Marine Pollution Bulletin 62 (2011) 1895–1904

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Baseline

Heavy metal concentrations and biomarkers of oxidative stress in native mussels (*Mytilus edulis chilensis*) from Beagle Channel coast (Tierra del Fuego, Argentina)

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ARTICLE INFO

Keywords:
Mussels
Heavy metals
Beagle Channel
Oxidative stress biomarkers

ABSTRACT

The aim of this study was to evaluate the usefulness of oxidative stress biomarkers of pollution in native mussels *Mytilus edulis chilensis* from the Beagle Channel. Spatial and seasonal variations of catalase, glutathione-S-transferase and lipid peroxidation in gills and digestive gland were analyzed in relation to environmental parameters, heavy metals in sediment and in tissue. Four sites with anthropogenic impact and a control site were selected and monitored during the four seasons of 2007. We found significant differences among sites in concentrations of dissolved nutrients and heavy metals in sediments, with the highest values recorded at sites with anthropogenic pressure. Different patterns were observed between concentrations of metals in tissues and in sediments suggesting differences in bioavailability. There were also significant differences in biomarker responses among sites, despite the strong seasonal variability. Our results showed relatively moderate levels of pollution in the study area as a result of urban influences.

During the last two decades, Ushuaia and Golondrina bays (54° 48' S, 68° 19' W Beagle Channel, Tierra del Fuego, Argentina) have been receiving anthropogenic inputs from Ushuaia city, whose population has been in continuous and rapid growth (INDEC, 2010). These anthropogenic sources include industrial and domestic effluents, stormwater runoff, streams, leaching from garbage, solid waste dumps and inputs related to maritime traffic.

Heavy metals are natural constituents of marine and freshwater environments, where they are generally found in low concentrations. Human activities have increased the levels of metal ions in many of these natural water systems. Particularly, industrial and domestic effluents have contributed to the increment of metal load in coastal waters being finally deposited into aquatic sediments (Ansari et al., 2004).

In this sense, previous studies conducted on sediments from Ushuaia and Golondrina bays revealed the deterioration of the coastal environment. The lack of a sewage treatment plant, the discharges from electronic assembling factories in the area and the intensive maritime traffic were reported as the main sources of heavy metals and other pollutants (Amin et al., 1996a; Esteves et al., 2006).

The use of biomarkers has become a common tool for environmental assessment as they can help to predict the effects of particular chemicals involved in monitoring programs (Gagné et al., 2008; Lam, 2009; Vlahogianni et al., 2007). The use of mussels of the genus *Mytilus* in routine biomonitoring programs has been proposed to evaluate the spatial and temporal presence of some pollutants, like heavy metals in coastal environments. Molluscs accumulate metals from their food and surrounding sea water in concentrations that exceed considerably those found in their natural environment (Rainbow and Phillips, 1993; Viarengo et al., 2007). *Mytilus edulis chilensis* is a conspicuous species along the intertidal coastal zone near Ushuaia city and it has already been used in biomonitoring programs in the area (Giarratano et al., 2010, 2011; Giarratano and Amin, 2010).

In addition, physiological changes in organisms can be related to the effects of toxic chemicals in water that are incorporated and accumulated in tissues of these filter-feeding molluscs. Regoli et al. (2002) and Viarengo et al. (2007) have reported that the toxicity of pollutants often depends on their capacity to increase the cellular levels of reactive oxygen species (ROS). This can happen either by the straightforward activation of processes that lead to their synthesis or indirectly acting on enzymes. Metal ions possess the ability to produce reactive radicals, resulting in DNA damage, lipid peroxidation and depletion of protein sulphydryls (Valko et al., 2005). When ROS levels exceed antioxidant defences, the cells go into oxidative stress which causes membrane lipid peroxidation and changes in the activity of the ROS defence system, among others. This system includes antioxidant enzymes like catalase and glutathione-S-transferase, whose activities are modified in response to cellular oxidative stress and they are particularly evaluated in target organs such as digestive glands and gills (Cheung et al., 2001; Fang et al., 2009; Lau et al., 2004).

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doi:10.1016/j.marpolbul.2011.05.031

The aim of the present study was to evaluate the biochemical response of native mussels (*M. edulis chilensis*) from five sites located in two bays near Ushuaia city, each one with different pollution level. For this purpose, biomarkers of oxidative stress were measured in gills and digestive gland of mussels during summer, autumn, winter and spring of 2007. In order to analyse the relationship between those biomarkers and the marine environmental conditions, abiotic factors in water and heavy metal concentrations in sediment and mussels were also measured.

In the present study, four sites influenced by intense anthropogenic activities and one less-impacted site were selected on the coastal zone of Ushuaia city (Fig. 1). Three of the sites were located along the shore of Ushuaia bay: one next to a fuel dock (FD), another one close to the nautical wharf (NW) and the third one at the industrial zone (IZ). A fourth site was located at the outlet of the sewage pipeline in the occidental coast of Ushuaia peninsula (UP). The fifth sampling site was on the shore of Golondrina bay (GB), far from the city in a relatively less-impacted area and considered a priori as control site. Water, sediment and mussel samples were collected in these stations in summer (February), autumn (May), winter (August) and spring (November) of 2007.

At each sampling site two measurements within a 7-day interval were taken per sampling period and the averages of these values were then used for analysis. Salinity, temperature, pH and dissolved oxygen were registered *in situ* by using a multiparameter device HORIBA U-10 during low tide. At the same time, coastal water samples were collected by hand using plastic bottles of 2 L according to the analytical specifications and were transported to the laboratory to be analyzed. A volume of 1000–1250 ml was filtered by GF/C filters to determine chlorophyll-*a* concentration according to Holm-Hansen et al. (1965) using a Sequoia Turner (model 450) fluorometer. Samples of 1000 ml were filtered by GF/C filters to determine particulate organic matter (POM) and dissolved inorganic nutrients. POM was analyzed following the method described by Strickland and Parsons (1972). Ammonia, nitrate, nitrite, phosphate and silicate were determined according to Strickland and Parsons (1972), Treguer and Le Corre (1975), Grasshoff et al. (1983), Eberlein and Kattner (1987) and Technicon® (1973), respectively. A Perkin Elmer UV–Vis lambda 25 spectrophotometer was used to perform the corresponding POM and ammonia determinations. A four-channel automatic Technicon® AA-II autoanalyzer was used for the other analyzed nutrients.

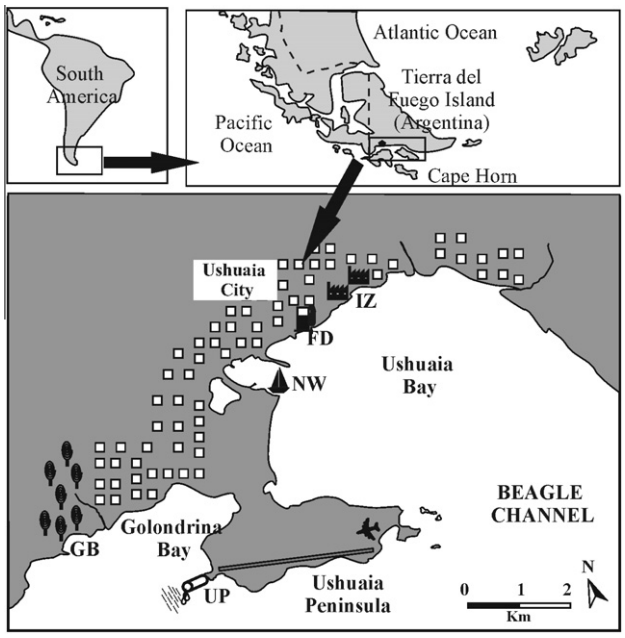

Fig. 1. Location of sampling sites. Golondrina bay (GB), industrial zone (IZ), fuel dock (FD), nautical wharf (NW) and Ushuaia peninsula (UP).

Native adult mussels of *M. edulis chilensis* (5.0 ± 0.5 cm shell length) were collected by hand during low tide at each site and season and transported to the laboratory in plastic buckets containing sea water from the corresponding sampling site. At the laboratory, digestive gland and gills were dissected and stored at −20 °C.

For enzymatic activity measurements, soft tissues of five mussels from each sampling site were homogenized at 4 °C in a 1:3 ratio (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. Homogenates were then centrifuged at 8000 rpm for 30 min at 4 °C. Catalase activity (CAT) was determined by the decrease in absorbance due to H_2O_2 consumption at 240 nm and 30 °C (Beutler, 1982). Activity was expressed as U CAT, meaning the amount of enzyme that hydrolyzes 1 μmol of H_2O_2 per minute per milligram of protein. Glutathione-S-transferase (GST) was determined at 340 nm and 25 °C using 1-chloro-2.4-dinitrobenzene (CDNB) as substrate (Habig and Jakoby, 1981). Activity was expressed as U GST which means that the amount of enzyme that conjugates 1 μmol of CDNB per minute per milligram of protein.

For lipid peroxidation (LPO) measurements, both gills and digestive glands were homogenized at 4 °C in a 1:3 ratio (w/v) of 0.1 M Tris buffer pH 7.8 and centrifuged at 9000 rpm for 10 min at 4 °C. In homogenates the generation of thiobarbituric acid reactive species was measured in a process conducted under elevated temperature (100 °C). LPO was quantified in terms of malondialdehyde equivalents (MDA) (Buege and Aust, 1978) and expressed as nanomols of MDA formed per milligram of protein.

All biochemical determinations were carried out by duplicate, then quantified and expressed in relation to soluble proteins content which was determined according to Markwell et al. (1978).

All these determinations were measured in a Perkin Elmer UV–Vis lambda 25 spectrophotometer.

To evaluate total heavy metal concentrations (Cd, Pb, Cu, Zn and Fe), intertidal sediment samples and mussels from each site and season were obtained. Sediment samples were collected using plastic spoons, then stored in polyethylene bags and transported to the laboratory. Samples were dried out in an oven until constant weight at 60 ± 5 °C and then were sieved in order to obtain the smallest fraction (<62 μm) where total metal concentrations were determined. This fraction has been considered the most indicated to analyse heavy metal concentrations in sediment (Amat Infante et al., 2002; Pekey, 2006). Whole soft tissues of five individuals from each location and season were carefully removed with a plastic knife and pooled. Samples were freeze-dried, homogenized and stored in polyethylene bags until analysis.

Subsamples (approximately 0.5 g) of sediment and tissues were taken to determine total metal concentrations following the method described by Marcovecchio et al. (1988). This technique includes a mineralization with a strong acid mixture ($HClO_4$:HNO_3, 1:3 v/v) under a controlled temperature glycerine bath (110 ± 5 °C). The extracts were diluted in 0.7% (v/v) HNO_3 up to 10 ml and metal concentrations were measured using atomic absorption spectroscopy (Perkin Elmer 2380) with air-acetylene flame.

Analytical quality (AQ) was checked against certified reference materials for each metal from National Institute for Environmental Studies (NIES), Tsukuba (Japan). 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. In all cases each sample was run by duplicate. Results were expressed as micrograms (except for Fe in sediment which was expressed as milligrams) per gram of dry basis.

Comparisons of physical and chemical parameters and heavy metal concentration in sediment and mussel tissues, among sites and seasons, were performed using the Kruskal–Wallis test. When significant differences were found, a post hoc multiple comparison of mean ranks test was used (Dunńs test – Daniel, 1978). Comparisons among sites and seasons for each biomarker and each tissue were performed by two-way analysis of variance (ANOVA) followed by Tukeýs test for multiple comparisons with unequal numbers of samples. Previously, biomarker data were tested for normality (Shapiro–Wilks) and for homogeneity of variances (Bartletťs test). If data did not meet these assumptions, they were $\log_{10}$ transformed. Values were reported as mean ± standard deviation.

An Industrial-Urban Contamination Index (IUCI) was calculated (IUCI = $\sum$ [Cu] + [Zn] + [Pb]) to describe the overall behavior of Cu, Zn and Pb, considering its anthropogenic source (Amat Infante et al., 2002). Spearman's correlation analysis was used to examine possible associations between heavy metals in sediments and those accumulated in tissues. In order to describe the studied sites, environmental and biological data were analyzed with Principal Component Analysis (PCA). For all statistical tests, the significance level was set at $p < 0.05$ (Sokal and Rohlf, 1981). All statistical analyses were performed using STATISTICA software package version 7.0.

Significant differences among sites were found in salinity, ammonia, nitrite, phosphate and silicate. The lowest values of salinity and the highest levels of these nutrients were measured in water samples from UP (Table 1). There were also significant differences among seasons in pH, temperature, chlorophyll-*a*, particulate organic matter and nitrate. A significant increment in pH was observed in spring compared to summer and autumn. At all sites the highest average temperatures were measured during summer and the lowest during winter. The concentration of particulate organic matter registered during summer was higher than those measured during winter and spring. The lowest concentration of chlorophyll-*a* was observed during winter. In the case of nitrate, an increasing tendency was found from summer to spring, being significantly higher the increment only in spring. There was no significant seasonal variation in ammonia, nitrite, phosphate and silicate concentrations.

Concentrations of Cd, Pb, Cu, Zn and Fe in sediment samples are given in Table 2. They can be arranged in the following sequence: Fe > Zn > Cu > Pb > Cd at GB and UP and Fe > Zn > Pb > Cu > Cd at IZ, FD and NW. No significant differences were found among seasons, but an increasing trend in Pb and Cu concentrations was observed from summer to spring. Regarding sites comparisons (Table 2), Cd was the only metal that did not show significant differences among sites. The concentration of Pb found at FD was higher than that from GB. Cu concentration measured at UP was greater than the level found at GB. Concentrations of Zn and Fe registered at UP were higher than at NW.

The IUCI index ranged between 63 and 340 (Fig. 2). The highest values were found at IZ (185 and 340), FD (150 and 254) and UP (199 and 311). A clear seasonal tendency was only observed at UP, increasing from summer to spring.

Heavy metal concentrations in soft tissue of mussels are listed in Table 3. The decreasing sequence was: Fe > Zn > Cu > Pb > Cd. Significant differences among sites were observed for Zn and Fe concentrations. Organisms from FD accumulated more Zn than those from GB, meanwhile organisms from GB showed higher levels of Fe than those from NW. No differences among sites were found in bioaccumulation of Pb, which was below the detection limit (2.15 μg/g) at FD and NW. Regarding seasonal variation, significant differences in Cd, Cu and Zn concentrations were observed. The values of Cd registered in winter and spring were higher than in summer and autumn. For Cu, the highest bioaccumulation was in the same periods than for Cd, but only significantly higher than summer. In the case of Zn, bioaccumulation in spring was higher than in summer.

Correlation analysis of heavy metal concentrations are listed in Table 4. There were several significant correlations but those higher than 0.6 in sediments were for Pb with Cu and Zn, for Cu with Zn and Fe and for Zn with Fe. In tissues, the highest correlations were found between Cd–Cu (0.67) and Cu–Zn (0.68). Despite significant positive correlations between the level of Cu (0.38) and Zn (0.24)

Table 1
Physical and chemical parameters among sites and among seasons.

	Sites					Seasons			
	GB	IZ	FD	NW	UP	Summer	Autumn	Winter	Spring
pH	7.90 a	7.92 a	7.85 a	7.92 a	7.84 a	7.72 a	7.67 a	7.90 ab	8.24 b
	(0.22)	(0.30)	(0.29)	(0.23)	(0.26)	(0.06)	(0.06)	(0.02)	(0.02)
DO (mg/L)	10.73 a	10.59 a	10.37 a	10.82 a	10.15 a	10.56 a	10.17a	10.43 a	10.96 a
	(0.18)	(0.68)	(0.54)	(0.82)	(0.60)	(0.94)	(0.47)	(0.25)	(0.53)
T (°C)	5.89 a	6.46 a	6.40 a	6.88 a	6.83 a	8.70 a	6.30 bc	3.84 b	7.12 ac
	(2.37)	(2.00)	(1.87)	(1.91)	(2.05)	(0.49)	(0.39)	(0.71)	(0.17)
Sal (%)	23.68 b	22.96 ab	23.65 b	24.28 b	21.26 a	23.72 a	22.48 a	23.10 a	23.36 a
	(0.59)	(0.79)	(0.47)	(1.56)	(1.30)	(2.43)	(1.00)	(1.14)	(0.37)
Chl-*a* (μg/L)	0.26 a	0.21 a	0.31 a	0.38 a	0.16 a	0.34 a	0.28 ab	0.07 b	0.36 a
	(0.25)	(0.14)	(0.22)	(0.22)	(0.03)	(0.15)	(0.15)	(0.06)	(0.23)
POM (mgC/L)	1147.01 a	1015.58 a	1381.30 a	930.51 a	1710.04 a	1799.95 a	1553.96 ab	793.97 b	799.67 b
	(688.54)	(598.01)	(692.92)	(182.18)	(1093.78)	(453.47)	(916.43)	(433.31)	(249.62)
NH_4^+ (μmol/L)	0.25 b	6.04 ab	1.21 ab	1.39 ab	88.52 a	29.15 a	23.35 a	17.76 a	7.65 a
	(0.43)	(5.06)	(0.42)	(1.27)	(48.96)	(62.73)	(49.63)	(30.67)	(11.96)
NO_2^- (μmol/L)	0.19 b	0.37 ab	0.23 ab	0.31 ab	1.05 a	0.42 a	0.46 a	0.48 a	0.36 a
	(0.09)	(0.20)	(0.08)	(0.21)	(0.16)	(0.48)	(0.30)	(0.55)	(0.42)
NO_3^- (μmol/L)	3.88 a	5.66 a	5.83 a	3.79 a	8.54 a	2.48 a	4.43 ab	5.06 ab	10.11 b
	(3.30)	(3.21)	(5.44)	(2.86)	(2.15)	(2.70)	(2.33)	(1.60)	(2.67)
PO_4^{-3} (μmol/L)	1.31 ab	2.03 ab	1.18 a	1.42 ab	9.64 b	3.56 a	3.35 a	3.32 a	2.23 a
	(0.37)	(0.87)	(0.39)	(0.31)	(4.14)	(6.06)	(4.17)	(3.18)	(1.30)
SiO_3^{-3} (μmol/L)	5.24 b	15.91 ab	5.31 b	4.83 b	15.33 a	6.83 a	11.93 a	8.94 a	9.60 a
	(2.36)	(9.58)	(1.35)	(2.39)	(5.37)	(7.98)	(7.03)	(8.71)	(9.16)

Values represent the mean and the standard deviation (in brackets) of pH, dissolved oxygen (DO) temperature (T), salinity (Sal), chlorophyll-*a* (Chl-*a*), particulate organic matter (POM), ammonia (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), phosphate (PO_4^{-3}) and silicate (SiO_3^{-3}). For each parameter, different letters identify significant differences (Kruskal–Wallis tests, $p < 0.05$), either among sites or among seasons.

Table 2
Heavy metal concentrations in sediments.

	Sites					Seasons			
	GB	IZ	FD	NW	UP	Summer	Autumn	Winter	Spring
Cd (μg/g)	0.44 a (0.17)	0.72 a (0.27)	1.02 a (0.49)	0.94 a (0.43)	1.59 a (0.59)	1.05 a (0.61)	0.88 a (0.31)	0.71 a (0.38)	1.12 a (0.81)
Pb (μg/g)	6.33 a (2.10)	45.19 ab (25.69)	44.18 b (6.38)	20.38 ab (15.08)	30.58 ab (4.83)	20.04 a (13.27)	25.16 a (16.11)	31.43 a (18.57)	40.70 a (26.88)
Cu (μg/g)	9.00 a (1.05)	27.00 ab (14.17)	26.57 ab (9.29)	11.81 ab (5.61)	54.54 b (30.86)	13.17 a (5.86)	20.86 a (14.30)	28.53 a (21.86)	40.58 a (32.11)
Zn (μg/g)	73.74 ab (22.39)	175.04 ab (28.51)	145.50 ab (34.92)	63.32 a (9.88)	183.00 b (18.91)	108.63 a (51.60)	134.75 a (49.87)	138.16 a (59.06)	130.93 a (74.89)
Fe (mg/g)	21.71 ab (3.76)	22.15 ab (1.43)	23.91 ab (6.42)	15.23 a (3.03)	35.43 b (12.22)	22.47 a (8.85)	22.02 a (5.95)	21.63 a (3.22)	28.62 a (14.58)

Values represent the mean and the standard deviation (in brackets). Concentrations are expressed in mg/g or μg/g dry weight. For each metal, different letters identify significant differences (Kruskal–Wallis tests, $p < 0.05$), either among sites or among seasons.

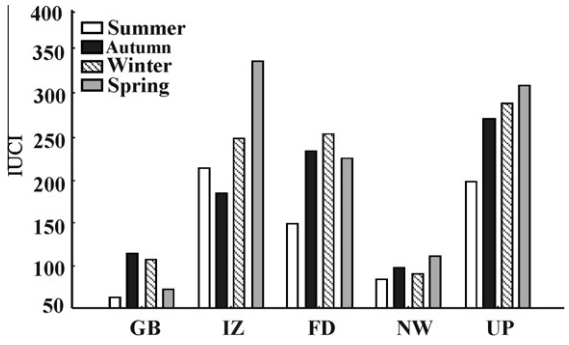

Fig. 2. Industrial-Urban Contamination Index (IUCI).

measured in sediment and that registered in tissue were found, the magnitude of correlations were relatively low.

Statistical results of two-way ANOVA for all studied biomarkers, in both gills and digestive gland, are presented in Table 5. CAT activities (Fig. 3a and b) were mostly higher in digestive gland than in gills. Significant seasonal variations were found in both organs, except in gills of mussels from NW and in digestive gland of mussels from FD. The highest mean activities in gills were recorded in spring, meanwhile in digestive gland no seasonal tendency was observed. Differences among sites were observed in both organs (Table 5). Gills of mussels from IZ and UP showed lower values than those from GB, meanwhile digestive gland of mussels from NW and UP had lower activity than those from GB. The two-way ANOVA revealed that the interaction of seasons and sites were also significant (Table 5). During winter,

Table 3
Heavy metal concentrations in mussel tissue.

	Sites					Seasons			
	GB	IZ	FD	NW	UP	Summer	Autumn	Winter	Spring
Cd (μg/g)	1.51a (0.71)	1.20 a (0.58)	1.20 a (0.89)	1.22 a (0.38)	1.71 a (0.34)	0.97 a (0.41)	1.01 a (0.67)	1.87 b (0.39)	1.63 b (0.33)
Pb (μg/g)	3.23 a (0.01)	3.13 a (0.01)	nd a	nd a	3.26 a (0.18)	3.23 a (3.23)	3.13 a (3.13)	3.13 a (3.13)	3.38 a (3.38)
Cu (μg/g)	5.27 a (1.96)	4.77 a (3.22)	6.74 a (4.04)	5.72 a (1.99)	5.41 a (2.21)	2.65 a (0.62)	4.52 ab (1.41)	6.85 b (1.21)	8.32 b (2.08)
Zn (μg/g)	99.27 a (27.06)	109.51 a (41.03)	189.39 b (75.31)	94.86 a (20.15)	98.32 a (19.52)	84.56 a (11.78)	102.06 ab (52.31)	136.07 ab (40.65)	150.40 b (71.66)
Fe (μg/g)	369.19 a (126.90)	212.45 ab (82.52)	224.68 ab (88.44)	132.63 b (27.93)	193.22 ab (60.01)	282.00 a (168.71)	146.23 a (59.98)	233.30 a (7.50)	244.20 a (95.41)

Values represent the mean and the standard deviation (in brackets). Concentrations are expressed μg/g dry weight. For each metal, different letters identify significant differences (Kruskal–Wallis tests, $p < 0.05$), either among sites or among seasons.

Table 4
Spearman correlation matrix among heavy metal concentrations in tissues and in sediments.

	Cd-s	Pb-s	Cu-s	Zn-s	Fe-s	Cd-t	Pb-t	Cu-t	Zn-t	Fe-t
Cd-s	1.00									
Pb-s	**0.33**	1.00								
Cu-s	**0.42**	**0.80**	1.00							
Zn-s	**0.20**	**0.62**	**0.82**	1.00						
Fe-s	**0.35**	**0.23**	**0.66**	**0.65**	1.00					
Cd-t	0.00	**0.22**	**0.44**	**0.31**	**0.31**	1.00				
Pb-t	0.05	0.01	**0.26**	**0.21**	**0.20**	**0.32**	1.00			
Cu-t	−0.14	**0.36**	**0.38**	0.09	0.02	**0.67**	−0.10	1.00		
Zn-t	−0.15	**0.57**	**0.44**	**0.24**	0.06	**0.50**	0.10	**0.68**	1.00	
Fe-t	−**0.47**	−0.13	−0.07	−0.01	0.11	0.14	0.07	0.17	0.10	1.00

Heavy metal concentration in mussel tissues (-t) and sediments (-s). Correlations are significant at $p < 0.05$ (bold).

Table 5
Results of two-way ANOVA for the biomarkers analyzed in gills and digestive gland of mussel *M. edulis chilensis*.

	CAT			GST			LPO		
	df	*F*	*p*	*df*	*F*	*p*	*df*	*F*	*p*
Gill									
Season	3	33.82	0.000*	3	11.80	0.000*	3	68.11	0.000*
Site	4	4.70	0.002*	4	4.90	0.002*	4	6.66	0.000*
Season*site	12	4.23	0.000*	12	10.65	0.000*	12	9.29	0.000*
Error	76			63			74		
Digestive gland									
Season	3	37.64	0.000*	3	2.71	0.052	3	46.49	0.000*
Site	4	2.61	0.043*	4	3.18	0.019*	4	15.51	0.000*
Season*site	12	10.26	0.000*	12	8.10	0.000*	12	9.21	0.000*
Error	72			68			62		

* Indicate significant result, $p < 0.05$.

gills of mussels from IZ and UP showed activities 4 to 6-fold lower than those from the other sites. In digestive gland, the lowest activities of this biomarker were measured in mussels from UP in summer and from IZ in winter.

GST activities (Fig. 3c and d) were higher in gills than in digestive gland in several cases. Significant differences among seasons were only recorded in gills. Higher activities were observed in summer and spring respect to autumn and winter. Differences among sites were significant in both analyzed organs (Table 5). In gills the activity was lower at IZ than at FD, NW and UP; meanwhile in digestive gland, lower values were observed in mussels from GB and FD than those from NW and UP. Regarding the interaction between sites and seasons (Table 5), GST activity in gills in summer was higher in mussels from IZ, FD and UP than in the other sites. In winter, the lowest activities were measured in organisms from GB and IZ. In spring, mussels from GB showed the highest values of GST. In digestive gland, the activity in summer was higher in mussels from IZ and UP; in autumn the highest values were measured in IZ and NW while in winter the highest activities were found in organisms from FD, NW and UP. No differences among sites were observed during autumn in gills and during spring in digestive gland.

Regarding LPO levels (Fig. 3e and f), a two-way ANOVA for gills and digestive gland, revealed that the effects of seasons and sites were significant, as well as the interaction between both variables (Table 5). In gills and in digestive gland we registered differences among seasons, being the highest levels in spring and the lowest in autumn. Differences among sites in gills showed higher activity in mussels from NW than those from IZ and UP. In digestive gland, the highest activity was recorded at IZ and the lowest at UP. The interaction between seasons and sites in gills revealed that the highest levels of LPO were in mussels from IZ in summer, from FD and NW in autumn and from GB and NW in spring. In digestive gland the highest LPO levels were registered at IZ in summer and winter, while the lowest one was measured in mussels from UP in spring. No differences among sites were recorded during winter in gills and during autumn in digestive gland.

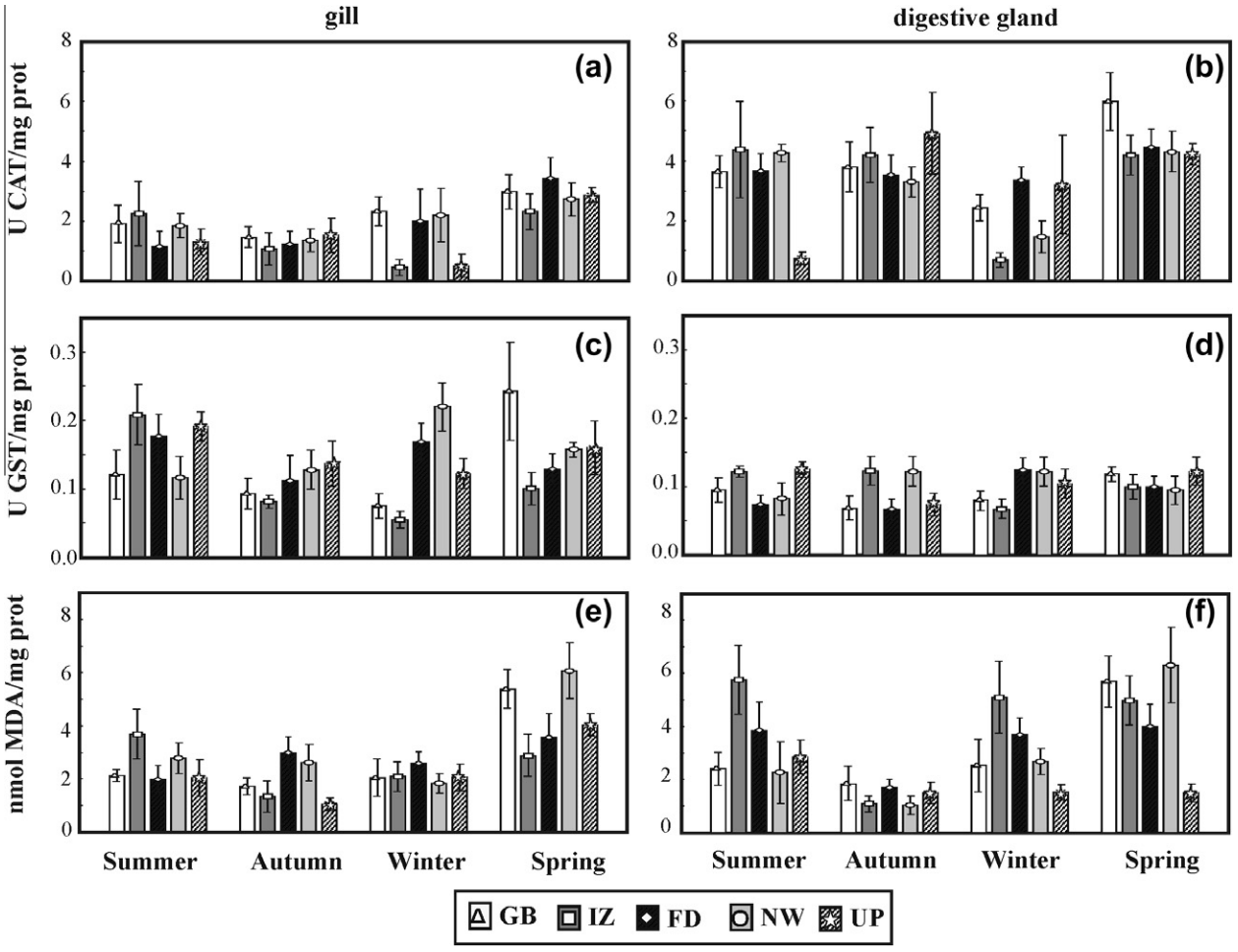

Fig. 3. Biomarkers responses at different sampling sites and seasons. CAT activity in gill (a) and digestive gland (b); GST activity in gill (c) and digestive gland (d) and LPO levels in gill (e) and digestive gland (f).

The PCA performed with biological and environmental data showed that 65% of the total variation is explained by the first four principal components (PC) (Table 6). The score for each case is presented in Fig. 4. The highest loadings of nutrients (except nitrate) and metals in sediment (except Pb) can be observed on the negative axis of PC 1 opposite to OD and salinity. According to mentioned parameters, UP was clearly separated from the other sites. PC 2 was represented by pH, nitrate, Pb, Cu and Fe in sediment, CAT and LPO in gills and Cd, Cu and Zn in tissue; all of them with positive signs (Fig. 4a). PC 3 was characterized by temperature and GST activity in digestive gland on positive axis. Finally, chlorophyll-*a* and Pb in sediment were positively correlated with PC 4, while GST activity in digestive gland was negatively correlated (Fig. 4b).

The differences recorded in some physical and chemical parameters such as pH, temperature, chlorophyll-*a*, particulate organic matter and nitrate concentration, were mainly related to seasonal changes and showed a similar trend at all sites for each sampling period. The registered low salinity values could be related to the input of rain water, industrial and/or domestic effluents and from thaw processes (Amin et al., 2011). Oxygen saturation could be directly related to the wind occurrence and intensity within the studied area, which may produce a strong mixing in the seawater column (Amin et al., 2011). Regarding inorganic nutrients, UP was the most affected site. The high concentrations of nutrient associated with low salinity are indicators of freshwater input from domestic and urban effluents.

According to the IUCI values, the highest concentrations of urban heavy metals (Cu, Zn and Pb) in sediment were recorded at IZ, FD and UP and this fact could be associated to several anthropogenic sources. These areas differ in their potential sources. In the case of IZ it may be attributed to industrial effluents and leaching of metals from garbage and solid waste dumps from the industrial area nearby. In FD, the high concentrations of Pb could be associated to the load and discharge of fuel that takes place at that port. Likewise, maritime traffic, hull corrosion and ship maintenance may be contributing to enhance concentrations of Cu, Zn and Pb and as well as Cd and Fe. Finally, UP receives metals principally from domestic effluents through sewage disperser, as well as, from stormwater runoff that could transport particles with adsorbed metals.

Table 6
PCA: correlations of environmental and biological variables with the first four principal components (PC).

Variables	PC 1	PC 2	PC 3	PC 4
pH	0.34	**0.87**	0.01	−0.03
DO	**0.64**	0.36	−0.01	0.23
Temperature	0.04	−0.14	**−0.83**	0.37
Chlorophyll-*a*	0.41	−0.03	−0.33	**0.63**
Particulate organic matter	−0.34	−0.46	−0.32	0.40
Ammonia	**−0.86**	−0.04	−0.20	0.00
Nitrite	**−0.93**	0.10	−0.21	−0.16
Nitrate	−0.31	**0.68**	−0.31	−0.10
Phosphate	**−0.88**	0.01	−0.15	−0.04
Silicate	**−0.65**	0.07	0.20	0.17
Salinity	**0.88**	−0.02	−0.05	−0.01
Cd-sediment	**−0.53**	0.31	−0.44	0.17
Pb-sediment	−0.18	**0.50**	0.34	**0.54**
Cu-sediment	**−0.54**	**0.70**	0.00	0.18
Zn-sediment	**−0.64**	0.37	0.25	0.35
Fe-sediment	**−0.59**	**0.54**	−0.29	0.01
CAT-gill	0.49	**0.65**	−0.36	−0.15
CAT-digestive gland	0.39	0.25	−0.38	0.32
GST-gill	0.08	0.11	**−0.65**	−0.41
GST-digestive gland	−0.12	0.26	−0.37	**−0.58**
LPO-gill	0.47	**0.54**	−0.47	−0.01
LPO-digestive gland	0.45	0.33	−0.10	0.06
Cd-tissue	−0.17	**0.58**	0.41	−0.34
Pb-tissue	−0.36	0.18	0.02	−0.01
Cu-tissue	0.18	**0.78**	0.37	−0.05
Zn-tissue	0.18	**0.55**	0.38	0.23
Fe-tissue	0.25	0.02	0.01	−0.23
Cumulative explained variance (%)	**26**	**45**	**57**	**65**

Correlations coefficients are significant when they are higher than 0.5 (bold coefficients).

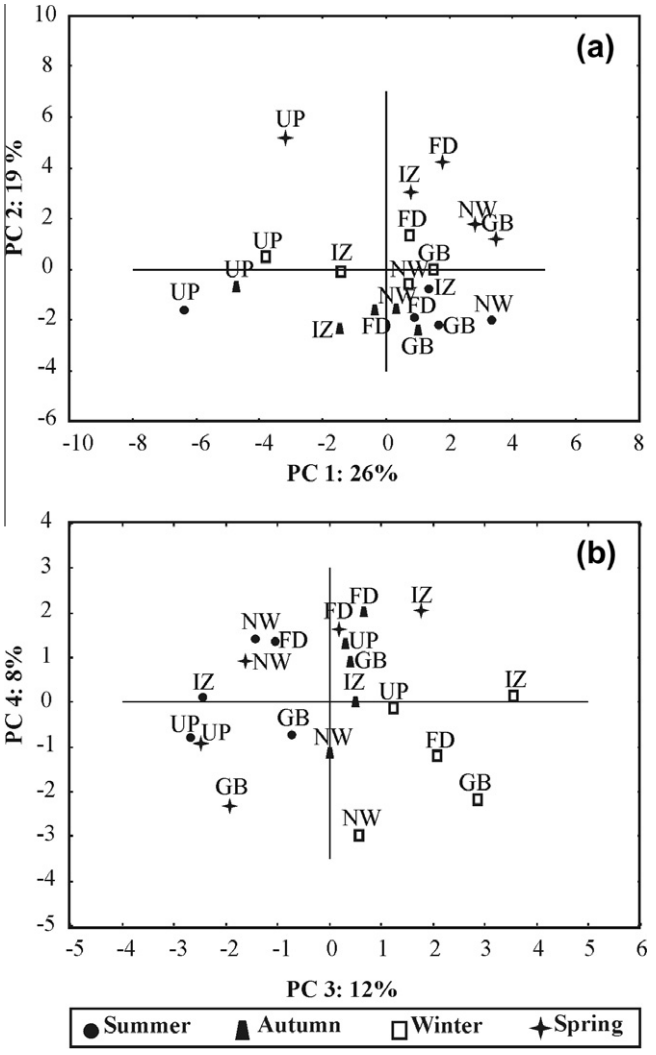

Fig. 4. Graphical representations of sites positions in the first four principal components of PCA, (a) PC 1 vs PC 2 and (b) PC 3 vs PC 4.

The levels of heavy metals in sediments at the present study were similar or even lower than those previously reported for the same environment (Amin et al., 1996b, 1997). They reported the highest values of Zn, Fe, Cu and Pb in sediments from FD and IZ. In addition to this, Giarratano et al. (2010) found high concentrations of Pb in sediments from IZ, FD and NW and high values of Cd in the south-western side of Ushuaia bay. Concentrations of Cd in sediments from Ushuaia and Golondrina bays found in this work were similar to those reported by the recent research of Giarratano et al. (2010) but clearly higher than the non detectable values reported by Amin et al. (1996a). This suggests that an input of this metal is actually affecting the environment. This is important, considering that Cd is highly toxic even at low concentrations (Marcovecchio and Ferrer, 2005; Sokolova et al., 2005). Presence of Cd in coastal and estuarine environments can be linked to both natural and non-point anthropogenic sources (Roesijadi, 1996). In the case of Ushuaia bay, Giarratano et al. (2010) explained that

its natural origin could be related to leaching from upwelling from marine sediment deposits; meanwhile the anthropogenic source could be linked to urban pollution.

On the other hand, the lowest concentrations of Cd, Pb and Cu in sediments found in GB indicated that these values could be considered as a baseline in comparison to those recorded at the other sites. Despite NW is close to the city, concentrations of all analyzed metals were similar or even lower than those found at GB. This could be attributed to the fact that the sampling point in NW is more distant from the direct discharge of effluents than at the other sites and a dilution process may be occurring. In this context, the IUCI revealed differences between sites indicating that GB and NW were the least urban influenced sites.

Metal accumulation in mussel's tissues revealed a different pattern from that observed in sediments. In fact, Spearman's correlation showed very low coefficients between both matrixes. Similar results were obtained with transplanted mussels by Giarratano et al. (2010) at the same area. Several environmental and biological factors such as salinity, seasonality, organic matter, tidal height, reproductive status and physiological condition of mussels could influence the accumulation of metals in mussel's tissues (Mubiana et al., 2006). However, effects of these factors in quantitative terms still remain unclear with different results reported depending on the studied species, element, location or season (Gil et al., 2006; Rainbow, 2002; Riget et al., 1996). According to Pempkowiak et al. (1999), metal speciation occurring in the sediments is expected to influence metal bioavailability and metal content in biota and, particularly, in the soft tissue of mussels. Heavy metals of anthropogenic origin are generally introduced into the environment as inorganic complexes or hydrated ions, which are adsorbed on surfaces of sediment particles through relatively weak physical and chemical bonds. Thus, heavy metals of anthropogenic origin are found predominately as labile and easily extractable fractions in sediments (Förstner, 1989).

Despite Zn concentration in sediments from IZ, FD and UP did not differ significantly, mussels from FD accumulated the highest levels of this metal. This fact reflects a different bioavailability of metals among sites. There are several factors that could affect the degree of bioavailability, such as type of source, granulometry, redox-potential and salinity. The source of Zn is probably different in each case. Zn would be contributed by urban effluent associated to organic matter in UP, in particles transported by Grande stream and industrial effluents in IZ and by port activities in FD. It is important to notice that the extraction technique used in this study allows the removal of total heavy metal; meanwhile mussels incorporate only the bioavailable fraction (Förstner, 1989).

High concentrations of Fe were observed in tissues of mussels from GB, IZ, FD and UP and the lowest concentration was registered in mussels from NW, coincidently where the lowest concentrations of this metal in sediment were also found. Particularly, in the present work and in agreement with previous data (Amin et al., 1996a, 1997; Giarratano et al., 2010), Fe was the most abundant metal in sediment with high concentrations even at unpolluted areas. Fe has a natural origin since the Andean rocks, that reaches our study area, are Fe-rich, principally present in the residual fraction associated to crystalline structures (Amin et al., 1996b; Dezileau et al., 2007) and it is not expected to be released. At most sites, Cd concentrations were higher in tissue than in sediment. This is in agreement with the results reported by Giarratano and Amin (2010) also for the Beagle Channel. In this sense, Gil et al. (2006) found Cd concentrations in tissue of molluscs even when no detectable levels of Cd were observed in sediment. This could be related to the increased solubility of this element in oxidized systems, like in Ushuaia and Golondrina bays, compared to other elements. 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).

In general, metal concentrations in mussels were found within the ranges reported by Gil et al. (2006) for Patagonian low impacted areas and did not exceed the permissible limits for human intake set by the National Service of Health and Food Quality (SENASA, 2008) which is 5 μg/g DW for Cd and 7.5 μg/g DW for Pb.

Differences observed in the responses of biomarkers between analyzed organs were consistent with other authors (Power and Sheehan, 1996) who indicated that GST activity is usually threefold higher in gills than in digestive gland; while CAT activity can be fourfold higher in digestive gland than in gills.

In agreement with previous studies (Lau et al., 2004), our results showed that oxidative stress is a highly seasonal phenomenon in bivalve molluscs from Ushuaia bays. Antioxidative enzymes are constitutive in nature and respond to variations due to intrinsic biological and ontogenic processes, physical and chemical parameters, anthropogenic pollutants (Lau et al., 2004) and food availability (Viarengo et al., 1991; Vidal et al., 2002). The interpretation of these responses in an environmental context is very complex taking into account all possible causes (Sheehan and Power, 1999).

Temperature has also been considered to be the most important factor affecting natural enzyme activity (Lau et al., 2004; Leiniö and Lehtonen, 2005) and also determining the reproductive cycle of *M. edulis chilensis* in Ushuaia and Golondrina bays. This species has a reproductive cycle with a prolonged spawning period which takes place principally in spring, after the sex cells are loaded with lipids, but partial spawning in summer is also possible depending on environmental conditions (Tortorelli, 1987). In the present study, the high values of biomarkers recorded in spring, which corresponds to the spawning period, would be mainly caused by biological processes. The increased metabolic rates during the spawning season (spring) could possibly increase the rate of ROS formation, resulting in oxidative stress (Verlecar et al., 2007). Leiniö and Lehtonen (2005) pointed out that, even under low pollutant conditions, seasonal factors may affect responses of biomarkers to a greater extent than pollutant stress. Except GST activity in digestive gland and LPO in gills, our results showed no differences in autumn among sites, which could be considered the post-spawning resting phase characterized by low metabolic activity. These results found in autumn are in agreement with regional biomarker studies (Amin et al., 2007). Similar results were also reported by Cancio et al. (1999) and by Sheehan and Power (1999) for other regions.

On the other hand, winter time marks the beginning of the reserve accumulation period (Tortorelli, 1987), so it could be considered a high exposure period. At this time, mussels rate of food uptake increases and thus, pollutant input rate also increases. In general, several authors reported higher metal levels in winter (Avelar et al., 2000; Odzak, 2002). The increase in winter or late winter may be due to life cycle differences that influence uptake, storage, and/or excretion or body condition (Burger and Gochfeld, 2006).

An inverse relationship was observed between CAT and LPO in digestive gland in mussels from GB and IZ in winter period. These sites showed a decrease in CAT activity and also an increment in LPO levels. Pampanin et al. (2005) observed an inverse proportion between LPO level and CAT activity. They suggested that CAT activity was reduced due to increased levels of pollution and that it was not sufficient to eliminate H_2O_2 before the formation of hydroxyl radicals leading to the enhancement of LPO levels. It has also been detected by Bebianno et al. (2005), who determined that high LPO levels could be the result of a deficiency in the antioxidant defence system.

Both inhibitory and stimulated effects were detected in CAT and GST activities. Whether a particular biomarker response is observable or not depends not only on the level of exposure, but also on how long after the exposure of the system is the measurement taken and if an efficient repair process occurs within the organism (Ansari et al., 2004). We must emphasize that we used native organisms. This factor has been mentioned by several authors (Cajaraville et al., 1997; Wepender et al., 2008) as responsible for low response in biomarkers, since mussels would be acclimated to the conditions in which they live, so the toxicity levels which they are continually exposed to may not be high enough to elicit a response.

PCA showed UP as the most impacted site, mainly by the highest concentrations of inorganic nutrients in water and some heavy metals in sediments. The main city effluent is discharged into the coastal marine environment precisely in UP and its colloidal and particulate matter after flocculation settles to the bottom, where is incorporated into the sediments. The major pollutants related to municipal effluents are organic matter, suspended solids, nutrients (nitrogen and phosphorus) and pathogenic micro-organisms, while other pollutants such as heavy metals and petroleum and chlorinated hydrocarbons, are also present in the effluents. Metals such as Cu, Zn and Fe are derived from human food consumption, while other metals can be introduced by cooking ware as well as a variety of household cleaning agents (Chambers et al., 1997). In addition, the lowest salinity evidences the freshwater input to this site, principally from domestic and urban effluents. The rest of the sites were associated in a separate group, but no clear correlations could be observed.

According with our results, Giarratano et al. (2010) found that CAT and LPO measured in mussels transplanted into Ushuaia Bay may be considered as good markers of urban pollution.

The results of PCA allowed establishing associations between biomarker responses and some biological and environmental variables studied. Increments in CAT activity and in levels of LPO in gills were correlated with increments in concentrations of Cd, Cu and Zn in tissues and Cu in sediment. There are several authors who reported that metals can both stimulate the formation of ROS and inhibit the normal via which these reactive species are removed of cells (Regoli and Principato, 1995; Stohs et al., 2000). In consequence, an induction of membrane lipid peroxidation process occurs in organisms (Stohs and Bagchi, 1995; Viarengo et al., 1990). On the other hand, Rainbow (1990) reported that metals like Fe, Zn, Cu, Cr, Co, Mn and Ni play an important role as biochemical and enzymatic cofactors so, correlations between metals and biological variables can be due to biochemical requirements and/or passive sequestration of these metals. GST activity in gills was positively associated with temperature. In this regard, Wilhelm Filho et al. (2000, 2001) found that the increment in temperature and food availability is followed by an increase in oxygen consumption and consequently in an increased cellular generation of ROS which can be compensated by increased antioxidant defences.

Ushuaia city and its bays are not immune to a decline in their environmental quality. In the present work oxidative stress biomarkers measured in gills and digestive gland of native mussels *M. edulis chilensis* were used successfully to monitor several sites of a coastal marine area with urban influences. In fact, the results obtained in this study showed impact of heavy metals, inorganic nutrients and particulate organic matter at the sampling sites.

This study constitutes the first work monitoring native mussels during a whole year from neighboring bays influenced by Ushuaia city. The results of a battery of biomarkers were evaluated in function of environmental data such as heavy metals, physical and chemical parameters.

The main city effluent, located at UP within Golondrina bay, has produced over the years the accumulation of organic matter and metals into this bay creating a non-point pollution source in this coastal marine environment. Cu, Zn and Fe were the metals which presented the highest enrichment in the sediments. It was also clear that water is impaired by high loading of nutrients.

The pollution of waters by municipal effluent remains an environmental issue since contaminants, such as metals, released into municipal sewer systems are more difficult to control than those released in industrial effluents. An urgent management is required to revert the negative impact of this chronic source of pollution.

The most affected sites by heavy metals in sediments were IZ, FD and UP due to industrial activity and Grande River, ship traffic and untreated urban waste discharges, respectively. The high Cd concentrations in comparison with previous data suggest an enrichment process. The sources could be natural such as upwelling or anthropogenic such as urban, domestic and industrial contributions. Heavy metal concentration in mussels and sediments were not correlated in all cases evidencing different bioavailability conditions.

The variability observed in biomarker responses could be assigned to differences in both pollution levels (intersite) and seasonal variability (intrasite). In spite of that, to be useful in environmental monitoring programs is necessary to take into account the natural variability of the biomarkers in relation to life cycle of the organisms as well as the incidence of another pollutants present in the environment which have not been analyzed in the present work.

Authors wish to declare that all of them have actively participated in the article preparation and approve this final version of the manuscript.

Acknowledgements

This study was supported with funds from grants by the Agencia Nacional Promoción Científica y Tecnológica (ANPCyT-PICT. PICT06 1261 and PICTR 090), Global Environmental Foundation, Proyecto de Naciones Unidas para el Desarrollo – GEF-PNUD ARG 02/018-B-CB-05. We are grateful to Sandra Botté, Nedda Chiarello, Raúl Asteasuain and Ricardo Saenz Samaniego for their valuable collaboration. We also wish to thank the reviewers for their comments which allowed to improve the manuscript.

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