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Effect of seasonality on oxidative stress responses and metal accumulation in soft tissues of *Aulacomya atra*, a mussel from the South Atlantic Patagonian coast

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ABSTRACT

This study investigated the effects of pollution and its interaction with temperature on the oxidative status of the ribbed mussel *Aulacomya atra* in the southern Atlantic Patagonian coast. Animals were collected from four sites with different degree and type of human activity impact, during the summer and winter of 2011. Seawater chromium, copper, manganese, nickel and zinc concentrations were measured, as well as metal accumulation, lipid peroxidation, protein oxidation, reduced glutathione levels, and enzymatic activities of superoxide dismutase and glutathione-S-transferase in gills and digestive glands.

Metal bioaccumulation and oxidative stress responses in both tissues were generally higher in mussels from harbor areas. Water temperature had a remarkable effect on gill SOD activity and protein oxidation during winter in mussels from all locations.

Methodologically, we conclude that measuring both metal bioaccumulation and oxidative stress responses allowed for a more accurate assessment of the biological effects of metal present in seawater.

1. Introduction

Marine environment receive increased amounts of environmental pollutants including trace metals due to human activities. Metals may be taken up by aquatic biota to toxic levels that increase cellular levels of reactive oxygen species (ROS) and cause oxidative damage to biomolecules (Livingstone, 2001; Regoli et al., 2002; Manduzio et al., 2005; Tsangaris et al., 2010). Moreover, trace metals can potentially increase ROS within cells through Haber-Weiss and Fenton-like reactions (Lloyd and Phillips, 1999; Eberhardt, 2001).

Like many others aquatic organisms, bivalves possess a detoxification system to neutralize ROS and protect against oxidative damage (Gorinstein et al., 2003; Valavanidis et al., 2006). Detoxification system comprises enzymatic and non-enzymatic components such as superoxide dismutase, catalase, glutathione peroxidases, glutathione reductase, glutathione-s-transferase, vitamin E, and reduced glutathione. Oxidative stress parameters related to detoxification mechanisms or toxicity, such as changes in antioxidant levels and oxidative damage to lipids and/or proteins, are frequently used as biomarkers for trace metals toxicity and to quantify environmental effects in bivalves (Viarengo et al., 1990; Regoli et al., 1998; de Almeida et al., 2004). In addition, seasonal factors such as temperature also affect the oxidative status of marine bivalves (Estevez et al., 2002; Abele et al., 2002; Abele and Puntarulo, 2004), for example, by influencing metabolic and physiological process.

Bivalves are adequate bioindicator species because they are sessile and filter feeders, they have a cosmopolitan distribution and they

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accumulate trace metals from the surrounding environment in their soft tissues (Ahn et al., 1996; Franco et al., 2002; Lincon-Smith and Cooper, 2002; Andral et al., 2004). In this study, the ribbed mussel *Aulacomya atra* was chosen as native bioindicator species to provide information about realistic environmental levels of trace metals and its effects. Although several studies on the effects of trace metals on other bivalves from the Argentine Sea have been conducted (Thompson and Sánchez de Bock, 2007; Arias et al., 2009; Massara Paletto et al., 2008; Amin et al., 2011), little is known about the influence of trace metals on antioxidant defenses and oxidative damage in *A. atra*. In Argentina, *A. atra* is common in rocky shores from Golfo San Matías, 40°47′–42°13′ S; 62°50′–63°48′ W to Beagle Channel, Tierra del Fuego (54°51′ S; 68°29′ W) where it forms extensive beds (Zaixso et al. 1998; Zaixso, 1999). *A. atra* plays an important role providing habitat for other aquatic invertebrates and algae, and is also an important food source for many aquatic animals and humans (Zaixso, 2003; Narvarte et al., 2007).

The city of Puerto Madryn is located on the coast of Golfo Nuevo, bounded to the north by Peninsula Valdes. In 1999, Puerto Madryn was declared a "World Heritage Site" by UNESCO for being the most biologically diverse protected area of Patagonia (Unesco, 1999). Moreover, this area is considered the second most important in the world for breeding and rearing of the southern right whale (*Eubalaena australis*). In addition, these shores are home for large colonies of elephant seals (*Mirounga leonina*), sea lions (*Otaria flavescens*) and Magellanic Penguins (*Spheniscus magellanicus*). According to the last census (2010), Puerto Madryn has ~82,000 inhabitants. The city has 2 large piers: the Almirante Storni pier, which is used by the aluminium metal industry since 1970 for entering their supplies and export their production, and the Comandante Luis Piedra Buena pier, which serves for the berthing of cruise ships, as well as of fishing and pleasure boats. Through Almirante Storni pier approximately 300,000 tons of aluminum per year are exported, and about 800,000 tons of aluminium oxide, 6000 tons of aluminum fluoride, 700 tons of silicon metal and 200 tons of steel are imported in the same period. On the other hand, in the Comandante Luis Piedra Buena pier a total of 3413 vessels have docked in the past five years (Puerto Madryn Port Administration, 2010). Sediments from these harbor locations have spatial trends of polycyclic aromatic hydrocarbons (PAHs) (Commendatore et al., 2000; Commendatore and Esteves, 2007; Massara Paletto et al., 2008) and trace metals (Harvey and Gil, 1988; Gil et al., 1999) concentrations.

The objective of this study was to determine the effects of environmental trace metals concentrations in the oxidative status of *A. atra* in relation to water temperature. To evaluate and identify temperature effects, animals were collected during summer and winter and metal content in digestive glands and gills were quantified as indicators of metal bioaccumulation. Reduced glutathione levels and superoxide dismutase and glutathione-S-transferase activity were measured as indicators of non-enzymatic and enzymatic antioxidants, respectively, and lipid peroxidation and protein oxidation were assessed as indicators of oxidative damage.

2. Materials and methods

2.1. Sample collection

Four sites were selected along the Patagonian coast in the south Argentine Sea, province of Chubut, Argentina, with different anthropogenic sources of trace metals, industrial waste, urban wastewater discharges, maritime traffic and port activity. The reference site is located in the area of Playa Fracasso (PF) in Golfo San José (42°24′47.6″ S, 64°19′1.3″ W), which is considered a pristine environment. The other three sites are located along the cost of Golfo Nuevo near the city of Puerto Madryn, Parque Piedras (PP) (42°43′57″ S, 65°1′53.9″ W), the harbor area of the Comandante Luis Piedra Buena pier (PB) (42°45′ 44.2″ S, 65°1′35.2″ W) and Punta Cuevas (PC) (42°46′45″ S, 64°59′34″ W) on the southern edge of the city, where the human impact is presumably lower than in the other city sampling sites (Fig. 1, location of the sampling sites). At each site, seawater samples were collected to determinate the total concentration of metals. Water samples were collected approximately 1 m above the mussels. Water samples were collected and stored in sterile plastic bottles (previously washed with 2 M nitric acid) and acidified to pH < 2 with (1:1) nitric acid (Martin et al., 1994) and kept refrigerated until the metal determinations were performed.

20 individuals were collected at each sampling site, by diving at a depth of 8–10 m during March (summer) and July (winter) 2011, and deep and water temperature were measured with a scuba diver computer Beuchat model Master Pro and by a temperature data logger (On Set-Tidbit).

Because bivalve body size and age are strongly related (Soldati et al., 2009), and because age has an important effect on the oxidative balance of organisms (Abele et al., 2009), we collected individuals of similar body size (range 6.84–9.52 cm shell length) in an effort to minimize the effect of body size on oxidative stress parameters.

After collection, specimens of *A. atra* were immediately anesthetized by placing on ice before they were killed. The digestive gland and gills were dissected and weighted, frozen and transported at constant temperature (−5 °C) to the University of Buenos Aires where metals levels and oxidative stress biomarkers were measured.

2.2. Preparation of the organs and sex determination

Digestive glands and gills were homogenized in 0.134 M KCl (1:5 w/v) containing 0.5 mM PMSF and 0.2 mM benzamidine (protease inhibitors). The homogenates were centrifuged at 11,000×*g* for 20 min at 4 °C, and the resulting supernatants were used for the biochemical determinations. Total soluble protein content was determined according to Bradford (1976), using bovine serum albumin as standard. Results were expressed as mg total protein per mL.

To identify the sex of the specimens of *A. atra,* mantle was observed using an optical microscope and the presence of oogonies and spermatogonies was determined. The males/females ratio was

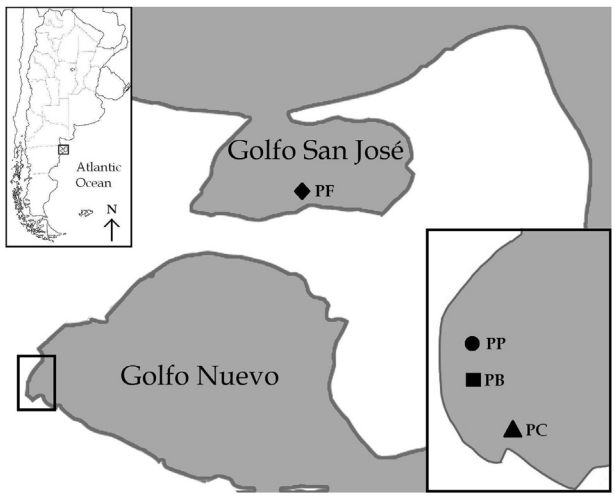

Fig. 1. Location of the sampling sites.

closer to 1 for all the sampling sites. No sex-related differences were observed for any of the measured variables.

2.3. Metals determination

Total trace metals in seawater samples (Martin et al., 1994) and in *A. atra* digestive gland and gills (Türkmen and Ciminli, 2007) were measured using a Perkin–Elmer model 3100XL inductively coupled plasma 160 optical emission spectrophotometer (ICP-OES). Tissues were homogenized in 0.134 M KCl (1:5, w/v), digested in 65% nitric acid (HNO_3) at 90 °C for 5 h until total evaporation, followed by further digestion in 32.5% HNO_3 containing 12% H_2O_2 for 10 h until total evaporation. Each acidification was repeated twice. The powders were re-suspended in 15 ml 5% HNO_3 and filtered through a 0.45 nylon filter. Results were expressed as μg metal per g tissue. Seawater samples were digested in 65% HNO_3 at 90 °C for 2 h until total evaporation, followed by digestions in 32.5% for 2 h and 5% HNO_3 for 3 h until total evaporation. The powders were re-suspended in 15 ml 5% HNO_3. Results were expressed as μg per 1000 mL water.

2.4. Oxidative damage

Lipid peroxidation in digestive glands and gills, estimated as thiobarbituric acid reactive substances (TBARS) content, was determined according to the Beuge and Aust procedure (1978). Briefly, 11,000×*g* supernatant from total homogenate was mixed with thiobarbituric acid (TBA) solution following by incubation at 95–100 °C for 45 min. After cooling, the reaction mixture was centrifuged and the 535 nm absorbance in the supernatant was measured. TBARS were determined using a molar extinction coefficient for the thiobarbituric acid–malondialdehyde complex of 156 mM^{-1} cm^{-1}. Results were expressed as micromoles of TBARS per mg proteins.

Oxidative damage to digestive gland and gill proteins (as carbonyl content) was quantified as described by Resnick and Packer (1994). Supernatants from 11,000×*g* homogenates were incubated with 10% streptomycin sulfate to eliminate DNA debris (15 min, room temperature). Two samples of extracted proteins were placed in glass tubes. To one tube 10 mM 2,4-Dinitrophenylhydrazine (DNPH) in 2.5 M HCl was added, while to the other tube 2.5 M HCl was added. Samples were incubated in the dark at room temperature for15 min, after which 20% trichloroacetic acid (TCA) (w/v) was added. After a 15 min incubation on ice, samples were centrifuged at 6000×*g* to collect the protein precipitates. The precipitates were dissolved in 6 M guanidine hydrochloride and incubated at 37 °C for 10 min. Carbonyl content was calculated from the peak absorbance (355–390 nm) using an absorption coefficient of 22,000 M^{-1} cm^{-1}. Results were expressed as nmol carbonyl per mg proteins.

2.5. Antioxidant defenses

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured using the procedure of Beauchamp and Fridovich (1971). The standard assay mixture contained 5, 10 and 15 μL enzymatic sample, 0.1 mM EDTA, 13 mM DL-methionine, 75 μM nitroblue tetrazolium (NBT) and 20 μM riboflavin, in 50 mM phosphate buffer (pH 7.5), to a final volume of 3 ml. Samples were exposed to intense cool-white light for 15 min, and kept in the dark until absorbance was measured at 560 nm. Enzymatic activity was expressed as SOD units per mg protein. A SOD unit was defined as the enzyme amount necessary to inhibit 50% the reaction rate.

Glutathione-S-transferase (GST, EC 1.11.1.9) activity was measured as described by Habig et al. (1974). Briefly, 10 mL of GSH solution (100 mM in phosphate buffer) and 20 mL of sample were combined with 960 mL of 100 mM phosphate buffer (pH 7.5). After adding10 mL of 100 mM 1chloro-2,4dinitro- benzene (CDNB) in ethanol, the change in absorbance at 340 nm was followed during 90s. One GST Unit was defined as the amount of enzyme needed to catalyze theformation of 1 mmol of GS-DNB per minute at 25 °C.

Reduced glutathione (GSH) content was determined following the Anderson procedure (1985) in presence of 5,5-dithiobis-(2-nitrobenzoic) acid (DTNB). Briefly, 100 μL supernatant from the 11,000×*g* sample was acidified with 50 μL of 10% sulfosalicylic acid. After centrifugation at 8000× for 10 min, supernatant (acid-soluble GSH) aliquots were mixed with 6 mM 5,5-dithiobis-(2-nitrobenzoic) acid (DTNB) in 0.143 M buffer sodium sulfate (pH 7.5) (containing 6.3 mM EDTA). Absorbance at 412 nm was measured after 30 min incubation at room temperature. GSH content was determined by a standard curve generated with a known GSH amount. Results were expressed as nmol thiols (GSH equivalents) per mg proteins.

2.6. Statistical analysis

The variations of each oxidative stress biomarker in digestive glands and gills between sites and seasons were tested by two-way analysis of variance (ANOVA) followed by a Bonferronis's post hoc test. Metal accumulation in each organ was compared by one-way analysis of variance (ANOVA) followed by a Dunnett's post hoc test. Normality and homogeneity of variances were tested by Lilliefors' and Bartlett's tests, respectively (Sokal and Rohlf, 1999). Graph Pad Prism 3 and Statistica v.7 software were used.

In order to ensure statistical independence of data, determinations for each metal and site were done using organs from different individuals. E.g. if the concentration of a metal in a given site was determined in the digestive gland of a particular individual, the determination of the same metal in gill was determined in samples from another specimen.

3. Results

3.1. Physicochemical features of the sampling sites

Table 1 shows the temperature range at the different sampling sites during March (summer) and July (winter) 2011 and the depth at which the samples were taken. There were no significant differences in average temperature among sampling sites in either season (Table 1).

Table 1
Thermic and Depth characterization of the 4 sampling sites (PF, PC, PB and PP) during summer and winter 2011.

	March (summer) 2011				July (winter) 2011			
	PF	PC	PB	PP	PF	PC	PB	PP
Average temperature (°C)	16,20	16,95	17,22	17,14	10,44	10,55	10,38	10,12
Maximum temperature (°C)	16,63	20,55	20,16	20,63	10,91	10,95	10,83	10,77
Minimum temperature (°C)	15,82	15,13	15,95	14,93	10,00	9,52	9,44	9,36
Depth (mts)	8–10	8–10	8–10	8–10	8–10	8–10	8–10	8–10

Table 2
Metal concentration (μg/L) in seawater from the 4 sampling sites (PF, PC, PB and PP).

Sampling sites	Metal (μg/L)				
	Mn	Zn	Ni	Cr	Cu
PP	23,34	126,15	5,1	5,25	5,85
PB	23,49	68,1	4,65	4,5	3,9
PC	13,44	49,95	0,3	0,15	0,9
PF	14,04	40,5	0,6	0,15	1,35

Sites located in the harbor area (PP and PB) show higher metal concentration in the water compared with the PC and PF (Table 2).

3.2. Tissue metals content

Individuals collected in the summer from the three sites in Golfo Nuevo (Punta Cuevas, Piedra Buena and Parque Piedras, PC, PB and PP, respectively) showed higher metal concentration in digestive gland for both manganese and zinc compared to those from the reference site (Playa Fracasso, PF) in Golfo San Jose (One-way ANOVA between sites for Mn and Zn $p < 0.05$ (Table 3). Furthermore, in winter digestive glands in individuals from the harbor areas (PB and PP) had higher concentrations of zinc (One-way ANOVA between sites $p < 0.05$) and nickel (One-way ANOVA between sites $p < 0.05$). However, copper concentration was significantly higher (One-way ANOVA between sites $p < 0.05$) only in the digestive gland of individuals from the PP site (Table 3).

During summer, manganese concentration in gills from specimens from PP was higher than in those from the other sites. (One-way ANOVA between sites $p < 0.05$) (Table 4). In contrast, in the same season, higher nickel values were recorded in individuals from PB (One-way ANOVA between sites $p < 0.05$) and zinc concentration was significantly higher in PC. Moreover, PC, PB and PP showed higher values than PF (One-way ANOVA between sites $p < 0.05$). Finally, the highest copper concentrations were recorded in PP and PB (One-way ANOVA between sites $p < 0.05$) (Table 4).

Manganese in gills during winter showed the same trend as in summer, in both cases individuals from PP had the highest concentration (One-way ANOVA between sites $p < 0.05$). A similar result was observed in copper concentration in gills in winter (One-way ANOVA between sites $p < 0.05$). Moreover, zinc concentration both gills and digestive glands were higher in individuals from the sites in the harbor area, where PB and PP showed the highest values and PF and PC the lowest (One-way ANOVA between sites $p < 0.05$) (Table 3).

Finally, concentration of accumulated chromium in digestive glands and gills was similar for all sampling sites during summer and winter (One-way ANOVA. between sites $p > 0.05$) (Tables 3 and 4).

Table 3
Metal accumulated (μg/g wet weight) in the digestive gland of *A. atra* from the 4 sampling sites (PF, PC, PB and PP) during summer and winter 2011. Data are expressed as means ± SD ($n = 2$). Significant differences between sites are indicated by asterisks: $^*p < 0.05$.

Digestive gland	Sampled sites			
Metals accumulated (μg/g ww)	Playa Fracasso	Punta Cuevas	Piedra Buena	Parque Piedras
Summer 2011				
Mn	0.135 ± 0.007	0.257 ± 0.032*	0.450 ± 0.014*	0.222 ± 0.013*
Zn	0.600 ± 0.064	1950 ± 0.028*	4563 ± 0.371*	2108 ± 0.057*
Ni	0.073 ± 0.039	0.150 ± 0.014	0.365 ± 0.156	0.143 ± 0.032
Cr	0.073 ± 0.032	0.113 ± 0.088	0.178 ± 0.067	0.105 ± 0.049
Cu	0.233 ± 0.039	0.315 ± 0, 005	0.463 ± 0.074	0.480 ± 0.170
Winter 2011				
Mn	0.198 ± 0.067	0.203 ± 0.081	0.315 ± 0.071	0.473 ± 0.173
Zn	0.408 ± 0.042	0.600 ± 0.057	9275 ± 1273*	4688 ± 1573*
Ni	0.065 ± 0.049	0.188 ± 0.057	0.420 ± 0.134*	0.425 ± 0.078*
Cr	0.031 ± 0.032	0.150 ± 0.057	0.285 ± 0.057	0.158 ± 0.085
Cu	0.260 ± 0.021	0.323 ± 0.103	0.418 ± 0.032	0.690 ± 0.141*

Table 4
Metal accumulated (μg/g wet weight) in the gills of *A. atra* from the 4 sampling sites (PF, PC, PB and PP) during summer and winter 2011. Data are expressed as means ± SD ($n = 2$). Significant differences between sites are indicated by asterisks: $^*p < 0.05$.

Gills	Sampled sites			
Metals accumulated (μg/g ww)	Playa Fracasso	Punta Cuevas	Piedra Buena	Parque Piedras
Summer 2011				
Mn	0.233 ± 0.124	0.218 ± 0.103	0.473 ± 0.053	0.840 ± 0.042*
Zn	1305 ± 0.297	2313 ± 0.042*	13,650 ± 0.042*	2848 ± 0.357*
Ni	0.105 ± 0.035	0.085 ± 0.035	0.310 ± 0.005*	0.223 ± 0.081
Cr	0.095 ± 0.005	0.103 ± 0.011	0.115 ± 0.000	0.125 ± 0.057
Cu	0.203 ± 0.060	0.228 ± 0.025	0.575 ± 0.004*	0.445 ± 0.064*
Winter 2011				
Mn	0.310 ± 0.057	0.313 ± 0.110	0.460 ± 0.028	0.583 ± 0.131*
Zn	1098 ± 1262	1828 ± 1623	6858 ± 0.965*	9538 ± 1609*
Ni	0.058 ± 0.018	0.113 ± 0.060	0.435 ± 0.346	0.253 ± 0.180
Cr	0.035 ± 0.014	0.080 ± 0.021	0.083 ± 0.018	0.083 ± 0.004
Cu	0.275 ± 0.035	0.300 ± 0.085	0.400 ± 0.028	0.488 ± 0.025*

3.3. Oxidative damage

TBARS content was not affected by season, neither in digestive gland (Fig. 2A) nor gills (Fig. 2B). However, both organs showed significant differences among sampling sites, with individuals from

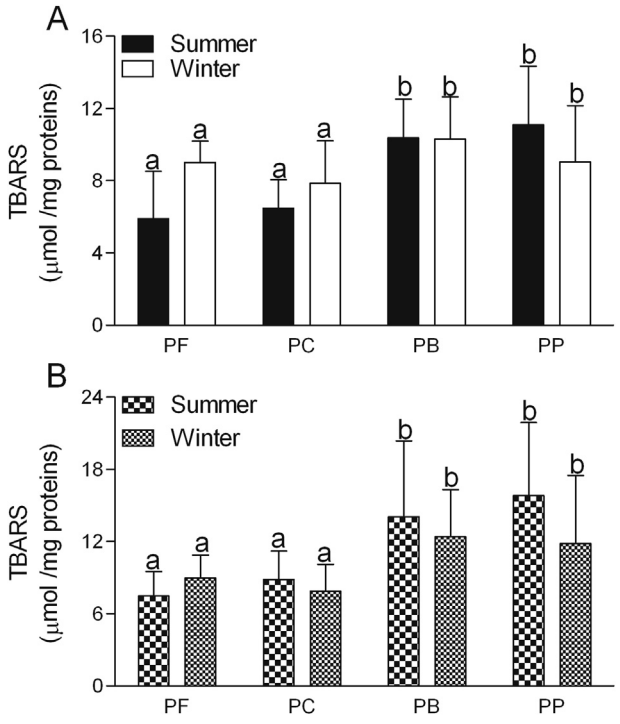

Fig. 2. Lipid peroxidation, expressed as μmol TBARS/mg prot, in digestive gland (A) and gills (B). Results are expressed as means ± S.D. ($n = 9$). Letters *a* and *b* indicate significant differences between sampling sites (PF, PC, PB and PP).

harbor areas (PP and PB) showing the highest values and PF and PC the lowest (Gills two-way ANOVA between sites $p < 0.01$ and between seasons $p = 0.058$, interaction $p < 0.05$; Digestive gland two-way ANOVA between sites $p < 0.01$ and between seasons $p = 0.312$, interaction $p < 0.05$) (Fig. 2A and B). The interaction effect is evident in digestive glands, since TBARS was higher in PP in summer than in winter, while in the other sites the trend is reversed (Fig. 2A). Moreover, in the gills of individuals from PF, TBARS values were higher in winter, contrary to what was observed in other sites (Fig. 2B).

Oxidative damage to protein (as carbonyl content) showed tissue-specific responses. In the digestive gland (Fig. 3A), the carbonyl content was higher during winter and no differences between sampling sites was observed (two-way ANOVA between sites $p = 0.726$ and between seasons $p < 0.01$, interaction $p = 0.629$). On the contrary, gills carbonyl content showed significant differences among seasons and sampling sites (two-way ANOVA between sites $p < 0.001$ and between seasons $p < 0.001$, interaction $p < 0.001$) (Fig. 3B). The interaction was likely because carbonyl content in PB and PP was higher during the winter than during the summer, while in the other sites there was no trend (Fig. 3B).

3.4. Antioxidant defenses

Superoxide dismutase (SOD) activity in digestive gland did not show significant differences between summer and winter. On the other hand, digestive glands from individuals collected at harbor areas (PP and PF) showed the highest SOD activity (two-way ANOVA between sites $p < 0.001$ and between seasons $p = 0.705$, interaction $p < 0.01$) (Fig. 4A). However, in gills, SOD activity showed significant differences between season and sampling sites. The higher values were registered in PB and PF sites during winter (two-way ANOVA between sites $p < 0.01$ and between seasons $p < 0.05$, interaction $p = 0.797$) (Fig. 4B). For digestive gland, interaction effect is significant because SOD values are higher in summer than in winter in PP, while in other places there is a reverse trend (Fig. 4A).

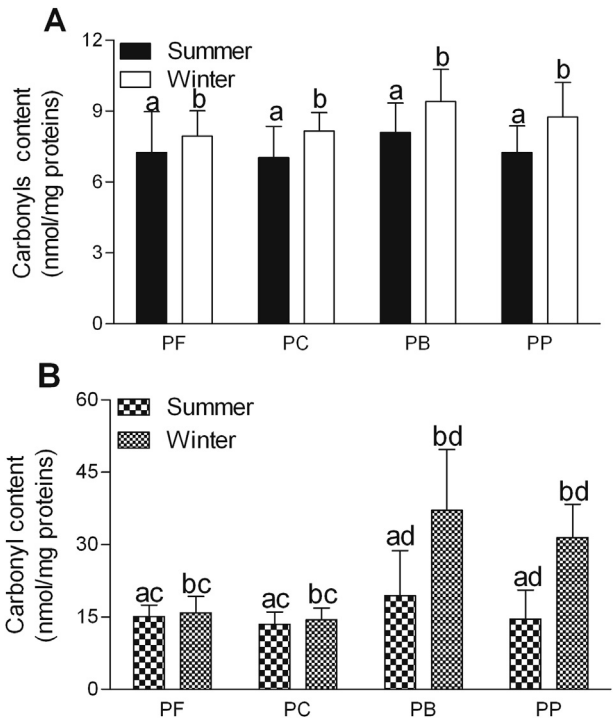

Fig. 3. Protein oxidation, expressed as nmol Carbonyls/mg prot, in digestive gland (A) and gills (B). Results are expressed as means ± S.D. ($n = 9$). Letters *a* and *b* indicate significant differences between sampling sites (PF, PC, PB and PP), and *c* and *d* between seasons.

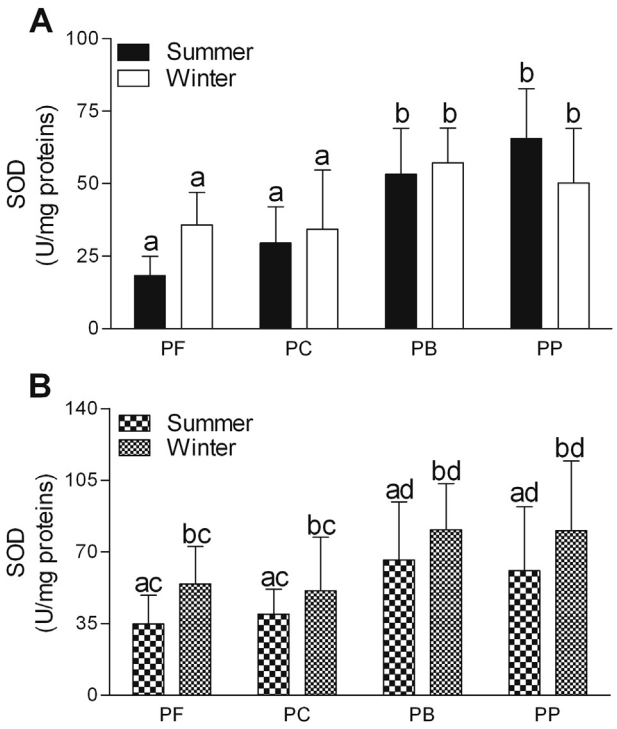

Fig. 4. Superoxide Dismutase (SOD) activity, expressed as U/mg prot, in digestive gland (A) and gills (B). Results are expressed as means ± S.D. ($n = 9$). Letters *a* and *b* indicate significant differences between sampling sites (PF, PC, PB and PP), and *c* and *d* between seasons.

Glutathione-s-transferase (GST) activity also showed similar responses in both tissues (Fig. 5A and B). Differences were observed only between sampling sites (digestive gland two-way ANOVA between sites $p < 0.05$ and between seasons $p = 0.29$, interaction $p = 0.81$ (Fig. 5A); gills two-way ANOVA between sites $p < 0.05$ and between seasons $p = 0.175$, interaction $p = 0.263$) (Fig. 5B). In both tissues, the higher values were recorded in PB and PP (Fig. 5A and B).

Reduced glutathione (GSH) showed higher values in individuals from the harbor area (PB and PP) in both organs (Fig. 6A and B), whereas no differences due seasonality were observed (digestive gland two-way ANOVA between sites $p < 0.01$ and between seasons $p = 0.216$, interaction $p = 0.114$ (Fig. 6A); gills two-way ANOVA between sites $p < 0.01$ and between seasons $p = 0.671$, interaction $p = 0.208$) (Fig. 6B). For both tissues the higher values were recorded in PB and PP (Fig. 6A, B).

4. Discussion

Our results provide evidence for metal bioaccumulation in the digestive gland and gills in the ribbed mussel *A. atra* from four sites impacted by human activity along the coast of the south Atlanctic Ocean, in Argentinean Patagonia. Marine mussels accumulate a wide variety of xenobiotics in their tissues, including trace metals, which are incorporated from the surrounding seawater. Therefore, they can be used to monitor the health of coastal waters (O'Connor, 1992; de Kock and Kramer, 1994; Roméo et al., 2003). Suspension-feeding bivalves bioaccumulate trace metals in their tissues in

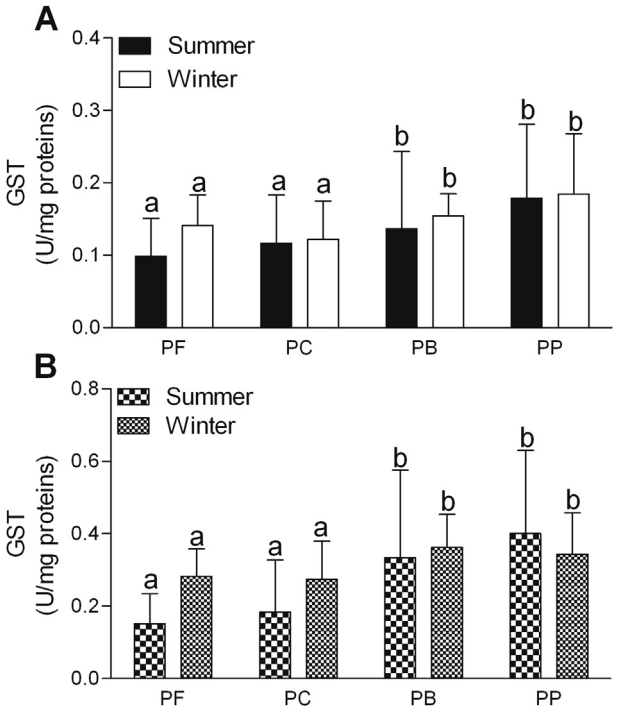

Fig. 5. Glutathione-S-transferase (GST) activity, expressed as U/mg prot, in digestive gland (A) and gills (B). Results are expressed as means ± S.D. ($n = 9$). Letters *a* and *b* indicate significant differences between sampling sites (PF, PC, PB and PP).

direct relation to environmental metal concentration (Elder and Mattraw, 1984). Metal uptake depends on ambient metal concentration, biological and environmental factors (Rainbow, 1990; Mubiana and Blust, 2007), exposure time and accumulation-elimination rate (Ying et al., 1993). In the present study, the analysis of metals in the soft tissues of *A. atra* revealed that individuals from the harbor areas (Piedra Buena and Parque Piedras), or near them (Punta Cuevas), where we also recorded the highest metal concentrations in water, have higher metal concentrations in digestive glands and gills compared to those from the reference site (Playa Fracasso).

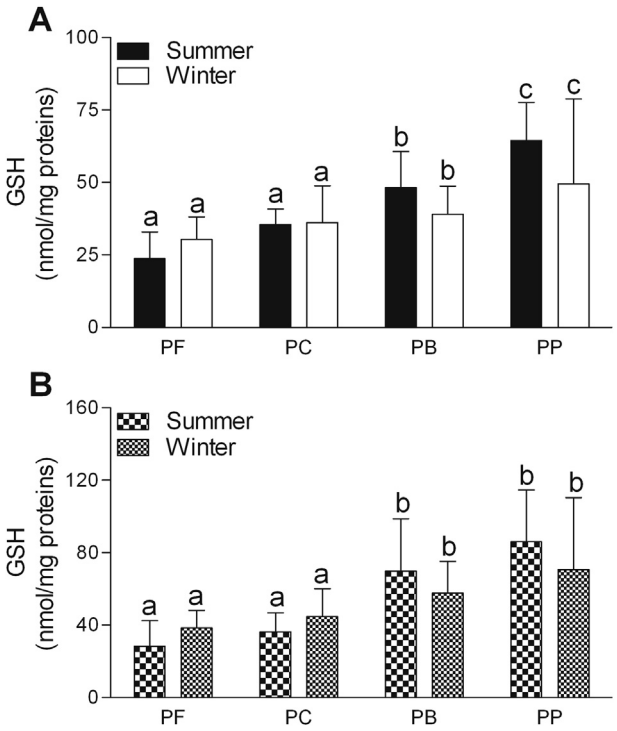

Fig. 6. Reduced glutathione (GSH) content, expressed as U/mg prot, in digestive gland (A) and gills (B). Results are expressed as means ± S.D. ($n = 9$). Letters *a*, *b* and *c* indicate significant differences between sampling sites (PF, PC, PB and PP).

Wang et al. (1995) postulated two major routes by which trace metals enter into bivalves: metals dissolved in the water can be absorbed through the gills, and metal associated with food particles cab be assimilated during digestion; the specific route depending on the type of metal (Wang and Fisher, 1999). Digestive gland and gills exhibited unequal accumulation of metals among the four sampling sites, both in term of concentration and type of metal. We hypothesized that the differences in metal bioaccumulation in soft tissues displayed by *A. atra* were related to metal bioavailability.

Among the five metals analyzed in this work (Mn, Zn, Ni, Cr and Cu), only two (Cr and Ni) had regulatory limits (maximum permitted concentrations according to the Ministry of Agriculture, Livestock, Fisheries and Food of Argentina, 2012). These limits were not exceeded in digestive gland and gills from any of the four sampling sites. Moreover, concentrations of Ni and Cr were found at concentrations two orders of magnitude lower than those set by the national regulations. Despite this fact, the metal concentrations measured in soft tissues of *A. atra* were high enough to induce oxidative stress responses.

Numerous studies have reported that exposure to trace metals induces oxidative stress in aquatic organisms (Winston and Di Giulio, 1991; Dovzhenko et al., 2005; Sabatini et al., 2011). Specifically, increased formation of reactive oxygen species (ROS) may cause oxidative damage to lipids and proteins (Regoli et al., 1998; Sheehan and Power, 1999; Stadtman and Levine, 2000; Levine et al., 2000; Livingstone, 2003). Previous studies have shown that exposure to metals induces oxidative stress in the clam *Ruditapes decussatus* (Geret et al., 2002) and in the vent mussel *Bathymodiolus azoricus* (Company et al., 2004), based on an increase in lipid peroxidation products. Similarly, our results showed that lipid peroxidation products (TBARS) were preferentially increased in gills and digestive gland of ribbed mussels from the polluted sites (harbor areas). Further evidence of increased oxidative damage to lipids due to metals exposure in mussels were observed in *Perna perna* in laboratory experiments (de Almeida et al., 2004) and in *Crenomytilus grayanus* in field studies (Belcheva et al., 2011). In addition, lipid peroxidation products may cause cellular damage by changing cell membrane integrity or by directly attacking proteins (Mattie and Freedman, 2001). Induction of protein carbonyl groups due to oxidation of certain amino acids residues has been widely used as a biomarker of oxidative stress (Stadtman, 1992; Berlett and Stadtman, 1997; Parveza and Raisuddinb, 2005; Sabatini et al., 2009). In this work, comparison among seasons showed markedly higher levels of oxidative damage to proteins in mussel digestive gland and gill during winter. Moreover, carbonyl content protein in gills also showed higher values in harbor sites. Tissue differences in antioxidant defenses and, therefore, in the susceptibility to oxidative stress, have been reported for other aquatic species (Ahmad et al., 2000; Oruc et al., 2004; Martínez-Canto et al., 2013). The differences in tissue responses reported in this study may be partially attributable to tissue-specific antioxidant systems.

Bivalves posses a complex system of antioxidant defenses against prooxidant pollutants that prevent oxidative damage by ROS (Adams et al., 2000; Valavanidis et al., 2006). This system includes both antioxidant enzymes and low molecular weight free radical scavengers like reduced glutathione (GSH). Our results indicate a high activity of superoxide dismutase (SOD) enzyme in digestive gland and gills of *A. atra* from the area with higher harbor

activity where higher metals concentrations are registered both in animals' tissues and water. These results are consistent to those reported by other authors, where SOD activity increased in aquatic animals exposed to trace metals in laboratory and field bioassays (Pellerin-Massicotte, 1994; Fournier et al., 2000; Manduzio et al., 2003; Vlahogianni et al., 2007). In addition, SOD activity in gills also showed significatively higher in winter.

Toxicity of oxidative pollutants in aquatic invertebrates can be related to changes of reduced glutathione (GSH) content (Regoli and Principato, 1995; Canesi et al., 1999; Cheung et al., 2001; Lehman et al., 2007; Wang et al., 2008). Moreover, GSH protects cells nonenzymatically by direct conjugation to oxyradicals (Regoli and Winston, 1999). Our results show highest GSH content in both digestive gland and gills from ribbed mussels from the polluted sites. Similar results were found in other bivalves mollusks exposed to trace metals, whereas GSH levels is increased (Yan et al., 1997; Cheung et al., 2002; Irato et al., 2003). Further, GSH participates as a substrate in detoxification reactions catalyzed by glutathione S-transferases (GST), trough the conjugation of xenobiotics with reduced glutathione (Hayes et al., 2005). Animals from the polluted sites showed a higher GST activity in both digestive gland and gills. Canesi et al. (1999) and Sabatini et al. (2011) observed similar responses after exposure to trace metals in different species of bivalves. However, we cannot rule out that these responses are at least partially due to other chemicals capable of ROS formation such as PAHs, pesticides and polychlorinated biphenyls (PCBs). Indeed, PAHs have been documented in sediments from the harbor areas in our study (Commendatore et al., 2000; Commendatore and Esteves, 2007; Massara Paletto et al., 2008). Therefore, it is important that further studies include PAHs determination in water and in *A. atra's* soft tissues to clarify whether the observed enhance oxidative stress response are specifically to trace metals and/or to PAHs present in water.

As ectothermic animals, *A. atra* cannot regulate their body temperature. Consequently, changes in seawater temperature critically affect their physiology and metabolic processes, including those related to production of free radicals (Dame, 1996; Kawall and Somero, 1996; Lushchak, 2011). In addition, temperature affects oxygen solubility. A decrease in environmental temperature increases oxygen dissolved levels in seawater and consequently also in the internal fluids of ectothermic animals (Wells, 1986), which may increase the risk of reactive oxygen species (ROS) formation (Abele and Puntarulo, 2004). In addition, an increase in oxidative stress due to a decrease in water temperature has been reported in fishes (Ansaldo et al., 2000; Malek et al., 2004) as well as in marine aquatic invertebrates (Viarengo et al., 1995; Niyogi et al., 2001). Moreover, Lushchak (2011) postulated that reduced temperature may increase oxidative stress in aquatic organisms through elevated ROS formation and/or reduced efficiency in the antioxidant defense system. In our study, temperature had a clear effect on oxidative stress biomarkers. Seasonal variations in gill's SOD activity and protein oxidation levels in the digestive gland and gills were observed in animals from unpolluted and polluted areas, both with higher values during winter season, when water temperatures were lower ($\sim$10 °C) than those recorded during the summer (16–20 °C). However, no statistically significant reductions in the levels of antioxidant defenses were seen associated with the decrease in temperature. The increase in oxidative stress observed in *A. atra* may be attributed to an increase in the rate of ROS formation, but further experimental work is necessary to ascertain this point.

5. Conclusions

The results obtained in the present study indicate that *A. atra* accumulates metals both in digestive gland and gills. The pro-oxidant effect of the metals induces activation of enzymatic and non-enzymatic antioxidant defense systems. However, these defense systems are not effective in preventing oxidative damage. Analyses of metal bioaccumulation in soft tissues of *A. atra* together with oxidative stress responses allows for a more accurate assessment of the actual biological effects of metal present in seawater from the South Atlantic Patagonian coast. Finally, metabolic responses related with oxidative stress are enhanced in winter, probably due to reduced temperature. All of these factors should be taken into account in future toxicological studios on bivalve species.

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