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Modulating effects of orally supplied *Euglena gracilis* on the physiological responses of the freshwater mussel *Diplodon chilensis*, exposed to sewage water pollution in a Patagonian river (Argentina)

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ABSTRACT

In order to test if orally supplied *Euglena* sp. cells modulate the physiological status of bivalves during bioremediation procedures, we evaluated the effect of *Euglena gracilis* diet on the immune response, oxidative balance and metabolic condition of *Diplodon chilensis* exposed to sewage water pollution. Mussels were fed for 90 days with *E. gracilis* (EG) or *Scenedesmus vacuolatus* (SV, control diet), and then exposed for 10 days at three sites along the Pocahullo river basin: 1) an unpolluted site, upstream of the city (control, C); 2) upstream (UpS) and 3) downstream (DoS) from the main tertiary-treated sewage discharge, in the city of San Martín de los Andes, Northwest Patagonia, Argentina. Our results show that the total hemocyte number decreases while pollution load increases along the river course for both, EG and SV mussels. Phagocytic activity is higher in EG mussels than in SV ones under all conditions. Reactive oxygen species (ROS) production in hemocytes increases with the increase in the pollution load, being significantly higher for EG mussels than for SV ones at DoS; no changes are observed for total oxyradical scavenging capacity (TOSC). Hemocytes' viability is increased for *E. gracilis* diet at C and remains unchanged in this group of mussels when exposed at the polluted sites. Lysosomal membrane stability is higher in EG mussels than in SV ones for all conditions, although it is decreased at polluted sites compared with that at C. Antioxidant (catalase) and detoxifying (gluthatione S-transferase) defenses are generally lower in gills and digestive gland of EG mussels than in SV ones. Lipid peroxidation (TBARS) is evident in gills of EG mussels at C, and in digestive gland of the same group, at all the sites. Gill mass factor (GF) is affected by the *E. gracilis* diet; it is increased at C and decreased at polluted sites when compared with that of SV ones. Digestive gland mass factor (DGF) is higher in EG mussels than in SV ones. In *D. chilensis*, continuous and long term feeding with *E. gracilis* cells favors immune response and reduces the damage caused by sewage pollution exposure on hemocytes. Nevertheless, diet and

Abbreviations: EG, Fed with *Euglena gracilis* cells; SV, Fed with *Scenedesmus vacuolatus* cells; C, River control site; UpS, Polluted site, upstream from the tertiary-treated sewage discharge; DoS, Polluted site, downstream from the tertiary-treated sewage discharge; WTM, Wet tissue mass; GF, Gill factor; DGF, Digestive gland factor.

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transplantation procedures may produce negative effects on the oxidative balance of gills and digestive gland and should be taken into account for bioremediation strategies.

1. Introduction

Euglena spp. cells are being considered as a promising dietary complement to be used in aquaculture activities [1,2]. These unicellular flagellates are able to synthesize and accumulate high amounts of paramylon, a β-1,3 glucan, which has been reported as an efficient immunostimulant for the rainbow trout [3,4] and for fingerlings of the fish *Labeo rohita* [5]. Orally supplied paramylon extracted from *Euglena gracilis*, enhances antioxidant responses in rat liver after carbon tetrachloride injection [6]. In addition, the high content of proteins in *Euglena* spp. cells promotes growth rates in farmed crustaceans and fish [3,7–9], while polyphenols, flavonoids, tannins, β-carotene, vitamin C and E may be present in these cells as a source of vitamins and antioxidant compounds, contributing to a healthy general status [2]. In hemocytes of bivalves, exposure to β-glucans increases nitric oxide production, peroxidase and antibacterial activity and phagocytosis during *in vitro* and injection-based experiments [10–13]. New evidence has been recently published for the immunostimulant effect of orally supplied *E. gracilis* cells on bivalves exposed to *Escherichia coli* [14].

Filtering bivalves are proposed as useful bioremediation tools against anthropogenic pollution, containing metals, organic matter, bacteria, algae and nutrients, eg. Refs. [15–17]. In particular, the freshwater mussel *Diplodon chilensis* is able to filter high amounts of particulate organic matter, coliform bacteria and algae, reducing microorganism and nutrient loads from eutrophicated and bacteria polluted water bodies [18–22]. Regarding this, Sabatini et al. [21] have found that *D. chilensis* may clear *Escherichia coli*, a typical bacterium found in sewage water, at a rate of 0.510 ± 0.036 L h^{-1} per gram of dry soft tissue mass (DTM), while Bianchi et al. [22] report that enteric bacteria are efficiently removed from sewage polluted water at a rate of 0.155 ± 0.01 L h^{-1} per gram of DTM. However, it has been shown that exposure to *E. coli* may cause hemocyte damage [14] and increased lipid peroxidation in gills and digestive gland of this bivalve [14,21].

In our previous work [14], feeding with *E. gracilis* cells has been evaluated in *D. chilensis* in order to improve its physiological responses against *E. coli*. The cited work brought promising results concluding that *E. gracilis* can be used as a nutritional and immune protective diet complement, suitable for filtering bivalves. Nevertheless, the variety of pollutants found in sewage water may have a different effect compared with those of the isolated bacteria. It has been reported that organic and inorganic pollutants contained in domestic effluents may cause alterations in the physiological status of bivalves, modifying cellular immune responses and viability [23–26] and causing genotoxic effects [27]. In addition, oxidative stress and detoxification mechanisms are increased while growth rate is altered in bivalves exposed to sewage polluted aquatic environments [26]. Similar results were obtained for wild and caged *D. chilensis* exposed to sewage water pollution in the field [21,22,28]. Thus, in order to test whether orally supplied *Euglena* sp. cells modulate the physiological status of bivalves during bioremediation procedures, we evaluated the effect of *E. gracilis* diet on the immune response, oxidative balance and metabolic condition of *D. chilensis*, after field exposure to different concentrations of sewage water pollution in a North-Patagonian river.

2. Materials and methods

2.1. Mussel collection and handling

Mussel collection, experimental feeding and field exposure were performed during the non-reproductive season of *D. chilensis* (May to August, 2012) in order to avoid physiological changes due to reproductive status [29]. Adult *D. chilensis* (n = 60; 68.13 ± 0.66 mm shell length) were collected by a diver from 1.5 m depth at an unpolluted area in the north coast of Lacar lake (Yuco, 40° 10′ S, 71° 31′ 30″ W). Mussels were immediately transported to the laboratory and placed in aerated tanks (150 individuals per m^2) containing dechlorinated tap water.

2.2. Strains

Lyophilized cells of *E. gracilis* (UTEX 753 strain, from the Culture Collection of Algae of Texas University, USA) and of the green algae *Scenedesmus vacuolatus* (BAFC CA4 strain from Laboratory of Phycology, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires) used in this work correspond to the same cultures previously used by Bianchi et al. [14]. Both experimental diets were set at 0.133 mg of lyophilized cells per mussel per feeding event, and each mussel received a ration of 0.128 mg paramylon per feeding, contained in lyophilized *E. gracilis* cells [14].

2.3. Experimental feeding

During 21 days of acclimation in laboratory, individuals were fed three times a week with *S. vacuolatus*. After acclimation, mussels were sorted into two groups: SV (n = 30), fed with *S. vacuolatus* (control diet) and EG (n = 30), fed with *E. gracilis*. Experimental diets were supplied three times a week for 90 days, performing water changes before each feeding. During this period, temperature was kept at 11.5 ± 1.0 °C.

2.4. Field exposure

After experimental feeding, both SV and EG mussels were sorted into six groups (n = 5 per group), which were placed into six cages (iron structure covered with plastic mesh) at a final density of 87 individuals per m^2. Two cages with SV or EG mussels were placed at each of three sites along the Pocahullo river basin, which crosses suburban and urban areas of San Martín de los Andes city, Northwest Patagonia, Argentina (40°09′24″S; 71°21′09″W). The sites were set as follows: C: upstream control, at a site with no significant sewage pollution (40° 7′ 7,4″ S; 71° 14′ 14.2″ W); UpS: 40 m upstream from the main tertiary-treated sewage discharge but downstream from diffuse discharge of untreated effluents (septic tank infiltrations and horse-cattle farming) and point source discharges of suburban primary treated sewage (40°09′32,1″S; 71°21′40.9″W); and DoS: 20 m downstream from the main tertiary-treated sewage discharge (40°10′S; 71°20′60″W). After 10 days of exposure, individuals were collected and transported in the cold to the laboratory, for immediate processing.

2.5. Water quality

At the time of mussel collection, water samples (n = 3) were collected at each site and transported in the cold to the laboratory. Water temperature (T °C), pH and dissolved oxygen (DO mg L^{-1}) were recorded at each site. Fecal coliform bacteria concentration (FC) was measured according to the Most Probable Number method (MPN/100 mL) [30]. Chlorophyll *a* was recovered from filtered samples (Whatman GFF, 0.45 μm pore) by acetone extraction and its concentration (Chl*a* μg L^{-1}) was calculated according to Lichtenthaler [31]. Particulate organic matter (POM mg L^{-1}) was measured in ashen filtered samples and calculated according to Juhel et al. [32]. Nitrate (NO_3^- μg L^{-1}) was measured by a cadmium reduction method (HACH method 8192) and nitrite (NO_2^- μg L^{-1}) by a diazotization method (HACH method 8507). Phosphate (PO_4^{-3} μg L^{-1}) was measured by the ascorbic acid method [30]. Sulphate (SO_4^{-2} mg L^{-1}) was measured by a turbidimetric method [30] and turbidity (FAU) by Attenuated Radiation Method (HACH Method 10047). Total cooper (Cu) content was determined by the bicinchoninic acid method (HACH Method 8506) and expressed as mg L^{-1}. Total iron (Fe) was determined by the FerroZine Method (HACH Method 8147) and expressed as mg L^{-1}. Absorbance measurements were carried out with a HACH DR/4000 spectrophotometer.

2.6. Hemocyte response

Hemolymph (2 mL) was withdrawn from the mussels' adductor muscle (n = 6, for each diet and site) using a sterile syringe. Hemolymph aliquots were placed on ice into sterile microcentrifuge tubes and analyzed within 1 h from collection.

2.6.1. Immune response and cytotoxicity

Total and viable hemocytes were counted using a Neubauer's chamber. Viable and nonviable cells were discriminated by the Trypan Blue exclusion method as described in Bianchi et al. [14]. Total hemocytes were expressed as cells mL^{-1} of hemolymph and hemocyte viability was presented as proportion of total hemocytes [23]. Hemocyte phagocytic activity was evaluated using Congo red stained *Saccharomyces cerevisiae* cells, as described in Bianchi et al. [14]. Phagocytic activity was calculated as phagocyted yeast cells/viable hemocytes (n = 300 for each mussel), observed in duplicate under light microscopy (100 − 400 ×).

Lysosomal membrane stability was evaluated in hemocytes by the Neutral Red Retention Time method (NRRT), as described in Bianchi et al. [14]. Results were expressed as neutral red retention time 50% (NRRT 50%, min), denoting the time at which 50% of the cells were stained, using a light microscope at 400 × for observation [33].

2.6.2. ROS and TOSC

Hemocytes were recovered by centrifugation (500 × g for 20 min), washed and re-suspended in anticoagulant solution (3 g glucose and 0.36 g trisodium citrate L^{-1}, 60 mOsm L^{-1}, pH 7). ROS and TOSC were measured in a Qubit fluorometer at 485/530 nm, using 2′,7′-dichlorofluorescin diacetate (H_2DCF-DA) as substrate [22]. ROS content was referred to a H_2O_2 standard curve and results were expressed as meq H_2O_2 10^{-6} viable cells. TOSC was calculated from the relative area between the curves obtained with and without the addition of ABAP (peroxyl radical source) and referred to 10^6 viable cells.

2.7. Tissue response

Gills and digestive gland were extracted, weighted (g) and separately homogenized (Omni 1000 motorized homogenizer at 20,000 rpm) in cold phosphate buffer (100 mmol L^{-1}, pH 7.0) 1:5 w/v, containing 0.2 mmol L^{-1} phenylmethylsulfonil fluoride (Sigma). Supernatants were obtained after centrifugation (11,000 × *g* for 15 min at 4 °C, Ependorf AG, Minispin centrifuge) and used for biochemical analysis. Since protein content in tissue samples varied significantly among treatments (Section 3.3.2), results for enzyme activities, and TBARS content were referred to grams of wet tissue mass (WTM) and not to protein concentration.

2.7.1. Enzyme activities

GST activity was estimated using 1-chloro-2,4-dinitrobenzene and reduced glutathione as substrates [34]. One GST Unit was defined as the quantity of enzyme that catalyzes the production of 1 μmol of GS-DNB per min at 25 °C. Results were expressed as U GST g^{-1} WTM. CAT activity was estimated by recording the hydrolysis of H_2O_2 [35]. One CAT Unit was defined as the amount of enzyme needed to catalyze the hydrolysis of 1 μmol of H_2O_2 per min at 25 °C. Results were expressed as U CAT g^{-1} WTM.

2.7.2. Lipid peroxidation

Lipid peroxidation was estimated by the thiobarbituric acid reactive substances method (TBARS) [36]. Results were expressed as μmol of TBARS g^{-1} WTM.

2.8. Metabolic condition

Mussels' metabolic condition was assessed using morphometric factors. Gill and digestive gland mass factor (GF, DGF) were calculated according to Bianchi et al. [14]. The wet mass (g) of each organ was divided by the shell length^3 and multiplied by 100. Protein content for each tissue was measured according to Bradford [37], using a bovine seroalbumin standard curve. Results were expressed as mg of protein g^{-1} WTM.

2.9. Statistical analysis

Data were presented as mean ± standard error of the mean (SEM). Normal distribution and homogeneity of variance were checked by Kolmogorov-Smirnov test and Levene test, respectively. One-way ANOVA and Newman-Keuls *post hoc* comparisons were applied to identify differences among physic-chemical and bacteriological data among sites. Two-way ANOVA (diet × site) and Newman-Keuls *post hoc* comparisons were used to identify differences between dietary groups (SV, EG) and among the different sites in the river (C, UpS, DoS). When statistical assumptions were not met, data were previously transformed by Log_{10} or Log_{10} (x + 1), when appropriate.

3. Results

3.1. Water quality

Water physic-chemical and bacteriological analyses reveal an increase in the pollution load along the river course (Table 1). Concentrations of fecal coliforms (FC), NO_3^-, PO_4^{-3}, SO_4^{-2}, total Fe, and turbidity values increase significantly as follows: C ˂ UpS ˂ DoS ($p < 0.05$ for all comparisons). The NO_2^- and POM contents are increased in the polluted sites (UpS, DoS) compared with those in the control site ($p < 0.05$; $p < 0.001$, respectively for both sites). Total Cu concentrations are higher in DoS than in C ($p < 0.001$). pH values decrease significantly in both polluted sites respect to the control ($p < 0.001$), while DO values increase under pollution conditions ($p < 0.001$). No significant changes are observed in water temperature (T°).

Table 1
Physic-chemical and bacteriological water quality at sampling sites in Pocahullo river basin. C: control, with no significant sewage pollution; UpS: upstream from the tertiary-treated sewage discharge; DoS: downstream from the tertiary-treated sewage discharge. Results for temperature (°C), pH, dissolved oxygen (DO), turbidity, fecal bacteria coliform (FC), chlorophyll *a* (Chl*a*), nitrate (NO_3^-), nitrite (NO_2^-), phosphate (PO_4^{-3}), sulphate (SO_4^{-2}), particulate organic matter (POM), total iron (Fe) and cooper (Cu) are expressed as mean ± SEM (n = 3). Different characters denote significant differences among sites (p between 0.0001 and 0.05).

	C	UpS	DoS
T (°C)	4.90 ± 0.03	4.01 ± 0.07	4.43 ± 0.07
pH	6.90 ± 0.03**a**	6.54 ± 0.05**b**	6.19 ± 0.06**c**
DO (mg L^{-1})	11.20 ± 0.06**a**	12.43 ± 0.03**b**	12.33 ± 0.03**b**
Turbidity (FAU)	5.6 ± 0.3**a**	10.0 ± 0.6**b**	14.0 ± 0.6**c**
FC (MPN/100 mL)	15 ± 4**a**	2350 ± 375**b**	7000 ± 1155**c**
Chl*a* (μg L^{-1})	0.49 ± 0.10**a**	2.79 ± 0.37**b**	3.30 ± 0.15**c**
NO_3^- (μg L^{-1})	0.025 ± 0.003**a**	0.038 ± 0.005**b**	0.050 ± 0.003**c**
NO_2^- (μg L^{-1})	0.0031 ± 0.0005**a**	0.0052 ± 0.0001**b**	0.0048 ± 0.0006**b**
PO_4^{-3}(μg L^{-1})	0.052 ± 0.003 **a**	0.153 ± 0.004**b**	0.201 ± 0.001**c**
SO_4^{-2} (mg L^{-1})	0.20 ± 0.03**a**	0.30 ± 0.03**b**	0.50 ± 0.03**b**
POM (mg L^{-1})	4.86 ± 0.41**a**	14.53 ± 0.55**b**	16.94 ± 0.94**b**
Fe (mg L^{-1})	0.072 ± 0.001**a**	0.087 ± 0.001**b**	0.083 ± 0.001**c**
Cu (mg L^{-1})	0.01567 ± 0.00003**a**	0.01967 ± 0.00003**ab**	0.03667 ± 0.00003**b**

3.2. Hemocyte response

3.2.1. Immune response

Hemocyte total number in *D. chilensis* hemolymph decreases along the river course, being significantly lower in DoS than in C (site effect, $p < 0.05$) (Fig 1a). Phagocytic activity is increased, by 36% at C and 65% at DoS, in EG mussels respect to SV ones (diet effect, $p < 0.05$) (Fig 1b).

3.2.2. ROS and TOSC

Diet × site interaction is significant for hemocyte ROS production ($p < 0.05$). This variable increases significantly in both groups of mussels at UpS and DoS compared to those at C ($p < 0.001$) and shows almost two-fold higher values in EG mussels than in SV ones at DoS ($p < 0.01$) (Fig 2a). No differences among sites or between diets are observed for TOSC (Fig 2b).

3.2.3. Cytotoxicity

Although statistically significant (diet × site interaction $p < 0.05$), hemocyte viability changes are not higher than 2% (Fig 3a). Lysosomal membrane stability, measured as neutral red retention time 50%, is about 81% higher in EG mussels than in SV ones (diet effect, $p < 0.001$), and decreases in mussels exposed at UpS and DoS when compared with C (site effect, $p < 0.001$) (Fig 3b).

3.3. Tissue response

3.3.1. Enzyme activities and lipid peroxidation

Gill GST activity is lower in EG mussels than in SV ones (diet effect, $p < 0.001$), and shows no site effect (Fig 4a). Diet × site interaction is significant for gill CAT activity ($p < 0.05$). This variable is increased only in SV mussels at UpS compared with the same group at the other sites and with EG mussels at the three sites ($p < 0.01$) (Fig 4b). Gill TBARS content also shows significant diet × site interaction ($p < 0.01$). This variable is increased in SV mussels at the polluted sites (DoS and UpS) compared with the same group at C ($p < 0.01$). TBARS content is slightly higher (about 15%) than in SV ones at the control site ($p < 0.05$) and is lower in EG than in SV mussels at UpS ($p < 0.05$) (Fig 4c).

Digestive gland GST (Fig 4d) and CAT (Fig 4e) activities are lower in EG mussels than in SV ones by about 19% and 18%, respectively (diet effect, $p < 0.05$ for both variables), while TBARS content (Fig 4f) is higher in EG than in SV by about 16% (diet effect, $p < 0.05$). In addition, GST activity in this organ is lower in mussels exposed at DoS than in those exposed at the other sites ($p < 0.05$). In the digestive glands of mussels exposed to both polluted sites, CAT activity decreases while TBARS content increases when compared with those at C ($p < 0.001$ for both variables).

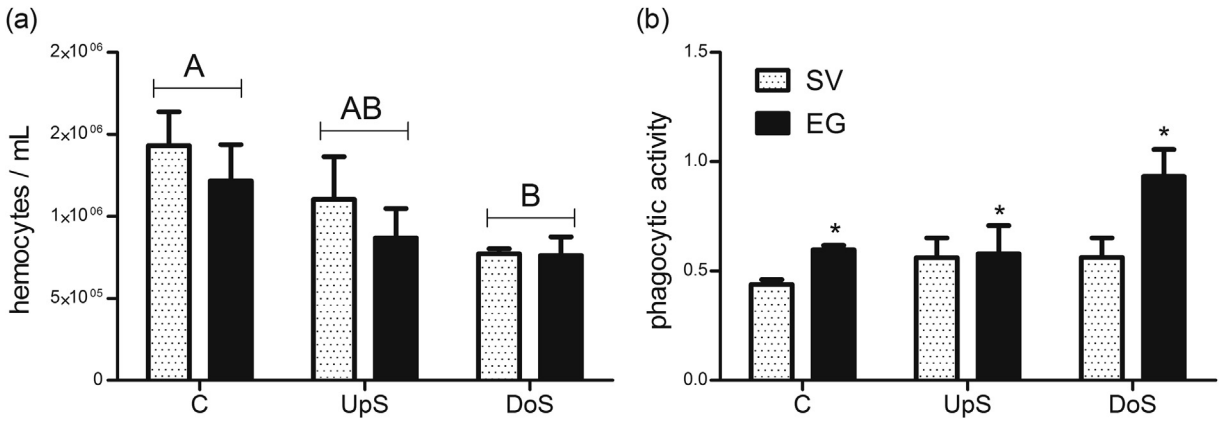

Fig. 1. Hemocyte total number (a) and phagocytic activity (b) in *Diplodon chilensis* previously fed with *Scenedesmus vacuolatus* (SV) or *Euglena gracilis* (EG) and exposed to sewage pollution at Pocahullo river basin, for 10 days. C: upstream control, with no significant sewage pollution; UpS: upstream from the tertiary-treated sewage discharge; DoS: downstream from the tertiary-treated sewage discharge. Results are expressed as mean ± SEM. Different characters denote significant differences among sites in (a) ($p < 0.05$). * denotes $p < 0.05$ between diets in (b).

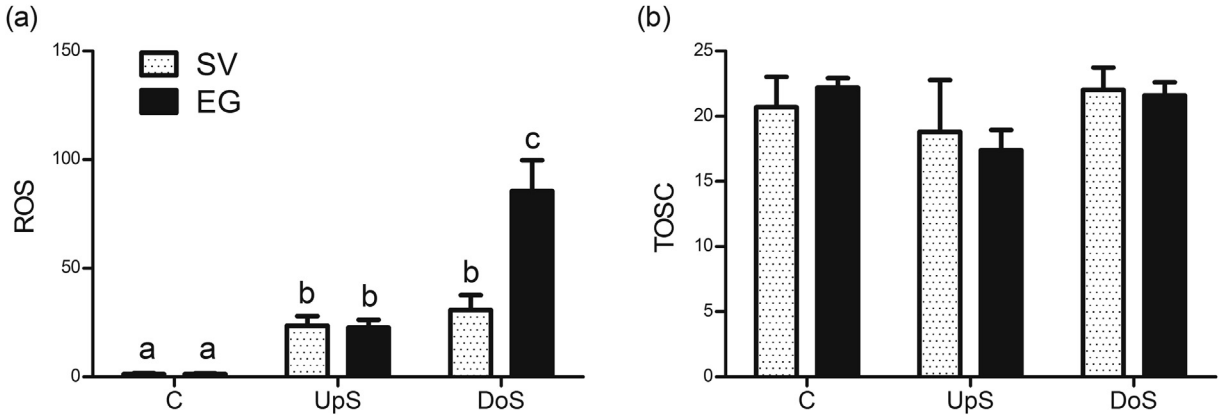

Fig. 2. Hemocyte reactive oxygen species (ROS) production (a) and total oxyradical scavenging capacity (TOSC) (b) in *Diplodon chilensis* previously fed with *Scenedesmus vacuolatus* (SV) or *Euglena gracilis* (EG) and exposed to sewage pollution at Pocahullo river basin, for 10 days. C: upstream control, with no significant sewage pollution; UpS: upstream from the tertiary-treated sewage discharge; DoS: downstream from the tertiary-treated sewage discharge. Results are expressed as mean ± SEM. Different characters denote significant differences among bars ($p < 0.01$).

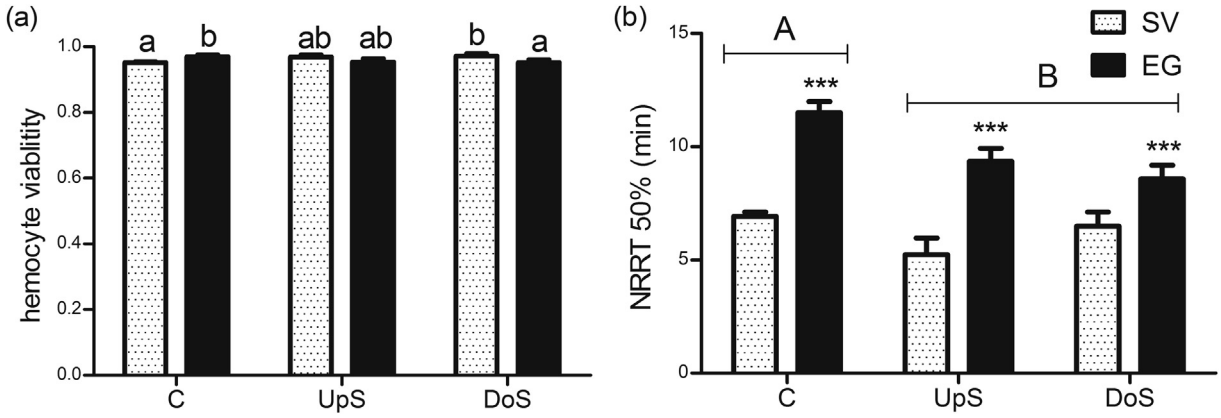

Fig. 3. Hemocyte viability (a) and lysosomal membrane stability (NRRT 50%) (b) in *Diplodon chilensis* previously fed with *Scenedesmus vacuolatus* (SV) or *Euglena gracilis* (EG) and exposed to sewage pollution at Pocahullo river basin, for 10 days. C: upstream control, with no significant sewage pollution; UpS: upstream from the tertiary-treated sewage discharge; DoS: downstream from the tertiary-treated sewage discharge. Results are expressed as mean ± SEM. Different characters (lower case) denote significant differences among bars ($p < 0.05$) in (a); among sites (upper case) in (b). *** denotes $p < 0.001$ between diets in (b).

3.3.2. Metabolic condition

Diet × site interaction is significant for GF ($p < 0.05$). This variable is higher in EG mussels transplanted to C ($p < 0.05$) and lower in those at UpS and DoS ($p < 0.05$, for both), compared with SV mussels (Fig. 5a). DGF is about 18% higher in EG mussels than in SV ones (diet effect, $p < 0.05$) (Fig. 5b).

Gill protein content is increased in mussels of both groups exposed at DoS, compared with those at C and UpS ($p < 0.05$) (Fig. 6a). No significant changes are observed for this variable in digestive gland (Fig. 6b).

4. Discussion

This work is the first field study on the modulating effects of orally supplied *Euglena* sp. cells on physiological responses of bivalves to anthropogenic pollution. Because of the scarce antecedents available, we discuss our present results based mostly on our previous work at laboratory conditions [14]. Nevertheless, the difference between exposure times from both studies should be taken into account (5 vs. 10 days).

4.1. Water quality

Sewage discharges are characterized by the presence of high loads of fecal bacteria and organic matter, which may increase water turbidity and lead to the eutrophication of water bodies [38,39]. Accordingly, concentrations of FC, NO_2^-, PO_4^{-3}, NO_3^- and POM, and turbidity values obtained in Pocahullo river basin are higher in the polluted sites (UpS, DoS) than in the control. With the exception of NO_2^- and POM, the levels of all these variables are increased by the point discharge of the main tertiary treatment plant of San Martín de los Andes City. In particular, FC concentration is more than two-fold higher in the DoS than in the UpS. The graded differences observed in these indicators indicate that mussels caged in the three sites chosen for the study have been exposed to relevantly different levels of sewage water pollution. Concentrations of other pollutants, such as SO_4^{-2}, total Fe and Cu, are also increased at polluted sites compared with the control but show little variation between both polluted sites. Fe and Cu are commonly found in sewage waste and can be accumulated and remobilized from sediment to water; e.g. in turbid rivers or during resuspension events near effluent discharges [40]. Finally, pH and OD levels are decreased and increased, respectively, from the control to the polluted sites. However, these statistical differences

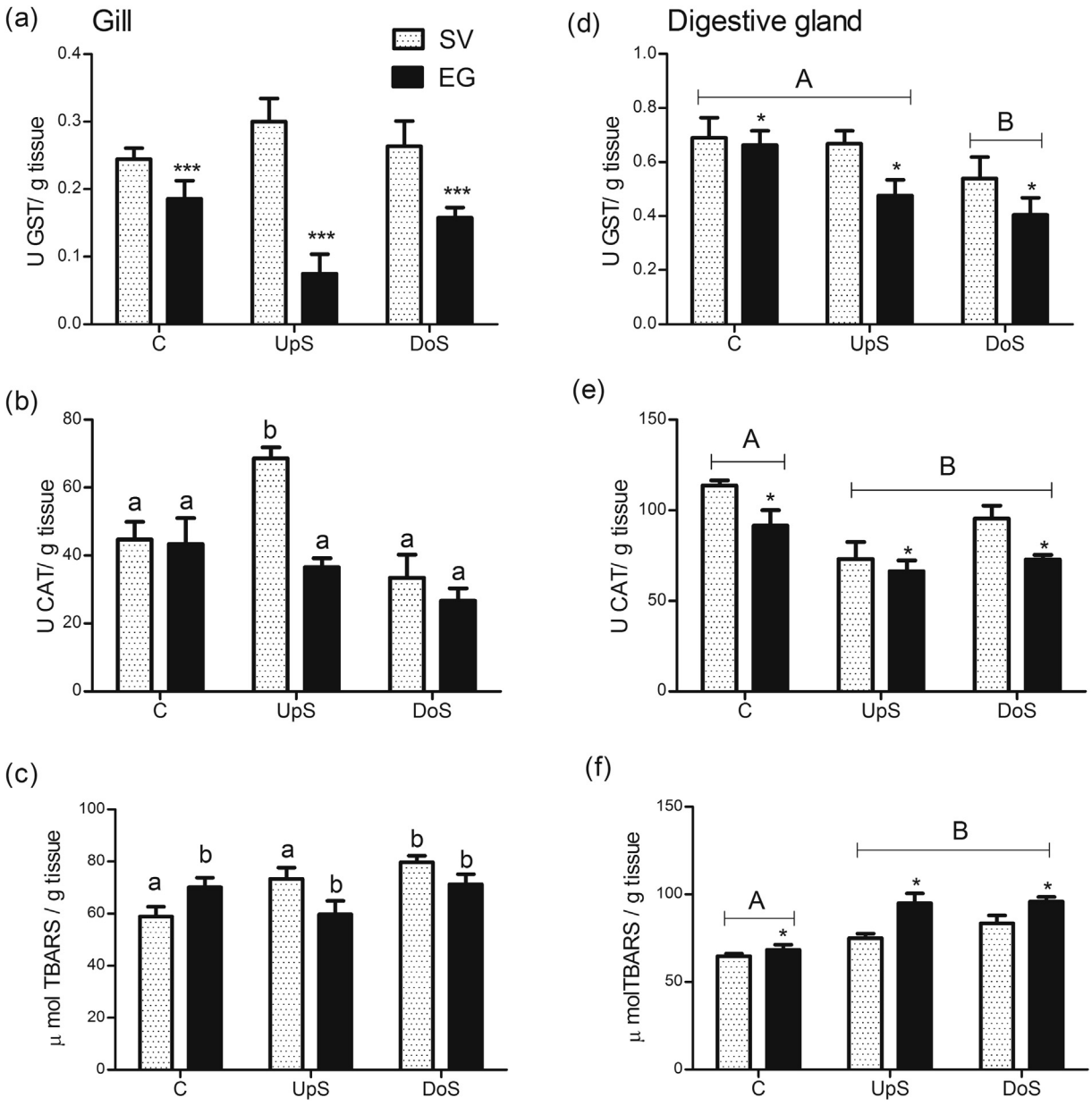

Fig. 4. Glutathione S-transferase (GST) and catalase (CAT) activities, and lipid peroxidation (TBARS) in gills (a–c) and digestive gland (d–f) of *Diplodon chilensis* previously fed with *Scenedesmus vacuolatus* (SV) or *Euglena gracilis* (EG) and exposed to sewage pollution at Pocahullo river basin, for 10 days. C: upstream control, with no significant sewage pollution; UpS: upstream from the tertiary-treated sewage discharge; DoS: downstream from the tertiary-treated sewage discharge. Results are expressed as mean ± SEM. Different characters (lower case) denote significant differences ($p < 0.05$) among bars in (b) and (c), and between sites (upper case) in (d) ($p < 0.05$), (e) ($p < 0.001$) and (f) ($p < 0.001$). Significant diet effects are denoted by * $p < 0.05$ and *** $p < 0.001$.

are not expected to be physiologically relevant, since the recorded values are within the normal range for North Patagonian Andean water bodies [41].

4.2. Hemocyte response

Hemocyte number in hemolymph of *D. chilensis* decreases in both dietary groups (SV, EG) in response to the increase of pollution load in Pocahullo river water. This is in accordance with the results published by Akaishi et al. [23] in *Mytilus edulis* exposed to increasing concentrations of untreated effluents, for 7 days. In contrast, in our previous work testing the modulating effects of dietary *E. gracilis* in *D. chilensis* exposed to *E. coli* for five days, no changes were observed in the total number of hemocytes, neither in SV nor in EG mussels [14]. This suggests that the decrease in this variable may be responding to the presence of other kind of pollutants, such as copper, which is present at higher concentrations in the polluted sites of Pocahullo river [42] or to combined effects of bacterial load and other pollutants. Alternatively, cellular migration from circulating system to tissues in response to microorganism invasion [43] and/or to damage produced by toxic exposure [44,45] may be causing this decrease in the total number of hemocytes in hemolymph. Previous results on increased accumulation of hemocytes in gills and mantle of *D. chilensis* after exposure to *E. coli* [14] would support the latter hypothesis.

Phagocytic activity of hemocytes is expected to increase upon microbiological stimuli, e.g. Refs. [46], while exposure to organic and inorganic pollutants may have an inhibitory effect on this

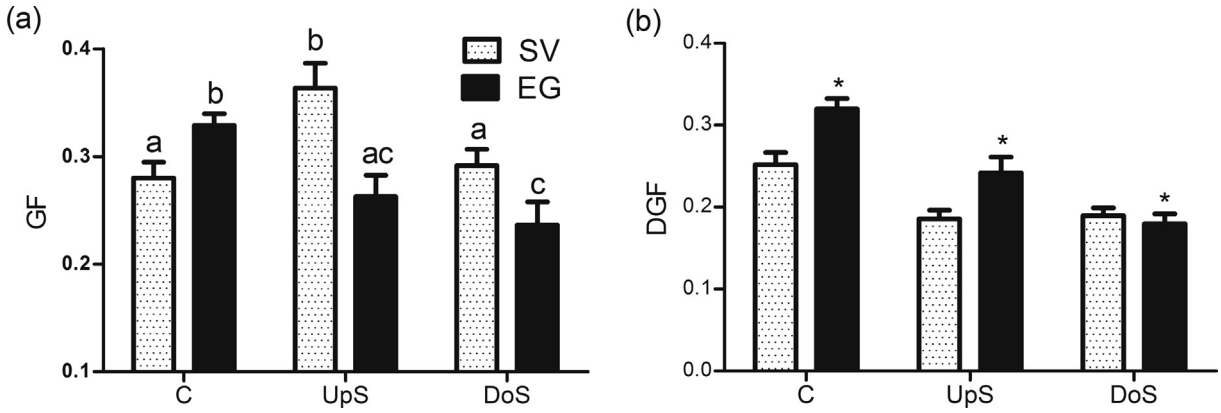

Fig. 5. Gill factor (GF, a) and digestive gland factor (DGF, b) in *Diplodon chilensis* previously fed with *Scenedesmus vacuolatus* (SV) or *Euglena gracilis* (EG) and exposed to sewage pollution at Pocahullo river basin, for 10 days. C: upstream control, with no significant sewage pollution; UpS: upstream from the tertiary-treated sewage discharge; DoS: downstream from the tertiary-treated sewage discharge. Results are expressed as mean ± SEM. Different characters denote significant differences ($p < 0.05$) among bars. * denotes $p < 0.05$ between diets in (b).

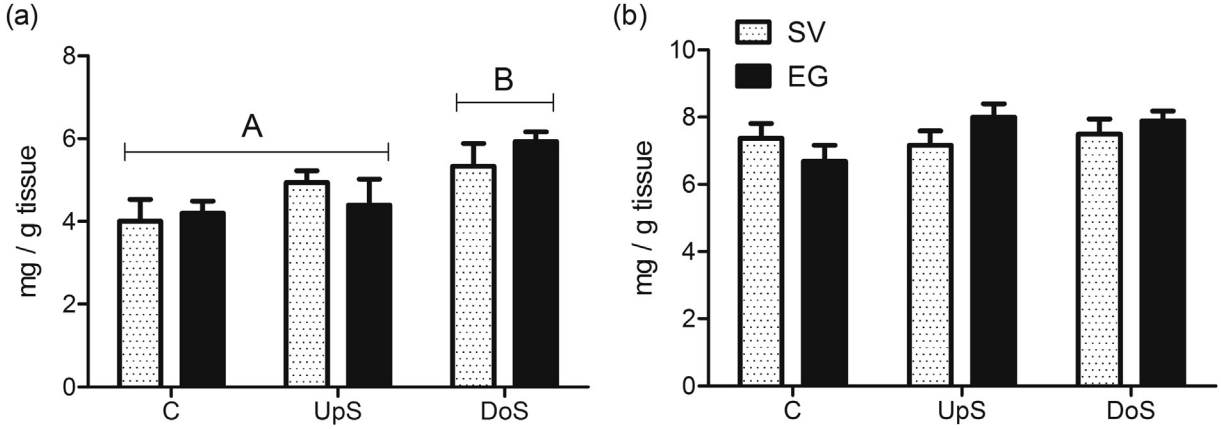

Fig. 6. Protein content in gill (a) and digestive gland (b) of *Diplodon chilensis* previously fed with *Scenedesmus vacuolatus* (SV) or *Euglena gracilis* (EG) and exposed to sewage pollution at Pocahullo river basin, for 10 days. C: upstream control, with no significant sewage pollution; UpS: upstream from the tertiary-treated sewage discharge; DoS: downstream from the tertiary-treated sewage discharge. Results are expressed as mean ± SEM. Different characters denote $p < 0.05$ between DoS and the other sites in (a).

activity [47,48]. In this work, phagocytic activity in hemocytes of SV mussels shows no change between control and polluted sites, after 10 days at Pocahullo river basin. In *M. edulis*, Akaishi et al. [23] observed a concentration-dependent increase in phagocytic activity but only after 14 days of exposure to untreated municipal effluents. Phagocytic activity in *D. chilensis* fed with *E. gracilis* is higher than that of SV mussels, in both control and polluted sites, with a clear increasing trend in EG mussels at the most polluted site (DoS). This improvement of phagocytic activity is comparable with that obtained in our previous work with *D. chilensis* under control and *E. coli* exposure conditions [14], and suggests a reinforcement of the immune response by β-glucan [10]; in this case, by EG diet.

ROS production in hemocytes may rise in response to both, activated immune response and as a product of the metabolism of toxic products included in effluents [49,50]. In this work, *D. chilensis* of both EG and SV groups show an increase in the cellular production of ROS in response to the increase in the pollution load along the river course. The effect of bacterial and fungal microbiological stimuli [51], metals [49], pharmaceutical drugs [52] and physiological stress caused by increased water turbidity [28], may be triggering this response. In addition, ROS production in hemocytes of EG mussels is higher than in those of SV ones at the most polluted site (DoS). This could be indicating a higher capacity of immune response activation, which correlates with the elevated phagocytic activity, described above for EG mussels. In addition, the total antioxidant capacity (TOSC) does not vary among sites in hemocytes of either dietary group, which suggests that increased ROS production in EG mussels is more likely related to an immunological response than to redox imbalance.

Exposure to sewage pollution has been shown to produce deleterious effects on bivalve's hemocytes. Hemocytes of *Mytilus galloprovincialis* exposed to landfill leachate for 4 days show oxidative damage to lipids and genotoxic effects [27]. Moreover, exposure to sewage pollution in the field causes loss of cellular viability in *Eliptio complanata* [25]. It is noticeable that *D. chilensis* fed with EG or SV maintain high levels (above 95%) of hemocyte viability irrespectively of the pollution level; however, this is in coincidence with the results obtained for mussels of this species chronically exposed to sewage pollution [22]. On the other hand, the lysosomal membrane stability decreases in polluted sites compared to the control; however, this variable is always higher in EG mussels than in SV ones, which indicates a protective effect of EG diet on cellular integrity of hemocytes.

4.3. Tissue response

After transplantation to polluted sites, gill GST shows lower activity in EG mussels than in SV ones, while CAT activity and

damage to lipids (TBARS) show no consistent differences between dietary groups nor among sites. These results suggest that *E. gracilis* diet has not significant effects on the oxidative balance of gills, when mussels are exposed to sewage water pollution. However, in our previous work with *D. chilensis* [14], challenge with *E. coli* in the laboratory has produced an increase in gill GST and CAT activities, compared with control mussels; although these activities remained lower in EG mussels than in SV ones as in the control groups. These differences between laboratory and field studies could be assigned to transplant effects [28] or to inhibitory effects of other pollutants, present in UpS and DoS sites, which would compensate a possible stimulatory effect of the increased bacterial load.

In the digestive gland, feeding with *E. gracilis* produces a general reduction of enzyme activity (GST, CAT) with a slight but significant increment in lipid peroxidation levels, in all transplanted mussels. This suggest transplant effects on antioxidant and detoxifying responses of EG mussels, when these are placed at control and polluted sites of the river. Similar effects were observed in *D. chilensis* transplanted to DoS for 30 days [28]. As it has been discussed in our previous work [14], the exogenous antioxidants, such as flavonoids, provided by *E. gracilis* cells [53] may increase the digestive gland antioxidant capacity, leading to the negative modulation of the intrinsic antioxidant response. Therefore, the interruption of this diet would temporarily increase the susceptibility of mussels to pro-oxidant conditions. Further studies, with more prolonged exposure to the river conditions should be performed, in order to assess whether the intrinsic antioxidant response can be restored or oxidative damage progresses and becomes harmful.

4.4. Metabolic condition

Morphometric and biochemical parameters are frequently used to assess the physiological and nutritional status of bivalves [21,54]. In this work, the GF is higher for EG mussels at control site but decreases at polluted sites, while SV mussels do not show a consistent trend. These results contrast with the augment in GF observed in *D. chilensis* challenged with *E. coli* in a previous work [14] and could be reflecting an impairment of inflammation processes in gills of EG mussels under stressful conditions in the Pocahullo river. Nevertheless, further testing is needed considering that gill protein content is increased in mussels exposed at DoS, suggesting the stimulation of metabolic responses or the loss of water in this organ. DGF is favored by *E. gracilis* diet. In contrast, mussels fed with this microorganism and challenged with *E. coli* have previously shown no changes in DGF [14]. However, the positive action of β-glucans on nutrient absorption [55] could be allowing mussels to take advantage on food availability in the river. In this way, *E. gracilis* diet could be stimulating digestive gland growth and/or inflammation processes, although no changes were observed in total protein content in this organ. Besides, the decreasing trend observed in DGF of mussels exposed to the most polluted site (DoS) may be explained by tissue damage exerted by pollutants in the digestive gland, e.g. Refs. [56,57].

5. Conclusions

Exposure to sewage water pollution stimulates immune response (phagocytic activity, ROS production), reduces hemocytes lysosomal membrane stability and causes moderate oxidative stress in gills and digestive gland of *D. chilensis* irrespectively of the previous diet. However, continuous and long term feeding with *E. gracilis* cells enhances this immune response, ameliorates the damage to hemocytes and tends to favor nutritive status of *D. chilensis*, which is reflected in the increase of digestive gland relative mass. Nevertheless, the obtained results also indicate that *E. gracilis* diet and transplantation procedures may produce negative effects on the oxidative balance of gills and digestive gland, and this should be taken into account during the application of bioremediation strategies.

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