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Long- term effects of water quality on the freshwater bivalve *Diplodon chilensis* (Unionida: Hyriidae) caged at different sites in a North Patagonian river (Argentina)

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Running head

Effects of water quality on a freshwater bivalve

Abstract

Water quality was monitored along an Andean river of global importance using the freshwater bivalve *Diplodon chilensis* as sentinel species. Bivalves were placed in cages at three sites (S1-3) in the Chimehuin river in order to evaluate the long-term effects of a trout hatchery (S2), and the open dump and sewage treatment plant of a nearby city (S3). Water samples and bivalves were collected at 0, 3, 6, 9, and 12 months, from S3, S2 and from a reference site upstream (S1), and physicochemical parameters and the biological response of the caged bivalves were studied. ROS production, antioxidant response, oxidative damage, energy status and morphometric ratios were included as response biomarkers. Most of biomarkers showed site-and time dependence including bivalves transplanted at S1, revealing natural variability. Both anthropogenic perturbed sites (S2 and S3) showed differences in the exposed-bivalves with respect to the reference site (S1) in their biomarker responses after 9 and 12 months of exposure (July and September, respectively). Multivariate analysis showed alterations in hemocytes (ROS, TOSC and NRRT50), energy balance in digestive gland (energy and DGF), and in the detoxification response (GST) and GSH values in gill when bivalves were exposed to hatchery waste; whereas metal and bacterial pollution (S3 in July) caused GSH increase and a reduction of lysosomal damage in hemocytes. Results show that changes in the water quality of Chimehuin river due to the anthropogenic impact can be detected using the biomarkers analyzed on *D. chilensis*, being a useful tool for studies of long-term monitories.

Keywords: *Diplodon chilensis*, Chimehuin river, biomarkers, bivalve caging

Accepted Article

## Introduction

Urban activities affect the quality of freshwater resources by discharging a variety of compounds, such as metals, pesticides, hydrocarbons, nutrients and pharmaceuticals. The impact of such compounds on aquatic biota has been analyzed through the response of biomarkers in several species. For example, macroinvertebrates and fishes are commonly used to evaluate aquatic ecosystem health (Carlisle et al., 2008). Particularly, bivalves have been recognized as excellent sentinels since they are easier to sample and monitor than fishes and other invertebrates (Hauer & Lamberti, 2006), they are tolerant to harsh environments but still reflect the stressful conditions (Turja et al., 2014), and have bioaccumulation capacities (Di Salvatore et al., 2013; Iummato et al., 2013, Ruiz et al., 2018). Additionally, bivalves have sedentary lifestyle and many of them are long-lived, which allows the study of long-term effects (Schwalb & Pusch, 2007). Transplantation of caged bivalves has been successfully applied in monitoring programs, where changes in nutritional and reproductive fitness were only attributable to environmental variables (Andral et al., 2004; Viarengo et al., 2007; Ruiz et al., 2018). The potential of a particular species to be used as sentinel in aquatic monitoring programs can be evaluated through the combination of several biomarkers.

In North-West Patagonia, Argentina, the river Chimehuin is one of the most important touristic attractions, receiving people from all over the world for the practice of fly fishing among other activities. The freshwater bivalve *Diplodon chilensis* is one of the most abundant macroinvertebrates in this river as well as in numerous oligotrophic freshwater bodies of the Argentinean and Chilean North Patagonia, and has also been reported to survive in severely eutrophic waters (Grandón et al., 2008, Rocchetta et al., 2014a). Sabatini et al. (2011b) have described the copper bioaccumulation capacity of *D. chilensis* under laboratory conditions. More recently, Bianchi et al (2014 a, b, 2015) have shown that immune response

biomarkers of this species are suitable for detecting domiciliary waste water contamination. Due to its abundance and longevity, which can exceed a century (Soldati et al., 2009; Rocchetta et al., 2014a; Yusseppone et al., 2015), *D. chilensis* has been proposed as a sentinel species for long-term monitoring of the environmental health of Patagonian freshwater bodies. A recent decline in *D. chilensis* river populations has been reported in Chile as a consequence of habitat deterioration (Parada & Peredo 2005; Parada, et al., 2009); but there are no reports on water quality deterioration in Argentinean rivers populated by this species (Yusseppone et al., 2017).

Based on this background, this paper aims to analyze the long-term response of a set of biomarkers in caged- *D. chilensis* transplanted to different sites in the Chimehuin river course, (a reference site, downstream a rainbow trout hatchery, and downstream the Junín de los Andes city open dump) after 3, 6, 9 and 12 months of exposure. We assessed reactive oxygen species (ROS) production, antioxidant response and oxidative damage to evaluate oxidative stress. The energy status was assessed through the content of main biochemical compounds and morphometric ratios. In addition, physicochemical and biological variables in water as well as organic matter content in sediments were included, in order to characterize the transplantation sites.

## Materials and Methods

### *Mussel collection and sampling*

Mussels were sampled by SCUBA diving from Paimún lake (39°44.78'S 71°37.48'W, S0).

After sampling, the mussels (75.1 mm mean  $\pm$  7.2 mm SD shell length) were transported and randomly assigned to plastic cages at a clean site (39°54.953'S; 71°06.363'W, S1),

downstream the hatchery discharge (39°54,973'S 71°06,340'W, S2) and downstream the

Junín de los Andes city open dump and the sewage treatment plant of the Junín de los Andes city (39°60,785'S 71°07,026'W, S3) at Chimehuin river (Fig. 1). The bivalve density in the cages was similar to that found in the natural population ( $S_0 = 12 \pm 3$  ind / m<sup>2</sup>). The cages' bottoms were filled with sediment from the superficial layer from each site and the cages were conditioned to allow water flux. The study was carried out from September 2011 (T0) to September 2012. Mussels were kept in cages for 3 (December), 6 (March), 9 (July) and 12 (September) months until collection for analysis (n = 12 per site and time).

#### *Water quality analysis*

Physicochemical analysis was performed using a HACH DR/4000 spectrophotometer (n = 3). Samples were collected in 5 % HCL pre-washed bottles and immediately processed.

Turbidity was measured by Attenuated Radiation Method (HACH Method 10047) and expressed as Formazin Attenuation Units (FAU). Total nitrogen (TN) and phosphorus (TP) were measured, after alkaline persulfate digestion, by the ascorbic acid and cadmium reduction methods, respectively (HACH Method 8192, 8048). An aliquot of the samples was filtered (Whatman GF / F 0.45 µm pore) and nitrite (mg / L), nitrate (mg / L), phosphate (mg / L), iron (mg / L), copper (mg / L), sulphate (mg / L) and silica (mg/L) levels were measured (HACH Method 8507, 8192, 8048, 8147, 8143, 8131, 8185, respectively). Dissolved Oxygen (mg / L), pH, conductivity (µS / cm) and temperature were measured *in situ* using a multi-parameter analyzer (Hanna HI 9828).

Chlorophyll *a* concentration was measured according to Lichtenthaler (1987) in filtered samples (n = 3) through glass fiber filters (Whatman GFF, 0.45 µm pore) as described above. Results were expressed as µg / L.

Total (TCB) and fecal (FC) coliform bacteria concentration were estimated by MPN 100 mL method (APHA, 1998) in water samples (n =3) collected in sterile containers and maintained at 4 °C until analysis. Results were expressed as MPN / 100 mL.

#### *Organic matter content in sediment*

Sediment was collected from the upper 5 cm (n = 3) and transported to the laboratory at 4 °C. For organic matter content (OM), the sediment was dried at  $90 \pm 5$  °C during 24 h until constant weight, then burned at 550 °C for 5 h and the difference between the initial and the final weight was considered as the OM. Results were expressed as % OM (Dean, 1974).

#### *Biomarkers*

##### *Tissue sample preparation*

Gills were excised from each bivalve, weighted and homogenized in 134 mM KCl solution (ratio 1:5 g tissue mass / mL) with protease inhibitors (0.2 mM benzamidine and 0.5 mM PMSF). A fraction of the homogenate was centrifuged for 15 min at 11,000 x g and the supernatant was used for enzyme assays (superoxide dismutase (SOD) and glutathione-S-transferase (GST) activities), reduced glutathione (GSH) content and thiobarbituric acid reactive substances (TBARS) content.

##### *Enzyme assays*

Superoxide dismutase (SOD) activity was measured by the technique of Beauchamp & Fridovich (1971) based on the inhibition of the reduction of nitroblue tetrazolium (NBT) which was monitored at 560 nm. One SOD Unit was defined as the amount of sample necessary for 50% inhibition. Data were expressed as SOD Units per gram of wet tissue mass (g WTM).

Glutathione-S-transferase (GST) activity was measured according to Habig et al. (1974). The change in absorbance was recorded at 340 nm during 90 s. One GST Unit was defined as the amount of enzyme needed to catalyze the formation of one  $\mu\text{mol}$  of DNB-SG per minute at 25° C. Data are expressed as GST Units per g WTM.

#### *GSH content*

Reduced glutathione (GSH) content was measured by the technique of Anderson (1985).

Using deproteinized samples (incubated with 10 % sulfosalicylic acid) GSH was oxidized by 5,5-dithiobis-2-nitrobenzoic acid (DTNB) in a reaction buffer (0.134 M Na-phosphate buffer, pH 7.5, 6.3 mM EDTA). The formation of 2-nitrobenzoic acid was monitored at 412 nm and the GSH content was quantified using a calibration curve. Data were expressed as nmol / g WTM.

#### *Oxidative damage*

Lipid peroxidation levels were measured by a technique modified from Beuge & Aust (1978).

Gill samples were incubated with reaction mixture (15 % trichloroacetic acid (TCA), butylated hydroxytoluene (BHT), 0.25 N HCl and 0.37 % thiobarbituric acid (TBA)) at 100 °C for 15 min and then cooled and centrifuged at 10,000 x g for 10 min. The thiobarbituric acid reactive substances (TBARS) were measured at 535 nm after. Data were expressed as  $\mu\text{mol}$  / g WTM.

#### *Lysosomal membrane stability*

Hemocytes lysosomal membrane stability was estimated by the neutral red retention time method (Mamaca et al. 2005, modified). Briefly, 1 mL of hemolymph was withdrawn from the adductor muscle of each one of 6 individuals per time and site using a sterile syringe and



working on ice. 50  $\mu$ L of hemolymph were placed on a slide (in duplicate), kept in a wet chamber for 5 min and then neutral red solution (50  $\mu$ L, 0.002 % in dimethyl sulfoxide) was added. The number of red cells was counted under microscope until stained cells reached 50 %. Results were expressed as neutral red retention time 50 % (NRRT50) (min).

#### *ROS production*

Aliquots of hemocytes pre-washed and resuspended in anticoagulant solution (3 g glucose and 0.36 g trisodium citrate / L, 60 mOsm / L, pH 7) were used to measure ROS production by a fluorometric method (Bianchi et al., 2014a modified from Moss & Allam, 2006). ROS content was referred to a H<sub>2</sub>O<sub>2</sub> standard curve with H<sub>2</sub>DCF-DA (2,7 dichlorofluorescein diacetate, 0.8 mM, Sigma). Results were expressed as nmol H<sub>2</sub>O<sub>2</sub> per mg protein.

#### *Total Oxyradical Scavenging Capacity*

Total Oxyradical Scavenging Capacity (TOSC) against peroxy radicals was measured in hemocytes fraction (6 individuals per site and time) obtained as was described above and following Amado et al., (2009) with modifications. Half-aliquot (50  $\mu$ L) was mixed with 4 mM ABAP solution (2,2-Azobis 2-methylpropionamide dihydrochloride) in another half, ABAP was omitted. H<sub>2</sub>DCF-DA was added in all the tubes and fluorescence reading was performed during 30 min. The relative area (RA) between the curves obtained with and without ABAP was calculated and TOSC was considered as RA<sup>-1</sup> and expressed as arbitrary units (a.u.) / mg protein.

#### *Energy status*

One portion of the digestive gland (DG) was dried at 65 °C, (n = 6) until constant weight to estimate the dry mass / wet mass ratio (DM / WM). In homogenates of DG (1:5 g tissue mass

/ mL of a 134 mM KCl, 0.2 mM benzamidine and 0.5 mM PMSF solution) total lipids were extracted by the technique of Bligh & Dyer (1959) using a chloroform-methanol mixture (2:1). Total protein content was measured by Bradford's technique (1976) using bovine serum albumin as standard. Glycogen content was determined by the spectrophotometric method of Van Handel (1965) using Anthrone as reagent and glycogen standard (SIGMA) for the standard curve. Energy conversion factors for aquatic invertebrates described by Beninger (1984) were used (carbohydrates 4.1 kcal / g, proteins 4.3 kcal / g and lipids 7.9 kcal / g). Data are expressed as cal / g DM. The digestive gland factor (DGF) was calculated as the ratio between wet digestive gland mass (g) and shell length (mm) (n = 12 per time and site).

#### *Statistical analysis*

Physicochemical and biological data were analyzed by two-way ANOVA followed by a Bonferroni's post hoc test with time and site as factors. Assumptions of homocedasticity and normality were tested by Bartlett's and Lilliefors tests, respectively (Sokal & Rohlf, 1999).

All data were expressed as mean  $\pm$  SD. Analyses were performed using GraphPad Prism 6 and Statistica v.7 software.

The relationship between environmental variables and biomarkers was studied by principal component analysis, in order to reduce dimensionality and to obtain equal number of variables in both groups. Only variables with full-data sets were included and the first four principal components were used as new variables (PCe for environmental variables and PCb for biomarkers) to analyze the correlation between the environmental characteristics and of the sites under study and the biochemical response of *D. chilensis* through canonical correspondence. This analysis was performed considering site\*time as case, using Infostat Software (v. 2017) (Di Rienzo et al. 2017).

## Results

### *Water quality and sediment organic matter*

The mean values of physic-chemical variables varied according to site and time of exposure ( $p_{\text{site*time}} < 0.0001$ ) (Fig.1-3S). An inverse relationship between temperature and dissolved oxygen was recorded in most cases while pH values ranged from ~6 to ~8 with no clear pattern. Conductivity was 20% higher at S1 than at S2 during the whole experimental period; while turbidity was highest at S2, and differed significantly from those in the other sites (~95% higher than S1 and S3). Nitrate and phosphate showed significantly higher values at S2 and S3 with respect to S1 but only in July and December, respectively. In addition, S2 showed 47% higher phosphate concentration than the other two sites in September. Copper concentration was 89% and 60% higher at S3 than at S1 and S2, respectively during the whole sampling period; while S2 presented 90% higher copper concentration than S1 in July and September. Silica, iron, sulphate and total nitrogen concentrations did not show significant trends. Total phosphorus was significantly higher at S2 compared to the other two sites in December, July and September. Chlorophyll a concentration changed depending on site and time ( $p_{\text{site*time}} = 0.0018$ ), remaining constant at S1 during the whole study and showing 70% higher levels at S2 and S3 (Fig. 3S). Higher values of total coliform bacteria, which corresponded in most cases to fecal coliforms, were recorded at S2 and S3 compared to S1 during the whole experimental period. The total coliform bacteria remained constant at site S1 during the whole experimental period whereas at S2 the values dropped in July to reach the initial levels in September. At S3 the total coliform concentration stepped up until July, reaching the highest values and then decreased in September ( $p_{\text{site*time}} < 0.001$ , Fig. 3S).

OM % showed a significant interaction between site and time ( $p_{\text{site*time}} < 0.0001$ ) with 49% and 70% higher values at S2 compared to S1 and S3, respectively for the whole study period.

These differences were significant for most of the comparisons (Fig. 3S).

### *Biomarkers*

Gill SOD activity remained constant throughout the year in mussels caged at the control site (S1), and increased significantly with respect to S1 in mussels caged at both contaminated sites in September, and only in S2 in December. (ANOVA  $p_{\text{site*time}} = 0.0006$ , Fig. 2A), (Fig. 2A). In contrast, GST activity increased significantly in the three sites in July, being significantly higher in S2 than in S1. In September, both, S2 and S3 mussels showed significantly higher GST activity than those from S1 for most of the comparisons (ANOVA  $p_{\text{site*time}} = 0.0002$ , Fig. 1B). No changes were observed between September (initial time at S0, natural population) and December (Fig. 2B).

Gill GSH content was 45% higher in mussels from S3 compared to those from S1 and S2 in July. On the other hand, in September, mussels from both, S2 and S3 showed lower GSH content than those from S1 ( $p_{\text{site*time}} = 0.0058$ , Fig. 2C). In September, gills from mussels exposed at S2 and S3 had higher TBARS levels (18% and 28%, respectively) than those from mussels exposed at S1 ( $p_{\text{site*time}} = 0.0177$ , Fig. 3).

There was a peak in hemocytes ROS production in S2 mussels in July, with higher level compared to those at the other sites at the same time, and also compared to the ROS production of S2 mussels hemocytes at the other times ( $p_{\text{site*time}} < 0.0001$ , Fig. 4A). TOSC levels were higher in S2 and S3 mussels than in those caged at S1 in March and September ( $p_{\text{site*time}} = 0.001$ , Fig. 4B). Hemocyte lysosomal membrane stability (as NRRT50) was lower

in hemocytes from S2 mussels compared with those from S1 and S3 in March, and July ( $p_{\text{site*time}} = 0.0008$ , Fig. 4C).

The calculated digestive gland energy content varied both with time of exposure and site, with no interaction effect (two-way ANOVA,  $p_{\text{site*time}} = 0.0681$ ,  $p_{\text{site}} < 0.001$ ,  $p_{\text{time}} = 0.001$ ), with 30% higher values at S2 and S3 compared to S1 (S1 =  $80.85 \pm 18.62$ ; S2 =  $115.9 \pm 27.34$ ; S3 =  $112.9 \pm 23.7$  cal/g DM). The highest values were recorded in March and July (December =  $89.922 \pm 3.696$ ; March =  $114.470 \pm 28.711$ ; July =  $126.051 \pm 25.685$ ; September =  $82.394 \pm 24.628$  cal/g DM) (Figure 6A). These results coincide with those obtained for total lipid content, which was 35% and 14% higher at S2 and S3, respectively, than at S1 (S1 =  $0.025 \pm 0.009$ ; S2 =  $0.039 \pm 0.002$ ; S3 =  $0.029 \pm 0.010$  g/g WTM). Total lipids were higher in March and July compared to December and September (December =  $0.023 \pm 0.005$ ; March =  $0.038 \pm 0.02$ ; July =  $0.044 \pm 0.007$ ; September =  $0.019 \pm 0.004$  g/g WTM) (two-way ANOVA,  $p_{\text{site}} = 0.0243$ ,  $p_{\text{time}} = 0.0001$ ).

There was a significant interaction between site and time on glycogen content (two-way ANOVA,  $p_{\text{site*time}} = 0.0008$ ) with higher values at S3 compared to S1 in July and September (S1<sub>Jul</sub> =  $28.56 \pm 11.69$  vs. S3<sub>Jul</sub> =  $68.43 \pm 19.45$ ; S1<sub>Sep</sub> =  $22.44 \pm 15.41$  vs. S3<sub>Sep</sub> =  $50.99 \pm 9.88$  mg/g WTM). In contrast, protein content varied significantly only with time (two-way ANOVA,  $p_{\text{time}} = 0.0003$ ) with the highest values in December and March (December =  $41.86 \pm 2.64$ ; March =  $38.99 \pm 7.38$ ; July =  $30.44 \pm 2.84$ ; September =  $30.27 \pm 5.87$  mg/g WTM).

There was significant interaction between site and time on DGF (two-way ANOVA,  $p_{\text{site*time}} = 0.001$ ). DGF was higher in S2 and S3 mussels in March, July and September than in December. At these 3 times, S2 and S3 mussels DGF was higher than that of S1 individuals, which showed little variation over time (Fig. 5B).

### *Relationship between environmental variables and biomarkers*

The first four principal component axes of environmental variables (PCe) cumulatively contributed to 82% of the total variance between treatments (site\*time) (see Appendix 1: correlation between the PCe and the environmental variables). PCe1 showed positive correlation between the PCe and the environmental variables). PCe1 showed positive correlation with temperature, conductivity and silica, and negative correlation with oxygen, pH, TP and chlorophyll *a*. PCe2 was positively correlated to turbidity and OM and negatively with copper and TCB. PCe3 was positively correlated to TN and chlorophyll *a*, whereas PCe4 was positively correlated to phosphate and sulphate.

The first four principal component axes for biomarkers (PCb) cumulatively contributed to 80 % of total variance between treatments (site\*time) (see Appendix 2: Correlation between the PCb and biomarkers). PCb1 showed a positive correlation with digestive gland GST and energy content, DGF and hemocyte ROS, and negative correlation with hemocyte TOSC. PCb2 was positively correlated to digestive gland GSH and hemocyte NRRT50. PCb3 was represented by digestive gland TBARS and SOD (positive correlation), and PCb4 was positively correlated to hemocyte ROS and negatively to digestive gland SOD.

The canonical correspondence analysis (CCA) showed that 80% of the total variation was explained by the first correlation between the environmental variables and biomarkers ( $R^2 = 0.80$ ,  $r = -0.9$ ,  $p < 0.001$ ). The first canonical function (F1) was represented by PCe1 on the y axis (canonical 1\_1) and PCb1 on the x axis (canonical 2\_1) (Appendix 3). Figure 6A shows that highest values of canonical 1\_1 (highest temperature, conductivity and silica and, lowest dissolved oxygen, pH, TP and chlorophyll *a*) are correlated with the lowest values of canonical 2\_1 (lowest GST, hemocytes ROS, Energy and DGF, and highest TOSC). This relationship was observed at S1\*March whereas the lowest values of canonical 1\_1 and the highest of canonical 2\_1 were observed at S2\*July, where lowest temperature, conductivity

and silica, and highest oxygen, pH, TP and chlorophyll a are correlated with highest GST, ROS, Energy and DGF, and lowest TOSC (Fig.6A).

The canonical function 2 (F2) explained 72% of total variability ( $R^2 = 0.72$ ,  $r = -0.84$ ,  $p < 0.001$ ) and is represented by PCe2 and PCe3 on the y axis (canonical 1\_2) and PCb2 on the x axis (canonical 2\_2) (Appendix 3). Figure 6B shows that highest values of canonical 1\_2 (highest turbidity, OM, TN and chlorophyll, and lowest copper and TCB) are correlated with the lowest values of canonical 2\_2 (lowest GSH and NRRT50). This relationship was observed at S2\*December and S3\*December whereas the lowest values of canonical 1\_2 and the highest of canonical 2\_2 were observed at S3\*July (lowest turbidity, OM, TN and chlorophyll a, and highest copper and TCB are correlated with highest GSH and NRRT50) (Fig.6B).

## Discussion

This study shows a site- and time-dependent biomarker response, which reveals a complex interaction between environmental conditions and bivalve responses. These results offer a realistic scenario of the *in situ* monitoring and make a contribution to future biomonitoring programs using the native bivalve *D. chilensis* as sentinel species.

We have analyzed the effects of the effluents of a fish hatchery and urban contamination on physicochemical variables of the Chimehuin river water, and their impact on *D. chilensis*. It has been reported that untreated aquaculture effluents increase suspended solids, organic matter and dissolved nutrients, especially phosphorus and nitrogen, which stimulate phytoplankton growth, and reduce dissolved oxygen concentration in receiving water bodies (Dalsgaard & Pedersen, 2011; Guilpart et al., 2012; Skoronski et al., 2018). In concordance, our study shows high levels of turbidity and total phosphorus and organic matter downstream

the hatchery outlet (S2). Nitrogen (as nitrate), total phosphorous and organic matter reach maximum values in winter (July) in this site. In addition, copper reaches high levels in July and September and fecal coliforms are highest for this site also in September. This could be related to the fact that the austral winter is the rainy season in this region. The increased precipitations rate favors the resuspension of nutrients, organic matter and pollutants from the aquaculture effluent and from the decay of the abundant aquatic and riparian vegetation of this area, which is accumulated during summer-autumn (dry season). Accordingly, chlorophyll is higher in S2 than in the other sites in July and reaches a peak in September, following the phosphorous trend, although phosphorous and organic matter are higher in S2 than in the other sites during most of the year.

Urban waste discharges can add a variety of pollutants to the environment, such as detergents, metals, pesticides, among others. In our study, S3 is strongly influenced by hydrological conditions related to the precipitation regime. The increase in water flow rate during winter-spring dilutes the pollutants and nutrients delivered by the sewage treatment plant and the leachates from the open dump. Despite this, S3 shows the highest levels of total coliform bacteria and copper concentration throughout the year. The most striking result for this site is the peak in fecal bacteria load recorded in July (7000 MPN /100 mL) together with a nitrate peak, which could be attributed to the release of untreated effluents from the sewage treatment plant, when it is overloaded by rain water. The chlorophyll a peak in March (dry season) coincides with a total nitrogen peak.

At S1 (reference site), the studied biomarkers show a time-dependent variation, which reflects seasonal variability. Similar results have been described for caged *Mytilus trosullu* (Turja et al., 2014) and *Mytilus galloprovincialis* (Roméo et al., 2003), highlighting the importance of recording natural variability in biomonitoring studies. There are no differences



between September 2011 (bivalves freshly collected from the lake) and December at S1 in the biomarker levels (excepting SOD and GSH), which suggests little transplantation effect, as has been previously described for *D. chilensis* by Bianchi et al. (2014a) in a three-month study. In winter (July) and/or early spring (September), the biomarker levels of *D. chilensis* caged at S2 and S3 differed significantly from those of S1 bivalves. This is in agreement with the increasing sewage disposal together with nutrient and pollutant resuspension during the rainy season, which affects water quality. SOD activity was induced at S2 and S3 compared to S1 in September, in coincidence with high levels of copper in both sites, as was previously observed in natural populations of bivalves from metal-polluted areas (*Aulacomya atra*: Di Salvatore et al., 2013; *Scrobicularia plana*: Ahmad et al., 2011) and in caged-bivalves transplanted to areas with multiple pollutants (e.g. metals, pesticides, hydrocarbons, Serafim et al., 2011; Turja et al., 2014). However, SOD activity was not significantly induced in March, when the peak of copper concentration was registered. In S3, the site with highest concentrations of copper and coliform bacteria, the GSH content was increased in July, coinciding with the peak in fecal bacteria. Increased GSH has been previously reported for bivalves exposed to trace metals (Irato et al., 2003; Di Salvatore et al., 2013) or fecal bacteria (Sabatini et al., 2011). The posterior drop in GSH content registered in September in S2 and S3 could be related to the increase of GST activity in the same period. GSH is a non-enzymatic antioxidant, which could be directly used to scavenge oxygen free radicals to protect the cells (Regoli & Principato, 1995), used as substrate by antioxidant enzymes or conjugated with various endogenous and exogenous substances in a biotransformation reaction catalyzed by GST (Regoli, Nigro & Orlando, 1998). This enzyme can be induced by organic compounds (Hoarau et al., 2001; Hernandez et al., 2018) and different metals, such as copper (Canesi et al., 1999; Roméo et al., 2003; Sabatini et al., 2011b), manganese and zinc (DiSalvatore et al., 2013). GST not only detoxifies anthropogenic chemicals by

biotransformation reactions but also plays an important role in the antioxidant defense (Awasthi et al., 2017; Dasari et al., 2017). Despite the activation of the antioxidant response, TBARS levels, which indicate lipid peroxidation, were increased in September in S2 and S3 bivalves, suggesting an oxidative stress condition by the end of the experimental period. Both S2 and S3 showed higher copper concentration in the water column than S1. Cu sulphate is a common algaecide in aquaculture (del Valle & Nagasawa, 1990), which was widely used during the 1990's. Despite it has not been applied in aquaculture during the last decades, our results denote the persistence of Cu in water samples from S2, probably due to slow release of metal ions trapped by sediments and/or vegetation. Lysosomal membrane stability (as NRRT50) is considered as a sensitive pollution biomarker (Regoli et al., 2004; Viarengo 2007; Guidi et al., 2010). Particularly, exposures to domiciliary waste (Parolini, Binelli & Provini, 2011; Toufexi et al., 2013; Tsarpali & Dailianis, 2012) as well as to metals (Domouhtsidou et al., 2004; Kournoutou et al., 2014) destabilize lysosomal membrane in bivalve hemocytes. We detected lower NRRT50 in hemocytes of S2 bivalves than in the other sites in March and July, with the clearest effect in July, in coincidence with a marked increase in ROS level and with the highest records of OM content and nitrate concentration, and highest copper concentration for this site. This could be related to resuspension of Cu associated to organic particles, which are ingested by the bivalves. Clearly, bivalves transplanted at S2 are negatively affected by seasonal pulses of nutrient incomes related to the trout hatchery and by the high content of organic matter accumulated in sediments and/or plants. Besides adsorbing copper, organic matter in the sediment can host different kind of decomposers and pathogens which can alter not only the metabolic activity but also the immune system of bivalves (Luo et al., 2014).

It has been reported that environmental stress disturbs the energetic balance of the organisms (Sokolova et al., 2012). The digestive gland energy status of *D. chilensis* changes along time

of exposure at S2 and S3 while little temporal change is evident at the control. The energy values calculated for proteins, lipids and carbohydrates, together with DGF are significantly increased in S2 and S3 with respect to S1 in March, July and September. Bacterial contamination at S2 and S3 provides an alternative food resource to *D. chilensis* enriched by saturated lipids (storage lipids) which could increase DGF (Sabatini et al., 2011a; Rocchetta et al., 2014a, b). In fact, most of the recorded increase in digestive gland energy corresponds to increased lipids energy. In addition, the chlorophyll *a* concentration recorded at S2 and S3 throughout the experiment indicates higher phytoplankton availability, which can account for the increased glycogen stores (at S3 in July and September) and DGF in bivalves from both polluted sites in March, July and September. Since green algae are able to accumulate metals like Cu (Patterson, Parker & Neves, 1999; Sabatini et al., 2009), the higher algal density in S2 and S3 could also favor metal incorporation by the bivalves through trophic transference, increasing the toxic potential of contaminants present in water (Sabatini et al., 2011b; Fassiano, 2016). Besides, metal accumulation has been reported to induce glycogen accumulation, through glycogen synthase activation and glycogen phosphorylase inhibition in *Perna viridis* (Diaz & García, 2001).

The interaction between physicochemical and biological variables was analyzed by multivariate analysis. The first canonical function (F1) predicts that bivalves exposed to normoxic ( $\sim 9 \text{ mgO}_2 / \text{L}$ ) environments with low nutrient load (mainly total phosphate, TP), and chlorophyll *a* concentration typical of oligotrophic waters, will have low gill GST activity, low hemocytes ROS production and TOSC, and low energy accumulation in digestive gland together with low DGF values. This relationship was strongly represented at S1 (reference site) in December. Environmental conditions characterized by high nutrient load, hyperoxic-waters and high chlorophyll *a* concentration, will have increased ROS production and a concomitant decreased in TOSC of hemocytes, induced gill GST activity

and increased energy stores and DGF in digestive gland as was observed at S2 in July. The second canonical function (F2) predicts that bivalves exposed to high nutrient load (total nitrogen), organic matter, turbidity and chlorophyll *a* consume GSH and have reduced lysosome membrane stability as was recorded at S2 and S3 in December, whereas under bacterial and Cu pollution bivalves accumulate GSH and there is no damage to hemocyte lysosomes as was observed at S3 in July.

Overall oxidative status and membrane damage biomarkers in hemocytes (ROS, TOSC and NRRT50), energy balance in digestive gland (energy and DGF), detoxification (GST) and non-enzymatic antioxidant response (GSH) in gill are more sensitive to the hatchery wastes whereas metal and bacterial pollution induce GSH accumulation preventing lysosomal damage of hemocytes.

Our results provide a holistic approach for evaluating the multiple interactions of environmental pollutants and physicochemical alterations and their consequences on *D. chilensis*, considering natural environmental influence. While several pollutants should be included to get an integrated study, the variables assayed explain the observed biological effects and the potential use of *D. chilensis* as sentinel species of long-term monitoring at the Patagonian region. Moreover, the multiple interactions of these variables reflect the anthropogenic impact on clean areas such as Chimehuin river with ecological and social importance. It represents one of the major threats in freshwater environments with implications for Unionida diversity in South America.

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## References

- Ahmad, I., Mohmood, I., Mieiro, C. L., Coelho, J. P., Pacheco, M., Santos, M. A., Duarte, A. C. & Pereira, E. (2011). Lipid peroxidation vs. antioxidant modulation in the bivalve *Scrobicularia plana* in response to environmental mercury--organ specificities and age effect. *Aquat Toxicol.* 103, 150-8, DOI: 10.1016/j.aquatox.2011.02.017.
- Amado L., Garcia L., Ramos P., Freitas R., Zafalon B. & Ribas Ferreira J. (2009). A method to measure total antioxidant capacity against peroxy radicals in aquatic organisms: application to evaluate microcystins toxicity. *Science of the Total Environment*, 407: 2115-2123, DOI: 10.1016/j.scitotenv.2008.11.038.
- Anderson E. (1985). Determination of glutathione and glutathione disulfide in biological samples. *Methods in Enzymology*, 70: 548-555, DOI: 10.1016/S0076-6879(85)13073-9.
- Andral B., Stanisiere J., Sauzade D., Damier E., Thebault H., Galgani F. & Boissery P. (2004). Monitoring chemical contamination levels in the Mediterranean based on the use of mussel caging. *Marine Pollution Bulletin*, 49: 704-712, DOI: 10.1016/j.marpolbul.2004.05.008.
- APHA (American Public Health Association). (1998). Multiple-tube fermentation technique for members of the coliform group. *Standard Methods for Examination of Water and Wastewater*, 20th ed; Clesceri L.S.; Greenberg A.E.; Eaton A.D., Eds; Madrid, Spain, section 9221, 53 pp.
- Awasthi, Y. C., Ramanaa, K. V., Chaudhary, P., Srivastava, S. K. & Awasthi, S. (2017). Regulatory roles of glutathione-S-transferases and 4-hydroxynonenal in stress-mediated signaling and toxicity. *Free Radical Biology and Medicine*, 111: 235-243, DOI: 10.1016/j.freeradbiomed.2016.10.493.

Beauchamp C. & Fridovich I. (1971). Improved assays and an assay applicable to acrylamide gels. *Analytical Biochemistry*, 44: 276-287, DOI: 10.1016/0003-2697(71)90370-8.

Beningher P. (1984). Seasonal variations of the major lipid classes in relation to the reproductive activity of two species of clams raised in a common habitat: *Tapes decussatus* L. (Jeffreys) and *Tapes philippinarum* (Adams & Reeve). *Journal of Experimental Marine Biology and Ecology*, 79: 79–90, DOI: 10.1016/0022-0981(84)90031-5.

Buege, J. A. & Aust, S. (1978). Microsomal lipid peroxidation. *Methods in Enzymology* 52, 302-310, DOI:10.101S0076-6879(78)52032-6.

Bianchi, V., Castro, J., Rocchetta, I., Bieczynski, F. & Luquet, C. (2014a). Health status and bioremediation capacity of wild freshwater mussels (*Diplodon chilensis*) exposed to sewage water pollution in a glacial Patagonian lake. *Fish and Shellfish Immunology*, 37: 268-277, DOI: 10.1016/j.fsi.2014.02.013.

Bianchi, V., Rocchetta, I. & Luquet, C. (2014b). Biomarker responses to sewage pollution in freshwater mussels (*Diplodon chilensis*) transplanted to a Patagonian river. *Journal of Environmental Science and Health, Part A*, 49: 1276-1285, DOI: 10.1080/10934529.2014.910065.

Bianchi, V., Castro, J., Rocchetta, I., Nahabedian, D., Conforti, V. & Luquet, C. (2015). Long-term feeding with *Euglena gracilis* cells modulates immune responses, oxidative balance and metabolic condition in *Diplodon chilensis* (Mollusca, Bivalvia, Hyriidae) exposed to living *Escherichia coli*. *Fish and Shellfish Immunology*, 42, 367-378, DOI: 10.1016/j.fsi.2014.11.022.

Bligh, E. & Dyer, W. (1959). A rapid method for total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, 37: 911-917, DOI: 10.1139/o59-099.

Bradford, M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72: 248-254, DOI: 10.1016/0003-2697(76)90527-3.

Canesi, L., Viarengo, A., Leonzio, C., Filippelli, M. & Gallo, G. (1999). Heavy metals and glutathione metabolism in mussel tissues. *Aquatic Toxicology*, 46: 67-76, DOI: 10.1016/S0166-445X(98)00116-7.

Carlisle, D., Hawkins, C., Meador, M., Potapova, M. & Falcone, J. (2008). Biological assessments of Appalachian streams based on predictive models for fish, macroinvertebrate, and diatom assemblages. *Journal of the North American Benthological Society*, 27: 16-37, DOI: 10.1899/06-081.1.

Dasari, S., Ganjayi, M. S., Oruganti, L., Balaji, H. & Meriga, B. (2017). Glutathione s-transferases detoxify endogenous and exogenous toxic agents-mini review. *Journal of Dairy, Veterinary & Animal Research*, 5: 157-159, DOI: 10.15406/jdvar.2017.05.00154.

Dean, W. (1974). Determination of carbonate and organic matter in calcareous sediments and sedimentary rocks by loss on ignition: comparison with other methods. *Journal of Sedimentary Petrology*, 44: 242-248, DOI: 10.1306/74D729D2-2B21-11D7-8648000102C1865D.

Del Valle, A. & Nagasawa, A. (1990). Bases para la salmonicultura. Centro de Ecología Aplicada del Neuquén (CEAN), Japan International Cooperation Agency (JICA). Ed. Hemisferio Sur S.A., 199 pp.

Dalsgaard, J. & Pedersen, P.B. (2011). Solid and suspended/dissolved waste (N, P, O) from rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, 313: 92-99. DOI: 10.1016/j.aquaculture.2011.01.037.



Díaz, A. & García, J. (2001). Concentration of the heavy metal Cu and Pb and their relationship with the synthetase glycogen and phosphorylase glycogen enzymatic activity in the mussel (*Perna viridis*). *Zootecnia Tropical*, 19: 115-129.

Di Rienzo, J. A., Casanoves, F., Balzarini, M. G., Gonzalez, L., Tablada, M. & Robledo, C. W. (2014). InfoStat versión 2014. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina. URL <http://www.infostat.com.ar>.

Di Salvatore, P., Calcagno, J., Ortíz, N., Ríos de Molina, M. & Sebastián, E. (2013). Effect of seasonality on oxidative stress responses and metal. *Marine Environmental Research*, 92: 244-252, DOI: 10.1016/j.marenvres.2013.10.004.

Domouhtsidou, G. P., Dailianis, S., Kaloyianni, M. & Dimitriadis, V. K. (2004). Lysosomal membrane stability and metallothionein content in *Mytilus galloprovincialis* (L.), as biomarkers: Combination with trace metal concentrations. *Marine Pollution Bulletin*, 48, 572-586, DOI: 10.1016/j.marpolbul.2004.01.013.

Fassiano, A. (2016). Toxicodinamia del del herbicida Imazetapir en *Scenedesmus vacuolatus*. Efecto de un formulado comercial, sus componentes y la mezcla con glifosato. Transferencia en una cadena trófica. Tesis de Doctorado, FCEN, Universidad de Buenos Aires, 174 pp.

Grandón, M., Barros, J. & González, R. (2008). Metabolic characterization of *Diplodon chilensis* (Gray, 1828) (Bivalvia: Hyriidae) exposed to experimental anoxia. *Revista de Biología Marina y Oceanografía*, 43: 531-537, DOI: 10.4067/S0718-19572008000300012.

Guidi, P., Frenzilli, G., Benedetti, M., Bernardeschi, M., Falleni, A. Fattorini, D., Regoli, F., Scarcellia, V. & Nigro, M. (2010). Antioxidant, genotoxic and lysosomal biomarkers in the freshwater bivalve (*Unio pictorum*) transplanted in a metal polluted river basin. *Aquatic Toxicology*, 100: 75-83, DOI: 10.1016/j.aquatox.2010.07.009.

Guilpart, A., Roussel, J.M., Aubin, J., Caquet, T., Marle, M. & Bris, H. (2012).

The use of benthic invertebrate community and water quality analyses to assess ecological consequences of fish farm effluents in rivers. *Ecological Indicators*, 23, 356-365. DOI: 10.1016/j.ecolind.2012.04.019.

Habig, W., Pabst, M. & Jakoby, W. (1974). Glutathione S-transferase: the first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry*, 249, 7130-7139.

Hauer, F. & Lamberti, G. (2006). *Methods in stream ecology*. Ed. Academic Press, 2nd edition, 877 pp.

Hernandez, E., Kusakisako, K., Talactac, M., Galay, R., Hatta, T., Fujisaki, K. Tsuji, N. & Tanaka, T. (2018). Glutathione S-transferases play a role in the detoxification of flumethrin and chlorpyrifos in *Haemaphysalis longicornis*. *Parasites and Vectors*, 11: 1-14, DOI: 10.1186/s13071-018-3044-9.

Hoarau, P., Gnassia-Barelli, M., Romeo, M. & Girard, J. (2001). Differential induction of glutathione S-transferases in the clam *Ruditapes decussatus* exposed to organic compounds. *Environmental Toxicology and Chemistry*, 20: 523-529, DOI: 10.1002/etc.5620200310.

Irato, P., Santovito, G., Cassini, A., Piccinni, E. & Albergoni, V. (2003). Metal accumulation and binding protein induction in *Mytilus galloprovincialis*, *Scapharca inaequivalvis*, and *Tapes philippinarum* from the lagoon of Venice. *Archives of Environmental Contamination and Toxicology*. 44, 476e484, DOI: 10.1007/s00244-002-1262-8.

Iummato, M., Sabatini, S., Cacciatore, L., Cochón, A., Ríos de Molina, M. & Juárez, A. (2013). Evaluation of biochemical markers in the golden mussel *Limnoperna fortunei* exposed to glyphosate acid in outdoor microcosms. *Ecotoxicology and Environmental Safety*, 95: 123-129, DOI: 10.1016/j.ecoenv.2013.05.024.

Kournoutou, G. G., Pytharopoulou, S., Leotsinidis, M. & Kalpaxis, D. L. (2014). Changes of polyamine pattern in digestive glands of mussel *Mytilus galloprovincialis* under exposure to cadmium. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 165: 1-8, DOI: 10.1016/j.cbpc.2014.05.005.

Lichtenthaler, H. (1987). Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods in Enzymology*, 148: 350-382, DOI: 10.1016/0076-6879(87)48036-1.

Mamaca, E., Bechmann, K., Torgrimsen, S., Aas, E., Bjørnstad, A. & Baussant, T. (2005). The neutral red lysosomal retention assay and comet assay on haemolymph cells from mussels (*Mytilus edulis*) and fish (*Symphodus melops*) exposed to styrene. *Aquatic Toxicology*, 75: 191-201, DOI: 10.1016/j.aquatox.2005.08.001.

Moss, B. & Allam, B. (2006). Fluorometric measurement of oxidative burst in lobster hemocytes and inhibiting effect of pathogenic bacteria and hypoxia. *Journal of Shellfish Research*, 25:1051-1057, DOI: 10.2983/0730-8000.

Parada, E. & Peredo, S. (2005). Relocation as a tool for biodiversity conservation and management. Lessons from *Diplodon chilensis* (Bivalvia, Hyriidae) studies. *Gayana*, 69: 41-47, DOI: 10.4067/S0717-65382005000100007.

Parada, E., Peredo-Parada, M., Quevedo, D. & Peredo, S. (2009). Establishment of suitable river reaches for relocation of vulnerable species: case *Diplodon chilensis*, DOI: 10.13140/2.1.3716.4484.

Parolini, M., Binelli, A. & Provini, A. (2011). Chronic effects induced by ibuprofen on the fresh water bivalve *Dreissena polymorpha*. *Ecotoxicology and Environmental Safety*, 74: 1586-1594, DOI: 10.1016/j.ecoenv.2011.04.025.

Patterson, M., Parker, B. & Neves, R. (1999). Glycogen concentration in mantle tissue of freshwater mussels (Bivalvia: Unionidae) during starvation and controlled feeding. *American Malacological Bulletin*, 15: 47-50.

Regoli, F., Nigro, M. & Orlando, E. (1998). Lysosomal and antioxidant responses to metals in the Antarctic scallop *Adamussium colbecki*. *Aquatic Toxicology*, 40, 375-392.

Regoli, F. & Principato, G. (1995). Glutathione, glutathione-dependent and antioxidant enzymes in mussel, *Mytilus galloprovincialis*, exposed to metals under field and laboratory conditions: implications for the use of biochemical biomarkers. *Aquatic Toxicology*, 31: 143-164, DOI: 10.1016/0166-445X(94)00064.

Regoli, F., Frenzilli, G., Bocchetti, R., Annarumma, F., Scarcelli, V., Fattorini, D. & Nigro, M. (2004). Time-course variations of oxyradical metabolism, DNA integrity and lysosomal stability in mussels, *Mytilus galloprovincialis*, during a field translocation experiment. *Aquatic Toxicology*, 68: 167-178, DOI: 10.1016/j.aquatox.2004.03.011.

Rocchetta, I., Lomovasky, B., Yusseppone, M., Sabatini S., Bieczynskic F., Ríos de Molina M. & Luquet C. (2014a). Growth, abundance, morphometric and metabolic parameters of three populations of *Diplodon chilensis* subject to different levels of natural and anthropogenic organic matter input in a glacial lake of North Patagonia. *Limnologica*, 44: 72-80, DOI: 10.1016/j.limno.2013.06.004.

Rocchetta, I., Pasquevich, M., Heras, H., Ríos de Molina, M. & Luquet, C. (2014b). Effects of sewage discharges on lipid and fatty acid composition of the Patagonian bivalve *Diplodon chilensis*. *Marine Pollution Bulletin*, 79: 211-219. DOI: 10.1016/j.marpolbul.2013.12.011.

Roméo, M., Hoarau, P., Garello, G., Gnassia-Barelli, M. & Girard, J. (2003). Mussel transplantation and biomarkers as useful tools for assessing water quality in the NW

Mediterranean. *Environmental Pollution*, 122: 369-378, DOI: 10.1016/S0269-7491(02)00303-2.

Ruiz, M.D., Iriel, A., Yusseppone, M.S., Ortiz, N., Di Salvatore, P., Fernández Cirelli, A., Ríos de Molina, M.C., Calcagno, J.A. & Sabatini, S.E. (2018). Trace metals and oxidative status in soft tissues of caged mussels (*Aulacomya atra*) on the North Patagonian coastline. *Ecotoxicology and Environmental Safety*, 155, 152-161, DOI: 10.1016/j.ecoenv.2018.02.064.

Sabatini, S. E., Chaufan, G., Juárez, A., Coalova, I., Bianchi, L., Eppis, M. R. & Ríos de Molina, M. C. (2009). Dietary copper effects in the estuarine crab, *Neohelice* (*Chasmagnathus*) *granulata*, maintained at two different salinities. *Comparative Biochemistry and Physiology*, 150, 521-527, DOI: 10.1016/j.cbpc.2009.07.006.

Sabatini, S., Rocchetta, I., Luquet, C., Guido, M. & Ríos de Molina, M. (2011a). Effects of sewage pollution and bacterial load on growth and oxidative balance in the freshwater mussel *Diplodon chilensis*. *Limnologica* 41: 356-362, DOI: 10.1016/j.limno. 2011.04.004.

Sabatini, S., Rocchetta, I., Nahabedian, D., Luquet, C., Eppis, M. & Ríos de Molina, M. C. (2011b). Oxidative stress and histological alterations produced by dietary copper in the freshwater bivalve *Diplodon chilensis*. *Comparative Biochemistry and Physiology, Part C*, 154: 391-398, DOI: 10.1016/j.cbpc.2011.07.009.

Schwalb, A. & Pusch, M. (2007). Horizontal and vertical movements of unionid mussels in a lowland river. *Journal of the North American Benthological Society*, 26: 261-272.

Serafim, A., Lopes, B., Company, R., Cravo, A., Gomes, T., Sousa, V. & Bebianno, B. (2011). A multi-biomarker approach in cross-transplanted mussels *Mytilus galloprovincialis*. *Ecotoxicology*, 20: 1959-1974, DOI: 10.1007/s10646-011-0737-7.

Sokal, R. R. & Rohlf, F. J. (1999). *Introducción a la Bioestadística*. Reverté, Barcelona,

Spain.

Sokolova, I., Frederich, M., Bagwe, R. & Lannin, G. (2012). Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. *Marine Environmental Research*, 79: 1-15, DOI: 10.1016/j.marenvres.2012.04.003.

Skoronski, E., Gonçalves, A.F., Hessa Melim, E.W., de Aguiar, A.R. Libardo, K., Fritzsche, W., Perez Fabrega, T. (2018). Evaluation of small-scale trout farming impact on water quality in Santa Catarina State, Brazil. *Latin American Journal of Aquatic Research*, 46. DOI: 10.3856/vol46-issue5-fulltext-11.

Soldati, A., Jacob, B., Schone, B., Bianchi, M. & Hajduk, A. (2009). Seasonal periodicity of growth and composition in valves of *Diplodon chilensis patagonicus* (D'Orbigny, 1835). *Journal of Molluscan Studies*, 75: 75-85, DOI: 10.1093/mollus/eyn044.

Toufexi, E., Tsarpali, V., Efthimiou, I., Vidali, M., Vlastos, D. & Dailianis, S. (2013). Environmental and human risk assessment of landfill leachate: an integrated approach with the use of cytotoxic and genotoxic stress indices in mussel and human cells. *Journal of Hazardous Materials*, 260: 593-601, DOI: 10.1016/j.jhazmat.2013.05.054.

Tsarpali, V. & Dailianis, S. (2012). Investigation of landfill leachate toxic potency: an integrated approach with the use of stress indices in tissues of mussels. *Aquatic Toxicology*; 124-125: 58-65, DOI: 10.1016/j.aquatox.2012.07.008.

Turja, R., Höher, N., Snoeijs, P., Baršienė, J., Butrimavičienė, L., Kuznetsova, T., Kholodkevich, S., Devier, M., Budzinski, H., Lehtonen, K. (2014). A multibiomarker approach to the assessment of pollution impacts in two Baltic Sea coastal areas in Sweden using caged mussels (*Mytilus trossulus*). *Science of the Total Environment*, 473-474: 398-409. DOI: 10.1016/j.aquatox.2012.07.008.

Van Handel E. (1965). Estimation of glycogen in small amount of soft tissue. *Analytical Biochemistry*, 11: 256-265.

Viarengo, A., Lowe, D., Bolognesi, C., Fabbri, E. & Koehler, A. (2007). The use of biomarkers in biomonitoring: A 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms. *Comparative Biochemistry and Physiology, Part C: Toxicology & Pharmacology*, 146: 281-300, DOI: 10.1016/j.cbpc.2007.04.011.

Yusseppone, M., Lomovasky, B., Luquet, C., Ríos de Molina, M. C. & Rocchetta, I. (2015). Age- and sex-dependent changes in morphometric and metabolic variables in the long-lived freshwater mussel *Diplodon chilensis*. *Marine and Freshwater Research*, 67; 1938-1947, DOI: 10.1071/MF15158.

Yusseppone, M. S., Márquez, F., Luquet, C. M., Brey, T., Ríos de Molina, M. C. & Rocchetta, I., (2017). Does shell shape variation play a role in conservation of the long-lived freshwater bivalve *Diplodon chilensis* (Bivalvia, Hyriidae)? *Ecohydrology*, 11:e1931, DOI: 10.1002/eco.1931.

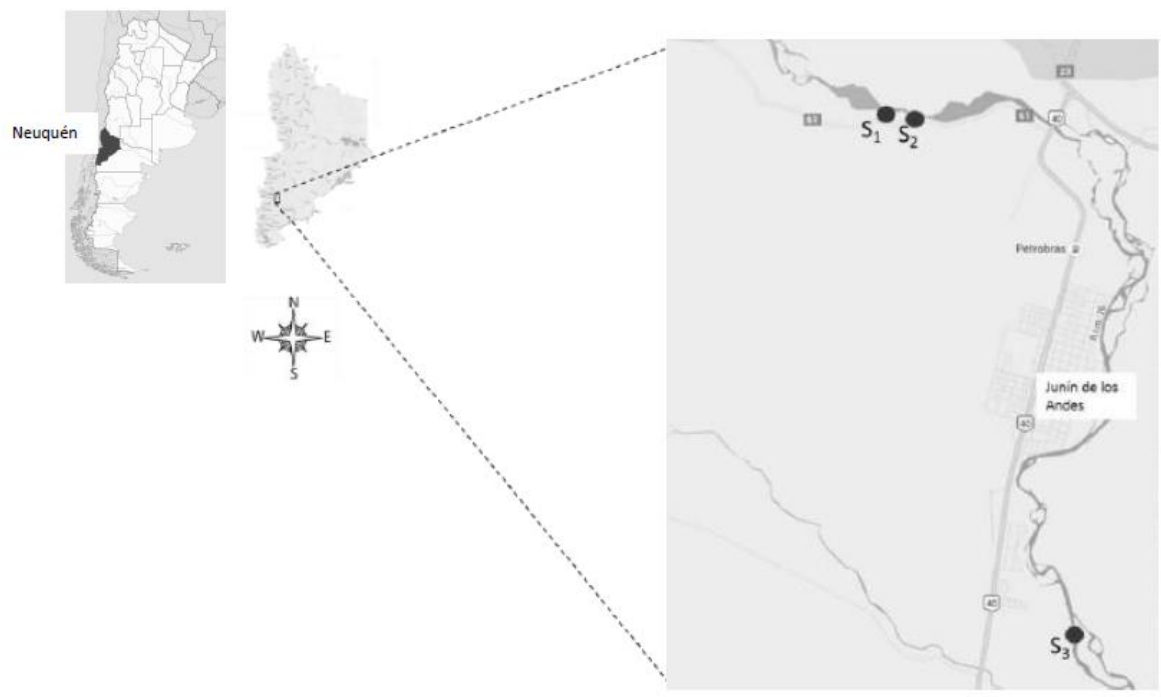


Figure 1. Location of sampling sites S1 ( $39^{\circ}54.953'S$ ;  $71^{\circ}06.363'W$ ), S2 ( $39^{\circ}54.973'S$   $71^{\circ}06.340'W$ ) and S3 ( $39^{\circ}60.785'S$   $71^{\circ}07.026'W$ ) at Chimehuin river, Neuquén province, Argentina.



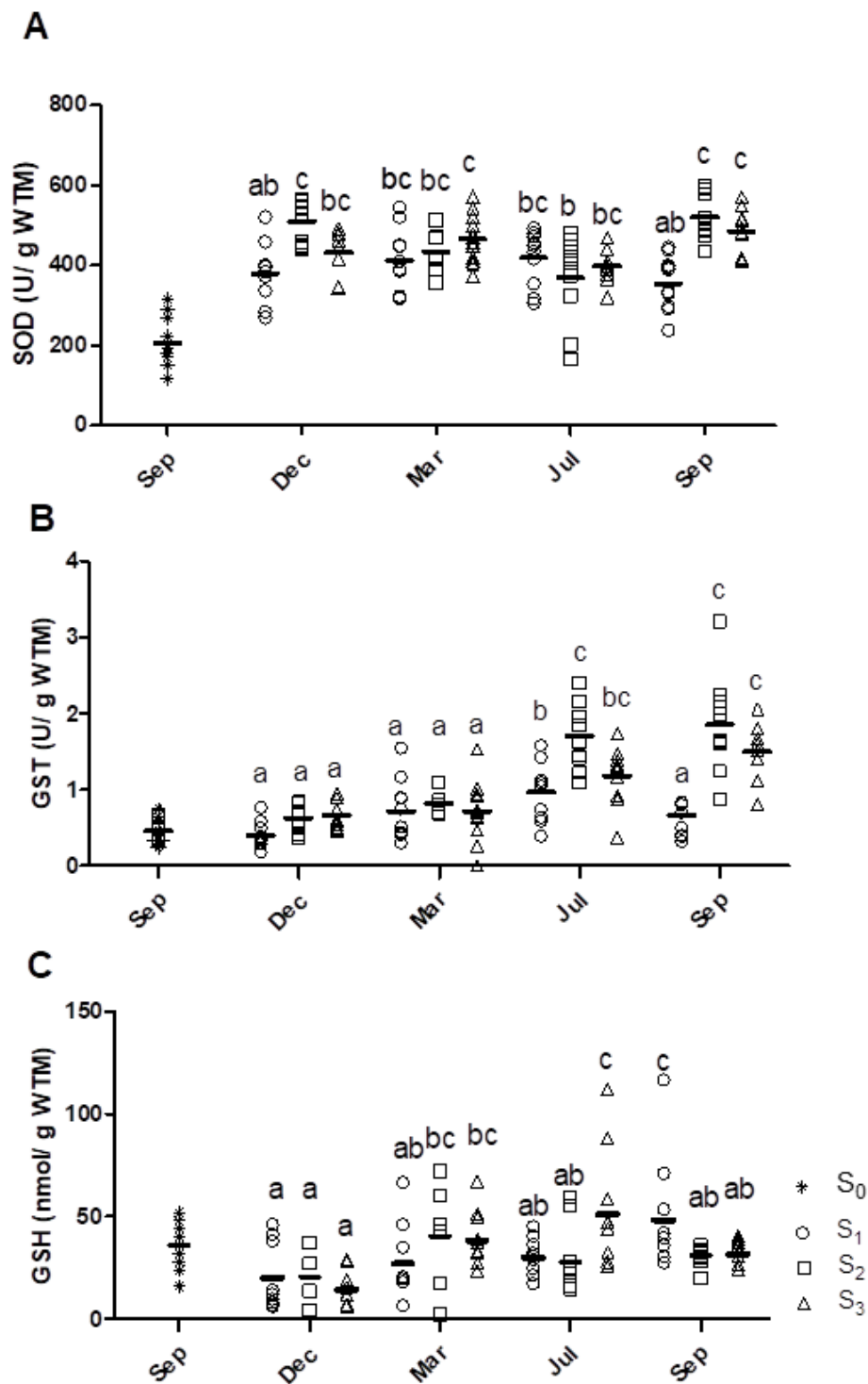


Figure 2. Enzyme activity of (A) superoxide dismutase (SOD) and (B) glutathione-S-transferase (GST), and (C) reduced glutathione (GSH) content in gill of *D. chilensis* exposed to S1 (clean site), S2 (downstream aquaculture waste) and S3 (downstream an open dump) at Chimehuin river before the transplantation in September (2011) and after 3 (December 2011), 6 (March 2012), 9 (July 2012) and 12 (September 2012) months, respectively. Different letters indicate significant differences with  $p < 0.05$ ,  $n=12$  (per site and time).

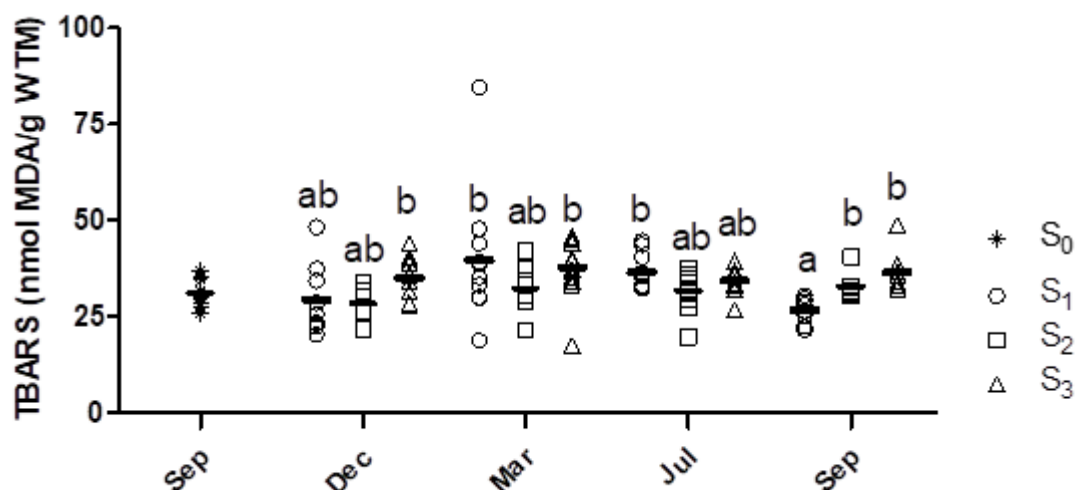


Figure 3. Thiobarbituric acid reactive substances (TBARS) levels in gill of *D. chilensis* exposed to S<sub>1</sub> (clean site), S<sub>2</sub> (downstream aquaculture waste) and S<sub>3</sub> (downstream an open dump) at Chimehuin river before the transplantation in September (2011) and after 3 (December 2011), 6 (March 2012), 9 (July 2012) and 12 (September 2012) months, respectively. Different letters indicate significant differences with  $p < 0.05$ ,  $n=12$  (per site and time).

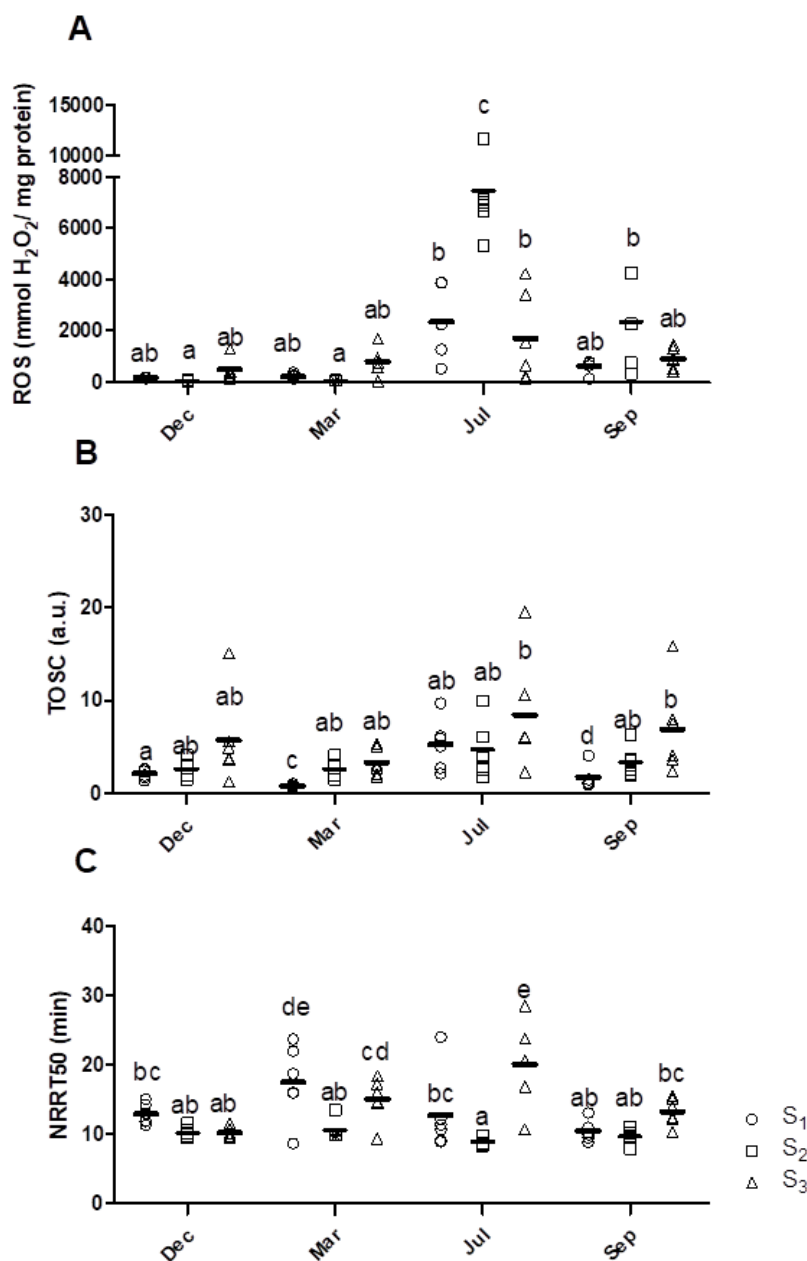


Figure 4. Reactive oxygen species (ROS) production (A), total oxyradical scavenging capacity (TOSC) (B) and neutral red retention time (NRRT50) in hemocytes of *D. chilensis* exposed to S1 (clean site), S2 (downstream aquaculture waste) and S3 (downstream an open dump) at Chimehuin river after 3 (December 2011), 6 (March 2012), 9 (July 2012) and 12 (September 2012) months, respectively. Different letters indicate significant differences with  $p < 0.05$ ,  $n=12$  (per site and time).

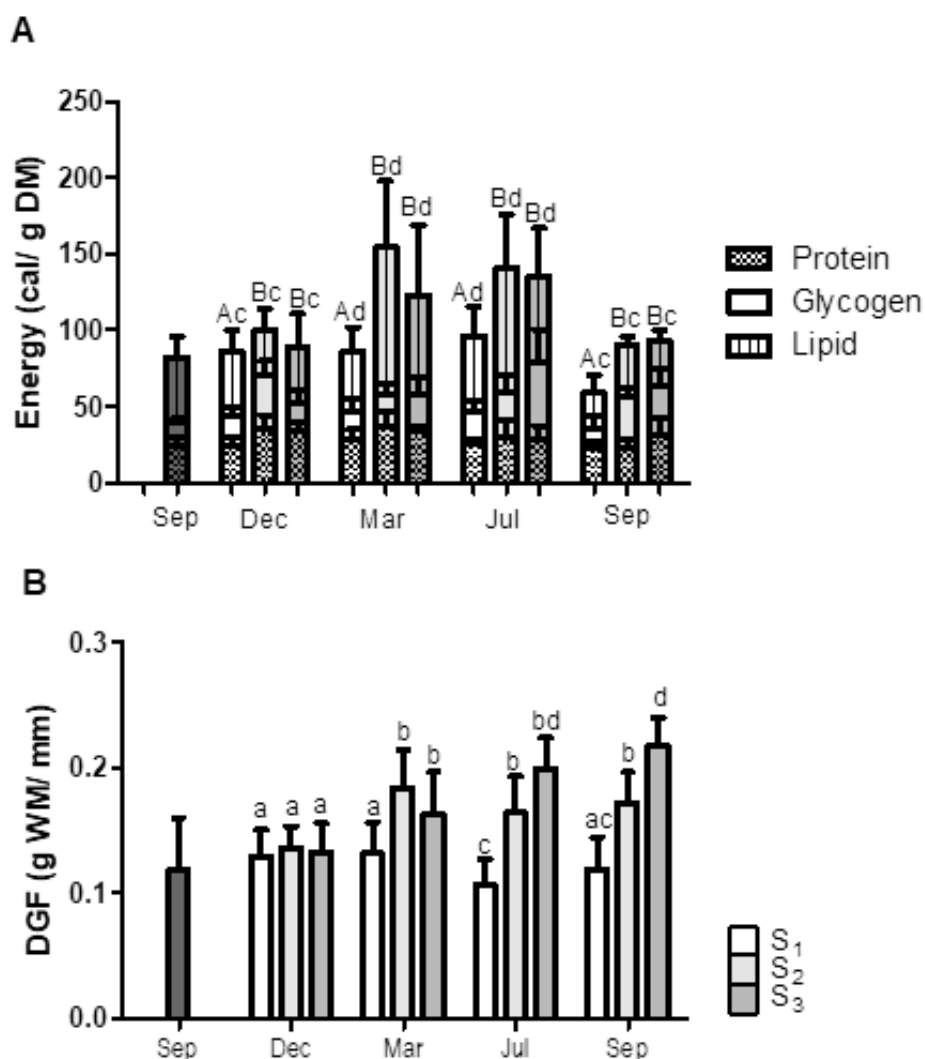


Figure 5. Energy values (A) and digestive gland factor (DGF) (B) in digestive gland of *D. chilensis* exposed to S1 (clean site), S2 (downstream aquaculture waste) and S3 (downstream an open dump) at Chimehuin river before the transplantation in September (2011) and after 3 (December 2011), 6 (March 2012), 9 (July 2012) and 12 (September 2012) months, respectively. In Fig. 5A letters A and B indicate significant differences between sites and c and d indicate significant differences between times ( $p < 0.05$ ),  $n=12$  (per site and time). In Fig. 5B different letters indicate significant differences with  $p < 0.05$ ,  $n=12$  (per site and time).

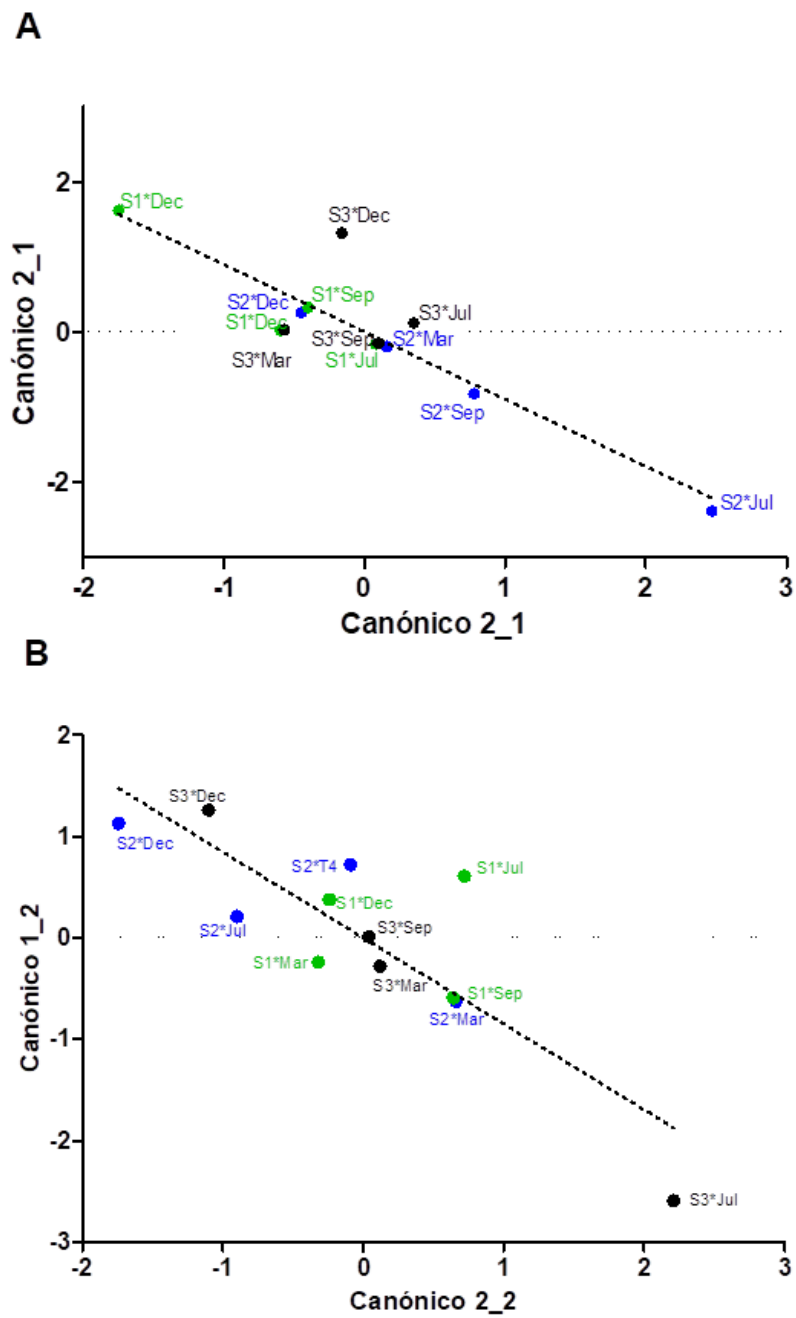


Figure 6. Canonical function 1 (A), y axis: environmental variables (canonical 1\_1), x axis: biomarkers (canonical 2\_1). Canonical function 2 (B), y axis: environmental variables (canonical 1\_2), x: biomarkers (canonical 2\_2).