Full length article

Health status and bioremediation capacity of wild freshwater mussels (*Diplodon chilensis*) exposed to sewage water pollution in a glacial Patagonian lake

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ABSTRACT

Deleterious effects on health and fitness are expected in mussels chronically exposed to sewage water pollution. *Diplodon chilensis* inhabiting SMA, an area affected by untreated and treated sewage water, shows increased hemocyte number and phagocytic activity, while bacteriolytic and phenoloxidase activities in plasma and reactive oxygen species production in hemocytes are lower compared to mussels from an unpolluted area (Yuco). There are not differences in cell viability, lysosomal membrane stability, lipid peroxidation and total oxygen scavenging capacity between SMA and Yuco mussels' hemocytes. Energetic reserves and digestive gland mass do not show differences between groups; although the condition factor is higher in SMA than in Yuco mussels. Gills of SMA mussels show an increase in mass and micronuclei frequency compared to those of Yuco. Mussels from both sites reduce bacterial loads in polluted water and sediments, improving their quality with similar feeding performance. These findings suggest that mussels exposed to sewage pollution modulate physiological responses by long-term exposure; although, gills are sensitive to these conditions and suffer chronic damage. Bioremediation potential found in *D. chilensis* widens the field of work for remediation of sewage bacterial pollution in water and sediments by filtering bivalves.

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1. Introduction

Municipal sewage waters contain pharmaceutical products, heavy-metals, ammonia, personal care products, endocrine disruptors and microorganisms [1,2], which are usually released to the aquatic environment, implying deleterious consequences for organisms' health and fitness [3—5]. Freshwater bivalves are benthic filter-feeding animals, which live in the sediment—water interface for long periods (sometimes many decades). Therefore these are exposed to contaminants from both dissolved and particulate compartments and their capability to overcome this long-term stress would eventually determine the population survival [6,7].

Mussels' health and fitness depends on the modulation of non-specific immune responses and oxidative balance against pollution exposure [8,9]. Cellular responses in mussels are performed by circulating hemocytes, which eliminates pathogens and foreign bodies by phagocytosis and encapsulation [10]. Additionally, activated hemocytes release large quantities of reactive oxygen species (ROS), as a source of local tissue damage (oxidative burst) [11,12]. Under these oxidative conditions, generated by both, the immune response and the effects of pro-oxidant pollutants, total scavenging capacity (TOSC) can be used to estimate the total defenses against

oxyradicals [13]. When antioxidant and detoxifying defenses are overwhelmed, lysosomal membrane destabilization, lipid peroxidation and DNA damage can occur in the cells [14,15]. In the context of humoral immunity, hemocytes can produce and release into the plasma anti-microbial factors, such as lysozymes and phenoloxidases, after pathogen recognition or under physiological stress [16–18].

Immune responses have been widely studied under laboratory conditions to elucidate the specific effect of microbiological and chemical challenges on bivalves of economic interest [19–22]. Other studies have evaluated the effects of industrial and agricultural effluents on transplanted and wild bivalves [23–25] as well as in laboratory studies [26]. Although the effects of sewage water on the immune response of marine and freshwater bivalves have been studied in the last years [27–31], the information available is still scarce. Particularly, the effects of long-time exposure to sewage pollution on bivalves' immune system and filtration capacity deserve further investigation [8].

In the last decades, water quality and biodiversity in some lakes and rivers of North-West Patagonia has started to be threatened by population growth and human activities [32–34]. Particularly, in the city of San Martín de los Andes, untreated effluents and primary treated sewage (sedimentation ponds) are discharged in different parts of Pocahullo river basin. A sewage tertiary treatment plant (STTP), serving about 20,000 people, releases its effluents near the river mouth. In the lake area next to the river mouth, Sabatini et al. [33] have detected chemical and bacteriological water deterioration together with oxidative damage in wild individuals of the native mussel *Diplodon chilensis*. The present work is the first study on *D. chilensis'* immune system.

As it has been reported for other bivalves [35–37], *D. chilensis* individuals extracted from clean areas are able to remove algae, suspended solids and nutrients from fish farming effluents [38,39], coliform bacteria from polluted wells [40] and *Escherichia coli* from the water column in laboratory exposures [33]. However, under stressing conditions, bivalves' filtering capacity can be altered, affecting both, their energy balance and growth and their bioremediation capacity [41–43]. The retention of filtering capacity in mussels chronically exposed to sewage water pollution or their ability for cleaning sediments has not been studied yet.

The main objectives of this work are to describe the health status of wild *D. chilensis* from a sewage polluted area in Lacar lake, through the characterization of its immunological response, both at cellular (total and viable hemocytes number, phagocytic potential, ROS) and humoral (bacteryolitic and phenoloxidase activities, and total protein) level. Additionally, total antioxidant capacity (TOSC) and oxidative damage indicators (lysosomal membrane stability, TBARS) are evaluated in hemocytes. DNA damage (micronuclei frequency) is analyzed in gills and energetic reserves (glycogen and total lipid) are evaluated in digestive gland as indicators of metabolic status. Somatic growth was estimated, together with digestive gland and gills mass, using morphometric factors. Finally, the capacity of this species to remove enteric bacteria, algae, particulate organic matter and nutrients (total nitrogen, total phosphorus) from polluted water and sediments is compared between individuals collected from a polluted area and a control area in the same lake. Chronically induced tolerance of *D. chilensis* to sewage water pollution and the suitability of this species for bioremediation purposes are discussed through the integration of physiological variables.

2. Materials and methods

2.1. Mussel collection

Sampling was performed during January and February 2013. Adult individuals of *D. chilensis* (n = 30 per site; 70.38 ± 0.57 mm

shell length) were collected by scuba diving (1–3 m depth) from an unpolluted area (Yuco) in Lacar lake (about 20 km from the city of San Martín de los Andes) (40° 10′ S, 71° 31′ 30″ W); and from the area in which Pocahullo river flows into the lake (SMA) (40° 10′ S, 71° 20′ 60″ W). The estimated age of the individuals collected from both sites ranged between 10 and 30 years old. This estimation was made according to von Bertalanffy growth curves and corresponding size-at-age data obtained by counting translucent growth bands in cross-sectioned shells of *D. chilensis* from the same population [44]. Mussels were transported on ice to the laboratory for immediate processing.

2.2. Field study

2.2.1. Water quality

Water samples (n = 3) were taken at each site, transported to the laboratory at 4 °C and analyzed in duplicate. Fecal coliform bacteria (FC) concentrations were measured by the More Probable Number method (MPN/100 mL) [45]. Chlorophyll *a* (Chl *a*) concentration (μg/L) was calculated according to Lichtenthaler [46]. Particulate organic matter was measured according to Juhel et al. [47] and expressed as mg OM/L. Water samples for physic-chemical analysis (HACH DR/4000 spectrophotometer) were collected in polyethylene bottles (pre-washed with 5% HCl) and kept at −20 °C. Total nitrogen (TN) was measured by a cadmium reduction method (HACH method 8192), after acid persulfate digestion (120 °C for 55 min) and expressed as μg/L. Total phosphorus (TP) was measured by the ascorbic acid method, after digestion with potassium persulfate [45] and expressed as μg/L. Turbidity was measured by Attenuated Radiation Method (HACH Method 10047) and expressed as Formazin Attenuation Units (FAU). Water temperature (°C) was recorded at each site.

2.2.2. Sediment quality

Sediment samples (n = 3) were taken from the superficial layer (5 cm depth) in sterile plastic containers, transported to the laboratory at 4 °C and analyzed in duplicate. A suspension of 1:9 (v/v) of sediment in sterile NaCl (0.9%) solution was used for FC analysis by MPN method [45]. The sediment was then dried (60 °C for 24 h) and bacteria content was expressed as MPN/g of dry sediment (gds). After that, samples were ashed at 550 °C for 4 h and weighted for estimating organic matter content (mg OM/gds). Chl *a* (μg/gds) was extracted in acetone 90% buffered with CaCO₃ (modified from Tett et al. [48]) and measured as described by Lichtenthaler [46]. Total nitrogen (TN) was measured by Kjeldahl's method [49] and Total phosphorus (TP) by the method proposed by Takeuchi [50]; both results were expressed as μg/gds.

2.2.3. Immune response

Working on ice, 2 mL of hemolymph were withdrawn from the adductor muscle of each individual (n = 6 per site) using a sterile syringe. Hemolymph was then aliquoted and placed in sterile microcentrifuge tubs for immediate analysis.

Total and viable hemocytes were measured by mixing 100 μL of hemolymph with 50 μL of Trypan Blue (0.2%) dissolved in sterile anticoagulant solution (3 g glucose and 0.36 g trisodium citrate per L, 60 mOsm/L, pH 7) (modified from Burkhard et al. [51]). After incubation (4 °C for 5 min), live (undyed) and dead cells (dyed) were counted within 15 min in a Neubauer chamber. Total hemocytes were expressed as cells/mL of hemolymph. Hemocytes' viability was presented as proportion of total hemocytes (modified from Akaishi et al. [8]).

Hemocyte phagocytic activity was evaluated using *Saccharomyces cerevisiae*. Yeasts were stained by suspending 5 mg of cells in 2.5 mL of dechlorinated tap water and 2.5 mL Congo red solution

(0.8%). The suspension was autoclaved (121 °C for 15 min), cooled and centrifuged (1500× g, for 5 min) discarding the supernatant. The pellet was washed from stain residues and kept at 4 °C. Hemolymph (100 μL) was mixed with a volume of stained yeast suspension containing twice the number of cells respect to the number of viable hemocytes in the sample. After incubation at room temperature for 30 min, a minimum of 300 cells was counted in duplicate under light microscopy (100−400×). Phagocytic activity was calculated as phagocyted yeast cells/viable hemocytes (modified from Kuchel et al. [18]).

Reactive oxygen species (ROS) were measured in hemocytes by a fluorometric method adapted from Moss and Allam [52]. Briefly, 300 μL of hemolymph were centrifuged at 500× g for 20 min. Plasma was discarded and hemocytes were re-suspended in the anticoagulant solution described above, centrifuged and re-suspended again to obtain washed cells. Three aliquots of this suspension (50 μL) were separately mixed with 140 μL of reaction buffer (Hepes 30 mM, KCl 200 mM; MgCl 1 mM, pH 7) and 10 μL of the fluorescent probe $H_2DCF-DA$ (2′,7′ dichlorofluorescein diacetate, 0.8 mM, Sigma) in fluorometer tubes (Qubit assay tubes Q32856, Invitrogen) and incubated at 35 °C for 12 min. The change in fluorescence (485/530 nm) was followed during 18 min in a Qubit™ fluorometer, Invitrogen. ROS content was referred to a H_2O_2 standard curve with the fluorescent probe and results were expressed as meq $H_2O_2/10^6$ viable cells.

Plasma was obtained after centrifugation at 500× g for 20 min and its total protein content (mg/mL) was measured fluorometrically (Quant-it protein assay Q33211, Invitrogen). Bacteriolytic activity (BA/mg prot) was measured by mixing 150 μL of plasma with 850 μL of *E. coli* JM109 strain suspension (0.5 in McFarland scale). Changes in absorbance at 625 nm for 5 min were recorded and one BA unit was defined as a 0.001 change (adapted from Das et al. [53]). Phenoloxidase activity (PO/mg prot) was measured by incubating 500 μL of plasma with 300 μL of L-DOPA (3 mg/mL L-DOPA, Sigma in phosphate buffered saline) and anticoagulant solution to a final volume of 1000 μL for 4 h. Negative controls were performed by adding tropolone (16 mM final concentration, Sigma) and spontaneous substrate oxidation was monitored by mixing anticoagulant solution and L-DOPA. Absorbance at 490 nm was followed for 20 min and one PO unit was defined as a 0.001 change in absorbance (modified from Aladaileh et al. [16]).

2.2.4. Antioxidant defense

Total oxyradical scavenging capacity (TOSC) was measured in hemocytes (n = 6 per site) obtained as described in Section 2.2.3. Three aliquots of cell suspension (50 μL) were separately mixed with 130 μL of reaction buffer (Section 2.2.3) and 10 μL of ABAP solution (2,2′-Azobis(2-methylpropionamidine) dihydrochloride, 4 mM, Sigma) in fluorometer tubes and incubated at 35 °C for 5 min. After this time, 10 μL of $H_2DCF-DA$ were added and the tubes were incubated at 35 °C for 12 min. Another set of three tubes was prepared in the same conditions but omitting the addition of ABAP. Fluorescence reading was performed as described for ROS. TOSC was calculated from the relative area between the curves obtained with and without ABAP and referred to 10^6 viable cells (modified from Amado et al. [54]).

2.2.5. Oxidative damage

Lysosomal membrane stability of hemocytes (n = 6 per site) was evaluated by the neutral red retention time method (modified from Mamaca et al. [15]). Briefly, 50 μL of hemolymph were placed on slides (in duplicate) and kept in a wet chamber for 5 min to let the cells attach. Neutral red solution (50 μL, 0.002% in dimethyl sulfoxide) was added and the number of red cells was counted every 10 min, using a light microscope at 400×, until stained cells

reached 50%. Results were expressed as neutral red retention time 50% (min). Lipid peroxidation (n = 6 per site) was estimated by the TBARS method [55] in hemocytes suspensions containing a known number of cells. Results were expressed as μmol TBARS/10^6 cells and μmol TBARS/mg prot.

2.2.6. Energetic reserves

Digestive glands (n = 6 per site) were extracted, weighted and kept at −20 °C until analysis. The organs were then homogenized in phosphate buffered saline (100 mM, pH 7). Total lipid content (mg/g of wet tissue) was extracted using Folch solution (chloroform−methanol 2:1 v/v) and quantified according to Bligh and Dyer [56]. Glycogen was extracted according to Van Handel [57] and referred to a glucose standard curve as meq Glu/g of wet tissue.

2.2.7. Morphometric factors

Soft tissues and shell (n = 12 for each site) were dried at 60 °C for 24 h for calculation of condition factor (CF = total dry mass/shell length^3 * 100) [39]. Digestive gland and gill factor (DGF, GF; n = 12 for each site) were calculated by replacing total dry mass for wet digestive gland mass or wet gill mass in the CF formula.

2.2.8. Micronuclei frequency

Gills (n = 6 per site) were extracted and dragged along a slide in a single layer of well-spread cells (in duplicate) and allowed to dry for a few minutes. Cells were then fixed with methanol for 30 s, stained with Giemsa stain (1:10 in distilled water) for 20 min and mounted with DPX medium (Sigma). Under light microscopy (1000×), about 1000 cells per mussel were counted [58,31] to estimate the micronucleated cells frequency. Small, circular or ovoid chromatin bodies showing the same staining pattern as the main nucleus were considered as micronuclei (MN). Results were expressed as MN/cell ± SE.

2.3. Laboratory study

2.3.1. Water bioremediation

Mussels from each site (n = 7) were placed in two aerated tanks with 5 L of dechlorinated tap water and starved for 36 h, without water changes. After that, individuals were individually placed in plastic containers, separated from the container's bottom by rigid plastic mesh. The containers were filled with 700 mL of water collected from 20 m downstream of the sewage tertiary treatment plant's discharge in Pocahullo river (PR). Mixing was provided by the aeration system. Controls were set in triplicate containers without mussels (Fig. 1(A)). Microbiological and physic-chemical variables were measured in each container at the beginning of the experiment (T_0) and after 6 h (T_6). Enteric bacteria (EB) concentrations were measured by the spread plate technique using McConkey's agar medium (Merck), incubating at 35 °C for 24 h. Results were expressed as colony forming units (CFU)/mL. Water samples for phytoplankton (Phyto) analysis were fixed in 4% formaldehyde, concentrated 20 fold and counted under light microscope (400×) in a Neubauer chamber. OM, TN and TP concentrations were measured and expressed as described in Section 2.2.1. Water temperature during the assay was kept at 14.5 ± 0.5 °C. Feeding rates calculation is described in Section 2.3.3.

2.3.2. Sediment bioremediation

Mussels from each site (n = 7) were starved for 36 h in aerated tanks filled with dechlorinated tap water. After that, mussels were placed in aerated glass containers with 340 cm^3 of sediment from the site PR and the same volume of dechlorinated tap water. Mussels were buried obliquely to ensure the maximum access to the sediment fraction [59]. Controls were set by triplicate in containers
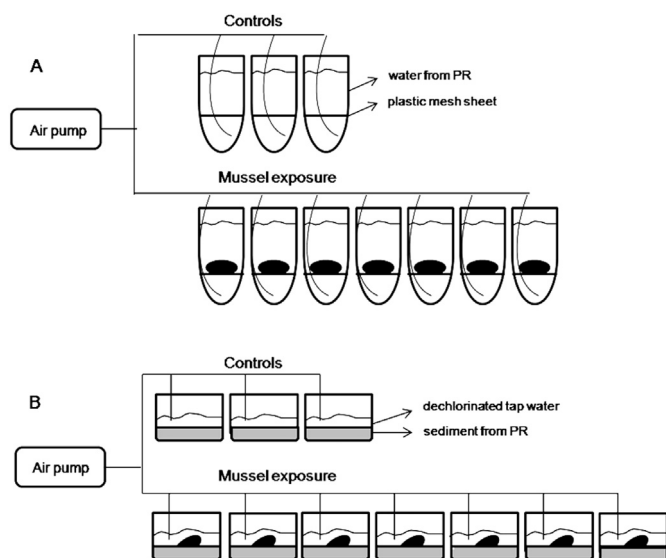
Fig. 1. Diagram of the static experimental system for the evaluation of *Diplodon chilensis* feeding rates and bioremediation effects on water (A) and sediment (B) collected from 20 m downstream of the sewage tertiary treatment plant's discharge in Pocahullo river (PR).

without mussels (Fig. 1(B)). Bacteriological and physic-chemical variables were measured by extracting samples from the superficial layer (0.5 cm) in each container at the beginning of the experiment (T_0) and after 24 h (T_{24}). EB were extracted as in Section 2.2.2 and counted by the spread plate technique (Section 2.3.1). Chl a, OM, TN and TP contents were measured and expressed as described in Section 2.2.2. Water temperature during the assay was $14 \pm 0.5 \,^\circ$C. Feeding rates calculation is described in Section 2.3.3.

2.3.3. Calculation of feeding rates

After bioremediation experiments, mussels were open by adductor muscle incision and soft tissues were dried (Section 2.2.7) and weighted. Clearance (CR) and filtration (FR) rates were calculated for bacteria and phytoplankton as follows (based on Soto and Mena [39]) and referred to g of dry soft tissue:

$$\text{CR} \left(\text{L} \times \text{h}^{-1} \times \text{gdt}^{-1} \right) = V/t \times [(\ln\text{Ci} - \ln\text{Cf}) - (\ln\text{Ci}' - \ln\text{Cf}')]/\text{gdt}$$

$$\text{FR} \left(\text{q} \times \text{h}^{-1} \times \text{gdt}^{-1} \right) = V/t \times [(\text{Ci} - \text{Cf}) - (\text{Ci}' - \text{Cf}')]/\text{gdt}$$

where Ci = initial concentration; Cf = final concentration; V = container volume (L); t = experimental time span (h); Ci' and Cf' = initial and final concentration in control container; q = quantity units; gdt = grams of dry soft tissue.

Sediment CR and FR were calculated replacing V, in the formula, for the dry mass of sediment considered to be available for benthic bioremediation (the total superficial layer with 0.5 cm depth). CR was expressed as gds $\times$ h^{-1} $\times$ gdw^{-1} and FR as q $\times$ h^{-1} $\times$ gdw^{-1}.

2.4. Statistical analysis

Data were presented as media ±standard error. Normal distribution and homogeneity of variance were checked by Kolmogorov–Smirnov and Levene's tests, respectively. *T*-test was utilized to identify differences between groups in the field study and for comparison of CR and FR. When statistical assumptions were not met, the non-parametric Mann–Whitney test was applied. Two-way repeated-measures ANOVA and Newman–Keuls *post hoc* comparisons were used to analyze the effects of mussels on water and sediment quality. When statistical assumptions were not met, data were previously transformed by $\text{Log}_{10} x$, $\text{Ln} x$, $\text{Log}_{10} (x + 1)$, or $\text{Ln} (x + 1)$, when appropriate.

3. Results

3.1. Field study

3.1.1. Water and sediment quality

Water and sediments from SMA have significantly higher loads of fecal coliform bacteria, organic matter and nutrients than those from Yuco; also showing elevated concentrations of Chl a in sediments and turbidity in water. Table 1 presents bacteriological and physicochemical variables measured in the areas of mussel extraction.

3.1.2. Health status

Mussels from SMA show significantly higher total number of hemocytes ($P < 0.05$) and phagocytic activity ($P < 0.01$) but lower hemocyte ROS production than mussels from Yuco ($P < 0.01$), while TOSC is similar in hemocytes of mussels from both sites (Fig. 2(a) and (b)). Plasmatic bacteriolytic (BA) and phenoloxidase (PO) activities are lower in SMA than in Yuco mussels ($P < 0.05$ for both activities) (Fig. 2(c)). No significant differences are observed for hemocytes' viability (0.90 ± 0.02 vs. 0.94 ± 0.008), lysosomal membrane stability (14.37 ± 1.55 vs. 14.66 ± 0.50 min), lipid peroxidation (293.7 ± 84.6 vs. 270.7 ± 97.7 μmol/10^6 cells; 24.45 ± 4.75 vs. 24.20 ± 5.11 μmol/mg prot) or plasma protein concentration (0.302 ± 0.01 vs. 0.296 ± 0.01 mg/mL) between groups (values for SMA and Yuco, respectively).

CF is significantly higher in mussels from SMA than in those from Yuco ($P < 0.05$), while there is no change in DGF (Fig. 3). Energetic reserves in digestive gland, as lipids (8.85 ± 0.77 vs. 9.77 ± 0.61 mg/g) and glycogen (94.12 ± 5.21 vs. 98.70 ± 11.21 meq Glu/g) are similar in both groups (values for SMA and Yuco, respectively). SMA mussels show significantly higher GF ($P < 0.05$) and micronuclei frequency than Yuco mussels ($P < 0.01$) (Fig. 4).
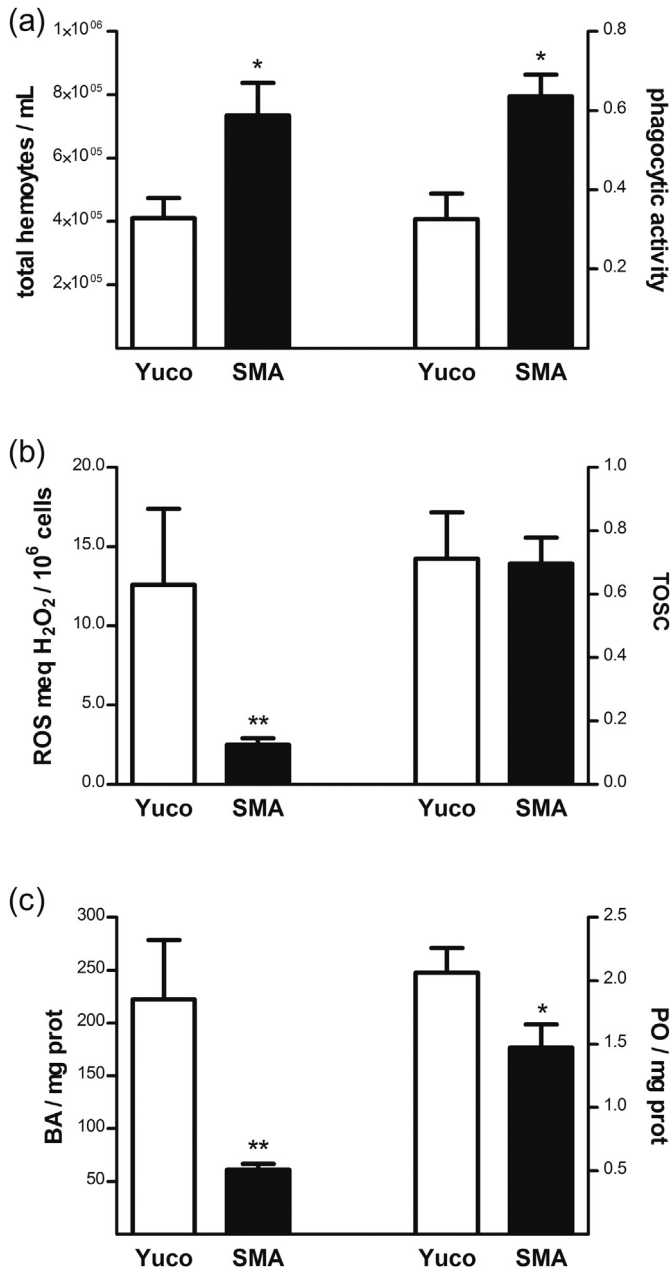
Table 1
Bacteriological and physicochemical variables measured in water and sediments from unpolluted (Yuco) and sewage polluted (SMA) areas in Lacar lake. Values for fecal coliforms (FC), clorophill a (Chl a), organic matter (OM), total nitrogen (TN), total phosphorus (TP), turbidity and temperature are expressed as mean $\pm$ SE.

	Water			Sediments		
	Yuco	SMA	P	Yuco	SMA	P
FC (MPN/100 mL or gds)	10 ± 10	950 ± 150	**0.01**	130 ± 3.6	2509 ± 150	**<0.01**
OM (mg/mL or gds)	7.56 ± 0.03	9.29 ± 0.56	**0.04**	22.37 ± 0.17	25.93 ± 1.41	**<0.05**
Chl a (μg/mL or gds)	$1.7 \times 10^{-3} \pm 0.0002$	$3.0 \times 10^{-3} \pm 0.0006$	**0.05**	3.88 ± 0.51	9.09 ± 0.77	**<0.01**
TN (μg/mL or gds)	285 ± 0.02	445 ± 0.01	**0.03**	1400 ± 12	3131 ± 606	**<0.05**
TP (μg/mL or gds)	10.86 ± 1.11	67.05 ± 0.37	**<0.01**	1461 ± 38	1774 ± 15	**<0.01**
Turbidity (FAU)	1.33 ± 0.33	7.67 ± 0.33	**<0.01**	–	–	–
T ($^\circ$C)	19.67 ± 0.33	20.5 ± 0.50	0.30	–	–	–

Fig. 2. Hematological variables measured in *Diplodon chilensis* collected from unpolluted (Yuco) and sewage polluted (SMA) areas of Lacar lake. (a) Total hemocytes and phagocytic activity; (b) bacteriolytic (BA) and phenoloxidase (PO) activity in plasma; (c) reactive oxygen species production (ROS) and total oxyradical scavenging capacity (TOSC) in hemocytes. Results are expressed as mean ± SE. * Denotes $P < 0.05$ and ** denote $P < 0.01$ between mussels from Yuco and SMA.

3.2. Laboratory study

3.2.1. Water and sediment bioremediation

After six hours (T_6), both water enteric bacteria concentrations ($P < 0.01$) and turbidity ($P < 0.05$) are significantly lower in the containers with Yuco or SMA mussels, respect to the control containers. Phytoplankton concentrations show a similar decreasing trend in both groups, but this effect is significant only for Yuco ($P < 0.001$). OM concentrations in water decrease at T_6 in controls and mussel containers ($P < 0.0001$ for both) while no significant difference could be observed between mussel and control containers. Water nutrient load is lowered in both mussel groups. TP is
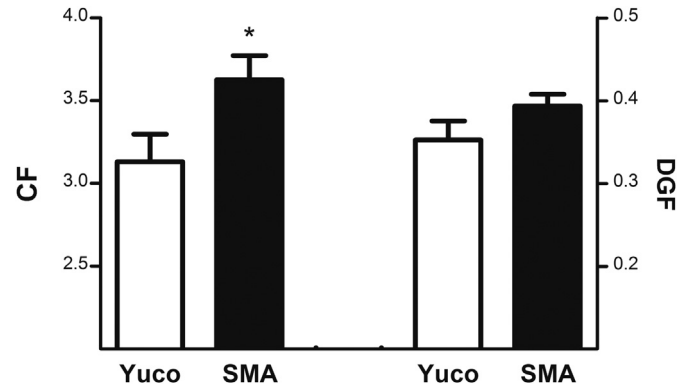

Fig. 3. Condition factor (CF) and digestive gland factor (DGF) in *Diplodon chilensis* collected from unpolluted (Yuco) and sewage polluted (SMA) areas of Lacar lake. Results are expressed as mean ± SE. * Denotes significant differences between mussels from Yuco and SMA ($P < 0.05$).

significantly lower than the control value in containers with Yuco mussels ($P < 0.05$) while in TN is significantly reduced by SMA mussels ($P < 0.05$) (Table 2).

Both groups of mussels significantly reduce enteric bacteria concentrations in sediments at T_{24} ($P < 0.05$). There are no changes in OM content in sediments of any of the containers. Changes in Chl *a*, TN and TP contents do not show any clear significant trend to be analyzed as mussel filtration effects (Table 2).

3.2.2. Feeding rates

Since in the bioremediation experiments (Table 2) enteric bacteria (in water and sediment) and phytoplankton (in water) were reduced significantly by the activity of mussels, clearance and filtration rates (CR and FR) were calculated. Mussels from Yuco and SMA removed enteric bacteria from PR water at similar clearance rates of 0.136 ± 0.03 and 0.155 ± 0.01 L $\times$ h^{-1} $\times$ gdt^{-1}, respectively. However, FR was significantly higher for SMA mussels ($2.64 \times 10^8 \pm 5.19$ vs. $7.61 \times 10^8 \pm 7.33$ CFU $\times$ h^{-1} $\times$ gdt^{-1}, for Yuco and SMA, respectively) ($P < 0.0001$) (Fig. 5(a)). There were no significant differences between mussels of both sites in CR or FR neither for phytoplankton in water nor for enteric bacteria in sediments (Fig. 5(b) and (c)).

4. Discussion

The present study integrates original information on the physiological status (immune response, oxidative balance, energetic
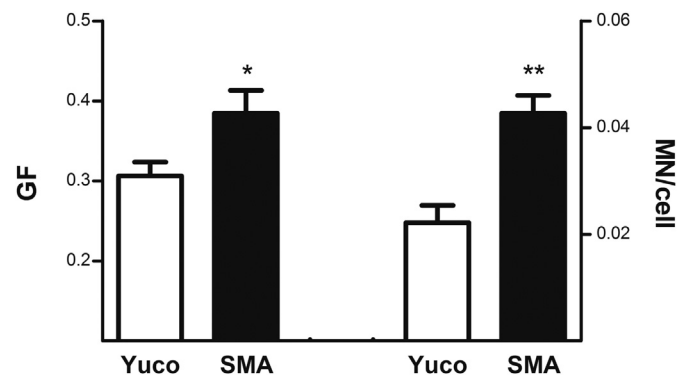

Fig. 4. Gill factor (GF) and micronuclei frequency (MN/cell) in gill cells of *Diplodon chilensis* collected from unpolluted (Yuco) and sewage polluted (SMA) areas of Lacar lake. Results are expressed as mean ± SE. * Denotes $P < 0.05$ and ** denote $P < 0.01$ between mussels from Yuco and SMA.

Table 2
Bacteriological and physic-chemical results from bioremediation experiments. *Diplodon chilensis* from unpolluted (Yuco) and sewage polluted (SMA) areas of Lacar lake were exposed to water (W) or sediments (S) collected from the highly polluted site, PR. Control data correspond to recipients without mussels. Values for enteric bacteria (EB), organic matter (OM), phytoplankton (Phyto), chlorophyll a (Chl a), total nitrogen (TN), total phosphorus (TP) are expressed as mean $\pm$ SE/mL of water or g of dry sediment (gds). Significant differences ($P < 0.05$) between each mussel group and its control are denoted by (*) and differences between initial and final conditions in each group are denoted by (+).

|	(W)	Control	Yuco	Control	SMA	(S)	Control	Yuco	Control	SMA
EB (CFU/mL or gds)	T_0	2763 ± 454	2929 ± 583	4070 ± 14	4070 ± 301	T_0	22,164 ± 1461	15,090 ± 2347	7312 ± 3325	12,694 ± 5165
	T_6	4000 ± 681	1003 ± 325*+	10,233 ± 3670	2253 ± 320*+	T_{24}	29,446 ± 5395	10,529 ± 3047*+	13,447 ± 6434	8371 ± 3532*+
OM (mg/L or gds)	T_0	37.88 ± 0.0005	37.88 ± 0.001	33.67 ± 0.001	34.36 ± 0.001	T_0	103.9 ± 2.05	118 ± 11.22	123.6 ± 7.83	122.9 ± 1.56
	T_6	27.48 ± 0.0005+	23.64 ± 0.001+	21.97 ± 0.002+	21.50 ± 0.001+	T_{24}	138.3 ± 21.21	122.5 ± 4.39	132.4 ± 12.70	183 ± 35.49
Phyto (cell/mL)	T_0	10,915 ± 292	8286 ± 393	8125 ± 851	10,575 ± 867	T_0	–	–	–	–
	T_6	10,375 ± 1491	5071 ± 484*+	9208 ± 458	8275 ± 396	T_{24}	–	–	–	–
Chl a (µg/mL or gds)	T_0	–	–	–	–	T_0	28.13 ± 10.52	22.70 ± 4.16	27.71 ± 0.63	25.06 ± 0.79
	T_6	–	–	–	–	T_{24}	23.78 ± 6.96	36.68 ± 6.59*+	25.06 ± 0.79	33.38 ± 2.40
TN (µg/L or gds)	T_0	477 ± 0.02	483 ± 0.03	150 ± 0.01	167 ± 0.01	T_0	2300 ± 731	3287 ± 236	3711 ± 1095	4097 ± 509
	T_6	381 ± 0.05	270 ± 0.07	213 ± 0.02*+	143 ± 0.01	T_{24}	3120 ± 291	6545 ± 299*+	4149 ± 593	3442 ± 884
TP (µg/L or gds)	T_0	309.5 ± 5.52	313.4 ± 13.22	245.6 ± 5.02	246.3 ± 33.52	T_0	2448 ± 122	2095 ± 114	2003 ± 58	2145 ± 320
	T_6	454.2 ± 17	190.1 ± 13*+	222.2 ± 3.28	173.1 ± 9.13	T_{24}	1267 ± 109*+	627 ± 190*+	1745 ± 451	1415 ± 374
Turbidity (FAU)	T_0	9 ± 4	11.57 ± 0.84	3 ± 1	4.43 ± 0.48	T_0	–	–	–	–
	T_6	8.5 ± 6.50	1.43 ± 0.30*+	4.67 ± 0.67	1.43 ± 0.20*+	T_{24}	–	–	–	–
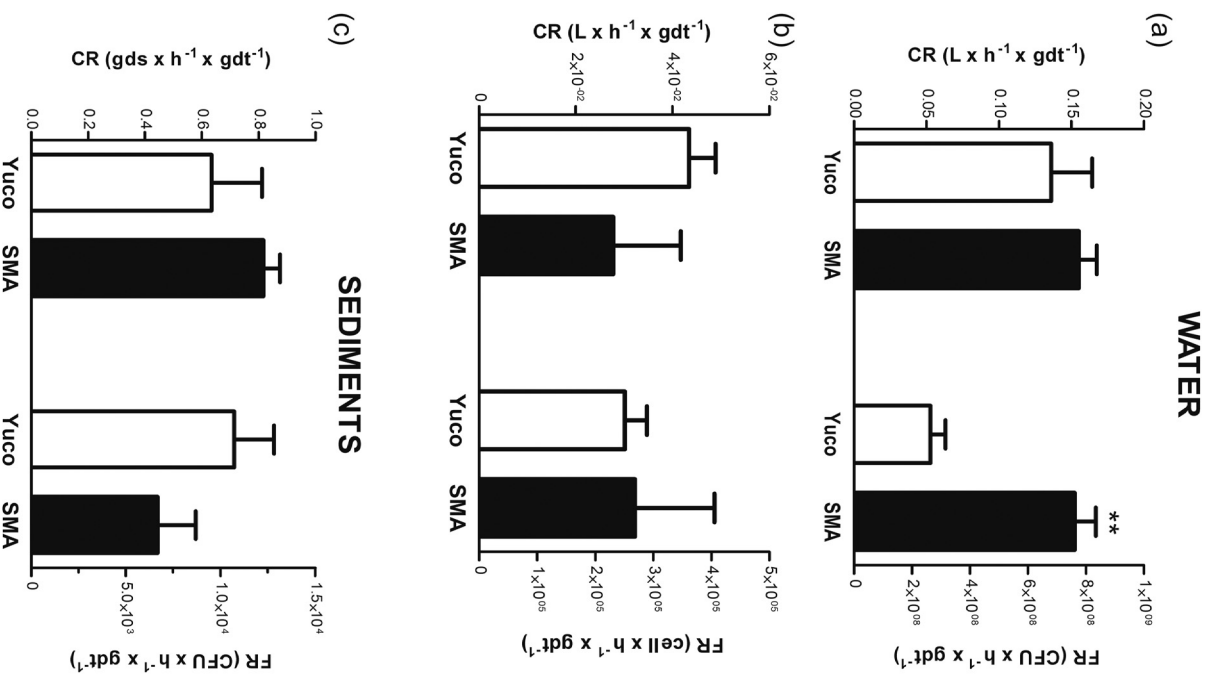
Fig. 5. Clearance (CR) and filtration (FR) rates of *Diplodon chilensis* from unpolluted (Yuco) and sewage polluted (SMA) areas in Lacar lake, calculated using water or sediments from the highly polluted site PR. CR and FR of enteric bacteria in water (a) and sediment (c). (b) CR and FR for phytoplankton in water. ** Denotes significant differences in FR between groups at $P < 0.001$.

Several studies have focused on physiological and immunological variables of bivalves exposed to sewage water under different conditions and for variable periods [7–9,27–29,63]. Studies on wild

reserves, feeding performance) and bioremediation potential of freshwater mussels chronically exposed to sewage water pollution, from field and laboratory studies. The SMA site has been affected by sewage–water pollution of varying intensity for more than 10 years [60]. The 10–30 years old individuals collected from this site have been exposed to pollution for at least one decade, which makes them a valuable model for chronic toxicity. Furthermore, considering a longevity of 60 years, for SMA and 70 years, for the reference site, Yuco [44], these are relatively young individuals, which are expected to display more active immune and antioxidant responses than older ones, as has been reported for the Antarctic bivalve *Laternula elliptica* [61,62].

populations have reported that chronic exposure to multiple contaminants negatively impacts mussel health and longevity [7,44], while immune and antioxidant responses are persistent and characteristic of the site pollution history [63]. The immune response, the oxidative balance and the feeding performance of *D. chilensis* collected from SMA suggest positive modulation of defensive mechanisms by long-term exposure to sewage pollution.

4.1. Immune response, oxidative balance and cytotoxic effects on hemocytes

Hemocytes may quickly react to maintain bivalve homoeostasis and integrity upon environmental changes [10]. Akaishi et al. [8] have reported that hemocytes' phagocytic activity can rise after long-term exposure to low sewage concentration and decrease upon short-term exposure to more concentrated sewage in the blue mussel *Mytilus edulis*. In addition, these authors have shown that, after a 90-day exposure, treated sewage may increase mussels' hemocyte number; while untreated sewage reduces this variable. In our work, phagocytic response and hemocyte number are almost twice as high in SMA mussels as in those from Yuco. Accordingly, cellularity and phagocytosis efficiency have been significantly increased in *Elliptio complanata* caged downstream of the outfall of tertiary-treated municipal effluents in Montreal Island, Canada [27] while Gillis et al. [7] have found increased phagocytic activity in wild freshwater mussels (*Lasmigona costata*) collected downstream of municipal wastewater discharges in southern Ontario, Canada. These immune responses could be associated to individual or mixed pollutants, commonly found in sewage water, such as bacteria, metals and estrogenic compounds [9,19,64]. Gagné et al. [29] suggested that the alteration of dopamine metabolism caused by the exposure to municipal effluents in *E. complanata* would mediate hemocytes activation.

Antibacterial activity responds primarily to bacterial exposure, but estrogenic compounds and metals may also stimulate this response [19,27,65]. Phenoloxidase can also be activated in bivalves by microbiological and physic-chemical stimuli [16,66]; although, as far as we know, the possible effects of sewage water on this enzyme have not been studied before. *D. chilensis* from SMA shows a reduction in both, bacteriolytic and phenoloxidease activities in plasma, which could be interpreted as indicative of a depressed immune response capacity as has been reported for *E. complanata* [9] and *Pinctada maxima* [66].

However, sewage pollution-related immunosuppression in bivalves has been reported to include: 1) decreased cell viability [8]; 2) decreased lysosomal membrane stability [30,31,67]; 3) increased lipid peroxidation in hemocytes [30,68]; 4) increased plasmatic protein content [23]. These deleterious consequences have been attributed, among others, to the exposure to bacteria, pharmaceutical products and metals (e.g. copper), which may cause oxidative stress, severe tissue damage and metabolic disorders. In this work, none of these effects is evident in hemocytes of SMA mussels. This, together with the increased cellular response suggests that the reduction in plasmatic bacteriolytic and phenoloxidase activities is not related to immunosuppression.

In particular, the values of neutral red retention time 50% (min) recorded in *D. chilensis*, as a lysosomal membrane stability indicator, are similar to those reported by Regoli et al. [69] for the land snail *Helix aspersa*. However, these values are considerably lower than those reported for other bivalves [15,67]. *D. chilensis* hemocytes might be sensitive to dimethyl sulfoxide (used for diluting neutral red). This compound may alter cell membrane integrity regulation, as seen in *S. cerevisiae* [70]. Nevertheless, the reduction of the reading time seems not to be a methodological impediment and our results are still recordable and analyzable.

ROS production by immune cells is considered to be linked to enhanced phagocytosis in order to potentiate microorganisms killing [71]. However, Donaghy et al. [10] suggest that such relationship is not always supported by published data in bivalves and despite the lowering in ROS production, the killing of microorganisms may be not affected. In addition, the increase in hemocytes' ROS production by sewage pollution exposure may indicate an excess of oxidant radicals [23] leading to cell damage [30]. In our work, despite the elevated phagocytic activity in SMA mussels, the ROS production is lower than in Yuco ones, which is in accordance with the lack of oxidative damage in hemocytes from this polluted site. Donaghy et al. [10] have also highlighted that ROS detection in bivalves is usually difficult to assess and depends on the chosen methodology. In this case, hemocytes ROS production in *D. chilensis* hemocytes has been effectively detected by using the fluorescent probe $H_2DCF-DA$ as substrate.

Compensatory activation of antioxidant mechanisms allows counteracting increased ROS formation during stressful conditions; thus, depletion of these defenses would make the organism more vulnerable to oxidative stress. The maintenance of TOSC at control levels in hemocytes of SMA mussels, even when ROS levels are lower than in Yuco mussels, suggests the existence of a high antioxidant potential, available to respond against increased levels of bacteria or other pollutants. The lack of oxidative damage in hemocytes from SMA mussels seems to be related to such antioxidant potential. Accordingly, Sabatini et al. [33] and Rocchetta et al. [44] have found increased enzymatic and non-enzymatic antioxidant activities in digestive glands of mussels from this population; although oxidative damage had not been fully compensated. In hemocytes of the snail *Lymnaea stagnalis*, the exposure to municipal effluents reduced the intracellular thiol levels, suggesting the loss of antioxidants, such as reduced gluthathione [71]. However, to the best of our knowledge, TOSC in hemocytes is evaluated for the first time in the present paper.

Our results suggest that the increase in hemocyte number and phagocytic activity is a long-term response, which enables the immune system to cope with mild bacterial load (e.g. 950 MPN/100 mL FC) as that measured in this study, maintaining low production of ROS and high TOSC defenses and thus, avoiding oxidative stress. Matozzo et al. [63] have suggested that different physiological responses shown by clams from unpolluted and polluted sites, upon copper challenge in the laboratory might respond to genetic adaptation according to their different ecological histories. We can speculate that *D. chilensis* is genetically adapted to deal with toxic products from bacteria, which are part of its natural diet, as is discussed in Section 4.2. This adaptation would permit SMA mussels to develop an antioxidant capacity in hemocytes, which compensates sudden increases in ROS production elicited by the presence of high bacterial loads, such as those detected in the same site in previous works (e.g. 24,000 MPN/100 mL FC, Sabatini et al., 2011). In addition, Rocchetta et al. [72] have suggested that the kind of fatty acids incorporated in the diet by SMA mussels could favor stress resistance and immunity upon chronic exposure to sewage discharges.

4.2. Energetic reserves

Energetic reserves of bivalves (lipids, carbohydrates) can be consumed under stressing conditions to fuel the up-regulation of detoxifying and antioxidant defenses [42,73], leading to lowered digestive gland mass and somatic growth. In contrast, digestive gland lipid and glycogen contents and digestive gland mass (DGF) are not altered in *D. chilensis* from SMA. Furthermore, CF is higher in these mussels than in those from Yuco. As seen before, the increase in food availability in sewage polluted waters seems to favor

somatic growth in these mussels, although it reduces longevity [44,74]. As benthic filter feeders, freshwater mussels are genetically adapted to feed on bacteria, which are abundant in the natural organic detritus. Rocchetta et al. [72] have detected abundant branched fatty acids (biomarkers of bacteria) in the lipid composition of *D. chilensis* both, in Yuco and SMA mussels, with significantly higher levels in SMA. This suggests that, besides feeding on bacteria, which naturally occur in the lake's sediment—water interface, SMA mussels can also feed on enteric bacteria (with different lipopolysaccharides) and pollutant-organic matter complexes, from sewage water, which are potentially toxic due to their pro-oxidant properties [33,75]. Our results suggest that SMA mussels can compensate this environmental stress without expending energy reserves, by consuming energy from food, which is more abundant in this site than in Yuco and favors a higher growth rate [44].

4.3. Micronuclei frequency and gill mass

MN arise as chromosome fragments after mitotic processes and their frequency has been reported to be increased by both organic and inorganic pollutants [76]. In our study, MN/cell in mussels' gills is higher in SMA than in Yuco. Tsarpali et al. [31] reported an increase of MN frequency in gills of *Mytilus galloprovincialis* exposed for 4 days to leachate from municipal solid waste and suggested that such genotoxic effects could be related to pro-oxidant conditions. Accordingly, *D. chilensis* have shown increased lipid peroxidation in gills after 7-month exposure to sewage water (Bianchi et al., unpublished results). Since almost no industrial activity is performed in the Lacar Lake area, the concentrations of mutagenic compounds from this origin (e.g. metal ions, phenols) are expected to be minimal. Thus, the cytotoxic effects observed in SMA mussels are more likely associated to chronic exposure to enteric bacteria and pro-oxidant compounds, such as pharmaceuticals, contained in sewage water [77—79]. In addition, GF of SMA mussels is increased compared to that of Yuco mussels. This increase in gill mass could be indicating: a — local inflammation, mostly responding to bacteria concentration [8,80] and/or b — tissue adaptations to water turbidity [81], e.g. increase in filtration area and mucus production in mussels from the polluted site. In addition, hyperplasia due to tissue damage and increased gill surface area responding to low oxygen tension could not be discarded [82]. In *D. chilensis*, the combination of MN and GF appears as a clear indication of long-term exposure to sewage water.

4.4. Feeding rates and bioremediation potential

Feeding rates are also considered as indicators of physiological status in bivalves [43], which can be negatively affected by pollutants and suspended material in water [83,84]. However, some bivalves can develop digestion and protection mechanisms, which enable them to feed on potentially harmful diets [33,85,86]. Our results show that mussels from both, unpolluted and sewage polluted areas are able to reduce water turbidity and enteric bacteria loads in water and sediments, with similar feeding performance. The CRs for enteric bacteria measured in sewage polluted water ($0.15 \; L * h^{-1} * g^{-1}$) are lower but still in the same order as those measured in the laboratory for the same species with *E. coli* ($0.510 \; L * h^{-1} * g^{-1}$) [33]. The higher CR in the cited study could be explained by the higher initial bacterial concentration (about 10^5 fold) and the higher assay temperature, compared to that in the present study (20 vs. 14 °C). Considering these methodological differences, the CRs obtained in both studies would be comparable; suggesting that the filtering capacity of *D. chilensis* is neither affected by long-term exposure to pollution nor by the presence of

particulate material in the water of the site PR used in the experiment. The similar CRs for phytoplankton calculated for SMA and Yuco mussels also indicate the absence of deleterious effects of chronic pollution on feeding performance. In contrast, the reduction of filtering capacity, reported for other bivalves exposed to sewage pollution, has been attributed to physical stress caused by suspended material in water [35,84].

As far as we know, bioremediation of sewage polluted sediments by filtering bivalves has not been reported before. The results of this study show that *D. chilensis* removes significant amounts of enteric bacteria from sediments. This capacity, measured as CR or FR, is not affected by long-term acclimation to the polluted site.

D. chilensis has been assessed as "Least Concern" for The IUCN Red list of Threatened species due to conservation problems related to euthrophication processes in some Chilean lakes [87,88]. In Argentina, Rocchetta et al. [44] have found reduced population density, with lack of young individuals (less than 7 years old) and reduced longevity in the polluted area of SMA, compared to populations located in clean areas of the same lake, such as Yuco and Nonthue. However, according to these authors, longevity of SMA mussels is still above 60 years and deleterious consequences observed at population level seem to be depending on lack of recruitment or juvenile mortality. Considering these data and the results of the present work, we can suggest that adult *D. chilensis* could be relocated to sewage polluted areas for remediating affected water and sediments and remain efficient for many years.

5. Conclusion

In *D. chilensis* chronically exposed to sewage pollution, the modulation of hematological defensive mechanisms (higher cell number and phagocytic activity, reduced ROS) results in higher capacity to respond to periodic increases of bacterial load without increasing hemocyte oxidative stress. These mussels preserve their capacity for feeding on bacteria, both from water and sediments, for several years or even decades, which widens the application possibilities of freshwater mussels for bioremediation strategies.

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