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Biomarkers of environmental stress in gills of ribbed mussel *Aulacomya atra atra* (Nuevo Gulf, Northern Patagonia)

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ABSTRACT

In this study, we assessed in gills of native ribbed mussels *Aulacomya atra atra* from three sites within Nuevo Gulf (Northern Patagonia) several biomarkers such as reactive oxygen species (ROS), lipid radicals (LR), malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione *S*-transferase (GST) and metallothionein (MT). Furthermore, concentrations of main trace metals (Fe, Al, Zn, Cu, Cd and Pb) were quantified in mussel tissue. Results showed significant induction of SOD, GST, MT and MDA, as well as, higher concentration of Fe, Al and Cd in winter than in summer. The high MDA content measured in mussels from Folías Wreck seemed to be caused by the very high levels of Fe that would come from the corrosion of the vessel. Mussels from the control site Punta Cuevas presented the lowest levels of Cd and the highest of Al in winter. Despite positive correlations were found between Al and GST and MT, no spatial differentiation was detected in those biomarkers. On the other hand, MT was only related to Al been most likely influenced by environmental variables than by the trace metals. It has to be highlighted that the relationship detected among water temperature, nutrients and antioxidant responses in gills is probably related to the fact that this tissue is in direct contact with water and it is sensitive to its fluctuations. Taking into account that mussel gill is a tissue actively proliferating and the first target of contaminants present in water, so that changes in its antioxidant system can provide an earlier warning signal than in other tissues.

1. Introduction

The recognition that free radical reactions are important both in normal biological processes, as well as in toxicity mechanisms induced by contaminants, has lead to a considerable increase in the application of oxidative stress biomarkers in several aquatic organisms (Livingstone, 2001). Reactive oxygen species (ROS) can be produced in organisms by exposure to contaminants causing numerous deleterious effects to cells, including lipid peroxidation, inactivation of enzymes, protein degradation and oxidation of DNA bases (Hermes-Lima, 2005; Manduzio et al., 2005; Valko et al., 2005). To prevent these injuries, enzymatic and non-enzymatic antioxidant systems are triggered to eliminate contaminant stimulated ROS, allowing the organism to overcome oxidative stress in polluted environments (Livingstone, 2001). Oxidative stress occurs in organisms when the rate of ROS production exceeds the rate of its decomposition by antioxidant systems, leading to an increase in oxidative damage to different cellular targets (Sies, 1993).

To protect against ROS, cells possess specific antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione-*S*-transferase (GST), among others (Halliwell and Gutteridge, 2006; Mohammed, 2014). The level of lipid peroxidation, which leads to the formation of secondary products such as MDA, has been largely measured as indicator of injury caused by ROS in different marine organisms (Almeida et al., 2005). Oxidative stress can be reduced through the complexation of free metals by MT. MT has a primary role in the homeostasis of essential metals and the detoxification of non-essential ones (Amiard et al., 2006), besides an antioxidant role as oxyradical scavengers (Gagné et al., 2008).

As free radicals cannot be directly detected due to their short life-times, a specific compound (spin-trap) is introduced into the system forming stabile paramagnetic species (spin adducts) with specific signature electron paramagnetic resonance (EPR) spectrum (Spasojević et al., 2011). Spin trapping-EPR analysis overcomes the limit of sensitivity of endogenous radical content in biological systems, and it has been proved to be the best method available to detect short-lived reactive free radicals generated in low concentrations in biological systems (Luo et al., 2006). Even though EPR detection of lipid radicals (LR) could be considered a fingerprint of radical presence, spin trapping studies cannot really distinguish among peroxyl (ROO●), alcohoxyl (RO●) and alkyl (R●)

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adducts owing to the similarity of the corresponding coupling constants (Jurkiewicz and Buettner, 1994).

Among the biomarkers used in environmental monitoring, those of oxidative stress measured by means of antioxidant responses is a promising biological-effect response (ICES, 2007), useful for assessing exposure and effects of pollutants in mussels providing the earliest warning signals (Box et al., 2007; Fernández et al., 2012; Rola et al., 2012). Nevertheless, oxidative stress biomarkers are also related to changes in physico-chemical and environmental factors such as temperature, salinity, food availability and dissolved oxygen levels (Lesser, 2006), as well as to intrinsic biological cycles such as gonadal development or the reproductive cycle (Alonso-Alvarez et al., 2004; Costantini, 2010). As these changes may influence normal metabolic activities, including antioxidant responses and ROS generation (Sheehan and Power, 1999), abiotic and biotic factors should be incorporated into the interpretation of biomarkers of oxidative stress.

Mussels have been employed as sentinel organisms in worldwide routine biomonitoring programs on the basis of their wide geographic distribution, their straightforward availability in the field and through aquaculture, and their suitability for caging experiments along coast lines (Viarengo et al., 2007). The study organism *Aulacomya atra atra* commonly known as ribbed mussel is a bivalve belonging to the family Mytilidae. It is a filter feeder with the ability to accumulate a wide range of contaminants. *A atra atra* is a typical species of the rocky shores along the Patagonian coast from Argentina forming extensive beds (Zaixso, 1999). It is an important habitat-forming species for numerous species of algae and animals and it is also a food source for gastropods, asteroids and sea birds as well as for humans (Zaixso, 2003).

Our objective in this paper was to evaluate a set of biomarkers in the gills of the ribbed mussel *Aulacomya atra atra* and to assess their spatial and seasonal variability. This kind of study is geographically and temporally limited but provides valuable information to evaluate the potential of biomarkers for environment quality assessment.

2. Materials and methods

Three sites within Nuevo Gulf were selected and are shown in Fig. 1. Storni Dock (S 42°44′10.5″ W 65°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 (S 42°47′24″ W 64°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 (S 42°46′18.5″ W 64°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 (Giarratano et al., 2013; Gil, 2001; Massara Paletto et al., 2008).

Data of physico-chemical parameters of seawater and concentration of labile trace metals in sediments from these sites have been previously published by Giarratano et al. (2013). Nevertheless, they are reproduced in this work in Supplementary Table 1 to facilitate comprehension of present results.

2.1. Animal collection

In August 2011 (winter) and February 2012 (summer), 50 individuals of *Aulacomya atra atra* were collected by scuba diving at each sampling site. Following collection, mussels were placed in isolated plastic containers previously filled with water from the sampling site and transported to the laboratory within 2 h of collection. No differentiation was made regarding sex. It was assumed a male/female ratio close to 1 according to Pérez et al., (2013). Gills were removed and stored at −80 °C until biochemical analysis.

2.1.1. Trace metals in organisms

Concentrations of Fe, Al, Zn, Cu, Pb and Cd were assessed in 3 pools of 10 gills of *A. atra atra* per site. Samples were dried at 60 °C to constant weight. 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. After the sample was cooled down; 2 ml of concentrated nitric acid (HNO_3) were added and evaporated to dryness on a sand bath at 80 °C. This procedure was repeated until white ashes were obtained and then were resuspended with a mixture of HNO_3 (3 percent v/v) and hydrochloric acid (HCl) (6 percent v/v) up to 10 ml (Boletín Oficial del Estado, 1991). Trace metals in samples and two blanks prepared as samples were assessed by an IL-457 atomic absorption spectrophotometer with air-acetylene flame, except for Al that was measured using a nitrous oxide-acetylene flame. Results are expressed in μg/g 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 percent and 6.6 percent. The accuracy for all metals, expressed as percentage of recovery, was between 89 percent and 102 percent. Detection limits were 1 (Fe), 10 (Al), 0.45 (Zn), 0.80 (Cu), 2 (Pb) and 0.10 (Cu) on a dry weight basis (μg/g dw).

2.1.2. Biochemical analyses

Production of ROS by gills ($n=6$) was evaluated after homogenization in 1:5 (w/v) 100 mM tris (hydroxymethyl)-aminomethane–HCl buffer pH 7.75, with 2 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM magnesium chloride ($MgCl_2$) (Gallagher et al., 1992). Measurements were conducted according to Viarengo et al. (1999) with modifications. 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 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) buffer pH 7.2, with 200 mM potassium chloride (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 was oxidized by ROS to the fluorescent compound, which is detected in wavelengths of 488 and 525 nm for excitation and emission, respectively. Results were expressed as arbitrary units per minute per mg of wet tissue.

Levels of lipid peroxidation products in gills were analyzed as MDA content according to Shaw et al. (2004). Levels of MDA were estimated by derivatization with 1-metyl-2-phenylindole and calibrated against a MDA standard curve. Samples were homogenized in 1:3 (w/v) in Tris–HCl buffer 20 mM pH 7.4 and centrifuged at 3000 × *g* for 20 min. Tissue MDA levels were derivatized in a 1 ml reaction mixture containing a final concentration of 10.3 mM 1-metyl-2-phenylindole (dissolved in acetonitrile/methanol 3:1), HCl 32 percent, 100 μl water or sample or standard (standard range 0–6 μM 1,1,3,3-tetramethoxypropane in Tris–HCl buffer 20 mM, pH 7.4). The tubes were vortexed and incubated at 45 °C for 40 min. Samples were cooled on ice, centrifuged at 15,000 × *g* for 10 min and read spectrophotometrically at 586 nm. Results were expressed as MDA nmol/g wet tissue.

Metallothioneins (MT) were analyzed in gills 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 phenylmethylsulphonyl fluoride (PMSF) and 0.01 percent β-mercaptoethanol. After acidic ethanol/chloroform fractionation of the tissue homogenate, MT was quantified by the spectrophotometric assay as described in Viarengo et al. (1997) using reduced glutathione (GSH) as standard to build a calibration curve. Results were expressed in terms of sulfhydryl groups (nmol-SH/g of wet tissue).

Upon return to the laboratory, gills ($n=6$) were removed and stored at −80 °C until enzymatic analysis of SOD, CAT and GST. 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) and supernatant were taken for enzymatic determinations. SOD activity was assayed by the epinephrine method (Misra and Fridovich, 1972), based on the capacity of SOD to inhibit the autooxidation of adrenaline to adrenochrome at 480 nm. One unit of SOD was defined as the amount of enzyme causing 50 percent inhibition of the autoxidation of adrenaline at 30 °C. CAT activity was quantified following the decrease in absorbance at 240 vnm due to consumption of hydrogen peroxide (H_2O_2) 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 min. GST activity was determined by increasing in absorbance at 340 nm, incubating GSH with 1-chloro-2,4-dinithrobenzene as substrate at 25 °C (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 standard.

Lipid-derived radical content (LR) was measured on homogenates from gills 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 2×10^5; 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 piperidin 1-oxyl (TEMPO), introduced into the same cell used for spin trapping. EPR spectra for both sample and TEMPO solutions were recorded at exactly the same spectrometer settings and the first-derivative EPR

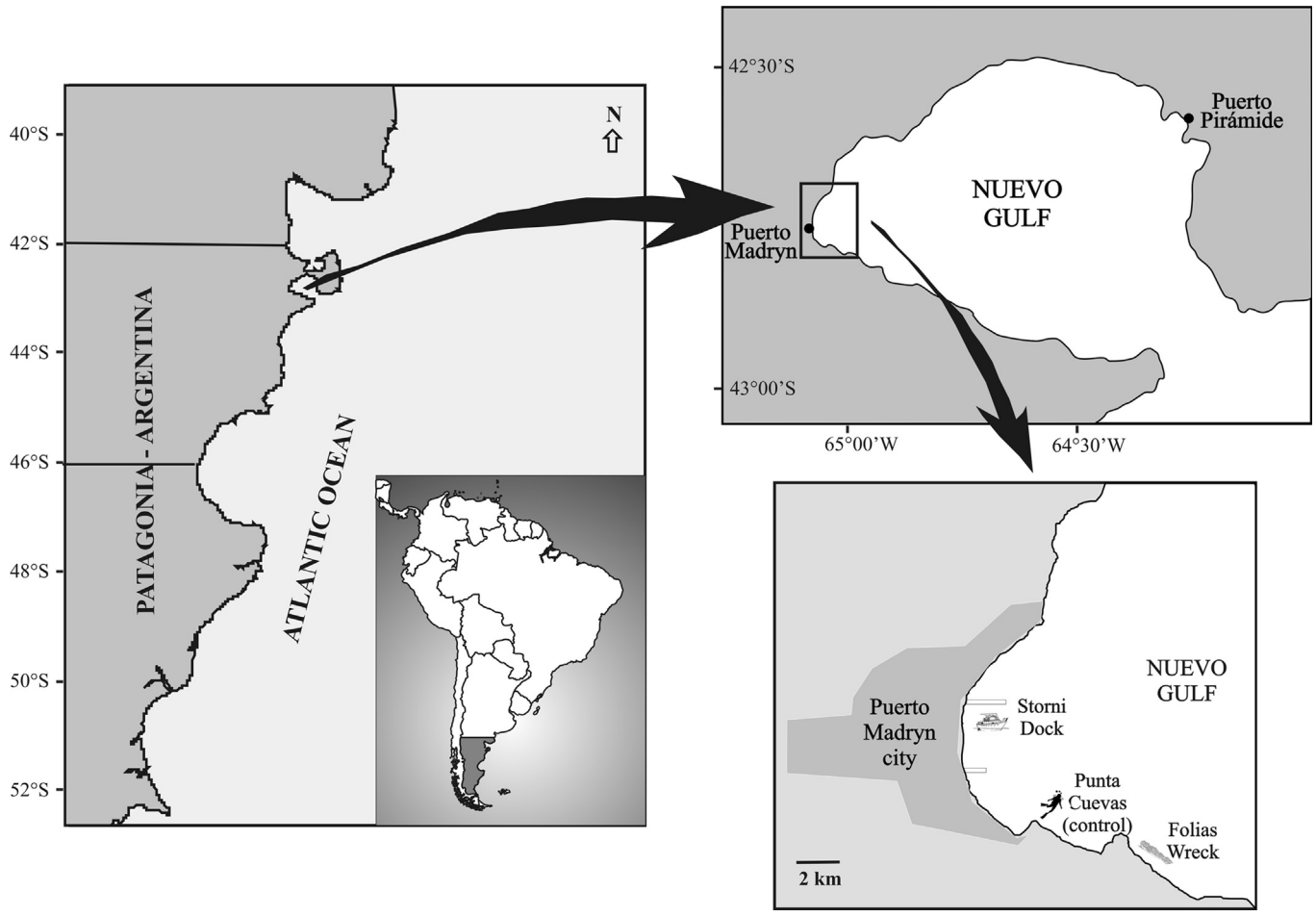

Fig. 1. Map of the study area.

spectra were integrated to obtain the area intensity, then the concentration of spin adduct was calculated according to Kotake et al. (1996) and expressed per mg of wet tissue.

2.2. Statistical analyses

Statistical analysis was carried out using a statistical package (STATISTICA 7.0). Results of all parameters were reported as mean ± standard deviation. The variation of each parameter among sites and between seasons was tested by two-way ANOVA ($p < 0.05$). Previously we tested the pre-requisites for analysis of variance (normality and homogeneity of variances). When significant differences were found, Tukey's test was applied to determine which values differed significantly. The existence and strength of relationships between parameters were determined by parametric Pearson correlation analysis using all data pooled.

3. Results

Concentrations of trace metals measured in gills of ribbed mussel *A. atra atra* for each site are presented in Fig. 2 (statistical results in Table 1). Fe was the metal detected with the highest concentrations. Mussels from Folías Wreck showed a mean of Fe in winter of 3852 ± 80 μg/g and in summer of 1091 ± 168 μg/g. Concentrations of Zn followed those of Fe in order of magnitude, but no significant differences were found among sites or between seasons. Al showed seasonal and spatial variations with higher concentrations in winter in Punta Cuevas (72.6 ± 3.1 μg/g) than in Folías Wreck (47.9 ± 6.0 μg/g). Cu registered the lowest values in mussels from Punta Cuevas, with an average concentration for both seasons of 11.7 ± 1.9 μg/g. Pb was below detection limit in all cases (< 2 μg/g). Cd showed the lowest concentration also in mussels from Punta Cuevas (2.24 ± 0.41 μg/g) and the average winter concentration (4.7 ± 1.7 μg/g) was higher than the average in summer (2.5 ± 0.6 μg/g). As summary, metals were found in the following decreasing order: Fe > Zn > Al > Cu > Cd > Pb.

ROS production, MDA level and MT content are presented in Fig. 3A, B and C, respectively. ROS production was the least in mussels from Storni Dock. As no differences were found between seasons, the mean value of ROS recorded for this site was 0.37 ± 0.16 a.u./min/mg wet tissue. MDA levels were significantly higher in mussels from Folías Wreck than in those from Storni Dock. This parameter showed seasonal variation, being the average of winter (13.86 ± 0.78 nmol MDA/g wet tissue) higher than that of summer (12.95 ± 0.44 nmol MDA/g wet tissue). MT concentration in gills of organisms did not vary among sampling sites, but they did between seasons being higher in winter (21.23 ± 3.33 nmol-SH/g wet tissue) than in summer (14.15 ± 3.19 nmol-SH/g wet tissue).

Mean values of antioxidant enzymes SOD, CAT and GST are displayed in Fig. 3D, E and F, respectively. Significantly higher activity of SOD was detected in ribbed mussel from Storni Dock than in those from Folías Wreck and Punta Cuevas. Moreover, SOD was higher in winter than in summer. CAT and GST activities were not significantly different among sites. Regarding seasonal variation, GST showed significant higher activities in winter than in summer.

As a measurement of oxidative stress in the lipid phase, LR content in gills of *A. atra atra* was assessed by EPR showing slight differences. No significant dependence on sites or seasons was found (Fig. 4A). LR in the samples combined with the spin trap PBN resulted in adducts that gave a characteristic EPR spectrum, in concordance with computer spectral simulated signals (Fig. 4B).

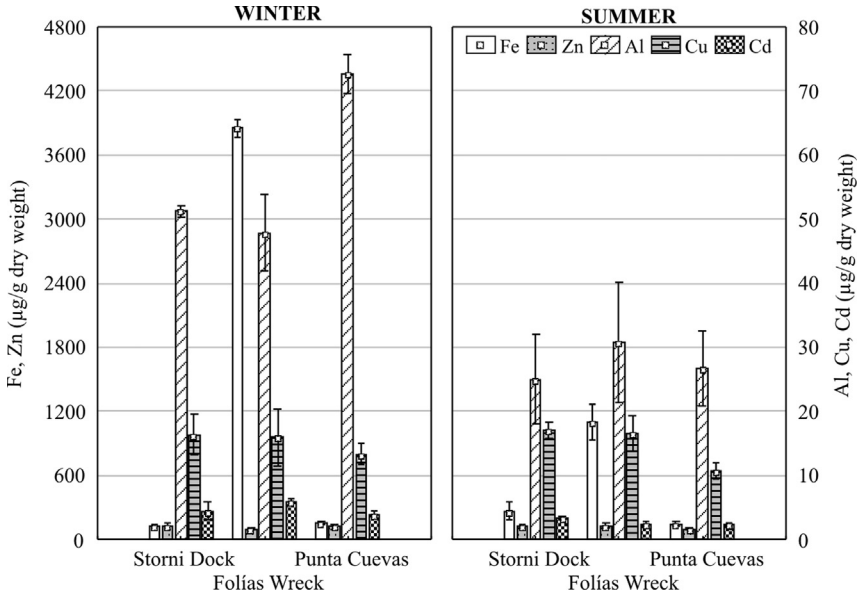

Fig. 2. Concentration of trace metals (µg/g dry weight) in gills of *A. atra atra* ($n=3$). Fe and Zn are represented on left axis; Al, Cu and Cd on right axis.

Table 1
Summary of the two-way ANOVA results for trace metal contents in gills of *A. atra atra*.

Parameter	Site	Season	Site × season
Fe	<0.0001, FW > others	<0.0001, W > S	<0.0001
Al	<0.05, PC > SD	<0.0001, W > S	<0.001
Zn	n.s.	n.s.	n.s.
Cu	<0.05, PC < others	n.s.	n.s.
Pb	n.s.	n.s.	n.s.
Cd	<0.01, PC < others	<0.005, W > S	<0.05

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

A Pearson correlation analysis was run to find possible associations between biochemical, chemical and environmental variables (Table 2). LR was positively correlated with MDA, GST and ROS. Positive correlations were also found between GST and ROS and between MDA and MT. With regard to the associations between biomarkers and metals were all positive as follow: GST and MT with Al, MDA with Fe and Cd and, SOD with Cu. MT and MDA showed positive correlations with most of the environmental variables, being only negative with temperature. SOD and GST showed similar correlations: positive with salinity and chlorophyll *a* and, negative with temperature. Compared to SOD and GST, CAT showed opposite relationships with the previously mentioned environmental variables. LR was positively associated with redox potential and phosphates. ROS was the unique biomarker not showing significant correlations with environmental parameters.

4. Discussion

Antioxidant enzymes are found in all tissues of bivalves, but are present in highest activities in the digestive gland, which is also considered the major site of xenobiotic uptake and oxyradical generating biotransformation enzymes and the most sensitive tissue of xenobiotic exposure (Ramos-Gómez et al., 2011). Gills represent a natural pathway for metals dissolved in seawater (Bustamante et al., 2002). The absence of protective structures in the gill epithelium accounts for the strong oxidative load experienced by this tissue during interaction with toxic compounds present in the marine environment (Soldatov et al., 2007). This fact makes gill a more susceptible tissue to changes in environmental conditions and its responses earlier signals of warning than those of digestive gland. Gill tissue reflects aquatic environment conditions the most adequately. Changes in the activity of antioxidant enzymes in gills weakly depend on the state of the mollusk. The use of the antioxidant complex of the digestive gland for diagnosing is less reasonable, because its state may be determined not only by the environment but also by certain internal factors (e.g., nutrition, spawning).

Mussels from Punta Cuevas (considered *a priori* as clean site) showed the lowest levels of Cd and Cu. However, these organisms presented the highest concentrations of Al in winter, coincidentally with the results found in digestive gland by Giarratano et al. (2013). Al is the third most abundant element on Earth and it is normally combined with oxygen and silica as aluminium silicates. In Punta Cuevas, the highest concentrations of inorganic silicates in seawater were found in both seasons. Hydes (1977) suggested that suspended particles in seawater are the main carriers of Al and its solubility depends on biological activity and vertical mixing of water. Despite this possible explanation for the higher bioavailability of Al in Punta Cuevas than in other sites, further studies are needed to elucidate the origin of this metal.

Trace metals found in gills of *A. atra atra* in this study were lower than the values found in digestive gland of the same ribbed mussel samples by Giarratano et al. (2013), probably due to the different physiological roles of these organs. The same tissular distribution of metals has been reported for different species of bivalves (Bustamante and Miramand, 2004; Géret et al., 2002; Husmann et al., 2012). Higher concentrations of trace metals in the digestive gland than in the gills suggest that food particles would be a major pathway of metal uptake (Bustamante and Miramand, 2005). Fe measured in gills from Folías Wreck was the only exception to this tissue distribution. Concentrations of Fe registered in gills in winter was four times higher than in the same period in digestive gland; while in summer the value measured in gills was two times higher than in digestive gland. Possibly there is a release of Fe from the corrosion of the wreck, which dissolves in water and becomes available for biota.

The higher concentration of Fe in winter than in summer was also found for Al and Cd, coinciding with the pattern found in digestive gland (Giarratano et al., 2013). Other authors have reported the same seasonal behavior for other species of mussels

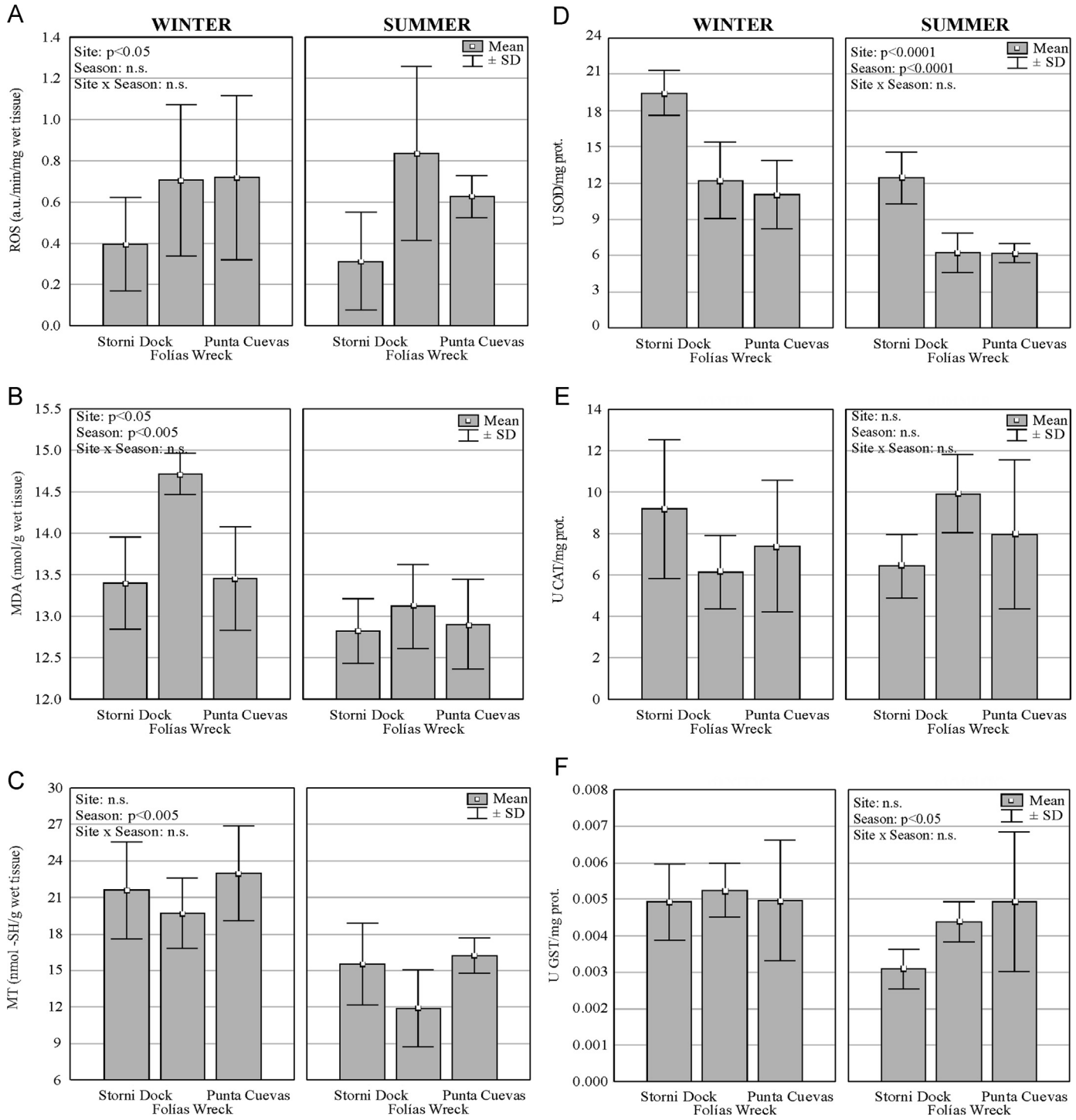

Fig. 3. (A) Reactive oxygen species production, (B) Malondialdehyde level, (C) Metallothionein content, (D) Superoxide dismutase activity, (E) Catalase activity and (F) glutathione *S*-transferase activity in gills of *A. atra atra* from Nuevo Gulf. The statistical significance was analyzed by two-way ANOVA test with site and season as factors. n.s.=not significant.

(Duarte et al., 2012; Giarratano et al., 2011; Odzak, 2002), oysters (Frías-Espericueta et al., 1999; Riba et al., 2005) and scallops (Bustamante and Miramand, 2005). Several biological and geochemical factors can cause seasonal variations in contaminant levels in mussels. Size, sex, reproductive season of individuals, water temperature, pH and salinity, among others, are factors which affect accumulation in mussels (Bryan, 1973; Cossa et al., 1980; La Touche and Mix, 1982; Lobel et al., 1991; Regoli & Orlando, 1994).

The purpose of this study was to test a battery of biochemical parameters usually associated to metal exposure and to analyze their correlation with metal accumulation in gills, as well as, with environmental variables. SOD and CAT, as the first lines of antioxidant defenses, are very responsive to increasing levels of ROS production stimulated by contaminants (Lima et al., 2007). In the present work, significant higher SOD activities were determined in mussel gills from Storni Dock which seemed to prevent MDA formation. Significantly higher MDA levels were found in gills of

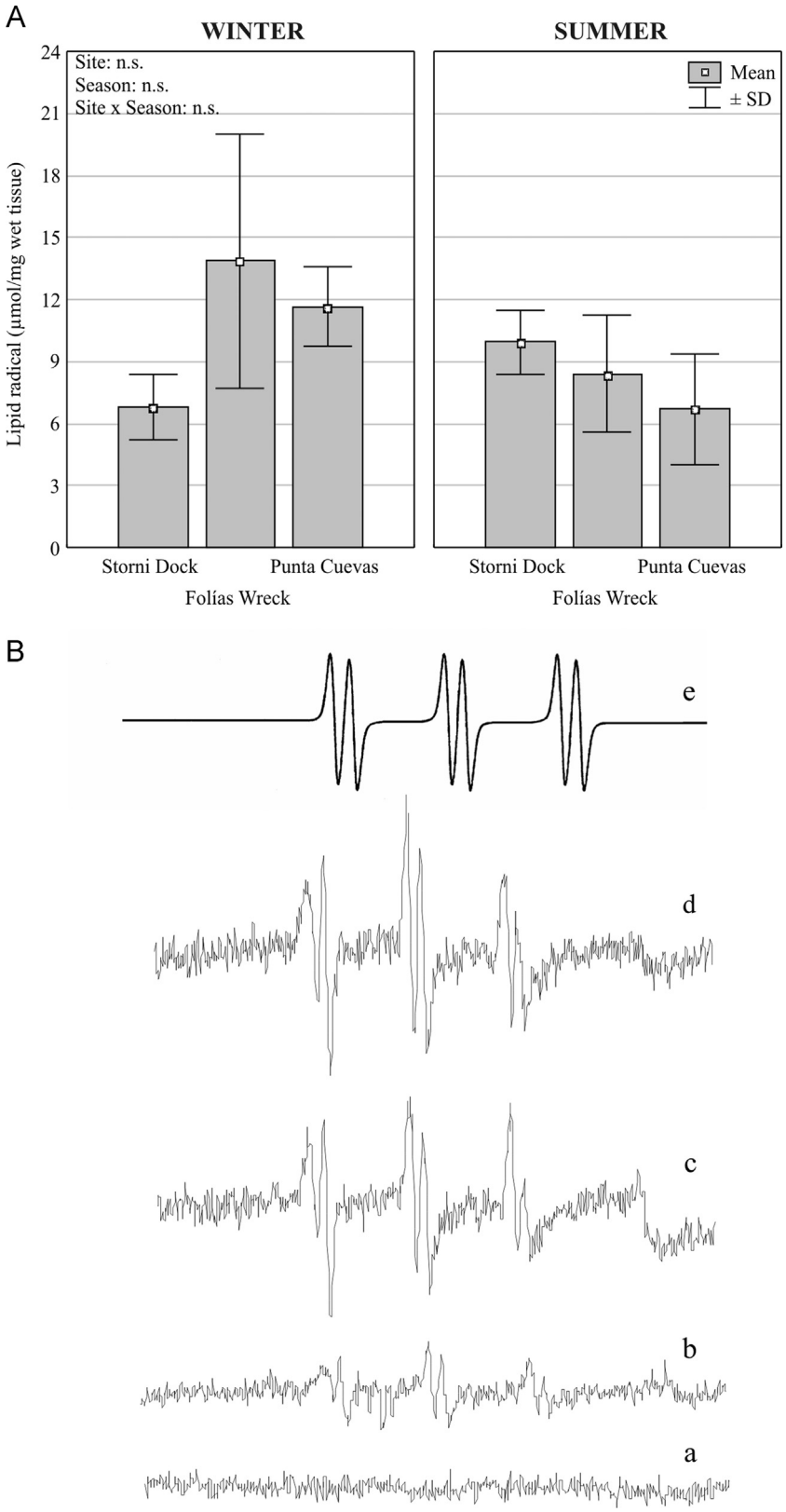

Fig. 4. (A) Lipid radical content in gills of *A. atra atra* from Nuevo Gulf. The statistical significance was analyzed by two-way ANOVA test with site and season as factors. n.s.= not significant. (B) EPR detection of lipid radical in gills of *A. atra atra* from Nuevo Gulf in winter of 2011. (a) EPR spectra of dimethylsulfoxide, (b) typical EPR spectra from Storni Dock, (c) typical EPR spectra from Folias Wreck, (d) typical EPR spectra from Punta Cuevas and (e) computer-simulated spectrum employing the following spectral parameters $g=2.005$ and $a_H=1.8$ G, are shown.

mussels collected at Folías Wreck, where the highest levels of ROS were measured. Folías Wreck, the most concentrated site with Fe in sediment, was also characterized by the highest levels of Fe in gills in winter. It is well established that Fe is an essential element implicated in many living processes. However, because it undergoes redox cycle it is involved also in initiation and propagation of

Table 2
Pearson correlation coefficients among biochemical, chemical and environmental variables.

Parameter	ROS	LR	MDA	SOD	CAT	GST	MT
ROS	1.00						
LR	**0.62**	1.00					
MDA	0.13	**0.58**	1.00				
SOD	−0.19	0.38	0.27	1.00			
CAT	0.11	−0.20	−0.45	−0.49	1.00		
GST	**0.56**	**0.63**	0.36	0.39	−0.24	1.00	
MT	−0.11	0.39	**0.61**	0.40	−0.49	0.30	1.00
Fe	0.27	0.51	**0.81**	0.13	−0.18	0.42	0.19
Zn	−0.27	−0.24	−0.27	0.34	−0.21	−0.16	−0.38
Al	0.29	0.42	0.46	0.43	−0.50	**0.59**	**0.69**
Cu	0.17	0.45	0.07	**0.61**	−0.11	0.36	−0.07
Cd	0.04	0.47	**0.88**	0.32	−0.34	0.27	0.27
Temperature	−0.24	−0.51	**−0.74**	**−0.58**	**0.66**	**−0.64**	**−0.71**
Dissolved oxygen	−0.13	−0.09	0.15	0.54	−0.25	0.32	0.12
Salinity	0.23	0.51	**0.75**	**0.56**	**−0.66**	**0.64**	**0.73**
pH	0.45	0.43	**0.63**	−0.22	−0.14	0.43	0.54
Eh	0.45	**0.66**	**0.79**	0.16	−0.44	0.54	**0.59**
NO_3+NO_2	0.31	0.54	**0.69**	0.39	**−0.62**	**0.57**	**0.78**
PO_3^4	0.30	**0.59**	**0.72**	0.40	**−0.61**	0.55	**0.75**
SiO_2^3	0.34	0.26	0.44	−0.14	−0.21	0.31	**0.59**
Chl. *a*	0.13	0.43	**0.76**	**0.57**	**−0.68**	**0.58**	**0.69**

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

free radical processes (Lushchak, 2011). Antioxidant enzymes were not able to compete with the high presence of Fe in Folías Wreck, where the increased concentration of MDA in mussel gills could be the result of the generation of HO● radicals induced by Fe via the Fenton reaction.

The significant positive correlation between ROS and LR, between LR and MDA as product of lipid peroxidation, and LR and MDA with GST suggest subsequent oxidative damage. Production of ROS gives rise to primary stage of lipid hydroperoxide formation (Girotti, 1998), resulting in increase markers of lipid peroxidation such as MDA in target tissues. Using electron paramagnetic resonance analysis, Abele and Puntarulo (2004) also detected higher lipid radical content in the polar clam *L. elliptica* and related the higher lipid radical formation rates to an elevated content of Fe in tissues. Fe initiates formation of highly toxic radicals from H_2O_2 via Fenton-type reactions and, moreover, exacerbates lipid peroxidation (Puntarulo and Cederbaum, 1988). Additionally, a positive correlation was also found between Fe and GST. This enzyme participates in the detoxification of lipid hydroperoxides using glutathione (GSH) and consequently, reducing the cellular pool of GSH (Winston and Di Giulio, 1991, van der Oost et al., 2003). The increase in GST activity probably was a strategy to prepare for oxidative stress in an effort to defend cells against oxidative damage, but it was not enough to prevent it.

In aquatic invertebrates, MT play an important role in many physiological processes including homeostasis, protection against trace metals and oxidant damage, metabolic regulation, sequestration and/or redox control (Mao et al., 2012). Although several studies with bivalves have demonstrated positive correlations between MT measured in gills and metal content (Géret et al., 2002; Khati et al., 2012; Trombini et al., 2010), our results showed no relationship between MT and metal concentrations, except for Al. This lack of correlation has been previously reported for *Mytilus galloprovincialis* (Fernández et al., 2010) and also for mussel *Mytilus edulis* (Geffard et al., 2005). Few researchers have found an induction of MT by Al (Jeffery et al., 1987; Santiago-Rivas et al., 2007; Zhang et al., 1998). MT readily associates with metal ions with low charge states such as Cd. They are not expected to associate as readily with 'hard' metal ions such as Al, which has high charge states (Desouky, 2012). In that sense, Desouky (2012) did not found an induction of MT by Al in two species of freshwater molluscs.

In addition, the significant correlation detected between MT and salinity, temperature and inorganic nutrients may indicate that its response is more related to these environmental factors than to pollutant exposure. In agreement with these results, Fernández et al. (2010) reported a positive correlation between MT and salinity. The seasonal influence on the MT and metal concentrations (higher values in winter than in summer) was pronounced, while site-specific influence was not detected. This seasonality is in agreement with the findings reported for mussel *M. galloprovincialis* (Ivanković et al., 2005; Pytharopoulou et al., 2006). The absence of differences between areas could be due to the low contamination gradient among studied sites.

SOD, GST, MT and MDA showed elevated levels in winter. The same seasonal tendency was found for different species of bivalves for GST (Borković et al., 2005; Manduzio et al., 2004; Power and Sheehan, 1996) and also for SOD (Borković et al., 2005). It has to be taken into account that the relationship detected between antioxidant responses in gills and water temperature is probably related to the fact that gill epithelium is in direct contact with water, and hence highly sensitive to its fluctuations (Fernández et al., 2010). The increase in temperature stimulates all metabolic processes in according with known thermodynamic principles (Lushchak, 2011). But under some circumstances the decrease in environmental temperature also may cause oxidative stress, such as those reported in zebrafish *Danio rerio* (Malek et al., 2004) and barnacle *Balanus balanoides* (Niyogi et al., 2001). Lushchak (2011) proposed two possible explanations: (i) temperature decrease weakens the systems of ROS elimination, and/or (ii) enhances ROS production. Unfortunately, there is no information on the mechanisms involved.

Ribbed mussels were subjected to different conditions of water temperature, salinity and nutrient supply at each season. Except for CAT that showed an opposite trend, most of biomarkers were positively correlated with inorganic nutrients and chlorophyll *a*. In this respect, oxidative changes have been reported as typical responses in bivalve molluscs during the periods of more intense feeding activities as a consequence of both higher oxidative metabolism and/or assumption of antioxidants by the diet (Regoli et al., 2002).

Comparing present results in gills with those found in digestive gland (Giarratano et al., 2013), SOD activity was higher in gills while CAT, GST, ROS and MT were lower. The higher SOD activity in gill than in digestive gland may be associated to the higher tissue exposure to oxygen due to the respiratory function (Box et al., 2009). CAT and SOD usually function together (Xu and Kuppusamy, 2005). CAT converts hydrogen peroxide, product of neutralization of anion superoxide by SOD, into water and molecular oxygen. In our study, we did not found a joint activation of SOD and CAT. It is possible that peroxidase, which is involved in similar processes than CAT, had neutralized hydrogen peroxide. Unlike some peroxidases that can reduce various lipid peroxides, CAT can only reduce hydrogen peroxide (Filho, 1996). GST are enzymes from a family of multi-functional proteins involved in the cellular detoxification of xenobiotic compounds that play a fundamental role in protection against endogenous and exogenous toxic chemicals (Sheehan et al., 2001). Previous studies found higher activity of this enzyme in gills than in digestive glands in mussels *M. galloprovincialis* (Bebianno et al., 2007, Fernández et al., 2010) and in *M. edulis* (Fitzpatrick and Sheehan, 1993). The activity of GST found in present study in gills was one order of magnitude lower than the activities found in digestive gland (Giarratano et al., 2013). The GST enzyme has been suggested as a useful index for conjugating activities and exposure to organic pollution in mussels (Fitzpatrick and Sheehan, 1993). Organic contaminants have not been considered in this work. Despite metals

are not natural substrata for this enzyme, a positive correlation was found between GST and Al. Other studies have also reported this kind of correlation for *M. galloprovincialis* (Fernández et al., 2010; Vidal-Liñán et al., 2010). The high GST activity in digestive gland compared to gills could be a response to the oxidative stress produced by the highest recorded metal exposure in winter in digestive gland. Besides, digestive gland is the main tissue involved in most biotransformation processes and redox-cycling generation. Based on the assumption that MT is induced by metals, it would be expected that tissues such as digestive gland with the highest accumulated metal concentrations should have the highest MT concentrations (Amiard et al., 2006).

5. Conclusions

Gills are located in the ventilated mantle cavity of molluscs and directly interact with the marine environment (Soldatov et al., 2007). Antioxidant responses measured in this tissue reflect the state of the aquatic environment more quickly than other tissues, since they do not depend on the physiological status of molluscs as do in digestive gland. Digestive gland is the main site of contaminant storage and oxyradical generation and its antioxidant system is affected not only by the environment but also by certain internal factors reflecting a pattern of seasonal variation as well as a long-term exposure. Keeping in mind the advantages and limitations for each tissue, biomarkers are valuable tools in pollution monitoring, especially when they are used together with bioaccumulation data of resident benthic macrofauna and are even more effective when are used to complement environmental monitoring through the assessment of sediment and water qualities.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2014.05.003.

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