food chains with possible ecotoxicological consequences for higher trophic levels.

San Antonio bay constitutes a case of study where despite its historical trace metal contamination, the application of biomarkers for environmental hazard and risk assessments has never been done. Our results corroborate that traditional analytical tools of reporting only contaminant concentrations in biotic and/or abiotic matrices are not enough to evaluate pollution status of this system, since the observed biochemical responses in the studied species does not necessarily correlate with metal content in sediments or in the crab itself. More research is required with respect to the use of biological tools like biomarkers in this and other protected areas of Argentina, in view of their incorporation into regulatory frameworks and better assessment of environmental condition.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2016.05.029.

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