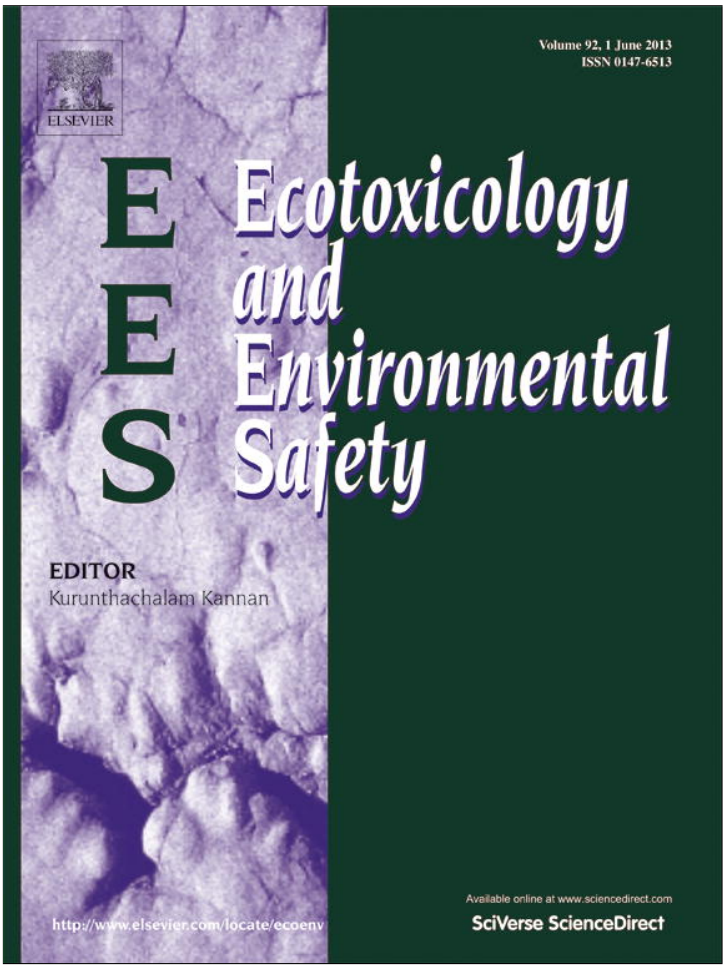

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Assessment of antioxidant responses and trace metal accumulation by digestive gland of ribbed mussel *Aulacomya atra atra* from Northern Patagonia

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ABSTRACT

Seasonal and spatial variability of trace metal concentrations and of a battery of antioxidant parameters were evaluated in digestive gland of the ribbed mussel *Aulacomya atra atra*. Fe, Al and Cu accumulated in tissue exhibited maximum values in winter, coinciding partially with the highest labile concentrations of Fe and Cu in sediment. Metals, as other pollutants, are known to influence the oxidative status of organisms and antioxidant enzymes have been often proposed as biomarkers of contaminant effects. Seasonal variations of trace metals did not appear to influence those of biochemical parameters, which generally showed an opposite trend with higher enzymatic activities in summer when trace metal concentrations were lower. Organisms from Punta Cuevas (control site) showed higher induction of reactive oxygen species production than those from both considered impacted sites, suggesting the possibility of some biochemical adaptation in organisms or a higher modulation of environmental and physiological factors on antioxidant responses than levels of trace metals. This study, which is the first in the area in this matter, showed that seasonal variations of potential biomarkers should be incorporated into interpretation of long-term biomonitoring studies in this marine coastal ecosystem.

1. Introduction

As transitional and coastal environments are among the most productive marine ecosystems in the world and are under continuous human pressure, it is critical that environmental contamination and its biological and ecological significance be properly and fully assessed (Chapman and Wang, 2001). The detection of contaminants in the environment by chemical analyses does not necessarily mean that they are bioavailable and their bioavailability does not imply that they have any effect on organisms, populations or communities. It is widely-accepted that biomonitoring in the marine environment plays a vital role in strategies and actions to identify, assess, control and reduce environmental issues, and ensures that ecosystems are being protected effectively (Wells, 1999).

Among adverse consequences of chemical exposure for aquatic organisms is the increase on cellular levels of reactive oxygen species (ROS). Metals and organic compounds can directly generate ROS due to their direct oxidative potential and by the straightforward activation of the processes that lead to their synthesis, or can indirectly generate ROS by acting on enzymes and cellular scavengers or due to poorly coupled biotransformation processes (Livingstone, 2001). In the event of oxidative stress, the antioxidant systems can be exceeded, causing the oxidation !of different molecules and leading to cellular dysfunctions (Manduzio et al., 2005).

Antioxidant defences, present in all aerobic organisms, include antioxidant enzymes whose function is to remove ROS, thus protecting organisms from oxidative stress. These antioxidant enzymes are important from the point of view of their potential use as pollution biomarkers (Cajaraville et al., 2000). Biomarkers are increasingly worldwide-recognized tools for the assessment of pollution impacts in the marine environment, and some are already incorporated in environmental monitoring programs (Cajaraville et al., 2000; Giarratano and Amin, 2010; Viarengo et al., 2007). Since antioxidant enzymes are interdependent in nature and are subjected to variations due to intrinsic biological cycles, physico-chemical environmental factors and anthropogenic pollutants, the interpretation of these responses is very complex (Sheehan and Power, 1999). The challenge is to integrate individual biomarker responses into a set of tools and indices capable of detecting and monitoring the degradation in health of a particular organism (Allen and Moore, 2004).

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Biomarkers complement contaminant analysis in aquatic organisms by providing first biological signals of exposure. Antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) are critically important in the detoxification of radicals to nonreactive molecules (van der Oost et al., 2003). SOD catalyzes the dismutation of superoxide anion radicals to hydrogen peroxide, which is subsequently degraded to water and oxygen by CAT (Fernández et al., 2012). Glutathione S-transferases (GST) are phase II biotransformation enzymes that enable the excretion of organic compounds by the enhancement of their polarity throughout their conjugation with glutathione (Sheehan et al., 2001). Metal-binding proteins such as metallothioneins (MT) are involved in heavy metal homeostasis being over-expressed in organisms experiencing high metal concentrations in their environment (Viarengo and Nott, 1993) and also as part of the antioxidant defence system of the cells through the scavenging of ROS (Figueira et al., 2012). All oxidizing species with higher redox potentials can be repaired by ascorbate, leading to the generation of the ascorbyl radical. This property makes ascorbyl radical probably the best endogenous marker of the oxidative status in biosystems, which can be easily detected by EPR spectroscopy since it gives a characteristic doublet (Spasojević et al., 2011). More stable radical species are also formed by adding exogenous spin traps, molecules that react with primary radical species to give more enduring radical adducts, such as lipid radicals, with characteristic EPR signatures (Malanga and Puntarulo, 2012).

Bivalve molluscs are widely used as marine pollution sentinels because of their sessile nature, filter-feeding habits, and their potential to accumulate high levels of trace metals in their soft tissues, providing a time-integrated indication of contamination with measurable cellular and physiological responses (Goldberg et al., 1978; Livingstone et al., 2000). We considered in this work the digestive gland, which is the main center for metabolic regulation in bivalves, participating in the mechanisms of immune defence and homeostatic regulation of the internal medium, as well as in the processes of detoxification and elimination of xenobiotics (Marigómez et al., 2002).

The present work aims to evaluate several biomarkers of oxidative stress in the digestive gland of the ribbed mussel *Aulacomya atra atra* from three sites in Nuevo Gulf (Northern Patagonia) and assess the environmental quality of surrounding water and sediment. In addition, the responses of all biomarkers as well as the formation of ascorbyl and lipid-derived radicals were studied at two different times of the year to explore possible seasonal variations that could confound the biomarker approach. This is the first study evaluating the use of oxidative stress biomarkers in the ribbed mussel *A. atra atra* in Argentina.

2. Materials and methods

Three sites within Nuevo Gulf were selected and are shown in Fig. 1. Storni Dock (S42°44′10.5″ W65°01′53.8″) is a deep commercial dock, unconditioned on tidal height, suitable for movement of cargo containers. Loading and unloading of fuel oil to a great variety and number of ships are done at this port. Folías Wreck (S42°47′24″ W64°56′24″) is a sunken ship of 60 m overall length located 300 m off shore of Paraná Beach, used for scuba diving activities. Punta Cuevas (S42°46′18.5″ W64°59′48.7″) is a diving area that hosts fishes, crabs, sea stars, among others, and it is located at the Southern end of the city. For its location, this last site was thought to be a relatively clean area according to previous works close to this area (Gil, 2001; Massara Paletto et al., 2008). Seawater, sediment and mussel samples were collected at the same time.

2.1. Physico-chemical parameters

Seawater samples were collected with a Van Dorn bottle from Storni Dock, Folías Wreck and Punta Cuevas in Nuevo Gulf to determine inorganic nutrients with a Skalar autoanalyzer and chlorophyll *a* according to Strickland and Parsons (1972). Temperature, dissolved oxygen, salinity, pH and redox potential (Eh) were measured *in situ* by a multiparameter device YSI 556 MPS.

Surface sediment samples (~2 cm) were collected by core samplers *ad hoc* in Storni Dock and Folías Wreck. It was not possible to obtain sediment samples from Punta Cuevas because of its rocky bottom. Samples were stored in polyethylene bags and transported to the laboratory, where they were dried until constant weight at 60 °C and sieved through nylon meshes in order to obtain the finest fraction (< 63 μm).

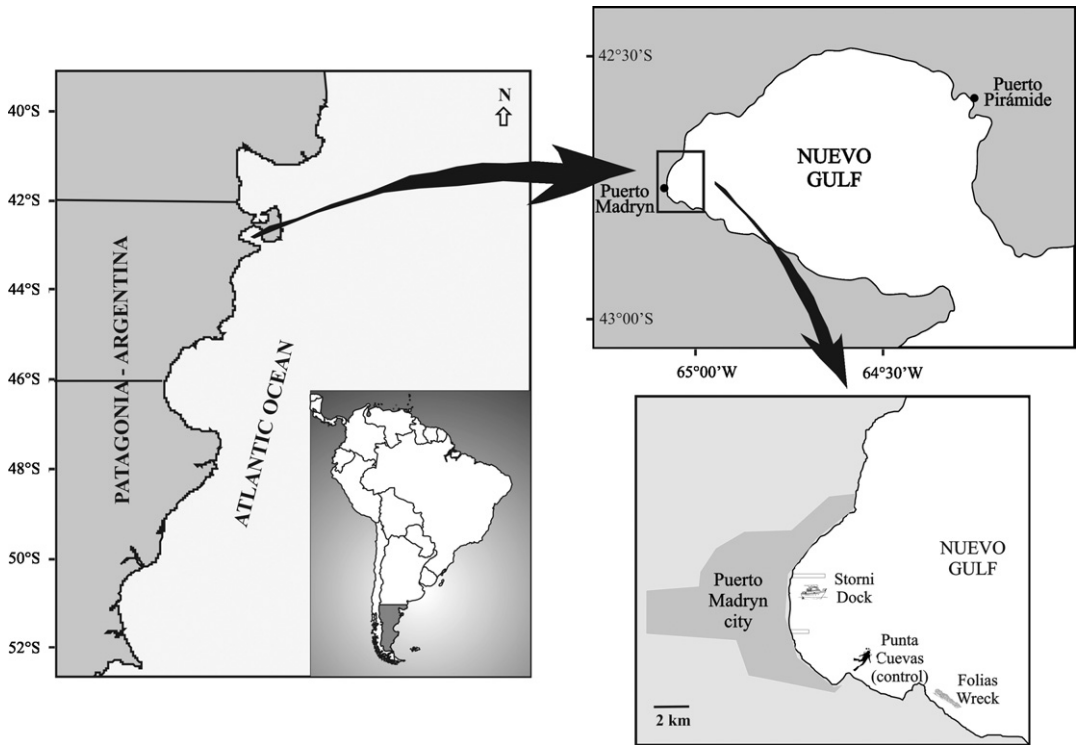

Fig. 1. Map of the studied area.

The cold extraction described by Agemian and Chau (1976) was applied to determine labile concentrations of Fe, Al, Zn, Cu, Cd and Pb in finest fraction of sediment. Briefly, 25 ml of 0.5 N HCl was added to 1 g of sediment and an orbital shaker at 2000 rpm was used to keep the sediment sample in suspension during extraction for 12 h at room temperature. Sediments were removed after centrifugation at 8000 rpm for 20 min at room temperature and the supernatants were stored at 4 °C pending analysis.

The concentrations of labile metals were determined by an IL-457 atomic absorption spectrophotometer (AAS) with air-acetylene flame, except for Al that was measured using a nitrous oxide–acetylene flame. Reagents of analytical-grade were employed for sediment mineralization as well as for blanks, and also to build up the standards for the calibration curve. Each sample was run in triplicate obtaining a mean value for each site, which was reported as μg/g on dry weight. Detection limits were Fe 2.5, Al 25, Zn 0.25, Cu 0.64, Cd 0.25 and Pb 2 μg/g.

2.2. Animal collection

In August 2011 (winter) and February 2012 (summer) 50 individuals of *A. atra atra* were collected by scuba diving at each sampling site. Following collection, mussels were placed in isolated plastic containers previously filled with seawater from the sampling site and transported to the laboratory within 2 h of collection. No differentiation was made regarding either sex or reproductive stage. Digestive glands were removed and stored at −80 °C until biochemical analysis.

2.2.1. Trace metals in organisms

Concentrations of Fe, Al, Zn, Cu, Cd and Pb in 3 pools of 10 digestive glands of *A. atra atra* per site were assessed. Approximately 0.5 g of dried tissue was placed in a crucible in a muffle furnace and the temperature was slowly increased from room temperature up to 400 °C for 6 h. When the sample was cooled down; 2 ml of concentrated HNO_3 were added and evaporated to dryness on a sand bath at 80 °C. This procedure was repeated until white ashes were obtained. Ashes were resuspended with a mixture of HNO_3 (3% v/v) and HCl (6% v/v) up to 10 ml (Boletín Oficial del Estado, 1991). Trace metals in samples and two blanks prepared as samples were assessed by AAS with an air-acetylene flame, except for Al that was measured using a nitrous oxide–acetylene flame. Each sample was run in duplicate obtaining a mean value for each site, which was reported as μg/g on dry weight. The reference material of oyster tissue NIST-SRM 1566a was used for the quality control of trace metal analysis in the ribbed mussel tissue. The precision for all metals, expressed as coefficient of variation, was between 0.8% and 6.6%. The accuracy for all metals, expressed as percentage of recovery, was between 89% and 102%.

2.2.2. Biochemical analyses

In order to measure enzymatic activities of superoxide dismutase, catalase and glutathione S-transferase, digestive glands ($n=6$) were removed upon return to the laboratory and stored at −80 °C until further analysis. Samples were homogenized in a 1:3 (w/v) ratio of buffer solution containing 20 mM Tris-Base, 1 mM EDTA, 1 mM DL-dithiothreitol, 0.5 M sucrose, 0.15 M KCl and 0.1 mM PMSF, with pH adjusted to 7.6. Homogenates were then centrifuged at 9000*g* for 30 min at 4 °C (Bainy et al., 1996). SOD activity was assayed by the epinephrine method (Misra and Fridovich, 1972), based on the capability of SOD to inhibit the autooxidation of epinephrine to adrenochrome at 480 nm at 30 °C. One unit of SOD was defined as the amount of enzyme that inhibits the rate of adrenochrome formation by 50% under the assay conditions. CAT activity was evaluated by the decomposition rate of H_2O_2 at 240 nm at 30 °C (Beutler, 1982). One unit of CAT was defined as the amount of enzyme catalyzing the elimination of 1 μM H_2O_2 per minute. GST activity was determined by incubating reduced glutathione with 1-chloro-2,4-dinithrobenzene as substrate at 25 °C and measuring the increase in absorbance at 340 nm (Habig et al., 1974). 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 as measured by the method of Lowry et al. (1951), with bovine serum albumin as a standard.

Metallothioneins were analyzed in digestive glands homogenized (1:3 w/v) in 20 mM Tris–HCl buffer pH 8.6, 0.5 M sucrose, 0.006 mM leupeptin, 0.5 mM phenylmethylsulphonyl fluoride (PMSF) and 0.01% β-mercaptoethanol. After acidic ethanol/chloroform fractionation of the tissue homogenate, MT were quantified by the spectrophotometric assay as described in Viarengo et al. (1997) using GSH as standard.

Production of reactive oxygen species by digestive glands ($n=6$) was evaluated after homogenization (1:5 w/v) in 100 mM Tris–HCl, pH 7.75 buffer, with 2 mM EDTA and 5 mM $MgCl_2$ (Gallagher et al., 1992). Measurements were conducted according to Viarengo et al. (1999) with modifications. The homogenates were then centrifuged at 4 °C for 20 min at 10,000*g* and 10 μl of the supernatants were employed. The reaction was followed in 30 mM HEPES, pH 7.2 buffer, with 200 mM KCl and 1 mM $MgCl_2$. Immediately before the reading, the fluorescent probe 2′,7′ dichlorofluorescein diacetate (DCFH-DA) was added to the buffer in a final concentration of 40 μM. The reaction mixture was incubated at 37 °C during 15 min. Thereafter, the nonfluorescent compound DCFH was oxidized by ROS to the fluorescent compound DCF, which is detected in wavelengths of 488 and 525 nm for excitation and emission, respectively.

Ascorbyl radical content ($A^{\bullet}$) was measured on homogenates from digestive glands ($n=5$) prepared in pure dimethylsulfoxide (DMSO) (1:10). A Bruker ECS 106 spectrometer was used for $A^{\bullet}$ measurements and the spectra were scanned at room temperature under the following conditions: 50 kHz field modulation, microwave power 20 mW, modulation amplitude 1 G, time constant 655 ms, receiver gain 1×10^5, microwave frequency 9.81 GHz and scan rate 0.18 G/s (Giulivi and Cadenas, 1993). Quantification was performed according to Kotake et al. (1996). The amount of spin adduct was calibrated using an aqueous solution of 2,2,5,5-tetramethyl piperidin 1-oxyl (TEMPO), introduced into the same cell used for spin trapping. Electron paramagnetic resonance (EPR) spectra of spin adduct solution and TEMPO solution were recorded at exactly the same spectrometer settings and the first-derivative EPR spectra were double integrated by a computer attached to the EPR spectrometer to obtain the intensity (area under the absorption spectrum), and then the concentration of spin adduct was calculated using the ratio of these areas.

Lipid-derived radical content (LR) was measured on homogenates from digestive glands ($n=5$) prepared in a fresh stock solution containing 40 mM N-t-butyl-α-phenyl nitrone (PBN) and pure DMSO (1:10). EPR spectra were obtained at room temperature using a Bruker spectrometer ECS 106, operating at 9.8 GHz with 50 kHz modulation frequency. 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 2×10^5; sweep with 100 G and conversion time 82 ms (Jurkiewicz and Buettner, 1994). Quantification was performed according to Kotake et al. (1996). The amount of spin adduct was calibrated using an aqueous solution of 2,2,5,5-tetramethyl piperidin 1-oxyl (TEMPO), introduced into the same cell used for spin trapping.

2.3. Statistical analyses

Results of all parameters were reported as mean ± S.D. Normality of the distributions and homocedasticity of variances were tested. When it was necessary, data was log transformed to meet homocedasticity of variances. The variation of each parameter among sites and between seasons was tested by two-way ANOVA. When significant differences were found ($p<0.05$), post hoc pair-wise comparisons were done using the Tukey's test to determine which values differed significantly.

3. Results

The physico-chemical characteristics of the seawater are shown in Table 1. Variables were similar among seasons, being the temperature, salinity and chlorophyll *a* the only parameters showing clear seasonal differences but not spatially. For these three variables, mean values were calculated integrating results for all sites. Mean winter temperature was 9.92 ± 0.28 °C, while in summer was 18.82 ± 0.25 °C. Salinity and chlorophyll *a* were higher in winter (34.21 ± 0.05 ppt and 3.08 ± 1.03 mg/m^3, respectively) than in summer (31.42 ± 0.04 ppt and 0.26 ± 0.18 mg/m^3, respectively). In both seasons, dissolved oxygen remained close to saturation (10.04 ± 1.59 mg/l), pH kept stable and slightly alkaline (8.20 ± 0.02) and redox potential presented typical levels of seawater (260.24 ± 19.26 mV). In the case of inorganic nutrients, concentrations tended to be higher in winter than in summer.

Sediments from Storni Dock showed lower percentages of silt-clay and organic matter than those from Folías Wreck in both seasons. The decreasing labile metal concentration in sediment was: Fe > Al > Zn > Pb–Cu > Cd (Table 2) and significant differences were found between sites and seasons (Table 3). Fe and Al were the most concentrated metals, one order of magnitude higher than the other ones. On the contrary, Cd was below detection limit (< 0.25 μg/g) at all sampling sites. Fe was the highest in winter at Folías Wreck (5.84 ± 0.09 mg/g), while at Storni Dock was higher in summer than in winter (3.80 ± 0.11 and 2.99 ± 0.07 mg/g, respectively). Al was maximum at Storni Dock in summer (3.13 ± 0.14 mg/g). Zn did not show differences between sites or seasons. Cu was higher in winter than in summer and at Storni Dock than at Folías Wreck. As Al, Pb was higher in summer than in winter, but it was maximum at Folías Wreck.

Table 1
Physico-chemical characteristics of seawater ($n=2$).

Parameter	Winter			Summer		
	Storni Dock	Folías Wreck	Punta Cuevas	Storni Dock	Folías Wreck	Punta Cuevas
Temperature (°C)	9.61 ± 0.03	10.00 ± 0.04	10.15 ± 0.06	18.28 ± 0.25	18.10 ± 0.36	18.59 ± 0.41
Dissolved oxygen (mg/l)	13.18 ± 2.58	10.09 ± 0.59	9.05 ± 0.09	9.04 ± 0.10	9.25 ± 0.07	9.63 ± 0.05
Salinity (ppt)	34.26 ± 0.01	34.20 ± 0.00	34.17 ± 0.01	31.40 ± 0.08	31.39 ± 0.06	31.46 ± 0.05
pH	8.18 ± 0.01	8.22 ± 0.13	8.22 ± 0.16	8.18 ± 0.05	8.19 ± 0.06	8.20 ± 0.07
Eh (mV)	242.30 ± 1.13	285.35 ± 1.20	283.15 ± 4.03	248.60 ± 1.27	257.05 ± 0.21	245.00 ± 0.36
NO_3+NO_2 (μM)	2.87 ± 1.51	3.38 ± 0.03	4.33 ± 0.02	0.08 ± 0.02	0.13 ± 0.02	0.17 ± 0.01
PO_4^{3-} (μM)	1.16 ± 0.17	3.03 ± 0.02	1.29 ± 0.03	0.97 ± 0.01	0.92 ± 0.02	0.94 ± 0.03
SiO_3^{2-} (μM)	2.95 ± 1.21	1.21 ± 0.33	4.86 ± 0.22	0.73 ± 0.11	1.40 ± 0.73	3.52 ± 0.16
Chl. *a* (mg/m^3)	3.05 ± 1.95	3.38 ± 0.82	2.80 ± 0.66	0.20 ± 0.12	0.44 ± 0.02	0.06 ± 0.01

Table 2
Physico-chemical characteristics of sediment and heavy metal contents ($n=3$).

Parameter	Winter		Summer	
	Storni Dock	Folías Wreck	Storni Dock	Folías Wreck
Silt-clay (< 63 μm) (%)	3.41	4.91	0.94	7.18
Organic matter (%)	1.58	3.12	3.25	4.54
Fe_{labile} (mg/g)	2.99 ± 0.07	5.84 ± 0.09	3.80 ± 0.11	3.39 ± 0.06
Al_{labile} (mg/g)	1.66 ± 0.02	2.10 ± 0.44	3.13 ± 0.14	2.11 ± 0.01
Zn_{labile} (μg/g)	43.22 ± 1.63	44.23 ± 0.34	43.29 ± 0.64	37.63 ± 4.49
Cu $_{labile}$ (μg/g)	13.95 ± 0.34	12.50 ± 0.00	9.23 ± 0.33	5.98 ± 0.01
Pb_{labile} (μg/g)	8.72 ± 1.75	14.99 ± 0.00	12.48 ± 0.03	17.45 ± 0.04
Cd_{labile} (μg/g)	< 0.25	< 0.25	< 0.25	< 0.25

There is no information for Punta Cuevas due to its rocky bottom.

Table 3
Summary of the two-way ANOVA results for trace metal contents in sediment.

Parameter	Site	Season	Site × season
Fe_{labile}	< 0.001, FW > SD	< 0.0001, W > S	< 0.0001
Al_{labile}	< 0.0001, SD > FW	< 0.0001, S > W	< 0.0001
Zn_{labile}	ns	ns	ns
Cu_{labile}	< 0.0001, SD > FW	< 0.001, W > S	< 0.01
Pb_{labile}	< 0.01, FW > SD	< 0.001, S > W	ns
Cd_{labile}	ns	ns	ns

SD Storni Dock, FW Folías Wreck, PC Punta Cuevas, W winter, S summer, ns not significant.

Descriptive parameters of the organisms are presented in Table 4. Organisms showed similar morphological parameters, except for those from Punta Cuevas that presented the highest widths and weights of total tissue and digestive gland (Table 6). Besides, these last two parameters were higher in summer than in winter.

The metal bioaccumulation pattern in digestive gland of *A. atra atra* from Nuevo Gulf was Fe > Al–Zn > Cu > Cd > Pb (Fig. 2). A two-way ANOVA (Table 6) revealed that the interaction between "site × season" was significant for Al and Cd. Al was higher at Punta Cuevas in winter than at the other sites; meanwhile Cd concentrations in bivalves from Folías Wreck were higher than at Punta Cuevas, for both seasons. Spatial differences were found in bivalves from Folías Wreck which showed the maximum level of Fe; meanwhile in those from Storni Dock the bioaccumulation of Zn and Cu was the uppermost. Seasonal differences were found in Fe and Cu concentrations, being the highest bioaccumulations in winter. Pb was below detection limit (2 μg/g dw) in all cases.

Mean values (± SD) of enzymatic activities are shown in Fig. 3, while the statistical results are summarized in Table 6. Significant interaction between "site × season" was found for CAT and GST. Both enzymes showed similar response with higher activities in bivalves from Punta Cuevas and Folías Wreck in summer than those from the same sites in winter. There were no significant differences between seasons in organisms from Storni Dock. SOD values varied significantly only among sites, being the activity higher at Storni Dock (6.90 U SOD/mg prot.) than at the other sites.

Metallothionein concentration in organisms varied with sampling site as well as with season (Fig. 4). Levels of metallothioneins were higher at Storni Dock (125.32 nmol-SH/g wet tissue) than at Folías Wreck (68.72 nmol-SH/g wet tissue) and Punta Cuevas (66.81 nmol-SH/g wet tissue) in summer. Similar spatial tendency was found in winter, being the registered values.70.67, 53.67 and 38.92 nmol-SH/g wet tissue for Storni Dock, Folías Wreck and Punta Cuevas, respectively.

DCFH-DA oxidation rate by digestive gland homogenate was evaluated as an index for the chemical ROS generation capacity. Data in Fig. 5 indicates that ROS generation capability at Punta Cuevas in winter (517.13 a.u./min/mg prot.) was significantly higher than at the other sites (253.50 ± 58.24 a.u./min/mg prot.). Interaction between "site × season" was statistically significant.

$A^{\bullet}$ content was assessed by quantification of EPR signals and results are presented in Table 5. Since no differences between seasons were found (Table 6), mean values were calculated integrating winter and summer results for each site. $A^{\bullet}$ contents were significantly lower at Storni Dock (8.27 μmol/mg wet tissue) than at Punta Cuevas (19.37 μmol/mg wet tissue) and Folías Wreck (23.85 μmol/mg wet tissue).

As an estimation of oxidative stress in the lipid phase, lipid peroxidation in the digestive gland was assessed by EPR as the tissue content of lipid radicals (Table 5). Lipid radicals in the digestive gland combined with the spin trap PBN resulting in adducts that gave a characteristic EPR spectrum with hyperfine coupling constants of $a_N=15.56$ G and $a_H=2.79$ G, in concordance with computer simulation of spectral signals obtained using the overall mentioned parameters (Fig. 6). Lipid radical content in

Table 4
Morphological and physiological characteristics of *A. atra atra* ($n=12$).

Parameter	Winter			Summer		
	Storni Dock	Folías Wreck	Punta Cuevas	Storni Dock	Folías Wreck	Punta Cuevas
Length (mm)	73.16 ± 4.56	73.92 ± 3.54	75.62 ± 4.81	74.86 ± 5.15	73.32 ± 2.57	75.08 ± 3.55
Width (mm)	36.97 ± 4.12	37.41 ± 2.92	39.66 ± 2.77	37.77 ± 3.68	37.16 ± 2.32	39.82 ± 2.65
Height (mm)	22.98 ± 1.77	25.06 ± 3.26	26.81 ± 4.60	25.87 ± 2.63	23.23 ± 1.61	24.30 ± 1.55
Total tissue weight (g)	6.32 ± 2.00	7.11 ± 1.75	7.68 ± 1.93	7.90 ± 2.44	6.85 ± 1.95	8.46 ± 1.55
Digestive gland weight (g)	0.36 ± 0.10	0.35 ± 0.07	0.58 ± 0.11	0.46 ± 0.15	0.45 ± 0.13	0.61 ± 0.11

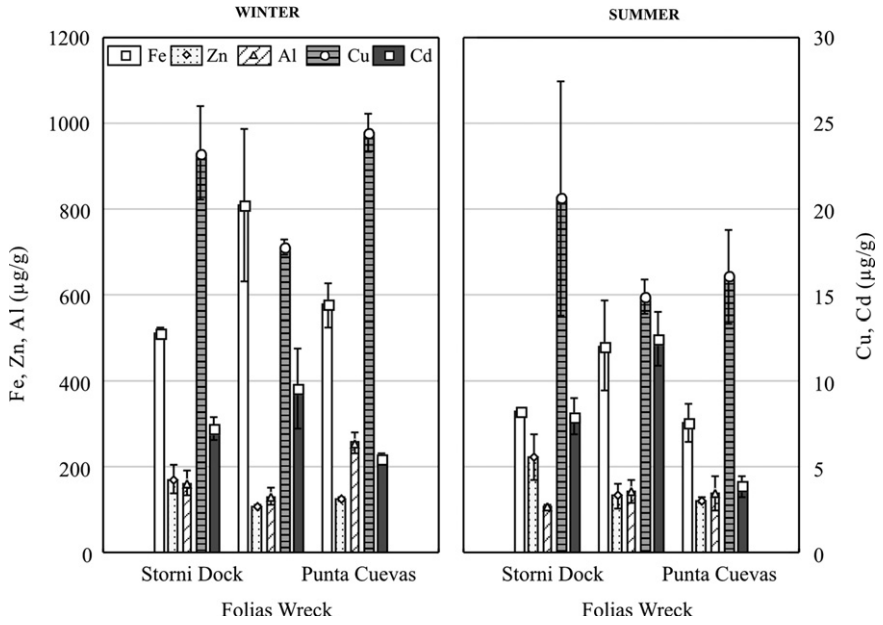

Fig. 2. Concentration of trace metals in digestive gland of *A. atra atra* ($n=3$). Fe, Zn and Al are represented on left axis and Cu and Cd on right axis.

digestive gland was significantly dependent on sites and seasons (Table 6). The lipid content measured in bivalves from Folías Wreck was superior to that from Storni Dock, in both seasons. Moreover, mean lipid content in digestive gland of mussels from Folías Wreck, Storni Dock and Punta Cuevas was 70%, 48% and 29% higher in summer than in winter, respectively.

Results from the correlation analysis among biochemical, chemical and environmental variables are shown in Table 7. Among biomarkers, positive correlations were found between GST and CAT, SOD and MT and, $A^{\bullet}$ and LR contents. Contrarily, negative correlations were found for ROS with GST and CAT and, for $A^{\bullet}$ content with SOD and MT. With regard to metals, GST and CAT displayed negative correlations with Cu and Fe levels, respectively. SOD and MT correlated positively to Zn content, while ROS showed the same tendency with Fe. Oppositely, $A^{\bullet}$ content was inversely related to Zn. In the case of environmental variables, GST and CAT showed positive correlation with temperature and negative with salinity, nitrates and phosphates. In comparison with GST and CAT, ROS showed the opposite relationships with the previously mentioned environmental variables.

4. Discussion

This study was carried out to evaluate, for the first time, several oxidative stress parameters in the wild ribbed mussel *A. atra atra*. Seawater physico-chemical characteristics and the levels of trace metals in sediment and mussels were also evaluated. Previous studies involving bioaccumulation of trace metals in the ribbed mussel *A. atra atra* from Nueva Bay have been reported (Gil et al., 1988, 2006; Mohamed, 2008) and also levels of trace metals in sediment (Gil et al., 1999; Harvey and Gil, 1988). It is important to remark that not all the sampling sites, the analyzed metals or the employed sediment fraction studied in present work coincide with those monitored in existing literature and/or the methodology is not the same, making difficult direct comparisons.

Results of physico-chemical characteristics of seawater were within the range of previously informed data (Gil, 2001; Solís, 1998). They were similar among sites, except for nitrates and silicates that were highest at Punta Cuevas in both seasons. Cold and more saline water enriched in dissolved nutrients was observed in winter. The higher salinity would be the result of high seawater evaporation in this time of the year, when air temperature exceeds that of seawater (Rivas and Beier, 1990; Scasso and Piola, 1988). Relative low nutrient concentrations in summer are in agreement with the expected higher primary production. However, this was not reflected by chlorophyll *a* concentrations, probably due to zooplankton grazing (Valiela, 2011).

Fe was more concentrated at Folías Wreck likely as a product of ship's corrosion, while Al was more concentrated at Storni Dock probably due to the biggest aluminum industry in Argentina developed precisely in the proximity of that dock. Fe concentrations measured in summer in the present study were 15–30% higher than the value reported by Mohamed (2008) for Storni Dock, but in the same range in winter and also similar to those published by Marinho (2011) for San Jorge Gulf, area considered

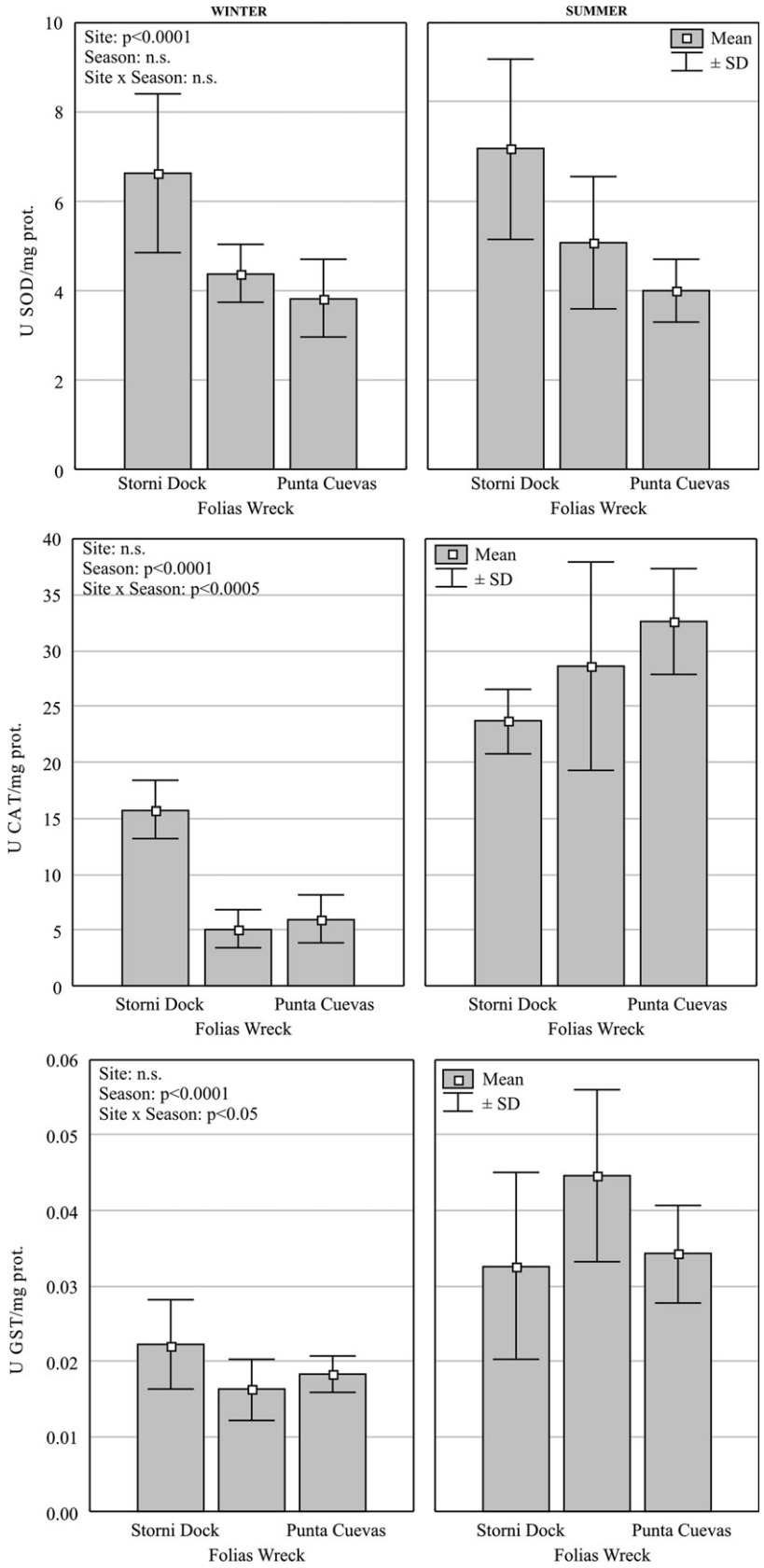

Fig. 3. Specific activities of superoxide dismutase, catalase and glutathione S-transferase in digestive gland of *A. atra atra* from Nuevo Gulf ($n=6$). The statistical significance was analyzed by two-way ANOVA test with site and season as factors. n.s.=not significant.

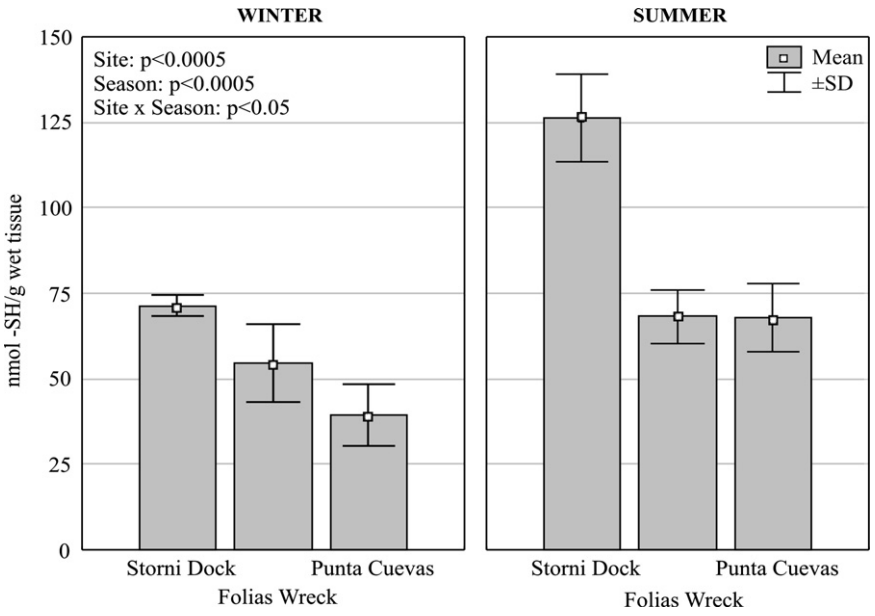

Fig. 4. Levels of metallothioneins in digestive gland of *A. atra atra* from Nuevo Gulf. The statistical significance was analyzed by two-way ANOVA test with site and season as factors.

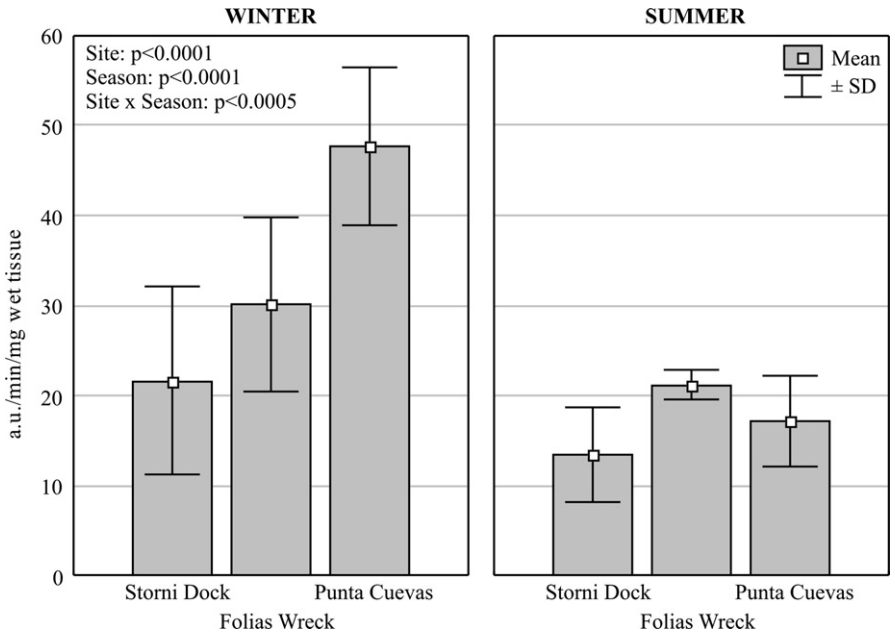

Fig. 5. ROS production in digestive gland of *A. atra atra* from Nuevo Gulf ($n=6$). The statistical significance was analyzed by two-way ANOVA test with site and season as factors.

Table 5
Ascorbyl and lipid radical contents (μmol/mg WW) in digestive glands of *A. atra atra* ($n=5$).

Parameter	Winter			Summer		
	Storni Dock	Folías Wreck	Punta Cuevas	Storni Dock	Folías Wreck	Punta Cuevas
Ascorbyl radical content	10.60 ± 2.76	21.69 ± 6.64	17.96 ± 1.45	5.94 ± 4.14	26.01 ± 3.09	20.77 ± 8.13
Lipid radical content	50.39 ± 21.50	82.01 ± 28.02	77.17 ± 17.06	74.94 ± 39.44	139.48 ± 25.86	99.81 ± 27.26

as low-polluted. In the case of Al, our values are the first ones of the mentioned area. Fe and Al are considered major elements on Earth, being the high values similar or even lower than those found in different areas of the world (Aubert et al., 2004; Gao et al., 2009; Wijsman et al. 2001).

Pb showed the highest concentrations at Folías Wreck, while Cu was highest at Storni Dock. Only Cu results were superior to those previously reported by Mohamed (2008) and also to those measured in San Jorge Gulf (Marinho, 2011). In the particular case of Pb, concentrations were similar or even lower than the values

Table 6
Summary of the two-way ANOVA results for biological and biochemical determinations.

Parameter	Site	Season	Site × season
Length	ns	ns	ns
Width	<0.05, PC > others	ns	ns
Height	ns	ns	<0.005
Total tissue weight	<0.05, PC > others	<0.05, S > W	ns
Digestive gland weight	<0.005, PC > others	<0.0001, S > W	ns
Fe	<0.005, FW > others	<0.005, W > S	ns
Al	<0.005, PC > others	<0.005, W > S	<0.01
Zn	<0.005, SD > others	ns	ns
Cu	<0.05, SD > FW	<0.01, W > S	ns
Pb	ns	ns	ns
Cd	<0.0001, FW > SD > PC	ns	<0.05
SOD	<0.0001, SD > others	ns	ns
CAT	ns	<0.0001, S > W	<0.0005
GST	ns	<0.0001, S > W	<0.05
MT	<0.0005, SD > others	<0.0005, S > W	<0.05
ROS	<0.0001, PC > others	<0.0001, W > S	<0.0005
Ascorbyl radicals	<0.0001, FW-PC > SD	ns	ns
Lipid-derived radicals	<0.01, FW > SD	<0.01, S > W	ns

SD Storni Dock, FW Folías Wreck, PC Punta Cuevas, W winter, S summer, ns not significant.

reported by Gil et al. (1999) and Mohamed (2008). Zn was similar in both studied sites and in the same range found by Mohamed (2008), but higher than the value reported by Gil et al. (1999) and Marinho (2011). Labile Cd in sediment was below detection limit in all cases. This finding is in agreement with Mohamed (2008) and also with other researches made in Patagonia (Gil et al., 1999; Vázquez et al., 2007). Labile Zn, Cu, Pb and Cd values measured in present study were within the range reported for sediments from Nueva Bay by Gil et al. (1999), who considered it as slightly polluted.

There are no published data of accumulated metals by *A. atra atra* or other bivalves, discriminated by tissues, from the studied area. In accordance with sediment data, Fe was the most bioaccumulated metal in digestive gland of *A. atra atra*, especially by mussels from Folías Wreck that would come from the corrosion of the ship. This highest value was 2–3 times lower than values reported for Antarctic clam *Laternula elliptica* (Ahn et al., 1996) and for Antarctic limpet *Nacella concinna* (Weihe et al., 2010) and similar to the maximum value measured in digestive gland mussel *Mytilus edulis chilensis* from an aquaculture area in South Patagonia (Giarratano and Amin, 2010). Zn and Cu were accumulated in higher proportion by mussels from Storni Dock, while Al by those from Punta Cuevas. Concentrations of Zn were higher than those reported for mussel by Giarratano and Amin (2010) and for limpet *N. concinna* (Weihe et al., 2010). Cu was slightly higher than the value measured in mussel, but 3 times lower than that found in Antarctic clam. In the case of Al, concentrations found in ribbed mussel from Punta Cuevas (256 μg/g) was one order of magnitude lower than the value found in limpet *N. concinna.*

Despite Cd was not detected in sediments, it was present in all digestive gland samples and in highest concentrations at Folías Wreck. This availability for biota, when no detectable levels of Cd were observed in sediment, has been previously found in different molluscs from Patagonia (Duarte et al., 2011; Giarratano and Amin, 2010; Gil et al., 2006; Vázquez et al., 2007). Under aerobic conditions, dissolved forms of Cd are dominant, providing greater availability of this metal to biota (Bewers et al., 1987). In general terms, Cd concentration in soft tissues of marine organisms could be many times greater than the concentrations of metal in the surrounding environment (Ansari et al., 2004; Apeti et al., 2009). Cd concentrations found in digestive gland of *A. atra atra* (3.20–13.89 μg/g) were higher than previous data reported for mussels *M. edulis chilensis* from Patagonia (Giarratano and Amin, 2010; Giarratano et al., 2010, 2011), but lower than 59 μg/g reported as natural background for *Chlamys tehuelcha* (Gil et al., 1988). Our results were also lower than those found in Antarctic bivalve *L. elliptica* (Husmann et al., 2012) and in different species of scallops (Belcheva et al., 2006; Bustamante and Miramand, 2004). Digestive gland in bivalves has been reported by many researchers as the main tissue of Cd accumulation (Hervé-Fernández et al., 2010; Husmann et al., 2012; Irato et al., 2003; Saavedra et al., 2008). The slower rate of excretion of Cd in the digestive glands has already been explained by the presence of metallothioneins (Hervé-Fernández et al., 2010; Viarengo et al., 1993). It is important to consider that the differences cited in Cd concentration may be due not only to different environmental levels of this metal, but also to the interspecific variation in the capacity of the bivalves examined to concentrate cadmium in the digestive gland.

The activity of antioxidant enzymes and antioxidant compounds are known to be under extensive seasonal control (Sheehan and Power, 1999). The first season-related factor is the increase in temperature, followed by an increase in oxygen consumption and by an increase in ROS generation; and the second factor in mussels seems to be the metabolic particularity of the stages of their reproductive cycle (Borković et al., 2005). Antioxidant defence enzymes are constitutive in nature and susceptible to variations due to intrinsic biological processes and cycles and ontogenic processes, beside the extrinsic influences, such as environmental changes and contaminants. The interpretation of these responses, in an environmental context, is very complex to account for all the possible causes (Sheehan and Power, 1999).

It is known that the exposure of aquatic organisms to metals may increase ROS generation, which can lead to an imbalance in antioxidant defences, enhance oxidative stress and generate lipid peroxidation, as has been reported in *Mytilus galloprovincialis* (Almeida et al., 2004; Vlahogianni and Valavanidis, 2007).

Studies on the seasonal variability of metallothioneins have provided conflicting results, showing in mussels from Adriatic coasts higher levels of these proteins in winter and lower in summer (Ivanković et al., 2005). Opposite results with a raise of MT during summer have been observed by Viarengo et al. (1997), which is in coincidence with our results. Concerning the role of MT in the homeostasis of trace metals and the elevated concentrations of these elements typical for mussel digestive gland, the

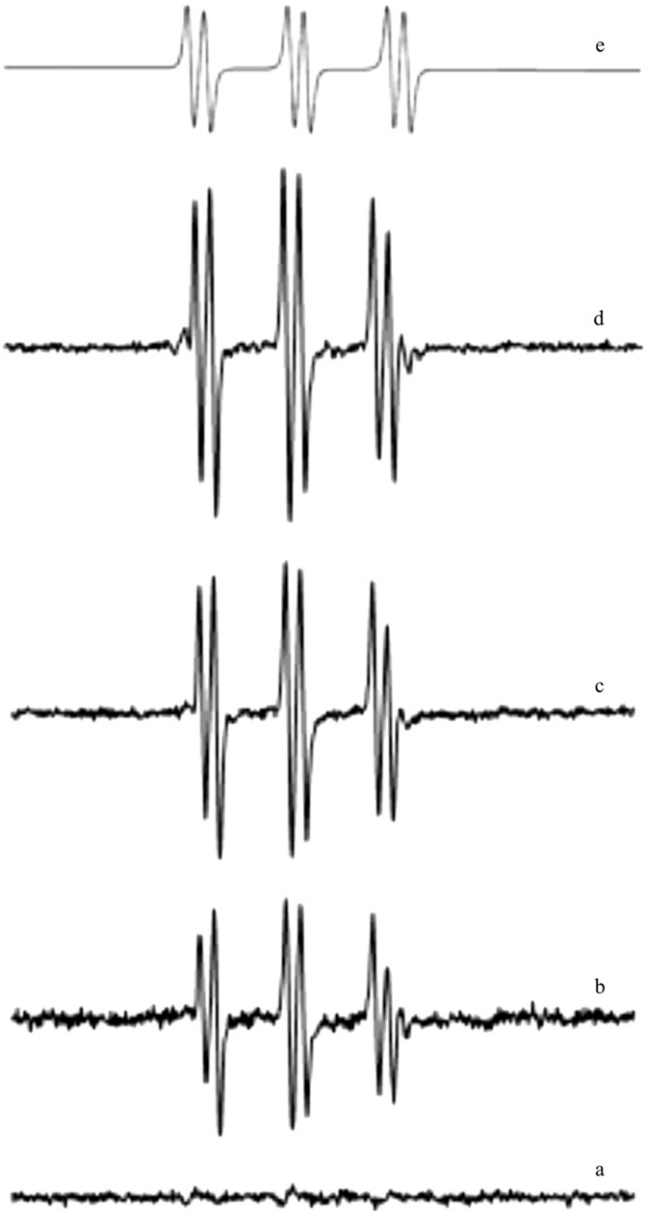

Fig. 6. Lipid radical detection by EPR in digestive gland of *A. atra atra* from Nuevo Gulf in winter of 2011: (a) EPR spectra of dimethylsulfoxide, (b) typical EPR spectra of digestive gland from Storni Dock, (c) typical EPR spectra of digestive gland from Folias Wreck, (d) typical EPR spectra of digestive gland from Punta Cuevas and (e) computer-simulated spectrum employing the following spectral parameters $g=2.005$ and $a_H=1.8$ G, are shown.

Table 7
Pearson correlation coefficients among biochemical, chemical and environmental variables.

	GST	CAT	SOD	ROS	Ascorbyl radicals	Lipid radicals	MT
GST	1.00						
CAT	**0.61**	1.00					
SOD	0.01	0.11	1.00				
ROS	**−0.63**	**−0.74**	−0.47	1.00			
Ascorbyl radicals	0.42	−0.12	**−0.66**	0.25	1.00		
Lipid radicals	0.31	0.09	−0.25	−0.05	**0.65**	1.00	
MT	−0.04	0.26	**0.94**	−0.45	**−0.67**	−0.12	1.00
Fe	−0.53	**−0.85**	−0.39	**0.85**	0.18	−0.15	−0.49
Zn	−0.21	0.02	**0.83**	−0.42	**−0.61**	−0.05	**0.81**
Al	0.04	−0.27	−0.24	0.12	0.10	−0.42	−0.41
Cu	**−0.74**	−0.31	0.36	0.06	−0.57	−0.23	0.34
Cd	0.02	−0.57	0.01	0.43	0.34	0.31	−0.10
Temperature	**0.63**	**0.78**	0.24	**−0.65**	0.09	0.54	0.42
Dissolved oxygen	−0.31	−0.35	0.06	0.12	−0.18	**−0.61**	−0.29
Salinity	**−0.64**	**−0.76**	−0.27	**0.65**	−0.09	−0.55	−0.45
pH	−0.13	−0.21	**−0.82**	0.57	0.51	0.28	**−0.70**
Eh	−0.41	**−0.68**	−0.43	**0.83**	0.30	0.12	−0.40
NO_3+NO_2	**−0.61**	**−0.69**	−0.30	**0.72**	−0.11	−0.56	−0.44
PO_4^{3-}	**−0.67**	**−0.66**	−0.23	**0.74**	−0.22	−0.60	−0.33
SiO_3^{2-}	−0.29	−0.08	**−0.70**	0.39	0.09	−0.32	**−0.68**
Chl. *a*	−0.60	**−0.80**	−0.25	0.58	0.03	−0.40	−0.42

Numbers in bold indicate significant correlations at $p<0.05$.

physiological pool of MT might compensate for seasonal oscillations of metal bioavailability. MT content in mussel tissues is not related to environmental metal concentrations only. Levels of these proteins could exhibit different variations toward biotic and abiotic factors including mussel reproductive state, age and sex, sea temperature and salinity, as well as the season of sampling (Ivanković et al., 2005). The positive correlation found in this study between MT and Zn could be related to the fact that MT may control the homeostasis of essential metals such as Zn, as they represent stocks able to fulfill enzymatic and other metabolic demands (Amiard et al., 2006). Moreover, regulation of MT genes by trace metals appears to be mediated by a zinc-sensitive inhibitor that interacts with a constitutively active transcription factor (Palmiter, 1994).

Metals are potent toxicants which have a wide spectrum of adverse effects. They accumulate in the bodies of organisms and increase formation of ROS which can cause protein, DNA and lipid damage (Regoli et al., 2004), enzyme inhibition, cell signaling impairment, disruption on calcium homeostasis and changes in gene regulation (Stohs and Bagchi, 1995). We found significant positive correlation between ROS and levels of Fe, being higher in winter. Coincidently with our results, an induction of ROS generation by Fe has been previously reported in digestive gland of bivalves *M. galloprovincialis* (Viarengo et al., 1999) and *Mya arenaria* (González et al., 2010).

On the other hand, the antioxidant activities of GST and CAT that were jointly induced in summer showed negative correlation with Cu and Fe, respectively. The simultaneous activation of GST and CAT activities suggests a similar pattern for hydrogen peroxide elimination. The same relationship has been reported for mussel *M. galloprovincialis* by Borković et al. (2005). The significant positive correlation detected between GST and CAT and temperature and negative with salinity, nitrates and the contents of Cu and Fe, respectively, may indicate that oxidative stress responses are more related to environmental factors than to metal exposure. Opposite results were found for ROS, which showed significant relationships with the same environmental parameters but inversely correlated. In this sense, it is known that trace metals are able to promote the intracellular formation of ROS in mussels (Verlecar et al., 2008; Vlahogianni and Valavanidis, 2007).

CAT induction has been regarded as an adaptive behavior to an unsafe environment and an important early indicator of oxidative stress (Cossu et al., 1997). Increased levels of GST, CAT, lipid-derived radicals and MT in ribbed mussels in summer may indicate the coordinated action of these responses to counteract different developmental, seasonal and environmental impacts during life cycle as it has been previously reported by Borković et al. (2005). In fact, MT has been regarded not only as a specific response to metal toxicity but also as an antioxidant defence as an

oxyradical scavenger that may functionally substitute antioxidant enzymes and/or help them to protect cells from oxidative stress (Viarengo et al., 1999).

Our results are in agreement with those reported by Viarengo et al. (1991) and Regoli (1998) for *M. galloprovincialis*, in which a general reduction of the antioxidant defence was found during the winter as a probable consequence of a change in the metabolic status of the animals. There is known to be a close functional relationship between the digestive system and gonad development and periods of food abundance (Mackie, 1984). Seasonal temperature variation is also thought to strongly influence the relationship between the digestive system and gonad development (Brey, 1995). In Northern Patagonian gulfs, the ribbed mussel reproduces from September to December, which coincides with the increased temperature seawater from the minimum in winter (8 °C). Spawning in January–February is followed by a resting sexual phase, when mantle is completely invaded by cellular tissue. The decrease in antioxidant defence level in digestive gland of *A. atra atra* in winter is probably due to factors such as the progressive infiltration of the organ by gonadic tissues during gametogenesis, previously reported by Niyogi et al. (2001) for the oyster *Saccostrea cucullata*.

Increased enzymatic activities during the reproductive cycle could be related to a higher metabolic activity of the organisms during the warmer spawning season and/or to the necessity during these phases of more efficient protection against peroxidative damages. In winter we found increased ROS generation coincidentally with Viarengo et al. (1991), who found enhanced peroxidative processes on *M. galloprovincialis*, when antioxidant enzymes activities were lower. Comparing seasonal variations of trace metals in digestive gland with those of antioxidant enzymes, an opposite trend was obtained with generally higher enzymatic activities in summer, when trace metal concentrations were the lowest. The same result was found by Regoli (1998) with mussel *M. galloprovincialis.*

SOD activity and ascorbyl radicals were the only oxidative stress parameters seasonally independent, that were inversely correlated and allowed a geographical differentiation of mussels. The organisms from Storni Dock were characterized by increased activity of SOD accompanied with low ascorbyl radical generation. These results partially matched those reported for limpet *Nacella magellanica* by Malanga et al. (2007), where SOD was seasonally independent but ascorbyl radicals increased with temperature. However, opposite results with higher activity of SOD in winter have been reported for *M. edulis chilensis* (Giarratano et al., 2011), *M. galloprovincialis* (Santovito et al., 2005) and *Perna viridis* (Verlecar et al., 2008). Great variability in the response of several antioxidant parameters in populations of aquatic organisms from polluted and unpolluted areas has been observed in other field studies. This fact makes difficult to draw accurate conclusions about how each parameter is affected by intrinsic and exogenous factors and which one is the most effective to explain the observed changes.

Except for Al that was highest in sediment from Punta Cuevas, the highest trace metals concentrations and biochemical responses were found at Storni Dock and Folías Wreck. There is for the former place one report of relatively high levels of hydrocarbons in sediment and in ribbed mussel *A. atra atra* (Massara Paletto et al., 2008). This factor is likely to modulate the responses measured at Storni Dock. In summary, our results show that the responses of the studied biomarkers are related to the accumulation of some trace metals and environmental factors. However, these responses may be related to the exposure to other pollutants, with synergistic or antagonistic effects, as well as the influence of other biological and environmental parameters that have not been considered in this study.

5. Conclusion

Overall results indicated a significant influence of seasonal variability on several biomarkers, being superoxide dismutase activity and ascorbyl radical production the only parameters seasonally independent. Except for lipid radicals, all biomarkers showed correlation with some essential metals Fe, Zn and Cu, but not with nonessential ones such as Cd. Folías Wreck was the most enriched site by Fe in sediment, as well as, in mussels. Further researches are necessary to determine the higher bioavailability of Al for mussels from Punta Cuevas. This work emphasizes the importance of setting how the seasonality affect the antioxidant status of ribbed mussel *A. atra atra* in order to discriminate the seasonal factor from the interpretation of biomonitoring data.

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