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Baseline

Seasonal and pollution-induced variations in biomarkers of transplanted mussels within the Beagle Channel

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ABSTRACT

The occurrence of biomarker variations linked to environmental factors makes it difficult to distinguish the effect of pollution. In an attempt to evaluate spatial and seasonal effects of environmental parameters on biomarker responses, mussels *Mytilus edulis chilensis* coming from an aquaculture farm were transplanted to several points within Ushuaia Bay (Beagle Channel) for 6 weeks in summer and winter. Activities of superoxide dismutase, catalase, glutathione-*S*-transferase and levels of lipid peroxidation were measured in gills and digestive gland. Cu, Zn, Fe, Cd and Pb concentrations were also assessed. Results indicated a significant effect of seasons on biological responses as well as in metal bioaccumulation showing the influence of natural factors such as dissolved oxygen, temperature and food availability. The interdependence of those environmental factors is important for the homeostasis of thermoconformers, especially regarding their oxidative metabolism and should also be taken into consideration to distinguish natural from pollution-induced variations.

Mussels, being the most widespread and cosmopolitan of all marine genera and inhabiting highly contaminated coastal waters, are extremely tolerant to fluctuations in salinity, temperature and other physicochemical parameters (Rainbow, 1995). Mussels are widely used as sentinel organisms in marine pollution monitoring programs due to their sessile and filtering habits, and ability to bioaccumulate organic pollutants and metals in their tissues (Goldberg, 1975). The exposure of marine molluscs to metals has been shown to induce oxidative stress throughout the formation of reactive oxygen and nitrogen species (ROS/RNS), which modulate the onset of several deleterious effects and cell damage (Almeida et al., 2004). Organisms have antioxidant enzymes that can intercept ROS/RNS, protecting molecular targets against oxidative injury. For example, superoxide dismutase (SOD) converts superoxide anion radical ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2), catalase (CAT) and glutathione peroxidase (GPx) detoxify H_2O_2, and glutathione-*S*-transferase (GST) conjugates xenobiotics with reduced glutathione (GSH) to facilitate the excretion (Almeida et al., 2004). The levels or activities of antioxidants and/or the determination of oxidative stress (DNA damage, protein oxidation, lipid peroxidation) are potential biomarkers revealing an effect mediated by contaminants in the organism (Regoli et al., 2002; Sureda et al., 2006; Valavanidis et al., 2006).

The physiological response of marine ectothermic organisms is strongly dependent on fluctuations of biotic and abiotic factors such as salinity, oxygen concentration, temperature and food availability, resulting in difficulties to interpret the biological effects exerted by xenobiotics (Camus et al., 2004; Manduzio et al., 2004; Sheehan and Power, 1999). Consequently, it is necessary to monitor the physical and chemical aspects of surrounding waters that will contribute to a better understanding of the biological responses. In addition, the genetic differences in susceptibility to pollution make comparisons among animals, originated from different populations, difficult due to the variability in biomarker responses (Astley et al., 1999). In our study, biomonitoring was carried out with animals coming from the same population (farmed organisms), grown in a clean site and transplanted into the area of environmental concern. This experimental design makes possible the reduction of the variability associated to the source, age and sexual stage.

Ushuaia city, located on the northern shore of Ushuaia Bay (54°48′S, 68°19′W, Tierra del Fuego, Argentina), is a very touristic area of the Beagle Channel. Being the southernmost city on Earth and also the gate to Antarctica, it receives all the touristic ships visiting the Antarctic Peninsula and many other sub Antarctic islands such as Malvinas (Falkland), South Orkney and South Georgia. Ushuaia Bay has been receiving significant inputs of contaminants

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from the homonymous city. Ushuaia has increased severely in the past years its urban wastewaters, industries, shipping and tourism. Previous reports indicated the existence of potentially toxic chemicals (heavy metals) in sediments and mussels of Ushuaia Bay (Amin et al., 1996a,b), as well as high loads of nutrients and organic matter (Amin et al., 2011; Gil et al., 2011; Solís et al., 2004a,b). Within this context, the development of tools for monitoring water quality and to assess the biological effects of pollution is an imperative need.

The main goal of the present study was to evaluate the spatial and seasonal variations of a battery of biomarkers in caged mussels *Mytilus edulis chilensis* at five sites characterized by different anthropogenic influences from Ushuaia city, as well as, to explore the possible relationships among biomarkers, physical and chemical parameters and bioaccumulated heavy metals. This is the first research that has studied the superoxide dismutase and glutathione-*S*-transferase activities and the seasonal variation of all selected biomarkers and heavy metals in mussels from Ushuaia Bay. This information will allow pointing out which biomarkers should be used in future monitoring programs in similar aquatic systems.

In the austral summer (January) and winter (July) of 2007, mussels *M. edulis chilensis* (shell size = 5.9 ± 0.4 cm) were brought from a mussel farm located at Brown Bay (Beagle Channel) in cold boxes to the laboratory in Ushuaia city. After a first sorting, mussels ($N = 45$) were placed in rectangular cages of 18 × 12 × 18 cm constituted by polypropylene netting. Three cages per site were immersed at a mean depth of 5 m in five sites of Ushuaia Bay (Fig. 1). Sites were chosen by direct anthropogenic inputs: Industrial Zone (IZ) is near electronic factories which dump their untreated wastes directly into the sea; Fuel Dock (FD) is located close to a military dock where several vessels load and unload fuel; Nautical Club (NC) receives domestic effluents and pluvial outflows; Aspirante Creek (AC) and Ushuaia Peninsula (UP) are the furthermost places from Ushuaia city with no direct human activity considered *a priori* as control sites. The exposure time was of 6 weeks on each season. Authors wish to declare that this study was conducted in accordance with national and institutional guidelines for the protection of animal welfare.

Temperature, pH, salinity and dissolved oxygen in water were recorded *in situ* at all sites by means of a multiparameter device Horiba U-10, at the same moment that the mussels were sampled. Water was sampled by hand into several bottles according to the analytical specifications and transported to laboratory for further filtration and preservation. Samples for the determination of dissolved nutrients (nitrite, nitrate, ammonia, phosphate and silicate) were filtered through Whatman GF/C filters, and analyzed using a four channels automatic Technicon® AA-II autoanalyzer (Strickland and Parsons, 1972). Chlorophyll *a* measurements were also performed at all sites and were evaluated using a fluorimetric method (Holm-Hansen et al., 1965).

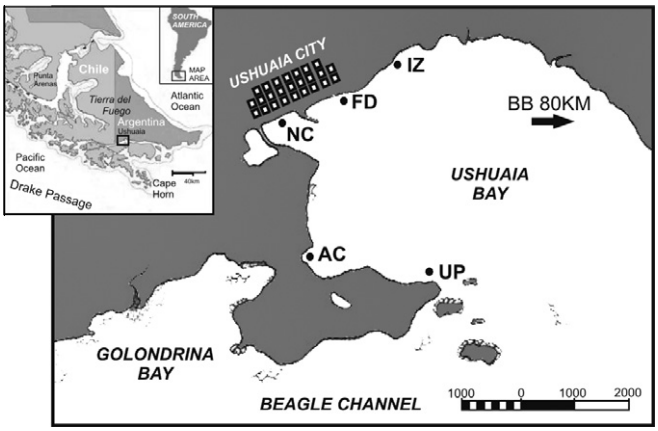

Fig. 1. Location of sampling sites in Ushuaia Bay (Tierra del Fuego, Argentina). IZ: Industrial Zone, FD: Fuel Dock, NC: Nautical Club, AC: Aspirante Creek, UP: Ushuaia Peninsula.

After 6 weeks of exposure, 10 mussels from each site were collected to determine total metal concentrations (Cu, Zn, Fe, Cd and Pb). Gills and digestive gland were dissected and dried at 60 °C until constant weight. Samples were homogenized with a porcelain mortar and stored in polyethylene bags until analysis. For each tissue, two aliquots of about 0.5 g were taken from the well-homogenized sample to determine total metal concentrations following the method described by Marcovecchio et al. (1988). This technique comprises a mineralization with a strong acid mixture ($HClO_4$:HNO_3, 1:3) under controlled temperature in a glycerin bath (110 ± 10 °C). The extract was diluted in 0.7% (v/v) HNO_3 up to 10 mL of final volume. Metal concentrations in these solutions were measured using a Perkin Elmer AA-2380 atomic absorption spectrophotometer with air-acetylene flame and deuterium background correction (D2BGC). Analytical grade reagents were utilized for tissue mineralization as well as for blanks and calibration curve standards build ups. In all cases bidistilled water was used to prepare the corresponding solutions. Each sample was performed in duplicate.

The certified reference material Mussel Tissue Flour R.M. No. 6 provided by the National Institute for Environmental Studies (NIES) of Tsukuba (Japan) were analyzed in addition to the studied organisms. The percentage of recovery for all metals ranged between 91% and 103%. The detection limits (μg/g dw) for these analysis were: Cu 0.77, Zn 0.88, Fe 2.73, Cd 0.27 and Pb 2.15.

For measurement of enzymatic activities, gills and digestive gland ($n = 5$) were removed upon return to the laboratory (less than 2 h) and immediately homogenized in relation 1:3 (w/v) 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 phenylmethylsulfonyl fluoride, with pH adjusted to 7.6. Homogenization was carried out at 4 °C using an Ultra Turrax T 25 homogenizer. Homogenates were then centrifuged at 9000*g* for 30 min at 4 °C (Bainy et al., 1996) and stored at −20 °C for short-term measurements. The activity of SOD was assayed by the epinephrine method (Misra and Fridovich, 1972), based on the capacity of SOD to inhibit the autooxidation of epinephrine to adrenochrome at 480 nm. CAT activity was evaluated by the rate of H_2O_2 decomposition at 240 nm (Beutler, 1982). GST activity was determined by measuring the increase in absorbance at 340 nm, incubating reduced glutathione and 1-chloro-2.4-dinithrobenzene as substrates, according to Habig et al. (1974).

For measurement of lipoperoxidation (LPO), gills and digestive gland ($n = 5$) were dissected and immediately homogenized in relation 1:3 (w/v) of 0.1 M Tris buffer pH 7.8 using an Ultra Turrax T25 homogenizer. Each homogenate was centrifuged at 9000*g* for 10 min at 4 °C and supernatants were stored for a short-term at −20 °C. LPO was measured in the supernatant fraction as malondialdehyde equivalent using trichloroacetic acid, thiobarbituric acid and hydrochloric acid reagents (TBA–TCA reagent, 0.375% w/v TBA, 15% w/v TCA and 0.25 N HCl) (Buege and Aust, 1978). An aliquot of 40 μL of that fraction diluted 10 times with homogenizing buffer was mixed with 800 μL of TBA–TCA reagent and heated in a boiling water bath for 15 min. After cooling, the flocculant precipitate was removed by centrifugation at 1000*g* for 10 min. Finally, the MDA concentration in the supernatant fraction was determined spectrophotometrically at 535 nm.

All biomarkers were carried out in duplicate and results were referred to the soluble protein concentration contained in the sample. Proteins were determined by Lowrýs method modified by Markwell et al. (1978) using bovine serum albumin as standard. A Perkin Elmer Lambda 25 UV/VIS spectrophotometer was used.

The geographical and seasonal variations of biomarker responses and heavy metal concentration were tested, in both gills and digestive gland by a two-way ANOVA with sites, seasons and interaction "sites × seasons" as variables. Post-hoc tests (Tukey) were used to discriminate between means of values. Prior to analysis, data were tested for normality and homogeneity of variance using the Kolmogorov–Smirnoff and Cochran C tests, respectively. Statistical significance was declared at $p < 0.05$.

Pearson correlations were used to study the influence of heavy metals and environmental variables on biomarkers. Analysis of principal components (PCA) of biomarkers and environmental data were applied to discriminate between different sites and/or sampling periods using a data matrix of 10 environmental parameters, four metals and four biomarkers in gill and in digestive gland as descriptors and five sites in two seasons as objects. Due to scale differences between variables, the analysis was based on standardized residuals (Legendre and Legendre, 1984). The analyses were carried out using the STATISTICA 7.0 program (STATISTICA, Microsoft Co.).

Physical and chemical characteristics of the sampling sites are summarized in Table 1. Temperature showed a clear seasonal variation being 9.78 ± 0.51 °C in summer and 4.84 ± 0.56 °C in winter. Average pH was 7.92 ± 0.09 and salinity was 31.78 ± 0.74 g/L. Slight differences between sites were found in salinity, being Nautical Club the site with the lowest values in both seasons, revealing the influence of freshwater coming from runoffs. Dissolved oxygen was in all cases near or above saturation being slightly higher in summer (11.48 ± 0.73 mg/L) than in winter (9.95 ± 0.60 mg/L). These high values reveal well oxygenated waters during the studied periods. Nutrients were higher in winter than in summer in most sites. Nautical Club showed the maximum levels of dissolved nutrients followed by Industrial Zone in both seasons. The chlorophyll *a* levels were relatively low but displayed marked seasonal changes, being superior in summer at all sites when temperatures are higher and nutrients are lower.

Table 2 shows the mean values of measured metal concentrations in mussels expressed in μg/g (dry weight). This table shows that gills accumulated mainly Zn > Fe > Cu > Cd, meanwhile in digestive gland the pattern of accumulation was slightly different Fe > Zn > Cu > Cd. Pb was above the detection limit (2.15 μg/g dw) only in gills of mussels transplanted to Industrial Zone, having a concentration of 3.48 ± 1.29 μg/g dw.

Two-way analysis of variance for heavy metal levels in gills and in digestive gland are shown in Table 3. Heavy metal concentrations in gills were significantly dependent on the season for all metals and on the site for Zn and Fe; the two-factor interaction was also significant, except for Cu. Cu, Zn and Fe bioaccumulation was higher in winter than in summer. In the case of Cd, seasonal variation varied with sites. At Industrial Zone bioaccumulation was higher in winter, meanwhile at Aspirante Creek and Nautical Club bioaccumulation was higher in summer. Differences among sites were found for Zn only in winter, where at Industrial Zone the level was the highest (341.04 ± 14.76 μg/g dw). Regarding Fe, in summer as well as in winter, differences among sites were found. In summer, mussels from Industrial Zone had concentrations higher than those transplanted to Aspirante Creek and Fuel Dock. In mussels exposed at Nautical Club bioaccumulation was superior to that of those transplanted to Aspirante Creek. Meanwhile in winter, the maximum level of Fe was measured in mussels exposed at Nautical Club (259.84 ± 0.42 μg/g dw). In the same sense, mussels from Fuel Dock and Industrial Zone had higher concentrations of Fe in gills than those from Aspirante Creek and Ushuaia Peninsula.

The analysis in digestive gland (Table 3) showed significant differences dependent on the season for all metals and on the sites for Cu, Zn and Fe; the two-factor interaction was also significant for all metals, except for Zn. Accordingly with the results for gills, Cu, Zn and Fe bioaccumulation was higher in winter than in summer. Also in agreement with results obtained for gills, Cd accumulation in digestive gland of mussels from Aspirante Creek and Nautical Club was higher in summer. In relation to variations among sites in summer, only for Fe we found significant differences being the highest bioaccumulation in mussels transplanted to Nautical Club (556.61 ± 9.54 μg/g dw). In winter, differences among sites were registered in Cu, Zn and Fe being the results variable for each metal.

The activity of SOD was of the same magnitude in gills and in digestive gland (Fig. 2). A two-way ANOVA revealed that seasons and the interaction of seasons and sites were significant. SOD levels were higher in winter in both organs assayed and no differences among sites were found.

CAT level was higher in digestive gland than in gills, seasonally independent (Fig. 3). Differences among sites were only found in gills, showing the highest activities those mussels transplanted to Nautical Club, Aspirante Creek and Ushuaia Peninsula (2.11 ± 0.47 U CAT/mg prot.). Significant interaction between sites and seasons was found in digestive gland of mussels coming from Fuel Dock, with maximum activity in summer (7.67 ± 0.96 U CAT/mg prot.).

GST activity was greater in gills than in digestive gland only in summer (Fig. 4). Two-way ANOVA showed that GST was seasonal dependent in gills, being superior in summer. Interaction between sites and seasons was also significant, resulting higher the activity in summer in organisms transplanted to Industrial Zone than those transplanted to Aspirante Creek and Ushuaia Peninsula.

Levels of LPO showed differences between tissues only in winter, being greater in digestive gland (Fig. 5). Two-way ANOVA, for gills and digestive gland, revealed that the effects of sites and seasons were significant, as well as the interaction between both variables. In gills, we registered in summer the lowest LPO levels in

Table 1
Physical and chemical parameters measured in the water.

Sites	Seasons	pH	Temperature (°C)	Salinity (g/L)	Diss. O_2 (mg/L)	Nitrite (μM)	Nitrate (μM)	Ammonia (μM)	Phosphate (μM)	Silicate (μM)	Chlorophyll a (μg/L)
IZ	Summer	7.93	9.4	31.6	11.76	2.12	6.09	1.60	0.33	5.92	0.305
FD	Summer	7.94	9.9	32.0	11.94	0.25	0.88	1.63	4.98	2.59	0.24
NC	Summer	7.86	10.5	30.6	10.81	0.76	5.84	94.83	0.43	21.48	0.48
AC	Summer	7.87	9.9	32.0	12.27	0.05	0.05	0.37	0.45	1.85	0.60
UP	Summer	7.76	9.2	32.3	10.62	0.21	1.72	0.70	0.48	2.22	0.142
IZ	Winter	8.00	5.1	32.4	10.06	0.47	18.70	564.77	1.11	15.92	0.036
FD	Winter	7.95	4.1	32.4	9.63	0.39	13.94	66.16	1.02	5.92	0.006
NC	Winter	7.85	4.4	30.3	9.15	0.76	16.79	1052.83	22.64	22.96	0.030
AC	Winter	7.99	5.4	32.1	10.76	0.25	9.42	3.36	1.02	4.44	0.024
UP	Winter	8.08	5.2	32.1	10.16	0.20	9.14	0.96	1.09	3.7	0.024

Table 2
Heavy metals concentrations (μg/g dw) in gill and digestive gland of mussel *M. edulis chilensis* transplanted to Ushuaia Bay in summer and winter of 2007.

Sites	Seasons	Cu		Zn		Fe		Cd	
		Gills	D. Gland	Gills	D. Gland	Gills	D. Gland	Gills	D. Gland
IZ	Summer	3.63 ± 0.44	3.86 ± 0.12	195.47 ± 2.54	50.78 ± 2.63	148.53 ± 7.69	369.65 ± 4.24	1.22 ± 0.35	0.96 ± 0.17
FD	Summer	3.08 ± 0.55	3.55 ± 0.11	230.86 ± 20.92	48.06 ± 3.33	81.04 ± 9.27	324.39 ± 3.43	1.28 ± 0.26	1.53 ± 0.26
NC	Summer	3.08 ± 0.33	4.34 ± 0.55	202.52 ± 3.74	48.66 ± 0.50	127.20 ± 0.18	556.61 ± 9.54	1.96 ± 0.17	1.78 ± 0.09
AC	Summer	1.91 ± 0.01	3.16 ± 0.00	206.64 ± 10.02	48.01 ± 0.18	74.24 ± 11.45	250.34 ± 17.19	2.14 ± 0.08	1.59 ± 0.35
UP	Summer	2.54 ± 0.44	3.32 ± 0.00	189.51 ± 0.78	42.19 ± 1.52	99.62 ± 13.12	366.22 ± 16.03	1.78 ± 0.26	1.65 ± 0.26
IZ	Winter	5.56 ± 0.82	8.79 ± 0.52	341.04 ± 14.76	70.39 ± 2.68	192.53 ± 3.53	967.50 ± 67.68	2.21 ± 0.09	1.20 ± 0.17
FD	Winter	5.21 ± 0.73	13.14 ± 0.32	256.45 ± 8.38	82.79 ± 13.76	199.88 ± 31.78	881.24 ± 31.25	1.39 ± 0.14	0.90 ± 0.13
NC	Winter	5.62 ± 0.62	10.65 ± 0.46	229.69 ± 3.66	82.63 ± 7.24	259.84 ± 0.42	1109.38 ± 29.62	1.07 ± 0.29	0.79 ± 0.06
AC	Winter	5.07 ± 0.48	6.75 ± 0.12	268.91 ± 17.12	77.36 ± 1.31	138.79 ± 5.32	637.76 ± 66.42	1.12 ± 0.15	0.71 ± 0.06
UP	Winter	4.67 ± 0.56	8.33 ± 0.27	231.57 ± 7.07	61.20 ± ∗1.19	103.03 ± 13.94	1332.36 ± 0.17	1.26 ± 0.17	1.27 ± 0.21

Table 3
Results of two-way ANOVA for the heavy metal concentrations in gills and digestive gland of mussel *M. edulis chilensis* transplanted to Ushuaia Bay in summer and winter of 2007.

Effect	Cu		Zn		Fe		Cd	
	Gills	D. Gland	Gills	D. Gland	Gills	D. Gland	Gills	D. Gland
Sites	n.s.	$p < 0.000001$	$p < 0.001$	$p < 0.05$	$p < 0.00001$	$p < 0.000001$	n.s.	n.s.
Seasons	$p < 0.000001$	$p < 0.000001$	$p < 0.00001$	$p < 0.000001$	$p < 0.000001$	$p < 0.000001$	$p < 0.05$	$p < 0.001$
Sites × seasons	n.s.	$p < 0.000001$	$p < 0.001$	n.s.	$p < 0.001$	$p < 0.000001$	$p < 0.001$	$p < 0.05$

n.s.: No significant.

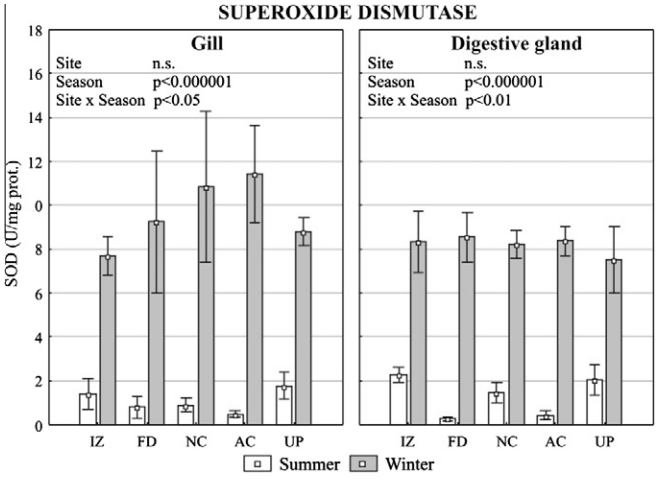

Fig. 2. Superoxide dismutase activity in gills and digestive gland of *Mytilus edulis chilensis* encaged for 6 weeks in all studied sites in summer and winter of 2007. Statistical comparisons (two-way ANOVA). n.s.: non significant. Values are mean ± SD.

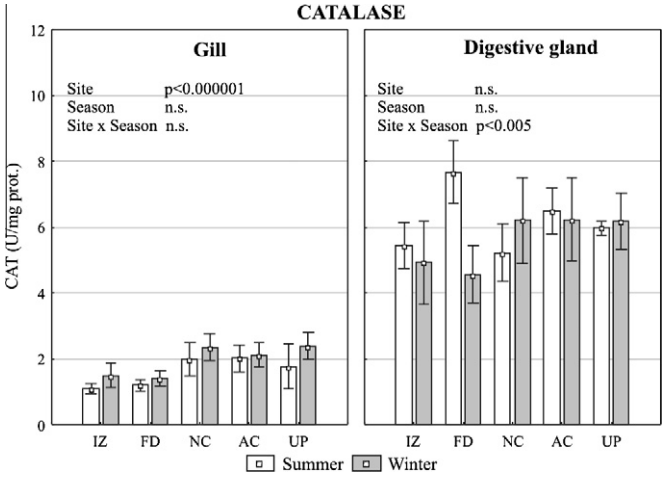

Fig. 3. Catalase activity in gills and digestive gland of *Mytilus edulis chilensis* encaged for 6 weeks in all studied sites in summer and winter of 2007. Statistical comparisons (two way ANOVA). n.s.: non significant. Values are mean ± SD.

mussels exposed at Industrial Zone (3.01 ± 0.67 μmol MDA/mg prot) . Meanwhile in winter, the highest levels were measured in mussels transplanted to Nautical Club (5.85 ± 0.96 μmol MDA/mg prot.). Regarding the interaction between sites and seasons, LPO levels in gills were significantly higher in Aspirante Creek and Ushuaia Peninsula in summer than in winter. In digestive gland, no differences among sites were found in summer. However, LPO values measured in mussels exposed at Nautical Club in winter (9.15 ± 1.39 μmol MDA/mg prot.) were significantly higher than those measured in the other sites. Contrarily, LPO level was the lowest in mussels transplanted to Ushuaia Peninsula (2.30 ± 0.23 μmol MDA/mg prot.).

The results of the correlation analysis among the biological, chemical and physical variables tested and heavy metal bioaccumulation, using Pearson's test ($p < 0.05$), are shown in Table 4. CAT activities in gills and digestive gland did not correlate with the studied variables. A positive correlation was found in gills and digestive gland, among SOD and Cu, Zn, Fe and nitrate. Negative correlations between SOD and dissolved oxygen, temperature and chlorophyll *a* were found. SOD activity in digestive gland showed a negative correlation with Cd. On the other hand, GST activity in gills presented a completely opposite behavior of SOD. LPO in digestive gland varied positively with Zn, ammonia and phosphate.

The PCA analysis with the whole set of data produced a two dimensional pattern which explained 68% of the total variance (Fig. 6A). The first axis PC1 represented a temporal tendency, showing a clear separation between summer and winter. Summer period was characterized by high values of temperature, dissolved oxygen, chlorophyll *a* and low of nitrate (probable indicator of higher photosynthetic activity). Cd in digestive gland and GST in gills were also higher in this period while the levels of Cu, Zn and Fe and SOD in both organs assayed were lower. The second axis PC2 mainly evidenced the continental discharges of freshwater. It

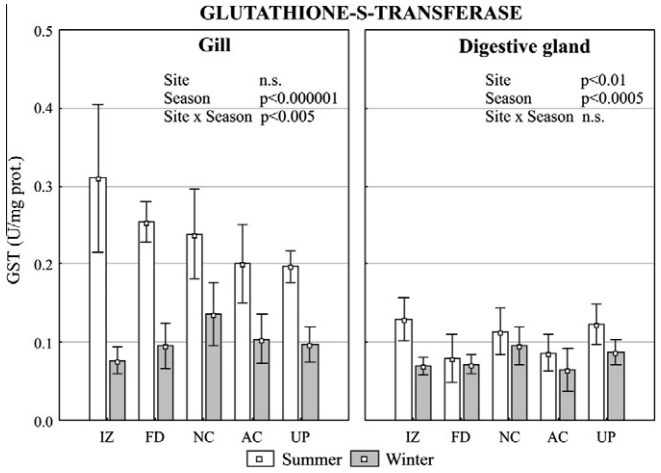

Fig. 4. Glutathione-*S*-transferase activity in gills and digestive gland of *Mytilus edulis chilensis* encaged for 6 weeks in all studied sites in summer and winter of 2007. Statistical comparisons (two-way ANOVA). n.s.: non significant. Values are mean ± SD.

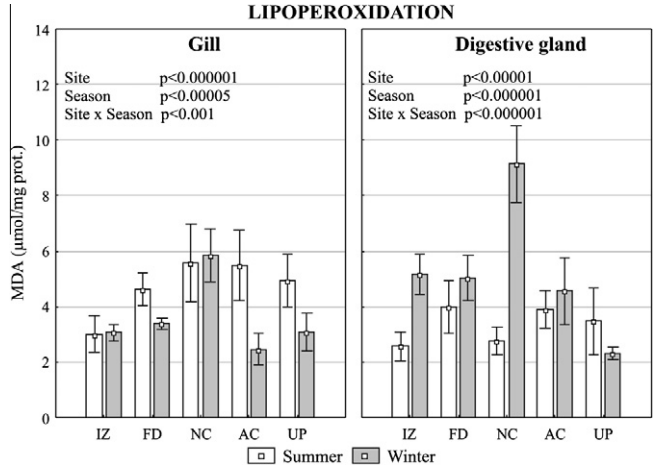

Fig. 5. Lipoperoxidation level in gills and digestive gland of *Mytilus edulis chilensis* encaged for 6 weeks in all studied sites in summer and winter of 2007. Statistical comparisons (two-way ANOVA). Values are mean ± SD.

was positively correlated with salinity and negatively with phosphate and silicate. LPO in gills was also negatively correlated with PC2. In both periods, Nautical Club was clearly separated from the other sites for having higher levels of ammonia, phosphate, silicate and LPO in gills and, lower values of salinity (Fig. 6B).

Among all studied sites, water quality from Nautical Club showed the highest impact from land. It receives pluvial and urban effluents that decrease the salinity and cause high concentrations of nitrate, ammonia, phosphate and silicate. This is in agreement with the previous study of Esteves and Amin (2004) who also found values of ammonia up to 1000 µM, but in our case it was not associated with low levels of dissolved oxygen. The impact of Grande Stream on Industrial Zone can be noticed through dissolved inorganic nutrients, especially by the high levels of nitrate, ammonia and silicate being higher than the values reported by Esteves and Amin (2004). In the same sense, when we compared with a more recently published work by Giarratano et al. (2010) only ammonia was higher, evidencing that somewhere along the course, Grande Stream is possibly contaminated with urban wastes and/or wastes coming from the pig slaughter house located nearby the mouth of the stream.

Trace metals (Cu, Zn and Fe) exhibited, in gills and digestive gland, strong seasonal variability, being the maximum values those registered in winter. These variations could be attributed to seasonal changes in reproductive cycle and food availability leading to alterations in body weight and composition. According to Regoli (1998), bioaccumulation in the gills probably reflects a different bioavailability of metals, while in the digestive gland it is influenced mainly by the progressive infiltration of the organ by gonadic tissues during gametogenesis. Such a trend evidences the natural influence of reproductive phases, which is known to "biologically dilute" the body burdens of trace metals in mussels. The same results and explanation were reported for mussel *M. galloprovincialis* (Bodin et al., 2004; Gorbi et al., 2008; Szefer et al., 2004). In the case of Cd, it was much more accumulated in summer than in winter in Nautical Club and Aspirante Creek, in both organs; meanwhile in Industrial Zone the opposite happened in gills. The concentrations of this metal were within the reported worldwide range of 1–2 µg/g for *M. edulis* (Zauke et al., 2003). The tissue distributions as well as the levels of Cu, Zn, Fe and Cd were similar to those reported in the same area by Giarratano et al. (2010). However, in present work, Pb was only detected in mussels transplanted to Industrial Zone showing a decreasing bioavailability in the other sites in comparison with previous data reported by Giarratano et al. (2010), where values ranged between 3 and 15 µg/g.

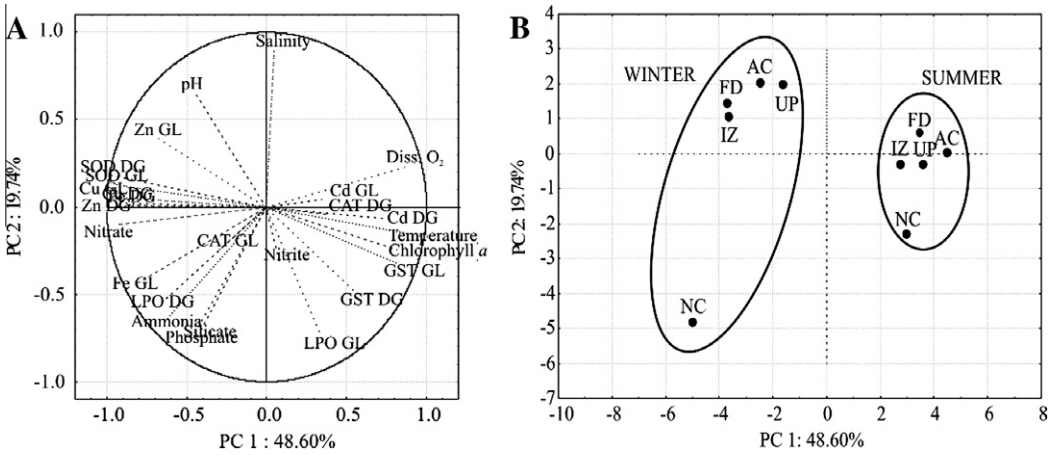

Fig. 6. Results of the PCA of the two dominant components. (A) Projection of the variables on the factor-planes 1–2. (B) Plots of scores for each site from PCA. Sampling sites were grouped by drawing arbitrary ellipses representing each studied season.

Table 4
Pearson correlation coefficients between biomarkers, heavy metal bioaccumulation in gills (GL) and digestive gland (DG) and physical and chemical data. Bold numbers indicate statistical significance at $p < 0.05$.

	Cu GL	Cu DG	Zn GL	Zn DG	Fe GL	Fe DG	Cd GL	Cd DG	pH	Diss.O2	Temp.	Sal.	Chl. a	NO_2^-	NO_3^-	NH_4^+	PO_4^{3-}	SiO_2^{2-}
CAT GL	0.16	0.19	−0.08	0.24	0.07	0.49	−0.06	−0.04	0.02	−0.38	−0.31	−0.36	−0.09	−0.44	0.14	0.29	0.35	0.26
CAT DG	−0.39	−0.50	−0.31	−0.36	−0.52	−0.31	−0.30	0.22	−0.06	0.49	0.35	−0.04	0.19	−0.32	−0.57	−0.13	0.25	−0.37
SOD GL	**0.91**	**0.84**	0.59	**0.93**	**0.66**	**0.80**	−0.45	**−0.80**	0.49	**−0.78**	**−0.96**	0.03	**−0.83**	−0.19	**0.80**	0.47	0.39	0.23
SOD DG	**0.95**	**0.88**	**0.68**	**0.92**	**0.71**	**0.84**	−0.32	**−0.76**	0.52	**−0.83**	**−0.98**	0.10	**−0.84**	−0.12	**0.89**	0.48	0.28	0.27
GST GL	**−0.72**	**−0.77**	**−0.72**	**−0.75**	−0.43	**−0.76**	−0.00	0.43	−0.46	**0.72**	**0.88**	−0.31	**0.70**	0.56	**−0.70**	−0.35	−0.12	−0.12
GST DG	−0.51	−0.51	**−0.77**	−0.62	−0.15	−0.36	0.03	0.37	−0.59	0.22	0.60	−0.41	0.41	0.60	−0.41	−0.12	−0.02	0.10
LPO GL	−0.49	−0.28	−0.52	−0.34	−0.05	−0.27	0.28	0.54	**−0.76**	0.08	0.45	−0.61	0.53	−0.16	−0.33	0.31	0.47	0.37
LPO DG	0.56	0.58	0.33	**0.69**	**0.78**	0.36	−0.23	−0.52	−0.20	−0.59	−0.56	−0.39	−0.40	−0.09	0.61	**0.88**	**0.87**	0.53

All biomarkers, except CAT, showed that season was a significant factor affecting the responses. SOD activity was higher in winter than in summer in both organs assayed and no differences among locations were found. This seasonal tendency is in agreement with activity measured in mussels *Perna viridis* (Lau et al., 2004) and *M. galloprovincialis* (Borković et al., 2005). The activity of SOD was the biomarker that better represented the seasonal bioavailability of metals, but it did not allow distinguishing among sites. Cu induced the activity of SOD in *M. galloprovincialis* (Manduzio et al., 2003; Regoli and Principato, 1995), as well as it has been enhanced by several metals in other marine bivalves and fish (Géret et al., 2002; Rodriguez-Ariza et al., 1993). Contaminant levels in tissues are likely to change during the season, reflecting variability in the discharge and metabolism of contaminants and/or changes in tissue weight (Sheehan and Power, 1999). Thus, the possibility of the observed seasonal biomarker patterns being partly driven by changes in contaminant levels cannot be totally excluded. However, possible seasonal changes in trace metal concentrations are not entirely explaining the observed patterns in the activities of SOD. Environmental factors, like food availability, dissolved oxygen and temperature, had a marked influence on the seasonal variability in this antioxidant defence.

CAT activity was the only biomarker seasonally independent. That characteristic would be a desirable feature of a bioindicator (Sheehan and Power, 1999), but it is unlikely to occur in thermoconformers like mussel (Wilhelm Filho, 1996). The activity of CAT was neither influenced by temperature at all in the Antarctic scallop *Adamussium colbecki* (Regoli et al., 1997) nor in mussel *M. edulis* (Power and Sheehan, 1996). In this work, CAT activity was higher in digestive gland, which is consistent with other studies on *M. edulis* (Giarratano et al., 2010; Power and Sheehan, 1996). Differences among sites were only found in gills, being the activity in Nautical Club, Aspirante Creek and Ushuaia Peninsula higher than that in Industrial Zone and Fuel Dock. The activity of CAT did not correlate with metals or with physical and chemical parameters. In other works, correlation were neither found between CAT activity and contaminant body-burden (PCBs, metals) in populations of *M. edulis* from the Venice Lagoon (Livingstone et al., 1995; Nasci et al., 1998) and in freshwater bivalve *Unio tumidus* (Cossu et al., 1997). The differences between sites found in gills could be related to parameters not evaluated in the present study. This response which seems not to be affected by this class of pollutants could be used to exclude the presence of studied metals and to verify the state of health of mussels (Regoli and Principato, 1995).

Contrarily to SOD, GST activity was superior in summer at all sites but only in gills, meanwhile in digestive gland the variation was only detected in Industrial Zone. In accordance with SOD, this enzyme did not show distinctions among sites. The same seasonal trend in the activity of GST was found in *P. viridis* (Lau et al., 2004) and in the scallop *A. colbecki* (Regoli et al., 1997). Kaaya et al. (1999) also found the highest GST activities in *M. galloprovincialis* and *P. perna,* in summer and autumn and the lowest levels in winter and spring. These variations could be linked to the mussel's reproduction cycle, where higher levels of GST activity would correspond with restoration of reserves and gametogenesis periods and lower values would coincide with spawning periods. Furthermore, summer variations of antioxidant efficiency have been indicated in several organisms as a typical short-term response to the seasonal increase of prooxidant challenge (Regoli et al., 2004). In this sense, we found positive correlation between GST activity and seawater temperature, food availability and dissolved oxygen.

GST activity was especially higher in gills rather than in digestive gland, in accordance with previous works on *M. edulis* (Fitzpatrick and Sheehan, 1993; Power and Sheehan, 1996). Only in gills, the levels of GST were inhibited by Cd and Zn. Considering that levels of metals were lower in summer; it is possible that the highest activity measured in summer would be more influenced by environmental factors than by metals. According to Orbea et al. (2002), under low pollution conditions like in this study, seasonal factors might affect biomarker responses to a greater extent than pollution stress. The increase in temperature is followed by an increase in oxygen consumption and by an increase in ROS generation (Borković et al., 2005). Reproductive activity and temperature associated changes in patterns of food storage and utilization could alter nutritional status which might also affect the levels of enzymatic activity (Orbea et al., 1999).

LPO levels varied with seasons, but there was not a defined tendency. Other authors have found little or no seasonality in MDA levels of *M. edulis* (Shaw et al., 2004), meanwhile a seasonal variation in *P. viridis* with lowest levels in winter have been reported (Lau et al., 2004). Both similarities and differences have been reported for seasonal changes of this biomarker even in populations of the same species which often exhibit opposite trends in the same periods. The variability of seasonal fluctuations confirms that individual antioxidants are difficult to predict and opposite changes in different areas can occur when the same environmental prooxidant factors have a different regional influence (Bocchetti and Regoli, 2006). LPO in digestive gland was positively correlated with ammonia and phosphate. This result is in agreement with data obtained by Charissou et al. (2004), who registered an induction of lipid peroxidation in places with the poorest quality environment. LPO in digestive gland was also positively correlated with Zn. A possible mechanism for such toxicity is by metal-mediated peroxidation of lipid components of membranes as a result of oxyradicals (Sheehan and Power, 1999). Several works have been carried out to assess the susceptibility of different tissues of marine organisms to LPO caused by pollutants. However, different responses were obtained in similar experiments making difficult the interpretation of such results. In general, organisms with lowered antioxidant status could be more susceptible to LPO, and therefore presenting higher levels of lipid peroxidation (Cossu et al., 2000; Géret et al., 2002). On the other hand, increases in

antioxidant defenses would be due to enhanced ROS/RNS production and elevated (Torres et al., 2002; Verlecar et al., 2008) or decreased (Niyogi et al., 2001; Rodriguez-Ariza et al., 1993) levels of MDA have been observed.

Seasonal changes in biomarkers and bioaccumulated heavy metals were very important, probably in interaction with the reproductive cycle of *M. edulis chilensis*. There is only one report in relation to reproductive cycle of *M. edulis chilensis* from Ushuaia Bay (Tortorelli, 1987). Tortorelli described for the mussel population from Ushuaia Bay a very long spawning period, from May to January. Partial asynchronous emissions may occur during other months of the year with a peak in May–June and a more important one in October–November. The sexual resting stage appears in alternate months during the whole year in very low percentages. Unfortunately, natural variations of selected biomarkers associated to reproductive stage has not been studied in *M. edulis chilensis*. However, reproductive activity and temperature-associated changes in patterns of food storage and utilization have been reported to cause changes in hormonal and nutritional status which might also be expected to affect the levels of bioindicator molecules in molluscs (Bodin et al., 2004; Borković et al., 2005; Sheehan and Power, 1999). This work evidences the need of knowing the natural variation of selected biomarkers in order to distinguish it from the effects of pollution.

The whole set of data allowed to separate the sites mainly as a function of seasons, bioavailability of heavy metals and physical and chemical parameters. Nautical Club was the most affected site evidencing the impact of urban wastes through highest concentrations of nutrients and lowest salinities.

The main novelty of this work is that SOD and GST activities have been studied for the first time in mussels transplanted to Ushuaia Bay, showing both enzymes seasonal dependence. The first one could be applied in winter season to monitor the bioavailability of heavy metals. The latter could be measured in summer season to reflect higher dissolved oxygen, temperature and chlorophyll *a*. At least within Ushuaia Bay, these biomarkers could not allow separation among sites.

Independence of seasonal variation is a desirable feature of a bioindicator molecule that was only reached by CAT activity. However, its variations could not be related to any of the physical and/or chemical variables studied.

The induction of LPO in digestive gland of mussels transplanted to Nautical Club in response to high levels of ammonia and phosphates allow us to propose this biomarker to monitor places which waters are receiving effluents with high nutrient concentration. This biomarker could evidence a deterioration of the water quality.

This field study could be useful as a regional pilot project to demonstrate the utility of such a biomonitoring assay strategy and the potential roles of biomarkers for assessing environmental stress levels. However, it is important to remark that in order to confirm the usefulness of selected biomarkers in this study, the number of tested samples should be increased.

It is clear from the data that significant changes of antioxidant parameters in gills and digestive gland of *M. edulis chilensis* are closely correlated with the seasonal variations of temperature, dissolved oxygen, chlorophyll *a* and bioavailability of heavy metals. This study shows the importance to include such variables in biomonitoring programs when analyzing data obtained from different sites and periods of the year, and especially when relating pollutant data to biological responses in places with contaminants at trace levels without known point sources.

Dr. Giarratano made the setting up of the experiment and conducted the field and laboratory works. The three authors participated actively in data analysis, interpretation of results, drafting the manuscript and approved the final version of the text.

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