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In situ experiment to evaluate biochemical responses in the freshwater mussel *Diplodon chilensis* under anthropogenic eutrophication conditions

M.S. Yusseppone[a,*], V.A. Bianchi[b], J.M. Castro[b], T. Noya Abad[a], Y.S. Minaberry[c], S.E. Sabatini[a], C.M. Luquet[b], M.C. Rios de Molina[a], I. Rocchetta[b,**]

[a] Departamento de Química Biológica, IQUIBICEN, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

[b] Laboratorio de Ecotoxicología Acuática, INIBIOMA, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET-UNCo), CEAN, Junín de los Andes, Neuquén, Argentina

[c] Departamento de Química Inorgánica, Analítica y Química Física, INIQUIMAE, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

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ABSTRACT

An *in-situ* experiment was performed to study metabolic responses of the freshwater mussel *Diplodon chilensis* to water contaminated by leachates from an open dump and cattle activity, in order to analyze both the effects of those contaminants on aquatic environments and the potential use of a native bivalve to evaluate the effects of anthropic influence and eutrophication. Bivalves from a reference site were cage-transplanted to a control site (site A) and to a temporal water pond (site B) over 30 and 60 periods. Water quality analyses revealed that the site B was affected by anthropogenic influence. Mussel's hemocytes from site B showed 50% lower reactive oxygen species production and 130% higher lysosomal membrane stability in the site B mussels. In addition, no oxidative stress was evident in gills, despite the elevated copper and iron concentrations recorded in the site B water samples (Cu_B = 0.3350 ± 0.0636 mg. L^{-1} *vs.* Cu_A = 0.0045 ± 0.0007 mg. L^{-1}; Fe_B = 3.8650 ± 0.4031 mg. L^{-1} *vs.* Fe_A = 0.0365 ± 0.0049 mg. L^{-1}). In contrast, the adductor muscle accumulated more Fe (~10–20-fold) than the gills and showed signs of oxidative stress, e.g. superoxide dismutase activity and TBARS levels were increased by 10% were 34%, respectively, in the site B compared with the site A after 60 days of exposure. Additionally, the adductor muscle showed signs of anaerobic metabolism activation. Cu is accumulated in gills from both sites' individuals, at 60 days, in concordance with the increase in the activity of the cu-containing enzyme cytochrome-c-oxidase. There was a reduction in the overall condition and digestive gland index in bivalves exposed at site B, associated with diminished levels of lipid and protein contents. Metal-pollution and eutrophication affects *D. chilensis* metabolism and is associated to tissue-specific exposure, anaerobic metabolism and general energetic condition depletion.

1. Introduction

The disposal of untreated domiciliary and industrial waste generates large amounts of leachates, which can alter biogeochemical processes with implications on aquatic communities (Miserendino et al., 2011). These leachates, which are composed by organic and inorganic substances, may migrate from the dumpsite by water superficial flow or by percolating through the underground layer to nearby freshwater bodies and affect the environmental health (Bakare et al., 2005; Ololade et al., 2019; Sokefun, 2008). In contrast with sewage water, which can be treated in specific plants with bacteriological and physicochemical processes (e.g. Sepehri and Sarrafzadeh, 2018, 2019), leachates generate diffuse inputs of organic matter and inorganic pollutants, which are only treated by natural plants and soil microorganisms before they reach the water bodies. Organic matter inputs, combined with elevated temperatures, promote stratification with long periods of oxygen depletion at the bottom of aquatic ecosystems (Diaz and Rosenberg, 2008; Paerl et al., 1998), which can cause hypoxia events. Hypoxia is a natural condition of many aquatic ecosystems produced in shallow waters with poor circulation or stratification periods with large loads of terrestrial organic matter (Diaz and Breitburg, 2009). Eutrophication-driven hypoxia events have been more frequently observed in

* Corresponding author.
** Corresponding author.
E-mail addresses: msyusseppone@gmail.com (M.S. Yusseppone), irocchetta@gmail.com (I. Rocchetta).

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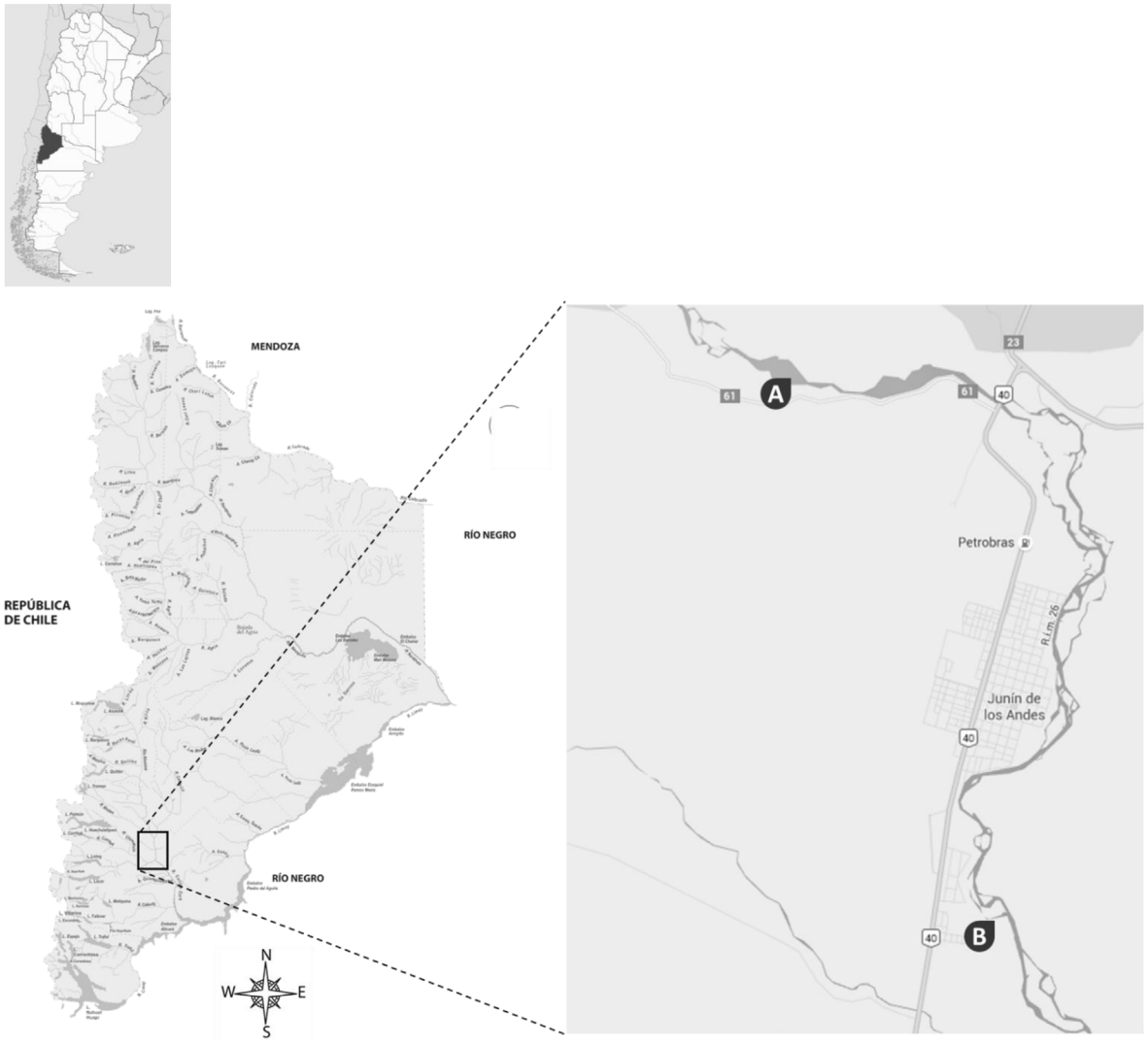

Fig. 1. Location of sampling sites, site A (39°54.953′S; 71°06.363′W) and site B (39°59.046′S; 71°04.079′W) at Chimehuin river, Neuquén province, Argentina.

freshwater and marine systems during the last decades, as result of human activity, being probably the main driving factor in the spreading of coastal dead zones (Diaz and Breitburg, 2009; Diaz and Rosenberg, 2008). There is abundant literature describing an induction of ROS production by hypoxia, which triggers metabolic signaling and improves the hypoxia-tolerance (Bell et al., 2007; Bickler and Buck, 2007; Clanton, 2007; Guzy et al., 2005; Hermes-Lima et al., 2015; Lushchak and Bagnyukova, 2006). On the other hand, several hypoxia tolerant species display ATP suppression or conservation through metabolic rate depression (MRD) to cope with this condition (Storey and Storey, 1990, 2007; Wheaton and Chandel, 2010). When hypoxia leads to MRD, respiratory rate and ROS production decrease; thus, no oxidative stress is produced (Yusseppone et al., 2018). The study of metabolic responses in natural environments altered by urban discharges that induce eutrophication and hypoxia is complex. High levels of organic matter, nutrients enrichment and elevated temperatures favor algal growth and the response of aquatic fauna can be controversial and species-specific.

The key species in many Andean Patagonian benthos communities is the freshwater bivalve *Diplodon chilensis* (Ribeiro Guevara et al., 2005; Torres et al., 2018). This bivalve can live in oligotrophic to eutrophicated waters at varied densities (Lara and Parada, 1988; Rocchetta et al., 2014). Its survival under experimental eutrophicated conditions was related to its anaerobic capacities (Grandón et al., 2008), and its hypoxia-anoxia tolerance in the laboratory involves the induction of the mitochondrial enzyme alternative oxidase together with MRD (Yusseppone et al., 2018). These attributes and the large number of studies on this species' ecology, growth, filter-feeding, reproduction, immune system, and toxicology, make *D. chilensis* an emerging model for the study of the effects of anthropic influence and eutrophication. Patagonian lakes and rivers are valuable resources not only from an ecological but also from a social and economic perspective. During the last years, untreated wastes have been discharged directly to the aquatic environments with consequent water quality degradation. Particularly, the open dump located downstream of Junín de los Andes city neither have a synthetic membrane liner at the bottom, nor a natural layer of compacted soil with the desired hydraulic conductivity or run-off control system. Despite it is not currently active, the leachates produced during the last decades have been discharged into the environment in close relation with the rainfall rates, as it was previously reported for other systems (Bakare et al., 2005), threatening one of the most important touristic rivers in Patagonia, the Chimehuin river. Environmental contamination due to untreated waste disposal is an important concern in developing countries, which deserves further investigation (Bakare et al., 2005; Ololade et al., 2019; Sokefun, 2008). The evaluation of contamination by using biomarkers may provide an insight on the current health status of aquatic environments and drinking water sources and a valuable tool for biomonitoring programs.

Based on this background, this paper aims to characterize the

metabolic response of *D. chilensis* to a temporal pond, which is contaminated by leachates from the open dump of Junín de los Andes city and by the occasional presence of cattle, by "*in situ*" exposure in cages during 30 and 60 days. This pond was selected as model environment because it is not inhabited by *D. chilensis* but is located near the margin of the Chimehuin river, where this species is abundant. The biological effects studied include metal accumulation in tissues, antioxidant response, oxidative damage and ROS production, lysosomal membrane stability, enzymes involved in aerobic and anaerobic metabolism and the energy status assessed through the contents of the main reserve compounds and morphometric ratios. These measurements were accompanied by analysis of physicochemical and biological variables in water and organic matter content in the sediment.

2. Materials and methods

2.1. Mussel collection and experimental design

Adult *D. chilensis* (75.1 mm mean ± 7.2 mm SD shell length) were collected by SCUBA diving in July 2012 from Paimún lake (39°44.78′S 71°37.48′W) and randomly allocated and caged in PVC containers in a temporal water pond, close to the Chimehuin river (39°59.046′S; 71°04.079′W, site B), and in a clean site in the same river (39°54.953′S; 71°06.363′W, site A) (Fig. 1). The cages were filled with sediment from the superficial layer from each site and conditioned to allow the water flux. Mussels were kept in cages for 30 and 60 days of exposure (n = 12 per site and time).

2.2. Water quality

Water samples were taken and analyzed at the beginning of the experiment (austral winter, July) at the two sites (A and B). The seasonal variability of physicochemical and biological quality in the area of this study are extensively described in Yusseppone et al. (2019).

Chlorophyll *a* concentration was analyzed according to Lichtenthaler (1987). For total and fecal coliform bacteria, water samples (n = 3) were collected in sterile containers and kept at 4 °C until the analysis was carried out using the Most Probable Number method (APHA, 1998). Results were expressed as MPN/100 mL. Dissolved oxygen (mg L^{-1}), pH, conductivity (μS cm^{-1}) and temperature were measured *in situ* using a multi-parameter analyzer (Hanna HI 9828). For physicochemical analysis, the samples were collected in 5% HCl pre-washed bottles and immediately processed by using spectrophotometric methods (HACH DR/4000 spectrophotometer) (n = 3). Turbidity was measured by Attenuated Radiation Method (HACH Method 10,047) and expressed as Formazin Attenuation Units (FAU). Total Nitrogen (TN) and Total Phosphorus (TP) were measured after alkaline persulfate digestion by the ascorbic acid and cadmium reduction methods, respectively (HACH Method 8192, 8048). Filtered water samples (GF/F 0.45 μm) were used to measure total ammonia concentration (mg L^{-1}, HACH Method 8155), nitrite (mg L^{-1}, 8507), phosphate (mg L^{-1}, 8048), iron (mg L^{-1}, 8147), copper (mg L^{-1}, 8506) and silica (mg L^{-1}, 8185), and chromium was measured with a LaMotte QW200 detector (Method 3645-SC).

2.3. Organic matter content in sediment

Sediment was collected from the upper 5 cm (n = 3) and transported to the laboratory at 4 °C, dried until constant mass at 90 ± 5 °C for 24 h, then burned at 550 °C for 5 h. The difference between the initial and final mass was considered as Organic Matter content (OM). Results were expressed as % OM (Dean, 1974).

2.4. Tissue preparation

The external gill and anterior and posterior adductor muscle from each animal were weighted and homogenized in 134 mM KCl solution (ratio 1:5 g tissue mass mL^{-1}) with protease inhibitors (0.2 mM benzamidine and 0.5 mM PMSF). A fraction of homogenate was centrifuged for 15 min at 11,000 × *g* and the supernatant was used for enzyme assays and the determination of thiobarbituric acid reactive substances (TBARS). Cytochrome-c-Oxidase (CcO) activity in both tissues was measured in total homogenates.

2.4.1. Metals content in tissues

Total homogenates of gill and adductor mussel were digested in 65% nitric acid (HNO_3) at 90 °C until total evaporation (5 h), followed by further digestion in 32.5% HNO_3 containing 12% H_2O_2 until total evaporation (10 h). Each digestion was repeated twice. The obtained powders were re-suspended in 15 mL 5% HNO_3 and filtered through a 0.45 nylon filter (Di Salvatore et al., 2013).

The labware was carefully cleaned and analytical grade (ACS specifications) reagents were used. Standard curves were constructed using Merck certified stock solutions. To overcome matrix interferences, all reagents used to treat the samples were added to the working standards in the same proportions. Every time a set of samples was digested a procedural blank was made to correct sample readings. Total trace metals in *D. chilensis* gills were determined using atomic absorption spectrometry with graphite furnace atomization (GFAAS, Shimadzu 6800) and flame atomic absorption spectrometry (FAAS). Instrumental parameters and graphite furnace programs were those provided by the manufacturer (Conti et al., 2011, 2012). Traceability of results was obtained from the analysis of the certified reference material Antarctic krill MURSTISS-A2 (Italian Research Program in Antarctica). The mean recovery percentages (five replicates) were around 90–110%. Results were expressed as mg metal g^{-1} wet tissue mass (WM).

2.4.2. Enzyme activity

Superoxide dismutase (SOD) activity was measured by the inhibition of nitro blue tetrazolium (NBT) reduction at 560 nm following the technique of Beauchamp and Fridovich (1971). One SOD Unit was defined as the amount of enzyme necessary to inhibit 50% NBT reduction rate. Glutathione-S-transferase (GST) activity was measured by the technique of Habig et al. (1974) in the presence of 1chloro-2,4dinitro-benzene (CDNB) and recording the changes in absorbance at 340 nm during 90 s. One GST Unit was defined as the amount of enzyme necessary to catalyze the formation of one μmol of DNP-SG per minute at 25 °C. Lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) activity were measured by the technique of Childress and Somero (1979). The rate of NADH oxidation was monitored at 340 nm during 90 s for LDH and 60 s for MDH, and a correction for unspecific NADH oxidation was performed. One enzymatic Unit was defined as the amount necessary to catalyze the formation of one μmol of NADH per minute at 25 °C. Cytochrome-c- Oxidase (CcO) activity was measured by the technique of Hardewig et al. (1999). The rate of cytochrome-c oxidation-was monitored at 550 nm. One CcO Unit was defined as the amount of enzyme needed to oxidize one μmol of Cytochrome-c per minute at 25 °C. Citrate synthase (CS) activity was measured by the technique of Srere et al. (1963). The formation rate of the chromophore complex CoA-SH and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) was monitored at 412 nm. One CS Unit was defined as the amount of enzyme needed to the formation of one μmol of DTNB-SH-CoA-complex per minute at 25 °C. Enzyme activity data were expressed as Units g^{-1} WM. All the enzymes activities were measured spectrophotometrically (UV-160A, Shimadzu).

2.4.3. Oxidative damage

Lipid peroxidation levels were estimated by spectrophotometric quantification at 535 nm (UV-160A, Shimadzu) of thiobarbituric acid reactive substances (TBARS) according to Buege and Aust (1978) (modified, Yusseppone et al., 2015). Data were expressed as nmol TBARS g^{-1} WM.

2.5. Effects on hemocytes

Hemolymph (1 mL) was withdrawn from the adductor muscle of 6 individuals per time and site using a sterile syringe and working on ice. Part of the hemolymph was used for lysosomal membrane stability and the remaining was washed and resuspended in anticoagulant solution (3 g L^{-1} glucose and 0.36 g L^{-1} trisodium citrate, 60 mOsm L^{-1}, pH 7, Castro et al., 2017) to obtain the hemocytes fraction for measuring ROS production. Hemocytes lysosomal membrane stability was measured by the neutral red retention time (NRRT50) method (modified from Mamaca et al., 2005). Briefly, 50 μL of hemolymph were placed on slides (in duplicate), kept in a wet chamber for 5 min and then 0.002% neutral red solution (50 μL in dimethyl sulfoxide) was added. The number of red cells was counted under microscope until stained cells reached 50%. Data were expressed as NRRT50 (min). Reactive oxygen species (ROS) production was measured with a Qubit fluorometer (Invitrogen) (Bianchi et al., 2014a, modified from Moss and Allam, 2006). ROS content was referred to a H_2O_2 standard curve with H_2DCF-DA (2,7 dichlorofluorescein diacetate, 0.8 mM, Sigma) and results were expressed as mmol H_2O_2 mg^{-1} protein.

2.6. Energy status in digestive gland

Digestive gland (DG) was homogenized (1:5 g tissue mass mL^{-1} of 134 mM KCl, 0.2 mM benzamidine and 0.5 mmol L^{-1} PMSF), to estimate total lipid, protein and carbohydrate content. Lipids were extracted by the technique of Bligh and Dyer (1959) using a chloroform-methanol mixture (2:1). Total protein content was measured by Bradford's (1976) technique using bovine serum albumin as standard. Glycogen content was determined by a spectrophotometric method (Van Handel, 1965) using anthrone as reagent and SIGMA standard glycogen for the standard curve. Data are expressed as mg g^{-1} dry tissue mass (DM). Energy contents were calculated using the conversion factors for aquatic invertebrates described by Beningher (1984) (carbohydates 4.1 kcal g^{-1}, proteins 4.3 kcal g^{-1} and lipids 7.9 kcal g^{-1}) and data were expressed as cal g^{-1} DM. The condition index (CI, Davenport and Chen, 1987) was calculated as the ratio between wet soft tissue mass (g) and shell mass (g) x 100. The digestive gland index (DGI) was calculated as wet digestive gland mass (g)/shell length (mm); (n = 12 per time and site). A fraction of the dissected digestive gland was dried (65 °C, n = 6) until constant mass to estimate the wet mass/dry mass ratio.

2.7. Statistical analysis

Water physicochemical and biological variables were compared between sites by Student's *t*-test. Biochemical data were compared between sites and times by two-way ANOVA followed by Bonferroni's post hoc test. Assumptions of homocedasticity and normality were tested by Bartlett's and Lilliefors tests, respectively (Sokal and Rohlf, 1999). Analyses were performed using GraphPad Prism 6 and Statistica v.7 software. Data were expressed as mean ± SD.

3. Results

3.1. Water quality

There were significant differences among sites in most of the analyzed variables. Temperature, conductivity, turbidity and concentrations of chlorophyll *a*, total ammonia, iron, copper and silica were higher in water samples from the site B than in those from the site A ($p < 0.05$). Besides, total nitrogen, total phosphorus and % OM were higher ($p < 0.05$), while oxygen concentration was lower ($p < 0.05$) at the site B than at the site A. There were no differences between sites in total coliform bacteria, and fecal bacteria were not detected (Table 1). Chromium was below the detection limits.

Table 1
Water physicochemical and biological variables in samples collected from the site A and the site B at the beginning of the experiment (austral winter, July).

Parameter	A		B	
	Mean	SD	Mean	*SD*
T (°C)	9.83	1.04	16.6*	1.00
pH	6.80	0.01	6.42*	0.25
DO (mg. L-1)	9.27	0.06	6.62*	0.06
Conductivity (μS. cm-1)	31.30	0.30	314.33*	7.37
Turbidity (FAU)	1.50	0.71	128.5*	0.71
Ammonia (mg. L-1)	0.20	0.02	3.63*	0.29
Nitrite (mg. L-1)	0.0032	0.0002	0.0032	0.0002
Phosphate (mg. L-1)	0.0100	0.0010	0.0100	0.0010
Iron (mg. L-1)	0.0365	0.0049	3.8650*	0.4031
Copper (mg. L-1)	0.0045	0.0007	0.3350*	0.0636
Silica (mg. L-1)	7.15	1.06	15.00*	0.85
Total N (mg. L-1)	0.70	0.14	3.20*	0.59
Total P (mg. L-1)	0.3395	0.0007	0.7170*	0.0099
Chlorophyll a (mg. L-1)	0.87	0.09	3.62*	0.09
Total coliform bacteria (MPN. mL-1)	26.5	4.9	10.0	1.0
Fecal coliform bacteria (NMP. mL-1)	ND		ND	
OM (%)	7.46	3.70	48.06*	5.02

Differences between sites were assessed by Test-T, *significant differences at $\propto = 0{,}05$, n = 3. ND: Non-detectable values.

3.2. Metals content in tissues

Iron content was higher at the site B than at the site A, in both, gill and adductor muscle tissue (two-way ANOVA between sites $p < 0.05$ for both tissues). Fe content reached levels about 10- to 20-fold higher in adductor muscle than in gills (Fig. 2A, C). Cu content was not affected by the site but changed with time of exposure. In the gills, Cu was about two-fold higher at 60 than at 30 days of exposure regardless of the site (two-way ANOVA between times $p = 0.05$) (Fig. 2B). The opposite trend was recorded in muscle, where Cu content levels were higher at 30 than at 60 days of exposure in both sites (two-way ANOVA between times $p < 0.01$) (Fig. 2D). Cr, Cd, Co and Pb were also measured but were below the blank of reaction in all the samples.

3.3. Enzyme activity

Gill SOD activity was not affected by time or site (Fig. 3A), while GST activity was 30% higher in the gills of the polluted site bivalves than in the control ones (two-way ANOVA between sites $p < 0.01$) (Fig. 3B). On the other hand, adductor muscle SOD activity was 10% higher at the site B than at the site A regardless of the time of exposure (two-way ANOVA between sites $p < 0.05$) (Fig. 3C), whereas GST activity was not altered by time or site in this tissue (Fig. 3D).

In gill tissue, CcO activity was 118% higher at 60 than at 30 days of exposure (Fig. 4A) (two-way ANOVA between times $p < 0.0001$), whereas no significant differences were observed in CS activity (Fig. 4B). In adductor muscle, neither CcO (Fig. 4C) nor CS activity (Fig. 4D) showed differences between sites or between times.

Gill MDH activity decreased 24% in bivalves at 60 days relative to that at 30 days (two-way ANOVA between times $p < 0.05$, Fig. 5A), while gill LDH did not vary with site or exposure time (Fig. 5B). In adductor muscle there was no significant change in MDH activity (Fig. 5C), whereas LDH showed 90% higher activity at the longest time of exposure and was 30% higher in site B bivalves than in site A ones ((two-way ANOVA $p < 0.01$ and $p < 0.0001$ between sites and times, respectively)) (Fig. 5D).

3.4. Oxidative damage

Gill TBARS levels did not differ significantly between sites or times (Fig. 6A), while in the adductor muscle there was a significant site-time

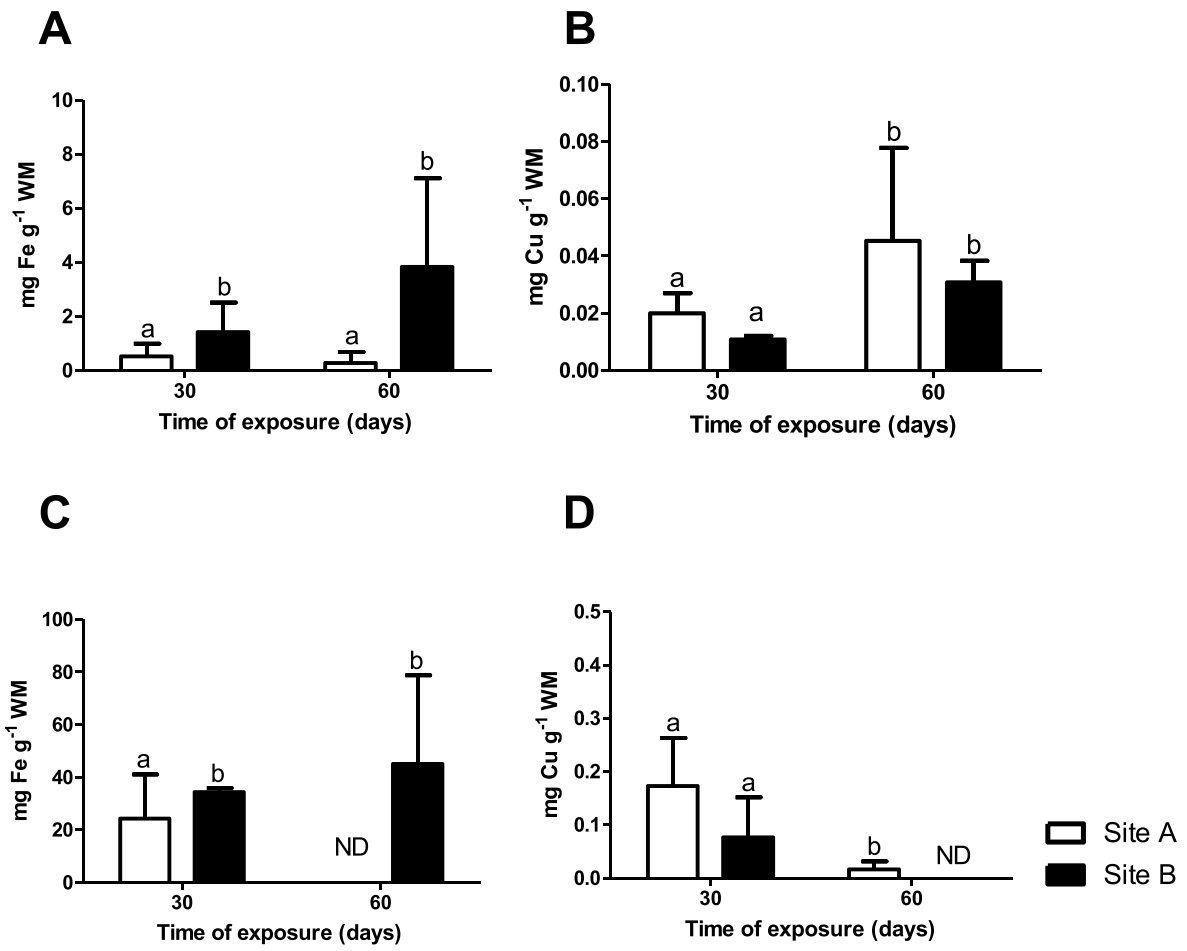

Fig. 2. Metal concentrations of iron (A) and copper (B) in gills and iron (C) and copper (D) in adductor muscle of *Diplodon chilensis* exposed at the sites A and B during 30 and 60 days. Different letters indicate significant differences with $p < 0.05$, $n = 3$ (per site and time). ND: non-detectable values.

interaction ($p < 0.01$) with significantly lower TBARS levels in the site A at 60 days of exposure than in any other site-time combination (Bonferroni test $p < 0.05$). TBARS levels were similar at both sites at 30 days, with a decrease of 33% only in the site A at 60 days of exposure, while it remained unchanged in the site B (Fig. 6B).

3.5. Effects on hemocytes

The hemocytes' lysosomal membrane stability, estimated as NRRT50 was 130% higher at the site B than at the site A regardless of the exposure time (two-way ANOVA between sites $p < 0.0001$) (Fig. 7A). In addition, the ROS formation rate in these cells was 50% higher at the site A than at the site B. The levels of this variable were lower at 60 than at 30 days of exposure (two-way ANOVA $p < 0.01$ for site and time factors, Fig. 7B).

3.6. Energy status

Although the total energy content did not change significantly; the analysis of each biochemical component showed a decrease along time of exposure of the digestive gland protein and lipid levels (two-way ANOVA between times $p < 0.05$ for both, Fig. 8A); while glycogen content did not differ between sites or times. The condition index decreased significantly with time of exposure and was higher in bivalves from the site A compared to those from the site B (two-way ANOVA $p < 0.05$ for both factors, Fig. 8B). In concordance with this result, digestive gland index was lower in the site B mussels than in those at the site A (two-way ANOVA between sites $p < 0.05$, Fig. 8C).

4. Discussion

The analysis of the temporal pond (site B) water quality shows a high nutrient load with high levels of N, especially as total ammonia, which is most probably due to the occasional presence of cattle. This is combined with elevated temperature and organic matter concentration in water and sediments, which lead to the reduction of the oxygen concentration, as it typically occurs in polluted eutrophicated waters. The increased phytoplankton productivity, inferred from the higher chlorophyll a level in this pond compared with the control site in the river, seems to partially counterbalance the oxygen depletion, thus the water is not strictly hypoxic. In addition, we have detected high Cu and Fe concentrations (about 100- and 75-fold higher in the site B than in the site A, for Cu and Fe respectively), most probably supplied by leachates from the city dump.

The study of caged individuals reveals tissue-specific effects of the polluted environment on *D. chilensis*. Despite the high Cu and Fe concentrations recorded in water from the site B and the important Fe accumulation in gills of site B individuals, no oxidative stress is evident in this tissue, except for an increment in GST activity. In contrast, the adductor muscle of site B mussels accumulates about 10 to 20-fold more Fe per gram of wet tissue mass than the gills and shows signs of oxidative stress (increased SOD activity) and oxidative damage (33% higher TBARS content) after 60 days of exposure. In fact, mussels exposed at both sites show similar TBARS levels at 30 days, which significantly decrease at 60 days in the site A mussels but not in the site B ones. This suggests that the oxidative damage recorded at 30 days reflects transplantation effect, as it has been previously reported for this

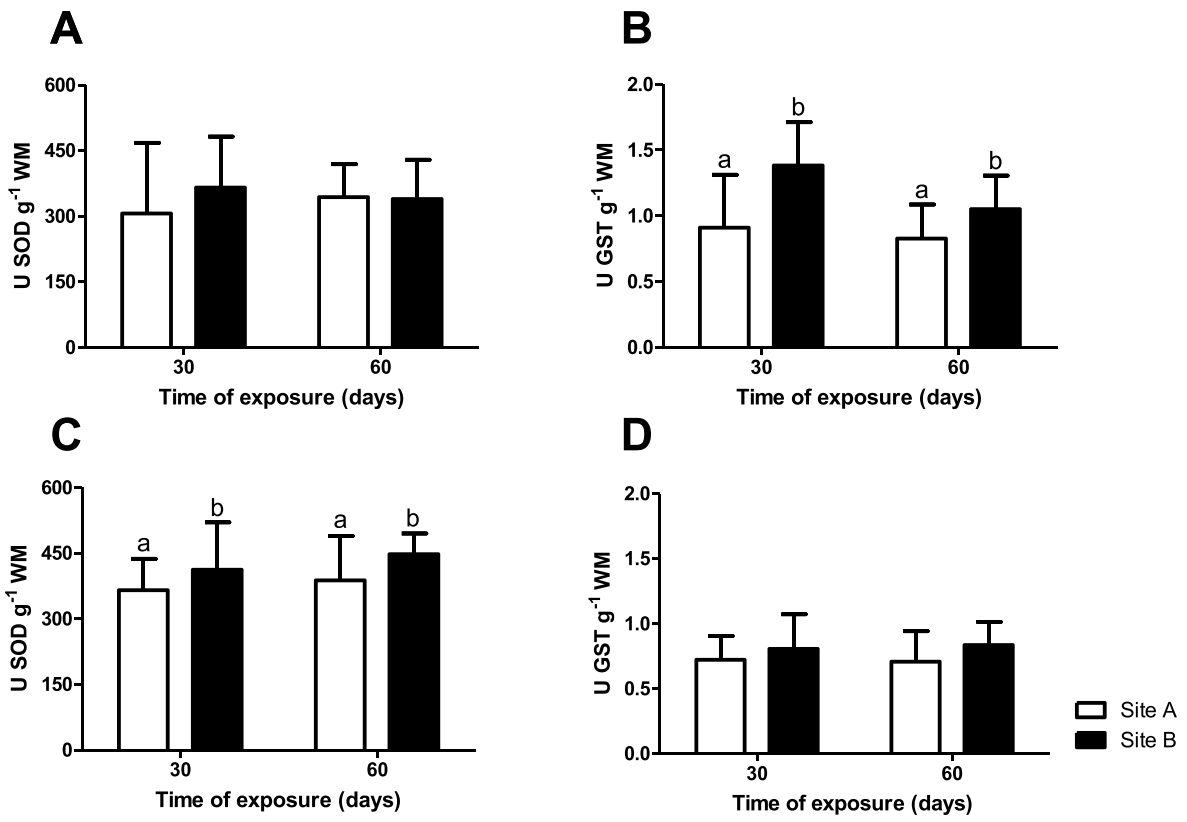

Fig. 3. Gill activity of superoxide dismutase (SOD) (A) and glutathione-S-transferase (GST) (B) and adductor muscle activity of SOD (C) and GST (D) in *Diplodon chilensis* exposed at the sites A and B during 30 and 60 days. Different letters indicate significant differences with $p < 0.05$, $n = 12$ (per site and time).

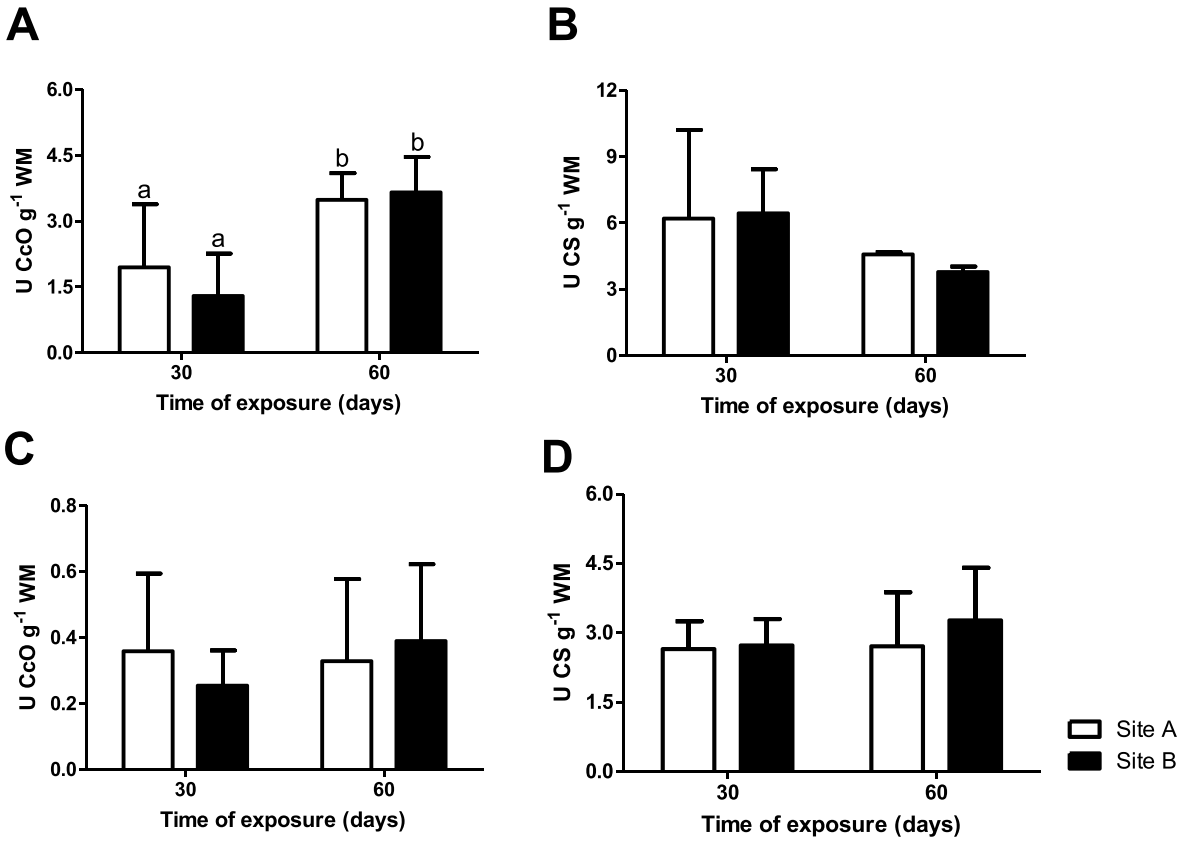

Fig. 4. Gill activity of citrate synthase (CS) (A) and cytochrome-c- oxidase (CcO) (B) and adductor muscle activity of CS (C) and CcO (D) in *Diplodon chilensis* exposed at the sites A and B during 30 and 60 days. Different letters indicate significant differences with $p < 0.05$, $n = 12$ (per site and time).

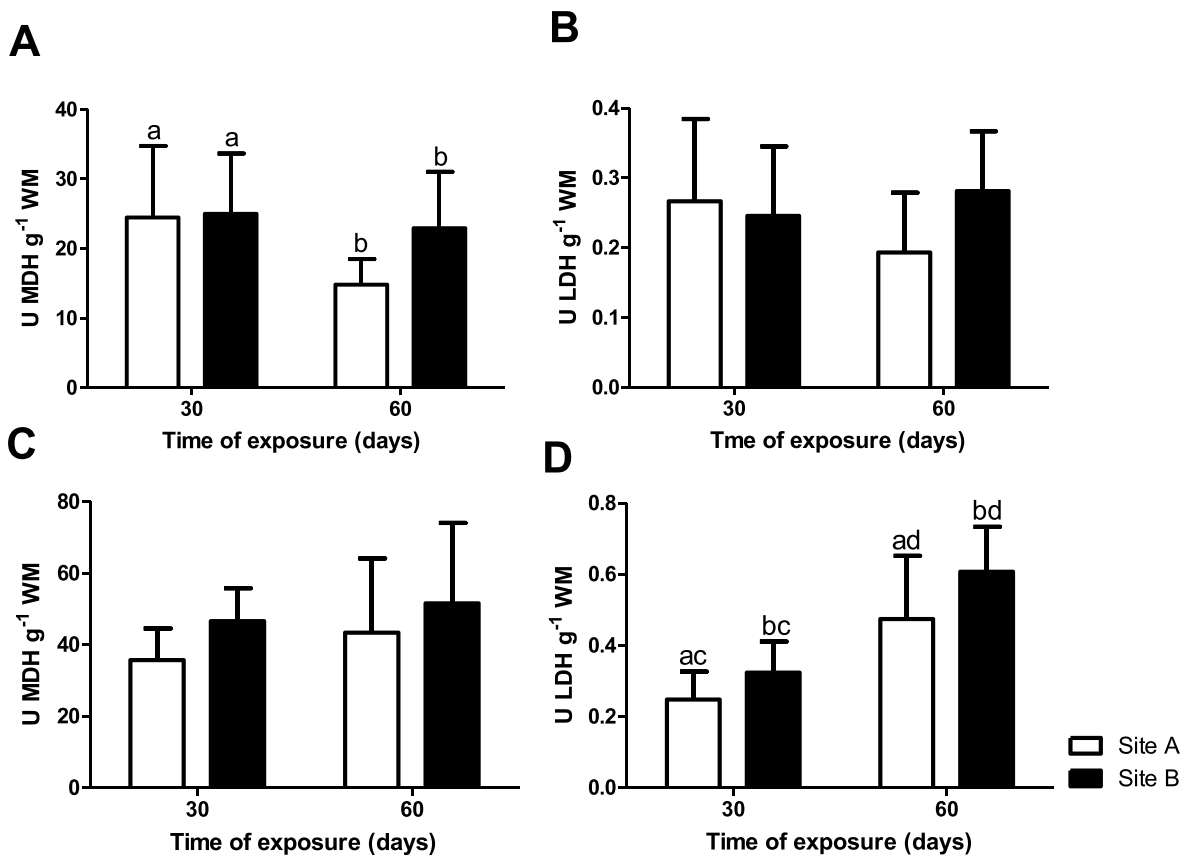

Fig. 5. Gill malate dehydrogenase (MDH) (A) and lactate dehydrogenase (LDH) (B) activity, and adductor muscle MDH (C) and LDH (D) activity in *Diplodon chilensis* exposed at the sites A and B during 30 and 60 days. Different letters indicate significant differences with $p < 0.05$, $n = 12$ (per site and time), a and b indicate differences between sites, c and d indicate differences between times.

species by Bianchi et al. (2014b), and that the site B individuals cannot compensate this effect. While the exposure to metals can stimulate ROS production through Haber-Weiss and Fenton-like reactions (Regoli and Giuliani, 2014; Sergent et al., 2018) and induce oxidative stress as has been reported for mollusks (Jara et al., 2004; Ruiz et al., 2018; Sabatini et al., 2011), metal bioaccumulation and toxicity in different tissues can be influenced by metabolic responses, including excretion and detoxification (Götze et al., 2014; Ivanina et al., 2014). Bivalves rapidly absorb and accumulate metals in soft tissues (Deb and Fukushima, 1999). Although the involved organs and transporters have not clearly identified for this group, both Fe^{2+} and Cu^{2+} uptake probably occur in the gills and/or in the digestive system. The divalent metal transporters (DMTs), which are apical metal/H^+ symporters expressed in various tissues of plants, fungi, fish and mammals, are the principal candidates for Fe^{2+} and Cu^{2+} entry to both, gill and digestive tract the cells, although Cu^+ can cross the membrane through Na^+ transporters (Bury and Grosell, 2003 for a review). In our experiment, the mildly acidic pH and the low oxygen content of the site B favor the predominance of Fe^{2+} over the insoluble Fe^{3+} and its uptake at the gill epithelium, probably through DMTs. According to Gobi et al. (2018, 2019), in fish, metals are initially accumulated in the gills and are later accumulated in other organs. Once inside the organism, Fe can be bound to specific storage and transport proteins, such as ferritin and transferrin (Zhang et al., 2003), while Cu can be bound to metallothioneins and used for the synthesis of metalloproteins (Dallinger et al., 2005). Although the Cu content in the gills of *D. chilensis* does not differ between sites, it is increased at 60 days in bivalves from both sites. This could be related to the synthesis of metalloproteins, which use this metal as cofactor, such as hemocyanin and oxygenase and oxidase enzymes (Andaman, 1991; Liu et al., 2014). In concordance, we have recorded an increase of the activity of CcO, which is a key metalloenzyme complex in aerobic metabolism, at 60 days, with no differences between sites. In rats, Dallman (1967) has reported that the synthesis of CcO associated to mitochondrial turnover or production of new cells depends on Cu availability. It should be considered that the 60 days sampling has been done in September coinciding with the start of the reproductive period of *D. chilensis*, which involves increasing metabolic demands in the gills. As other unionoids, *D. chilensis* develops a marsupium in the internal hemibranch where larvae are bred; thus, storage compounds are moved to the gills and several gill enzymes, including CcO, are activated (Parada et al., 1990; Yusseppone et al., 2015). These changes in gill structure and metabolism probably lead to a hemolymph accumulation and increased concentration of Cu-containing proteins, which is not related to external Cu concentration. The decreased Cu content in the adductor muscle of mussels from both sites after 60 days could also be explained by hemolymph and metalloproteins movement to the gills. However, further evidence is needed to support these ideas. The lack of Cu accumulation in tissues from the site B mussels with respect those of site A also suggests that this metal could be excreted at the gills, possibly through a mechanism depending on GST (Bigot et al., 2010; Martin-Diaz et al., 2008), which shows higher activity in the site B mussels, and/or at the digestive gland, as has been previously reported for this species (Sabatini et al., 2011). On the other hand, Fe content diminishes in adductor muscle after 60 days only in site A mussels and seems not to be mobilized to the gills. This result is difficult to explain with our experimental results but it could involve the mobilization of Fe to other organs (Gobi et al., 2018, 2019) and could be associated with the reduction of the lipid peroxidation level in this tissue. In site B individuals, the lack of change in adductor muscle Fe content coincides with sustained lipid peroxidation level and SOD activity. Additionally, the increase in gill GST activity in site B mussels, both at 30 and 60 days can also respond to Fe accumulation.

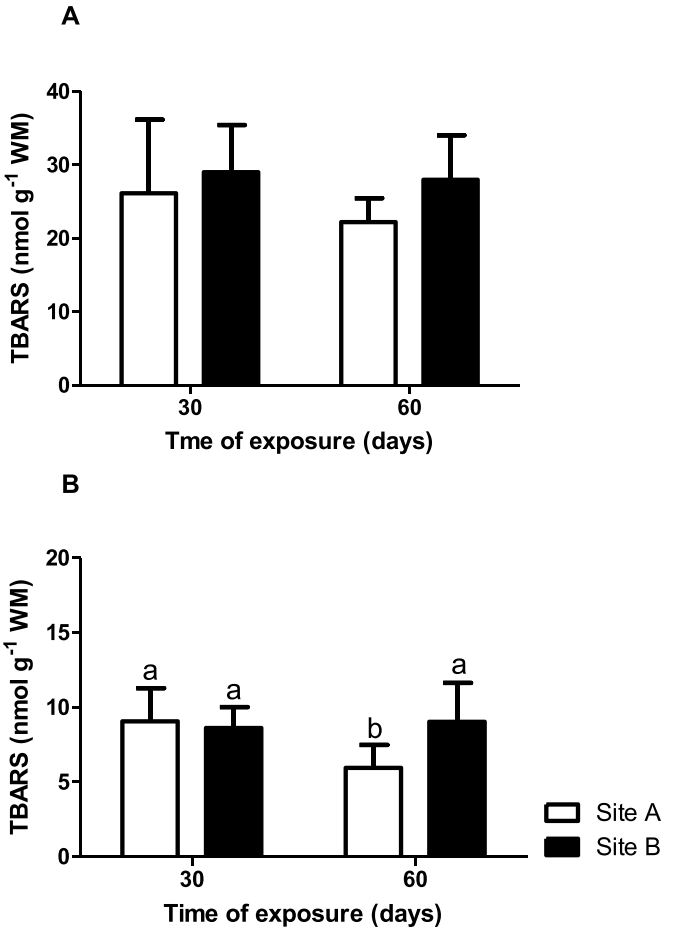

Fig. 6. Levels of thiobarbituric acid reactive substances (TBARS) in gills (A) and in adductor muscle (B) in *Diplodon chilensis* exposed at the sites A and B during 30 and 60 days. Different letters indicate significant differences with $p < 0.05$, $n = 12$ (per site and time).

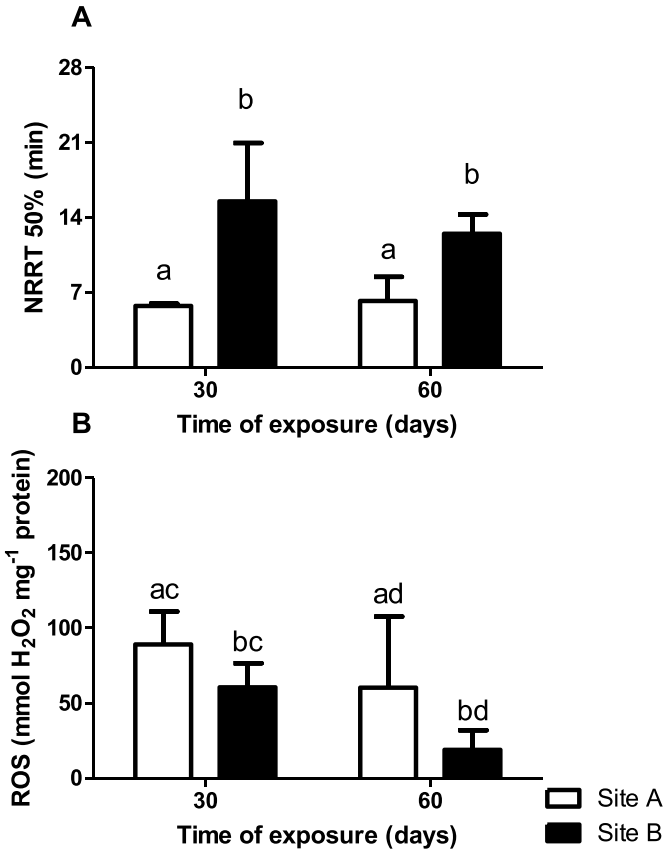

Fig. 7. Neutral red retention time (NRRT) (A) and reactive oxygen species (ROS) production (B) in hemocytes of *Diplodon chilensis* exposed at the sites A and B during 30 and 60 days. Different letters indicate significant differences with $p < 0.05$, $n = 12$ (per site and time), a and b indicate differences between sites, c and d indicate differences between times.

Despite the significant accumulation of Fe in muscle and gills of the site B mussels, which is expected to induce oxidative stress, hemocytes show lower ROS production and higher lysosomal membrane stability in the site B. Accordingly, Sheir and Handy (2010) have reported increased hemocyte lysosomal stability and normal phagocytic activity together with histological damage in gills, adductor muscle of *Mytilus edulis* exposed to cadmium. Ivanina et al. (2015) have found low intracellular levels of free metal ions in hemocytes of *Crassostrea virginica* and *Mercenaria mercenaria* exposed to Cu and Cd and proposed that this was related with metallothionein induction in hemocytes and high detoxification activity. The enhancement of lysosomal membrane integrity in *D. chilensis* exposed at site B could also be related with induction of protein synthesis. Besides metalloproteins, heat shock proteins (HSPs), particularly HSP70 have been reported as stabilizing lysosomes in a cancer cell line (Kirkegaard et al.,2010). In marine and freshwater bivalves, HSP-70 and HSP-90 are induced by exposure to metals (e.g. Valenzuela-Castillo et al., 2019; Radlowska and Pempkowiak, 2002). Particularly, in *D. chilensis*, experimental hypoxia and anoxia induce the expression of HSP-90 (Yusseppone et al., 2018).

Besides Fe and Cu, the site B water is characterized by its high ammonia concentration, 18-fold higher than that in the site A. A recent study shows that the freshwater bivalve *Unio pictorum* has low sensitivity to combined effects of ammonia and temperature stress (Beggel et al., 2017). These authors suggest that *U. pictorum* responds to elevated concentrations of ammonia by reducing its filtering activity; thus, reducing the exposure to ammonia. In addition, reduced ventilation activity has been reported by Wang (2009) as a response of the green mussel *Perna viridis* to the exposure to metals under different hypoxic and anoxic conditions. Lowered ventilation rate can result in the activation of anaerobic metabolism. In *D. chilensis*, induction of an anaerobic pathway enzyme, MDH activity in gills has been suggested as contributing to the long-term hypoxic survival (Grandón et al., 2008; Yusseppone et al., 2018). Accordingly, in the present work, *D. chilensis* adductor muscle LDH activity is increased in the site B individuals in a time dependent manner, while MDH shows a similar pattern but with no significant changes. The combined exposure to ammonia, metals and moderately low oxygen concentration is probably inducing a reduction in the ventilation rate, which in 60 day-exposed individuals is probably accompanied by higher gill oxygen consumption and thickening of the diffusion barrier due to the marsupium development. This would result in reduced hemolymph oxygen tension, which, in turn, could explain the lower ROS production in hemocytes from site B individuals, although we have no direct measurements of oxygen tension.

Lipid, carbohydrate and protein reserves are important for fueling the elevated energy demands under stressful conditions (Sokolova et al., 2012). Particularly, bivalves store lipids and complex carbohydrates, such as glycogen, for the gonad development and to satisfy enhanced needs by anaerobic activity (De Zwaan and Wijisman, 1976). Despite differences are not reflected in the total energy levels, protein and lipid contents in the digestive gland decrease along the time of exposure in both sites, probably due to the mobilization of these macromolecules to the gills and gonads in the reproductive period. This time effect is also evident in the condition index (CI). In turn, CI and digestive gland index (DGI) are lower at the site B. This site effect could be explained by two nonexclusive causes, the increased energetic demands for coping with stressful conditions (Sokolova et al., 2012),

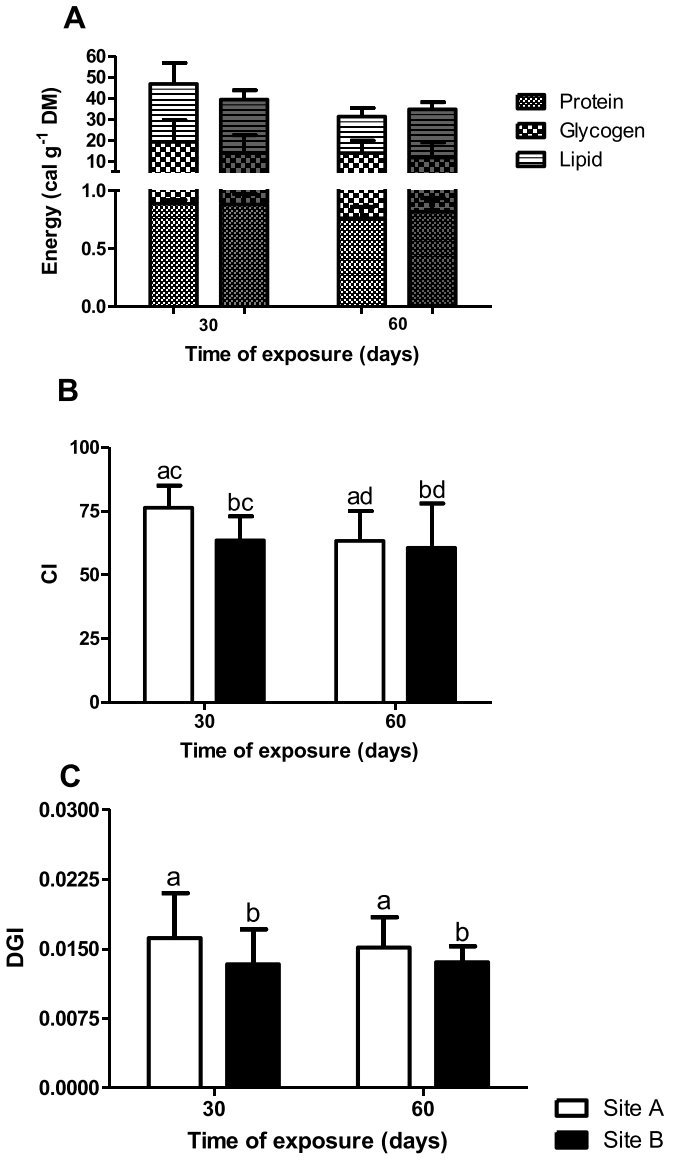

Fig. 8. Energy values (A), condition index (CI) (B) and digestive gland index (DGI) (C) in digestive gland of *Diplodon chilensis* exposed at the sites A and B during 30 and 60 days. Different letters indicate significant differences with $p < 0.05$, $n = 12$ (per site and time), a and b indicate differences between sites, c and d indicate differences between times.

including metal excretion at the digestive gland (Sabatini et al., 2011), and/or reduced energy intake due to lower ventilation/filtration rate (Sokolova and Lannig, 2008).

5. Conclusions

Metal-pollution and eutrophication effects on *D. chilensis* metabolism are evidenced as tissue-specific responses. Gills and adductor muscle accumulate Fe but not Cu when the mussel is exposed to high environmental concentrations of both metals. Gills do not suffer oxidative stress or damage despite it is directly exposed to aquatic pollutants while muscle shows signs of oxidative stress (SOD activation) and mild oxidative damage. The overall effects of pollution can be observed as signs of anaerobic metabolism in muscle and in the reduction of the condition and digestive gland indexes in mussels from the polluted site, associated with diminished levels of reserve molecules.

The results support the study of bivalve metabolism and its alterations in the assessment of environmental pollution, especially in areas under the pressure of multiple stressors and contaminants.

CRediT authorship contribution statement

M.S. Yusseppone: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **V.A. Bianchi:** Methodology, Investigation. **J.M. Castro:** Methodology, Investigation. **T. Noya Abad:** Methodology, Investigation. **Y.S. Minaberry:** Investigation, Resources. **S.E. Sabatini:** Methodology, Investigation, Formal analysis. **C.M. Luquet:** Conceptualization, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision, Funding acquisition. **M.C. Rios de Molina:** Conceptualization, Resources, Supervision, Funding acquisition. **I. Rocchetta:** Conceptualization, Methodology, Resources, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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