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Effects of sewage pollution and bacterial load on growth and oxidative balance in the freshwater mussel *Diplodon chilensis*

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ABSTRACT

The effect of chronic exposure to *Escherichia coli* on morphometrical parameters, different antioxidant defenses, lipid peroxidation and protein oxidation has been studied in digestive gland of the freshwater mussel *Diplodon chilensis* in the laboratory during 6 weeks. In, a parallel field study, we evaluated morphometrical and oxidative stress parameters in digestive glands of mussels collected from a sewage polluted area (SMA) and from a pristine area (control). Both sites were characterized by analyzing bacteriological and physic-chemical parameters. In the laboratory *D. chilensis* was able to filter bacteria at high concentrations with a clearing rate of 0.510 ± 0.036 L/h per gram of dry soft tissue mass. The digestive gland mass to shell length ratio (DGM/SL), reduced glutathione (GSH), lipid peroxidation, as concentration of thiobartituric acid reactive substances (TBARS), and glutathione-S-transferase (GST) activity of mussels fed with bacteria were significantly higher than those of control mussels after the fourth week. Fecal bacteria in lake water samples were undetectable in the control, and higher than 24,000 MPN (most probable number)/100 mL in SMA. DGM/SL was higher in SMA. No differences between sites were observed in total lipid and protein content, neither in superoxide dismutase activity. GSH content was higher in SMA, with no difference in the oxidized form. GST activity and MDA were significantly higher in SMA but protein oxidation was not affected.

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Introduction

Bivalve mollusks are capable of removing large amounts of phytoplankton and other suspended particulate matter, helping to clear the water column (Nakamura and Kerciku 2000; Yamamuro et al. 2000). For this reason, these filter-feeding animals have become useful biological tools for mitigating the eutrophication and pollution of coastal ecosystems (Nelson et al. 2004; Gifford et al. 2005; Lindahl et al. 2005). Besides these decontaminating capacities, the sessile way of life and filter feeding habits of bivalves imply constant exposure to pollution and accumulation pollutants. Thanks to these characteristics, these animals have been extensively used for biomonitoring programs (Viarengo et al. 2007).

The freshwater mussel *Diplodon chilensis* (Hyriidae) is abundant in lakes and rivers of west Patagonia, Argentina and is widely distributed in temperate and cold lakes of Chile (Bonetto 1973; Valdovinos and Pedreros 2007). This species has been considered as a potential bioremediation tool, since it is able to reduce chlorophyll *a* and nutrient levels in co-cultures with salmon (Soto and Mena 1999) as well as to clearing coliform bacteria from domiciliary wells (Lara et al. 2002). In a recent work, Parada et al. (2008) have shown the suitability of *D. chilensis* as a biofilter for treating effluents from trout culture tanks. At an experimental scale, these authors have demonstrated the ability of this species to reduce the impact of fish farming on several water quality parameters such as total suspended solids, total phosphorous, nitrite and chemical oxygen demand.

It has also been demonstrated that *D. chilensis* accumulates heavy metals even when they are present at very low concentrations in the environment (Ribeiro Guevara et al. 2004). Several studies related to growth, age and life span of this species have been

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developed in this region (Parada et al. 1989; Viozzi and Brugni 2001; Semenas and Brugni 2002) but, besides the study of Valdovinos and Pedreros (2007), who relate the population decrease of this species in polluted lakes of Chile to increased turbidity, the effects of organic pollution on these animals have not been addressed so far.

Lake Lacar is a medium sized lake of glacial origin, with a surface of 50.28 km^2 and a maximum depth of 277 m. The city of San Martín de los Andes (about 30,000 people) is situated on the shore of the lake's eastern Bay. The river Pocahullo crosses the city, receiving untreated domestic effluents in different parts of its course and the discharge of the municipal sewage treatment plant (a tertiary treatment facility serving about 15,000 people), near its mouth. Since San Martín de los Andes economy depends almost exclusively on tourism, there are no significant industrial pollutants flowing into the lake. Considering the high capacity of *D. chilensis* to filter and digest bacteria reported by Lara et al. (2002), we assume that in the polluted zone, next to the sewage discharge, coliform bacteria constitute an important dietary source for this species. If this is so, these mussels should be involved in the reduction of the bacterial numbers and also in recycling the nutrients supplied by the domestic discharge. As a negative result of this kind of diet, this population of *D. chilensis* must deal with bacterial toxins.

It is well known that, in mammals, gram negative bacteria, typically *Escherichia coli*, produce toxic lipopolysaccharides (LPS or endotoxins), which cause inflammatory reactions mediated by cytokines, leading to the production of reactive oxygen species (ROS) by phagocytic cells. The resulting oxidative stress is evidenced by the increase of lipid peroxidation and reduction of reduced glutathione content, among other changes (Kheir-Eldin et al. 2001).

Oxidative stress parameters of marine and freshwater bivalves have been widely studied as pollution biomarkers. Almeida et al. (2003) have reported that the mussel *Perna perna* suffers lipid peroxidation in mantle tissue but not in digestive glands after chronic exposure to sewage-contaminated seawater. These authors suggest that the higher content of antioxidants in the digestive gland accounts for the lack of oxidative damage in this tissue. Bainy et al. (2000) have shown significant increase of GST activity in the digestive gland of the same species caged in an area subjected to domestic pollutants. In *Perna viridis*, changes in GST/GSH correlate with chlorinated hydrocarbons while lipid peroxidation, measured as thiobarbituric acid-reactive substances, is correlated with chlorinated pesticide concentration (see Nicholson and Lam 2005). For freshwater environments, lipid peroxidation and DNA damage in the digestive gland and gills of *Unio tumidus* have been considered as good biomarkers of degraded water quality (Charissou et al. 2004). However, as far as we know, oxidative stress variables have not been studied in relation to the capacity of bivalves to filter bacteria in sewage contaminated waters.

The present study was carried out in order to evaluate the effect of coliform bacteria (*E. coli*) and sewage pollution on the digestive gland of the freshwater mussel *D. chilensis* on growth, antioxidant defense system, detoxifying enzymes and oxidative damage to lipids and proteins through laboratory and field studies

Materials and methods

Sample collection

Individuals of *D. chilensis* and water samples (triplicates) were obtained at two different locations during winter. The control sample was obtained from Yuco, an unpolluted area of the Lacar lake, situated about 20 km from the city of San Martín de los Andes. The second sampling site was established on the Eastern Bay shore of the same lake, approximately 50 m from the discharge of San Martín de los Andes (SMA) sewage treatment plant (40°10′S, 71°20′60″W). The distance between sampling sites was 20 km. Mussels and water samples were obtained by a diver from banks located at a depth of 5–8 m and 3–5 m at Yuco and SMA, respectively. The mussels were transported alive at low temperature to the University of Buenos Aires, where they were used for field or laboratory studies. Water samples were collected from the sampling sites, in sterile plastic containers, for bacteriological and physicochemical analysis. The concentration of bacteria was measured by the most probable number (MPN) method per 100 mL of water (Guinea et al. 1979). The following variables were measured in situ: temperature, pH, dissolved oxygen and conductivity with a portable electronic meter (Hanna, Hach). Samples for chemical analysis were collected and cold preserved until further processing. Sodium, potassium, magnesium and calcium concentrations and total hardness were determined by ionic chromatography using a Dionex column ICS 3000-DX500. Total phosphorus (TP) and nitrogen (TN) were performed by Koroleff (1983). Zinc and copper were analyzed by atomic absorption spectrometry (Shimadzu 6800) with a graphite furnace atomizer (GFA6500). Chlorophyll *a* concentrations were calculated according to the equations given by Marker et al. (1980). Alkalinity, total suspended solids (TSS), biological oxygen demand (BOD_5) and chemical oxygen demand (COD) were estimated by Standard Methods (APHA 1992).

Field study

Thirty animals (6.41 ± 1.66 cm shell length) from each sampling site were immediately processed after collection. They were measured, and anesthetized by placing on ice before they were killed. We recorded shell length and then dissected and weighed the digestive gland, which was then homogenized to study oxidative stress parameters. Soluble protein was measured by the method of Bradford (1976).

Laboratory study

Animals collected at the unpolluted site (Yuco) were kept for a week in the laboratory and fed with the green alga *Chlorella kessleri*. The mean size ± standard error of the group of mussels used for the laboratory experiments was 6.13 ± 1.49 cm shell length.

Filtration rate

Each one of seven mussels was placed in an individual beaker with 500 mL of dechlorinated tap water, with constant aeration and a temperature of 20 °C. Each beaker was replicated but with the mussel omitted, in order to correct the obtained values for bacterial growth (control). Bacteria were kept in suspension by means of gentle stirring with a magnetic device. After 48 h of acclimation with no food, when each individual had the valves opened, *E. coli* strain JM 109, kindly provided by Dr. Sandra Russal (Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires), was added at a final concentration of 2.8×10^8 CFU/mL, the maximum concentration at which the mussels filtrated in preliminary trials. One sample per beaker was taken at 0, 1, 2 and 3 h and present bacteria were estimated by plate count in agar-nutritive broth medium (Merck 105450). Filtration rate (F) for each individual, expressed as L/h per dry soft tissue mass (g), was calculated according to Jorgensen (1990):

$$F = V * \frac{(\log \text{final bacterial concentration in control} - \log \text{final bacterial concentration in the experiment})}{0.434} * \frac{\text{time}}{\text{dry soft tissue mass}}$$

where V is the beaker volume, control is the beaker without mussel, and experiment, the beaker with mussel. 0.434 is a conversion factor for logarithms. Dry soft tissue mass was measured after dissecting out and drying the soft tissues in an oven for 48 h at 60 °C, until constant mass. Time is the interval after which a sample was taken. Since the F values calculated for each one of the 3 intervals were similar, only the results for 3 h were reported.

Effects of filtering E. coli

Animals collected at Yuco were kept for a week in the laboratory, fed with the green alga *C. kessleri* and then fasted for 48 h before the start of the experiments. A total of 64 mussels, randomly sorted into two groups, control and treated, were used for this study. Eight mussels of each group were then placed in each of 4 glass containers with 5 L of aerated dechlorinated tap water. The resulting density was similar to the observed in the Lacar lake (SMA site), 12 individuals/m^2. Laboratory conditions were 20 ± 2 °C and 12:12 light/dark photoperiod.

The control group was fed with *C. kessleri* (final concentration 4×10^6 cells/mL). The treated group was fed with *E. coli* strain JM 109 at a final concentration of 6.4×10^6 CFU/mL. We used a bacterial concentration about 40-fold lower than the maximum concentration at which these mussels are able to filter, in order to ensure the survival of the mussels throughout the experiment. Water was changed and fresh *C. kessleri* or *E. coli* were added daily for 6 weeks, in the control and in the treated group, respectively.

Groups of 4 control and 4 treated individuals were sacrificed at the following times: 24 h, 48 h, and after 1, 2, 3, 4, 5 and 6 weeks. Digestive gland glutathione-S-transferase activity, lipid peroxidation and reduced glutathione levels were measured as described below.

Analysis

Determination of antioxidants

For determination of antioxidants, digestive gland tissue from each animal was homogenized in the cold with phosphate buffer, 50 mM (pH 7) and containing protease inhibitors (phenylmethylsulfonil fluoride, PMSF, 0.5 mM and benzamidine, 10 mM). The homogenate was centrifuged 15 min at $7000 \times g$; and the resulting supernatant was used for the assays.

Total glutathione, reduced and oxidized (GSH + GSSG), was estimated by the GSSG reductase recycling assay as described by Anderson (1985). The reaction for GSH–GSSG determination was initiated by adding GSSG reductase, diluted in Na-phosphate–EDTA buffer (pH 7.5). The acid-soluble sulfhydryl content (non protein thiols, NPT, of which GSH forms ca. 90%) was then determined by the method of Sparnins et al. (1982). Briefly, the sulfhydryl content was quantified spectrophotometrically by recording the stoichiometric formation of 5-thio (2 nitrobenzoic acid) (TNB) from DTNB (5,5′-dithiobis-(2-nitrobenzoic) acid) at 412 nm. Values were expressed as nmoles per gram of digestive gland. Dithiobis (2-nitrobenzoic acid) DTNB was prepared in Na-phosphate buffer (pH 8.0). GSH was determined in the same way but omitting the GSSG reductase step. GSSG was estimated as 1/2 (total glutathione–GSH).

Superoxide dismutase (SOD) activity was determined by measuring the inhibition of the reduction of nitroblue tetrazolium (NBT) by the light activated generation of O_2^- by riboflavin in the presence of methionine. The rate of reduction of NBT was measured at 560 nm (Fridovich 1997).

Glutathione-S-transferase (GST) activity was measured by the technique of Habig et al. (1974). Briefly we mixed 10 μL of GSH (100 mM in phosphate buffer) and 20 μL of sample in 960 μL of 100 mM phosphate buffer (pH 6.5). After adding 10 μL of 100 mM 1-chloro-2,4-dinitrobenzene (CDNB) in ethanol, the change in absorbance at 340 nm was followed during 90 s. One GST Unit was defined as the amount of enzyme needed to catalyze the formation of 1 μmol of GS-DNB per minute at 25° C.

Oxidative damage

Total lipids were extracted from the homogenate with a chloroform–methanol mixture (2:1 v/v) and were quantified according to the Bligh and Dyer (1959). The level of lipid peroxidation was determined by the thiobarbituric acid reactive substances method (TBARS) (Fraga et al. 1988). Briefly, the $11{,}000 \times g$ supernatant from total homogenate was mixed with thiobarbituric acid (TBA) solution and incubated at 95–100 °C for 45 min. After cooling, the reaction mixture was centrifuged and the supernatant absorbance was determined at 535 nm.

Oxidative damage to proteins was evaluated by the carbonyl content of the digestive glands (Reznick and Packer 1994). Homogenate supernatants were incubated with 10 mM 2,4-dinitrophenylhydrazine for 1 h at room temperature. Proteins were precipitated with 20% (w/v) trichloroacetic acid, washed twice with ethanol/ethyl acetate (1:1), and dissolved in 6 M guanidine hydrochloride. Carbonyl content was evaluated by measuring absorbance at 372 nm ($\varepsilon = 22{,}000\ M^{-1}\ cm^{-1}$) and expressed as μmol carbonyls per milligram of protein.

Statistics

Data are presented as mean values ± standard error of the mean (SEM). Results for each analyzed variable in the field study were compared between sites of sampling by Student's t test. Results from laboratory experiments were analyzed by two way ANOVA followed by a Dunnett's post hoc test. Differences were considered significant with $P < 0.05$. Normality and homogeneity of variances were tested with Lilliefors and Bartlett tests, respectively (Sokal and Rohlf 1984). Graph Pad Prism 3 software was used for statistical analysis.

Results

Laboratory study

In the filtration experiment, *D. chilensis* cleared *E. coli* from water at a rate of 0.510 ± 0.036 L/h per gram of dry soft tissue mass. There were no significant differences (ANOVA $P > 0.05$) in total lipid content and in DGM/SL between mussels fed with *E. coli* and controls during the 42 days of experiment (data not shown).

There was no effect of filtering *E. coli* on the mussel's digestive gland, neither on enzymatic and non enzymatic antioxidants nor in oxidative damage to lipids during the first week. GSH level started to increase at day 14 in treated individuals and became significantly higher than the control at day 42 (ANOVA $P < 0.01$) (Fig. 1) while GST activity and lipid peroxidation increased since day 35 and 42, respectively, in mussels fed with bacteria (ANOVA $P < 0.001$ and $P < 0.05$, respectively) (Figs. 2 and 3). At the end of the experiment GSH was 43% higher in treated than in control mussels (308.63 ± 24.18 vs. 216.14 ± 42 nmol/mg protein). GST activity was increased in treated mussels by 68% (0.848 ± 0.034 vs. 0.505 ± 0.106 U/mg protein) while lipid peroxidation was increased by 37% (2.42 ± 0.27 vs. 1.77 ± 0.23 μmol TBARS/mg protein).

Field Study

Physic-chemical variables and bacteria counts

Characteristics of both sampling sites are presented in Table 1. Control and SMA sites presented soft waters due to the hardness levels, lower than 60 mg/L $CaCO_3$ (Romero Rojas 1999), with similar cations and metals concentrations. The low values of BOD and

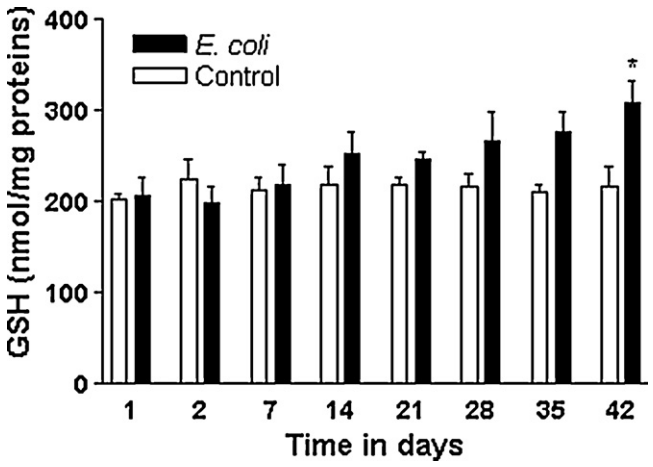

Fig. 1. Reduced glutathione (GSH) content (nmol/mg protein) in mussels fed with a non toxic green alga, *Chlorella kessleri*, as control, and with the fecal bacteria *Escherichia coli*. Data are expressed as means ± SEM ($n = 4$). Asterisk denotes significant differences between control and treated mussels: $^{*}P < 0.05$.

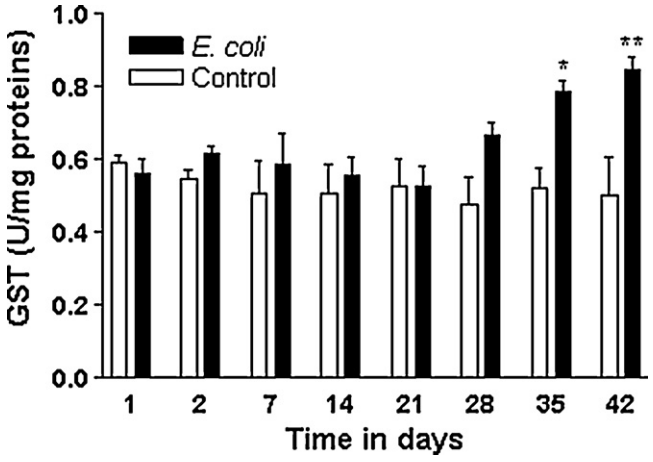

Fig. 2. Glutathione-S-transferase (GST) activity (Units/mg protein) in mussels fed with a non toxic green alga, *Chlorella kessleri*, as control, and with the fecal bacteria *Escherichia coli*. Data are expressed as means ± SEM ($n = 4$). Asterisks denote significant differences between control and treated mussels: $^{*}P < 0.05$ and $^{**}P < 0.01$.

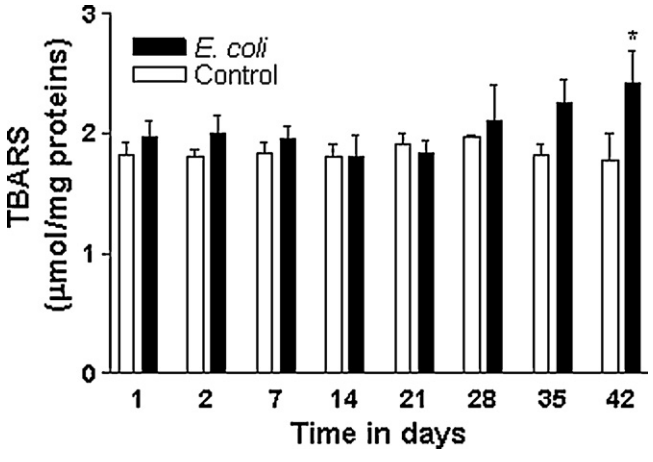

Fig. 3. Lipid peroxidation (µmol TBARS/mg protein) in mussels fed with a non toxic green alga, *Chlorella kessleri*, as control, and with the fecal bacteria *Escherichia coli*. Data are expressed as means ± SEM ($n = 4$). Asterisk denotes significant differences between control and treated mussels: $^{*}P < 0.05$.

Table 1
Physic-chemical variables of water samples collected from Yuco (control) and SMA sites.

Parameter/site	Control	SMA
pH	6.9 ± 0.5	7.1 ± 0.4
Conductivity (µS cm^{-1})	50 ± 2.5	67 ± 2.8
TSS (mg L^{-1})	2.5 ± 0.1	6.6 ± 2.1*
DO (mg L^{-1})	11 ± 0.6	10.6 ± 0.7
Total P (µg L^{-1})	2.2 ± 0.1	3.0 ± 0.3
Total N (µg L^{-1})	118 ± 5.9	305 ± 35.4*
Alcalinity (mg L^{-1} HCO_3^-)	31 ± 1.6	46.5 ± 0.7*
Total hardness (mg L^{-1} $CaCO_3$)	30 ± 1.5	52 ± 5.7*
Ca (mg L^{-1})	6.0 ± 0.3	8.0 ± 1.4
Mg (mg L^{-1})	4.0 ± 0.2	5.5 ± 0.7
K (mg L^{-1})	0.8 ± 0.1	0.9 ± 0.2
Cu (mg L^{-1})	<0.05	<0.05
Zn (mg L^{-1})	0.02	0.03
Chl a (µg L^{-1})	1.0 ± 0.1	1.4 ± 0.4
BOD_5 (mg L^{-1})	1.6 ± 0.1	1.8 ± 0.1
COD (mg L^{-1})	3.0 ± 0.2	3.1 ± 0.1

Samples were obtained during winter. Data are expressed as means ± SD ($n = 3$).
* Significant differences between control and SMA are indicated by asterisks: $P < 0.05$.

COD indicated a very good water quality (Romero Rojas 1999). The COD/BOD ratio suggested the presence of organic matter biodegradable (control: 1.9 ± 0.1, SMA: 1.7 ± 0.2, $P > 0.05$). The main differences between both places presented in this Table were the TN, TSS, alkalinity and total hardness values ($P < 0.05$).

The mean number of total coliform bacteria at the control sampling site was 17 ± 4.4 MPN/100 mL, with absence of fecal bacteria, whereas in SMA most counts yielded numbers of total and fecal coliform bacteria higher than 24,000 MPN/100 mL. The counts from SMA resulted much higher than the maximum value recommended for recreational use, 400 or 200 MPN/100 mL, for total and fecal coliform bacteria, respectively (Municipal regulation number 6564 City of San Martín de los Andes).

Growth

Mussels collected at the polluted site near the sewage plant of SMA were larger than the controls collected at Yuco (7.46 ± 0.61 and 5.31 ± 0.71 cm, respectively) ($P < 0.001$, $n = 30$). The digestive gland mass also showed differences between mussels collected at both places, being 1.74 ± 0.17 g for SMA and 0.69 ± 0.09 g for the controls ($P < 0.001$, $n = 30$). The most significant difference was observed for the digestive gland mass to shell length ratio (DGM/SL). This ratio was 0.23 ± 0.003 for SMA mussels and 0.13 ± 0.01 for the controls ($P < 0.0001$, $n = 30$) (Fig. 4). Correlation analysis for DGM/SL vs.

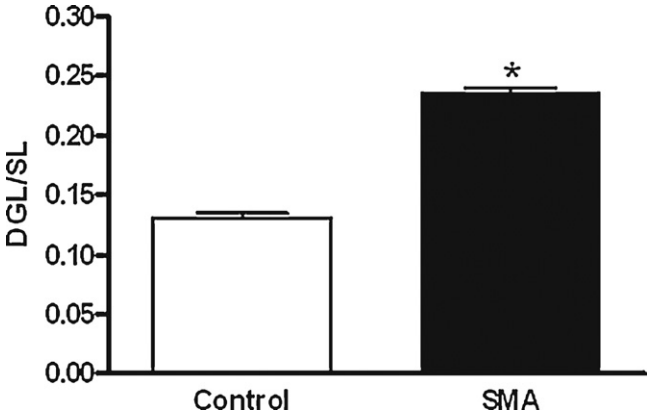

Fig. 4. Digestive gland mass to shell length ratio (DGM/SL) in control and SMA samples. Data are expressed as means ± SEM ($n = 30$). Asterisk denotes significant differences between control and SMA: $^{*}P < 0.0001$.

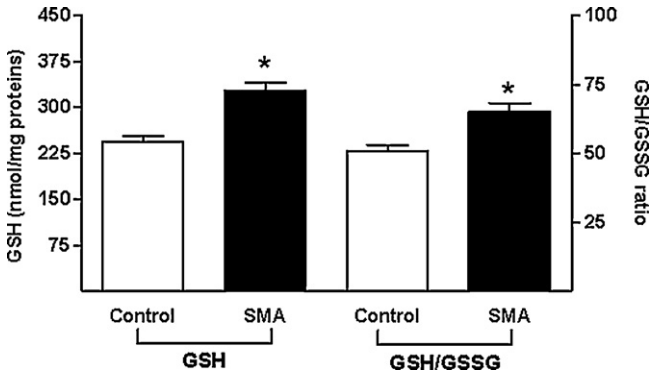

Fig. 5. Reduced glutathione (GSH) content (nmol/mg protein) and reduced to oxidized glutathione ratio (GSH/GSSG) in control and SMA samples. Data are expressed as means ± SEM ($n = 30$). Asterisks denote significant differences between control and SMA: $^*P < 0.00001$ for GSH and $^*P < 0.005$ for GSH/GSSG ratio.

SL resulted not significant for mussels from both sites (data not shown).

Antioxidant defense

Individuals collected from SMA showed higher GSH level than control animals (327 ± 13.29 and 243.3 ± 9.96 nmol/mg protein for SMA and control, respectively, $P < 0.00001$) (Fig. 5, bars on the left). The calculated oxidized glutathione (GSSG) level was not significantly different between sites (2.73 ± 0.18 and 2.59 ± 0.15 nmol GSSG/mg protein for SMA and control, respectively, data not shown in graph). The GSH/GSSG ratio was significantly higher in SMA (130.5 ± 5.95 vs. 102.0 ± 4.08, $P < 0.005$) (Fig. 5, bars on the right). The percentage difference between SMA and control was 34% and 27% for GSH content and GSH/GSSG ratio, respectively. The GST activity was increased by a 19% in the digestive gland of SMA animals (0.69 ± 0.044 vs. 0.58 ± 0.034 U/mg protein, for SMA and controls, respectively, $P < 0.01$) (Fig. 6, bars on the left). In contrast, SOD activity was not statistically different between groups (0.0207 ± 0.001 U/mg protein for SMA and 0.0213 ± 0.0009 U/mg protein for the controls) (Fig. 6, bars on the right).

Oxidative damage

The oxidized proteins level, assayed as carbonyl groups in digestive gland homogenates from SMA animals was 0.87 ± 0.010 nmol/mg protein. This value was almost identical to the obtained from control animals, 0.90 ± 0.0071 nmol/mg protein (data not shown in graph). No significant differences

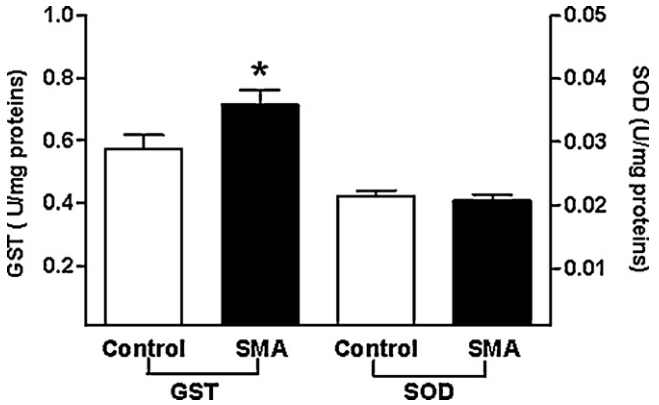

Fig. 6. Glutathione-S-transferase (GST) (Units/mg proteins) and superoxide dismutase (SOD) (Units/mg proteins) activity in control and SMA samples. Data are expressed as means ± SEM ($n = 30$). Asterisk denotes significant differences between control and SMA: $^*P < 0.01$.

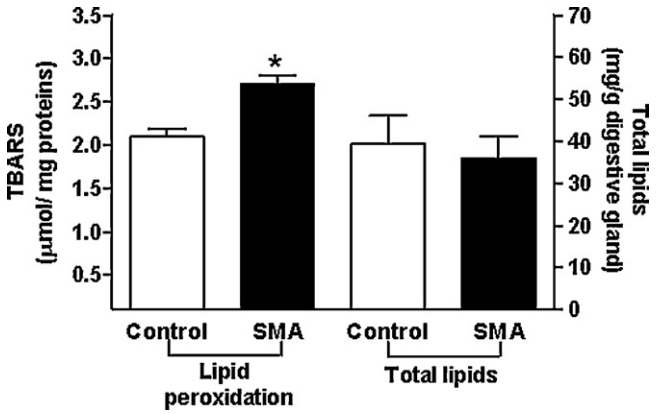

Fig. 7. Lipid peroxidation (µmol TBARS/mg protein) and total lipid content (mg lipid/g digestive gland) in control and SMA samples. Data are expressed as means ± SEM ($n = 30$). Asterisk denotes significant differences between control and SMA: $^*P < 0.001$.

were found in the total protein content between control and SMA samples (2.57 ± 0.069 and 2.48 ± 0.068 mg/mL, respectively).

On the other hand, the exposure to the polluted environment of SMA produced a 30% increase in lipid peroxidation (2.702 ± 0.105 vs. 2.094 ± 0.096 µmol TBARS/mg protein $P < 0.001$, $n = 30$) (Fig. 7, bars on the left). The total lipid content per g of digestive gland was similar between groups (Fig. 7, bars on the right).

Discussion

D. chilensis is a filter feeder that feeds on bacteria-phytoplankton, benthos and organic matter. It is capable of digesting coliform bacteria (Lara et al. 2002) and can remove large quantities of microalgae and nutrients from eutrophic lakes (Soto and Mena 1999). Our experimental results show that the filtration rate of *E. coli* by *D. chilensis* is similar to filtration rate of microalgae by this species, measured by Soto and Mena (1999). The ability of *D. chilensis* to feed on coliform bacteria could possibly explain the larger size individuals collected at SMA, from a muddy substrate where fecal bacteria are abundant, compared to those sampled at an oligotrophic sandy site (Yuco). In contrast, Lara and Parada (1991) have reported lower growth rate and condition index in mussels obtained from muddy substrates, rich in organic matter than in mussels obtained from sandy substrates.

Our results could also respond to differences in the age structure between the studied populations. Parada et al. (1989) have reported differences in growth rate, longevity and size between a lentic and a lotic population of *D. chilensis*, which are subject to different seasonal environmental variations.

On the other hand, the capacity of *D. chilensis* to feed on coliform bacteria is probably restricted to adult mussels, since young individuals, which can be more sensitive to water pollution, have not been frequent in our samples from SMA. Preliminary results of our group show that all the individuals collected for this study had been recruited to the population before the pollution problems had been evident.

The higher DGM/SL ratio recorded in SMA individuals may also indicate increased metabolic activity related to higher food availability and/or higher detoxifying activity. In the laboratory, the DGM/SL ratio has increased both in mussels fed green microalgae and in bacteria, after being fasted for 48 h. After 1 and 2 weeks, both groups have reached similar DGM/SL values to that obtained for control mussels in the field study. Thus, the higher DGM/SL observed in SMA mussels should likely respond to another kind of pollutants present in sewage water or to the combination of such pollutants with high bacterial concentration. Oligotrophic glacial

deep lake with low conductivity and low nutrients are the main characteristic of the Lacar lake (Diaz et al. 2007). Our results are in concordance with those for both sampling sites. Although the physic-chemical parameters are within the values recommended for recreational use (Romero Rojas 1999), total nitrogen and total suspended solids showed higher values in SMA probably due to the urban waste discharges, as indicative for eutrophication process (OECD 1982). The NT values measured in our study are not toxic for *D. chilensis* (Valdovinos and Pedreros 2007).

In many species, an increase in body or digestive gland mass can be accounted for by the synthesis of saturated lipids as reserve energy for the activation/synthesis of antioxidant compounds under environmental stress conditions (Helm et al. 1991). However, the total lipid content of the *D. chilensis* digestive gland does not differ among individuals collected from the polluted and the clean site. Similarly, in the laboratory study, this variable was insensitive to the kind of food source, *E. coli* or green algae. Thus, if *D. chilensis* stores saturated lipids in the digestive gland for activation/synthesis of antioxidant compounds, this function is neither stimulated by exposure to sewage pollution nor by feeding on *E. coli*. A previous study on this species has shown differences in the fatty acid composition in relation to food quality but only small changes in the lipid levels and distribution (Pollero and Brenner 1981).

Feeding on gram-negative bacteria exposes the mussels to LPS present in the bacterial cell walls. In mammals, bacterial LPSs induce acute oxidative stress responses (Sugino et al. 1987). Repeated injections of sublethal doses of LPS produce oxidative stress but subsequently increase the antioxidant capacity, decreasing lipid peroxidation (Ben-Shaul et al. 1999). In our laboratory study, *D. chilensis* was able to consume great numbers of *E. coli* without showing any acute change in lipid peroxidation or in antioxidant defenses. However, long term exposure (4–6 weeks) enhances the antioxidant defense by increasing GSH levels and GST activity. In spite of this enhancement of the antioxidant capacity, some lipid peroxidation is evident at this time. Accordingly, in the field study, GSH content, GSH/GSSG ratio and GST activity are augmented in digestive glands of *D. chilensis* taken from the sewage polluted site. These and the laboratory results suggest that chronic exposure to sewage pollution or to *E. coli* alone induce de novo synthesis of GSH in parallel with an increase of GST activity, as has been earlier reported for stressed plants (May and Leaver 1993; Jain and Bhalla-Sarin 2001).

For bivalves, Torres et al. (2002) report increased activities of several antioxidant and detoxifying enzymes (CAT, GPx, GST) in the mussel *Mytella guyanensis* taken from sites polluted with waste water and trace metals. In contrast with our results, these authors report low levels of GSH and high levels of GSSG, indicative of an acute oxidative stress condition. Other field studies have shown increased GST activity and oxidative damage in bivalve species exposed to organic pollutants (Fitzpatrick et al. 1997; Vidal et al. 2002). In particular, long-term exposure to municipal effluents elevates GST activity in digestive gland of freshwater mussels (Gagne et al. 2004). However, these authors have related this enzymatic response to the presence of organic toxicants other than LPSs, such as polyaromatic hydrocarbons, pharmaceutical drugs and personal-care products. Since some of these pollutants are probably present in the sewage water of SMA; their contribution to oxidative stress responses observed in *D. chilensis* cannot be disregarded.

The results of this work indicate that the sewage effluent from the city of San Martín de los Andes affects the metabolism of *D. chilensis*. The increase of the non-enzymatic and enzymatic antioxidants, like GSH and GST activity, together with the lack of significant protein oxidation, suggest an adaptive response to chronic exposure to wastewater pollution. However, there is an increased damage to lipids revealed by augmented MDA levels under these conditions, which shows that the oxidative stress is not fully compensated. Although this oxidative status seems to be well tolerated by adult individuals, the sensitivity of juveniles should be studied in further works in order to assess possible effects of sewage pollution at a population dynamics level.

The capability of adult *D. chilensis* to live in environments heavily polluted by sewage, and its capacity to filter and digest fecal bacteria at high concentrations demonstrated by Lara et al. (2002) and by our laboratory study, make this species a promising tool for bioremediation of urban pollution. Although further studies are needed to clarify whether the biochemical changes observed in the field study respond specifically to bacteria or to toxic products present in sewage water, the analysis of oxidative stress responses in *D. chilensis* can be a useful method for in situ monitoring studies in Patagonian lakes and rivers.

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References

Almeida, E.A., Bainy, A.C.D., Loureiro, A.P.M., Medeiros, M.H.G., Di Mascio, P., 2003. DNA and lipid damage in the brown mussel *Perna perna* from a contaminated site. Bull. Environ. Contam. Toxicol. 71, 270–275.

Anderson, E.M., 1985. Determination of glutathione and glutathione disulfide in biological samples. Meth. Enzymol. 70, 548–555.

APHA, 1992. American Water Works Association and Water Pollution Federation. Standard Methods for Examination of Water and Wastewater, 17th ed. American Public Health Association, Madrid, Spain.

Bainy, A.C.D., Almeida, E.A., Müller, I.C., Ventura, E.C., Medeiros, I.D., 2000. Biochemical responses in farmed mussel *Perna perna* transplanted to contaminated sites on Santa Catarina Island, SC, Brazil. Mar. Environ. Res. 50, 411–416.

Ben-Shaul, V., Sofer, Y., Bergman, M., Zurovsky, Y., Grossman, S., 1999. Lipopolysaccharide induced oxidative stress in the liver: comparison between rat and rabbit. Shock 12, 288–293.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917.

Bonetto, A.A., 1973. Nayades de la Patagonia. Rev. Asoc. Cienc. Nat. Litoral. 4, 117–185.

Bradford, M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Charissou, A.M., Cossu-Leguille, C., Vasseur, P., 2004. Relationship between two oxidative stress biomarkers, malondialdehyde and 8-oxo-7,8-ihydro-29-deoxyguanosine, in the freshwater bivalve *Unio tumidus*. Sci. Total Environ. 322, 109–122.

Diaz, M., Pedrozo, F., Reynols, C., Temporetti, P., 2007. Chemical composition and the nitrogen-regulated trophic state of Patagonian lakes. Limnologica 37, 17–27.

Fitzpatrick, P.J., O'Halloran, J., Sheehan, D., Walsh, A.R., 1997. Assessment of a glutathione-S-transferase and related proteins in the gill and digestive gland of *Mytilus edulis* (L.) as potential organic pollution biomarkers. Biomarkers 2, 51–56.

Fraga, C.G., Leibovitz, B.E., Tappel, A.L., 1988. Lipid peroxidation measured as thiobarbituric acid-reactive substances in tissue slices. Characterization and comparison with homogenates and microsomes. Free Radic. Biol. Med. 4, 155–161.

Fridovich, I., 1997. Superoxide anion radical, superoxide dismutases, and related matters. J. Biol. Chem. 250, 18515–18517.

Gagne, F., Blaisea, C., Hellou, J., 2004. Endocrine disruption and health effects of caged mussels, *Elliptio complanata*, placed downstream from a primary-treated municipal effluent plume for 1 year. Comp. Biochem. Physiol. C 138, 33–44.

Gifford, S., Dunstan, R.H., O'Connor, W., Macfarlane, G.R., 2005. Quantification of in situ nutrient and heavy metal remediation by a small pearl oyster (*Pinctada imbricata*) farm at Port Stephens, Australia. Mar. Pollut. Bull. 50, 417–422.

Guinea, J., Sancho, J., Pares, R., 1979. Análisis microbiológico de aguas. Aspectos aplicados. Universidad de Barcelona, Ediciones Omega, Barcelona, pp. 11–100.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.

Helm, M.M., Holland, D.L., Utting, S.D., East, J., 1991. Fatty acids composition of early non-feeding larvae of european flat oyster *Ostrea edulis* L.J. Mar. Biol. Assoc. U.K. 71, 675–691.

Jain, M., Bhalla-Sarin, N., 2001. Glyphosate-induced increase in glutathione S-transferase activity and glutathione content in Groundnut (*Arachis hypogaea* L.). Pest. Biochem. Physiol. 69, 143–152.

Jorgensen, C.B., 1990. Bivalve Filter Feeding: Hydrodynamics, Bioenergetics, Physiology and Ecology. Olsen & Olsen, 140 pp.

Kheir-Eldin, A.A., Motawi, T.K., Gad, M.Z., Abd-ElGawad, H.M., 2001. Protective effect of vitamin E, β-carotene and N-acetylcysteine from the brain oxidative stress induced in rats by lipopolysaccharide. Int. J. Biochem. Cell Biol. 33, 475–482.

Koroleff, F., 1983. Simultaneous oxidation of nitrogen and phosphorus compounds by persulfate. In: Grosshoff, K., Eberhadt, M., Kremling, K. (Eds.), Methods of Seawater Analysis. Verlag Chemie, Weinheimer, pp. 168–169.

Lara, G.P., Parada, E., 1991. Seasonal changes in the condition index of *Diplodon chilensis chilensis* (Gray, 1828) in sandy and muddy substrata. Villarica lake, Chile (39° 18′S; 72° 05′ W), vol. 62. Boletín Sociedad de Biología de Concepción, Chile, pp. 99–106.

Lara, G., Contreras, A., Encina, F., 2002. La almeja de agua dulce *Diplodon chilensis* (Bivalvia, Hyriidae) potencial biofiltro para disminuir los niveles de coliformes en pozos. Experimentos de laboratorio. Gayana 66, 113–118.

Lindahl, O., Hart, R., Hernroth, B., Kollberg, S., Loo, L.O., Olrog, L., Rehnstam-Holm, A.S., Svensson, J., Svensson, S., Syversen, U., 2005. Improving marine water quality by mussel farming: a profitable solution for Swedish society. Ambio 34, 131–138.

Marker, A.F.H., Crowther, C.A., Gunn, R.J.M., 1980. Methanol and acetone as solvents for estimating chlorophyll a and phaeopigments by spectrophotometry. Ergerb Limnol. 14, 52–69.

May, M.J., Leaver, C.J., 1993. Oxidative stimulation of glutathione synthesis in *Arabidopsis thaliana* suspension cultures. Plant Physiol. 103, 621–627.

Nakamura, Y., Kerciku, F., 2000. Effects of filter-feeding bivalves on the distribution of water quality and nutrient cycling in a eutrophic coastal lagoon. J. Mar. Syst. 26, 209–221.

Nelson, K.A., Leonard, L.A., Posey, M.H., Alphin, T.D., Mallin, M.A., 2004. Using transplanted oyster (*Crassostrea virginica*) beds to improve water quality in small tidal creeks: a pilot study. J. Exp. Mar. Biol. Ecol. 298, 347–368.

Nicholson, S., Lam, P.K.S., 2005. Pollution monitoring in Southeast Asia using biomarkers in the mytilid mussel *Perna viridis* (Mytilidae: Bivalvia). Environ. Int. 31, 121–132.

OECD, 1982. Eutrophication of Water: Monitoring, Assessment and Control. Organisation for Economic Co-operation and Development, Paris.

Parada, E., Peredo, S., Lara, G., Valdebenito, I., 1989. Growth, age and life span of the freshwater mussel *Diplodon chilensis chilensis* (Gray, 1828). Arch. Hydrobiol. 115, 563–573.

Parada, E., Peredo, S., Cárdenas, S., Valdebenito, I., Peredo, M., 2008. Diplodon chilensis a potential residual waters depurator on inland water salmonid fishfarms: a laboratory scale study. Gayana 72 (1), 68–78.

Pollero, R.J., Brenner, R.R., 1981. Effect of the environment and fasting on the lipid and fatty acid composition of *Diplodon patagonicus*. Lipids 16, 685–690.

Reznick, A.Z., Packer, L., 1994. Oxidative damage to proteins: spectrophotometric methods for carbonyl assay. Methods Enzymol. 38, 357–363.

Ribeiro Guevara, S., Bubach, D., Vigliano, P., Lippolt, G., Arribere, M., 2004. Heavy metal and other trace elements in native mussel *Diplodon chilensis* from Northern Patagonia lakes, Argentina. Biol. Trace Elem. Res. 101, 1–19.

Romero Rojas, J.A., 1999. Calidad del agua 2 da edición. Alfaomega Mexico. Ley provincial 899. Reglamentación del código de aguas de la Provincia del Neuquén. Anexo del boletín oficial edicion No. 2641-19, decreto 790/99.

Semenas, L., Brugni, N., 2002. Características poblacionales y ciclo de vida de *Diplodon chilensis* (d'Orbigny, 1835) (Hyriidae, Bivalvia) en el Lago Gutierrez (Patagonia, Argentina). Ecol. Austral 12, 29–40.

Sokal, R.R., Rohlf, F.J., 1984. Introducción a la bioestadística. In: Reverté, S.A. (Ed.), Serie de Biología Fundamental. España.

Soto, D., Mena, G., 1999. Filter feeding by the freshwater mussel, *Diplodon chilensis*, as a biocontrol of salmon farming eutrophication. Aquaculture 171, 65–81.

Sparnins, V.L., Venegas, P.L., Wattenberg, L.W., 1982. Glutathione S-transferase activity: enhancement by compounds initiating chemical carcinogenesis and by dietary constituents. J. Natl. Cancer Inst. 68, 493–496.

Sugino, K., Dhi, K., Yamada, K., Kawasaki, T., 1987. The role of lipid peroxidation in endotoxin-induced hepatic damage and the protective effect of antioxidants. Surgery 101, 746–752.

Torres, M.A., Testa, C.P., Gaspari, C., Masutti, M.B., Neves Panitz, C.M., Curi-Pedrosa, R., Alves de Almeida, E., Di Mascio, P., Wilhem Filho, D., 2002. Oxidative stress in the mussel *Mytella guyanensis* from polluted mangroves on Santa Catarina Island, Brazil. Mar. Pollut. Bull. 44, 923–932.

Valdovinos, C., Pedreros, P., 2007. Geographic variations in shell growth rates of mussel *Diplodon chilensis* from temperate lakes of Chile: implications for biodiversity conservation. Limnologica 37, 63–75.

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. Comp. Biochem. Physiol. C 146, 281–300.

Vidal, M.L., Basseres, A., Narbonne, J.F., 2002. Seasonal variations of pollution biomarkers in two populations of *Corbicula fluminea* (Müller). Comp. Biochem. Physiol. C 131, 133–151.

Viozzi, G.P., Brugni, N.L., 2001. Relación parasitaria y nuevos registros de gloquidios de *Diplodon chilensis* (Unionacea:Hyriidae) en peces de la Patagonia argentina. Neotropica 47, 3–12.

Yamamuro, M., Hiratsuka, J., Ishitobi, Y., 2000. Seasonal change in a filter-feeding bivalve *Musculista senhousia* population of a eutrophic estuarine lagoon. J. Mar. Syst. 26, 117–126.