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Delayed response of microbial epipelic biofilm to nutrient addition in a Pampean stream

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ABSTRACT: In streams and rivers, the first organisms which directly receive and respond to nutrients are primary producers (algae, macrophytes) and microbial heterotrophs (bacteria, fungi) since they rely on available inorganic nutrients from the water column. The aim of the present study was to analyze the response of the epipelic microbial biofilm in a Pampean stream submitted to a continuous input of inorganic nutrients (nitrogen and phosphorus). For this purpose, we measured the effects of moderate nutrient addition during 14 mo on the epipelic biofilm community of a meso-eutrophic stream that runs through the Pampean plain. The effects of nutrient enrichment were tested by analyzing the difference in algal and bacterial biomass and 2 extracellular enzymatic activities (β-glucosidase, phosphatase) at an enriched reach compared with those measured at an unmodified upstream reach. Overall, the response of the epipelon of this Pampean stream produced a slow and delayed effect on algal biomass increases, which might result in a delayed effect on the increase of bacterial densities. Neither phosphatase activity nor β-glucosidase activity exhibited significant changes due to nutrient addition. This may be due to the fact that the phosphatase activities measured were basal activities, uninhibited by enrichment, and that the epipelic β-glucosidase activity was regulated more by substrate availability than by any nutrient imbalance. Although changes are slow, if some of these changes were to become chronic, they would affect the functioning and services of the whole stream ecosystem.

KEY WORDS: Epipelon · Bacterial biomass · Phosphatase · β-glucosidase

INTRODUCTION

Nutrient enrichment is a global pressure for all types of aquatic ecosystems (UNEP 2007), since it drives increases in algal biomass and community changes in freshwater and coastal marine ecosystems (Smith 2003). Rising human pressure on water resources, derived from changes in land use and combined with climate change scenarios, can alter runoff patterns, leading to a greater discharge of water in streams and rivers (Hulme & Sheard 1999). These effects can lead to a greater input of nutrients into streams and rivers and may produce a cascade of effects on the functioning of the whole ecosystem, with accompanying economic repercussions (Dodds et al. 2009). The first organisms which directly receive and respond to nutrients are primary producers (algae, macrophytes) and microbial heterotrophs (bacteria, fungi), since they rely on available inorganic nutrients from the water column (Stelzer et al. 2003).

In low-order streams (<4), the benthic microbial community is responsible for most of the organic matter processing and nutrient dynamics, and its biomass is more significant than is planktonic biomass (Pusch et al. 1998). Streambed biofilms are made up of bacteria, algae, fungi, and microfauna, which are attached on benthic surfaces (sand grains, rocks or cobbles, leaf litter) or macrophytes. Biofilm microorganisms live in close physical contact and are usually embedded in a polymeric matrix. Because biofilms may be later used by consumers, they constitute an obligate step in the integration of nutrients in the

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cycling of matter and energy in the river (Allan 1995). Within the biofilm, algal growth relies on the availability of inorganic nutrients and light, while bacteria might make use of algal exudates or decaying macrophytes, as well as organic compounds, in the flowing water. The heterotrophic activity within the biofilm is responsible for the hydrolysis and/or oxidation of organic compounds by means of extracellular enzymes. The extracellular enzymes, mainly produced by bacteria and fungi, and, in some instances, algae (Sinsabaugh et al. 1991, Romaní et al. 2012), convert high-molecular-weight molecules to low-molecular-weight ones, which are then available for microorganisms (Chróst 1992).

The effects of nutrient additions in temperate forested low-order streams showed substantial increases in both algal and bacterial biomass of the biofilm, with hardly any significant effects in the animal groups (Sabater et al. 2011). The enrichment also caused enzymes associated with nutrient acquisition (such as phosphatase) to decrease and those linked to the use of algal-related material (such as peptidase and β-glucosidase) to increase. Similar results were obtained in a pristine tundra river with long-term phosphorus addition (Peterson et al. 1993).

However, little is known about the biofilm metabolism and its potential response to the addition of nutrients in Pampean plain streams, which possess singular characteristics in their streambed and energy flow. These streams run through depositional areas and usually show a streambed made of fine sediments (silt and clay) covered by epipelic biofilms. Their low current velocities, the absence of riparian vegetation, and the high concentrations of nutrients favor the development of dense and highly diverse macrophyte and/or floating macroalgal communities which might provide organic matter sources for microbial heterotrophs (Rodrigues Capítulo et al. 2009, Gómez et al. 2011). Most Pampean streams are described as not being limited by either light or nutrients (Giorgi 1998, Feijoó et al. 1999), although they show a clear seasonal pattern of primary production (increasing in spring and summer; Giorgi 1998). The highest primary production in a Pampean stream was measured in the floating macroalgae, and this was especially higher during low-flow periods; the epipelic biofilm showed much higher respiration than macrophytes and floating macroalgae, underlying the relevance of the epipelic biofilm as a relevant compartment for heterotrophic activity and organic matter use (Acuña et al. 2011).

The Pampean plain contains the highest demographic and industrial concentrations in the country, the greatest agriculture and livestock production, as well as the most intense use of agrochemicals. The expected anthropogenic pressure in Pampean streams might lead to increases in nutrient content.

The aim of the present study was to analyze the response of the epipelic microbial biofilm in a Pampean stream submitted to a continuous input of inorganic nutrients (nitrogen and phosphorus). For this purpose, we measured the effects of moderate nutrient addition (3-fold) during 14 mo on the epipelic biofilm community of a meso-eutrophic stream that runs through the Pampean plain. The experimental design was consistent with a BACIPS design (before–after/control–impact paired series). As explained by Underwood (1991, 1994), the comparison between a single impact and a single control location is confounded by any other cause of different time courses of abundances in the 2 locations that is not due to the identified human activity (in this case, the nutrient addition). Despite its limitations, this field experimental design is still more applicable than micro-/mesocosm approaches, since it provides greater ecological relevance. Since using multiple reference sites in large-replicated experiments is often not feasible, BACIPS designs are still useful in predicting impacts (Stewart-Oaten & Bence 2001). Moderate and long-term nutrient additions mimicked potential, gradual increases in nutrient loadings and allowed the detection of possible effects on the microbial epipelic biofilm that were delayed and/or chronic (Sabater et al. 2011). Nutrient enrichment effects were tested by analyzing the algal and bacterial biomass and 2 extracellular enzymatic activities (β-glucosidase, phosphatase) of epipelic biofilms at an enriched reach and were compared with those analyzed at an unmodified upstream reach. We hypothesized that inorganic nutrients might cause an increase in algal growth that would lead to a greater availability of organic matter to be decomposed and/or an enhancement of decomposing activities. This might lead to an increase in the extracellular enzymes involved in organic matter degradation (i.e. β-glucosidase, degrading polysaccharides), but a decrease in those involved in the acquisition of nutrients (i.e. phosphatase).

MATERIALS AND METHODS

Study site

La Choza is a Pampean stream located in the lowland prairies of Buenos Aires province, Argentina, within the lower part of Río de La Plata watershed

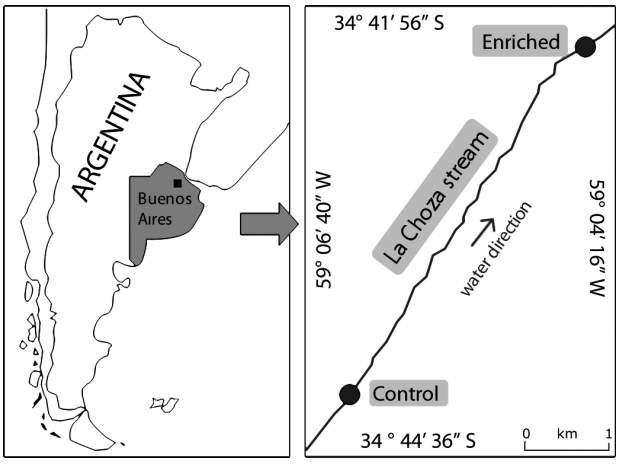

Fig. 1. Location of La Choza Stream (■) in Buenos Aires province, Argentina, and site coordinates

(Fig. 1). It drains a 48 km^2 basin on deep loess deposits. Native vegetation is temperate grassland with diverse gramineous species, though one-fourth of the land is devoted to agriculture, and 75% to extensive cattle grazing. The climate is temperate continental, resulting in wet springs and autumns. Average annual rainfall is 925 mm, and average temperatures range from 10°C in July to 22°C in January. The streambed of the Pampean streams is composed of calcium carbonate precipitates and deposits of silt and clay. Turbidity is high after storms due to the erosion of the organic-rich surface soil horizons. The low water velocities, high nutrient levels, and high light inputs favor macrophyte and plankton development, especially during spring and summer (Giorgi 1998, Bauer 2009). The nutrient concentrations in La Choza indicate that the stream can be classified as eutrophic, when total phosphorus is considered (TP > 75 µg l^{-1}) or meso-eutrophic, when total nitrogen is considered (TN > 700 µg l^{-1}) (USEPA 2000). The nutrient addition experiment was conducted in a third-order section, naturally devoid of riparian trees.

Experimental setup

The experimental manipulation consisted of artificially enriching the nutrient concentration in a 100 m reach (designated E for enriched), in comparison to a geomorphologically and hydrologically similar downstream reach that served as a control (C). The C reach was located 5.3 km upstream to ensure that the samples taken in both reaches were independent. Both reaches (C and E) were sampled for 6 mo prior to the addition of nutrients. In the E reach, N and P concentrations were increased 3 times compared to the concentrations in the C reach. The sampling period during the enrichment lasted 14 mo (October 2007 to December 2008). Samples were taken monthly, and sample collection was always conducted around mid-day (10:00 to 13:00 h).

Nutrient enrichment was achieved by the use of fertilizer bags (12% P and 12% N; Nitrofoska) distributed along the reach. Nutrient addition was adjusted twice a week in order to follow the natural nutrient dynamics in-stream. This was achieved by analyzing the soluble reactive phosphorus (SRP) concentration in both reaches in triplicate, and then refilling the fertilizer bags accordingly so that the nutrients in the E reach remained higher than those in the C reach.

Epipelic biofilm sampling

The epipelic biofilm samples were collected monthly by pipetting 10 aliquots of 4 ml streambed samples at random from each stream reach, each aliquot corresponding to 1 cm^2 of the superficial sediment layer (Gómez & Licursi 2001). Samples for chlorophyll analysis and bacterial density were collected in triplicate, while 5 replicates were collected for extracellular enzyme analysis. A granulometry analysis in both reaches was also performed to characterize the sediment (Folk 1959).

Physical–chemical parameters

Dissolved oxygen, pH, temperature, and conductivity were measured using a Horiba-U10 multiparametric sensor. Nutrient samples were filtered through pre-combusted glass fiber filters (Whatman GF/F, Whatman International) and analyzed for ammonia, nitrate, and SRP concentrations according to standard methods (APHA 1998). Total dissolved inorganic nitrogen (DIN, µg N l^{-1}) was calculated as the sum of nitrate and ammonia. Photosynthetically active radiation (PAR) was measured above the water surface using a LI-COR LI-250A.

Chlorophyll *a*

Epipelic biofilm samples were filtered through Sartorious GF/C filters. Filters were stored in the dark, dried, and frozen until they were analyzed. Samples were sonicated for 2 min in a Cleanson CS-1106 sonicator, and chlorophyll *a* (chl *a*) was then extracted

with 90% acetone for 12 h. The supernatant was read in a UV-VIS Auto 2602 spectrophotometer, and the concentration was calculated according to Strickland & Parsons (1968).

Bacterial biomass

Epipelic biofilm samples were stored in sterile glass vials with formalin 2% v/v. Bacterial density was estimated after sonication (three 2 min cycles) and appropriate dilution (1:100 to 1:400) of the samples. Diluted samples were stained for 10 min with DAPI (4′,6-diamidino-2-phenyilindole) to a final concentration of 1 µg ml^{-1} (Porter & Feig 1980), and filtered through a 0.2 µm black polycarbonate filter (GE Osmonics). Bacteria were then counted using an epifluorescence microscope (Olympus BX-50) under 1000× magnification. Twenty fields were counted for a total of 400 to 800 organisms per replicate. Bacterial biovolume was calculated assuming a 0.1 $µm^3$ constant volume per bacterial cell (Romaní et al. 2009).

Extracellular enzyme activities

The extracellular enzyme activities of β-glucosidase (β-GLU, EC 3.2.1.21) and phosphatase (PHA, EC 3.1.3.1–2) were measured using the fluorescent-linked substrates methylumbelliferyl (MUF). Samples were incubated at substrate saturation conditions of 0.3 mM. Epipelic biofilm samples were incubated for 1 h in the dark, at river temperature, immediately after sampling, along with blanks and standards of MUF. At the end of the incubation, a glycine buffer (pH 10.4) was added (1/1 vol/vol) and the fluorescence was measured at 365/455 nm excitation/emission (Shimadzu RF-540). Values are expressed as nanomoles of MUF per square centimeter of sediment surface area per hour.

The β-GLU efficiency, i.e. the amount of enzyme produced per bacterial cell, was calculated and expressed as nanomoles of MUF per square centimeter of sediment surface area per hour per cell. The standard deviation for this value was calculated by dividing each β-GLU value by the mean bacterial density for the same sampling date.

Data analysis

The effects of nutrient enrichment on the measured variables were analyzed following a BACIPS ANOVA design between the control and enriched reaches (Stewart-Oaten et al. 1986). As a measure of the effect of size, partial η^2, which explains the strength of the association between a predictor and the dependent variable, was calculated with the sums of squares as:

SS effect/SS effect + SS error

Cochran's test (Cochran 1941) was used to test for homogeneity of variance on the transformed data, and then a 3-factor ANOVA was performed: (1) Periods: *before*, *after* (BA); (2) Reach: *control*, *enriched* (CE); and (3) Time: *sampling times*, nested within periods [TIME(BA)].

Impacts can cause different patterns in the resulting tables of these analyses. The interaction between the factors Period and Reach (BA × CE) will be significant if there are immediate or '*press*' effects, i.e. if the variables in the impacted site are shifted to a new average condition in the *after* period. Alternatively, if there are gradual or '*pulse*' effects, the interaction between the factors Reach and Time [CE × TIME(BA)] will be significant, with no significant interaction in BA × CE. Both interactions will be significant if there is not only a substantial difference between the sites and between periods, but also a large variation within each period in each site.

Also, Pearson's product moment correlations were performed between the biological variables to try to establish the relationship between them.

RESULTS

Physical–chemical parameters

The physical–chemical parameters obtained from both stream reaches throughout the sampling periods (*before* and *after* the fertilization) are shown in Figs. 2 & 3.

Before fertilization, the conductivity values (means ± SD) were slightly higher in the E (1159.5 ± 500.6 µS cm^{-1}) than in the C reach (866.6 ± 511.8 µS cm^{-1}), and differences were enhanced after fertilization (1272.6 ± 101.5 µS cm^{-1} in the C reach and 1748.4 ± 96.4 µS cm^{-1} in the E reach; Table 1).

The temperature varied along with the incident PAR, following a typical seasonal variation, and no differences between the control and enriched sites were measured.

Before the fertilization had started, the dissolved oxygen concentration averaged 9.8 mg l^{-1} in both

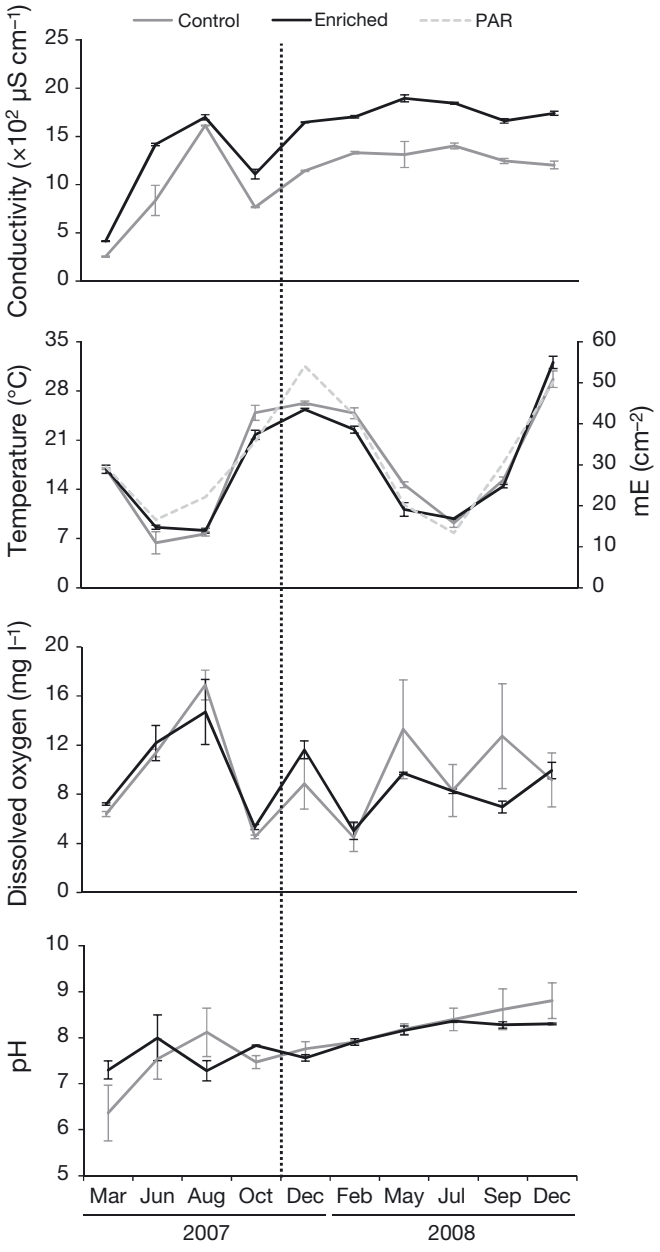

Fig. 2. Means (±SD, n = 3) of the physical–chemical parameters in control and enriched reaches of the La Choza stream during the study period. Dotted line marks the beginning of the fertilization period

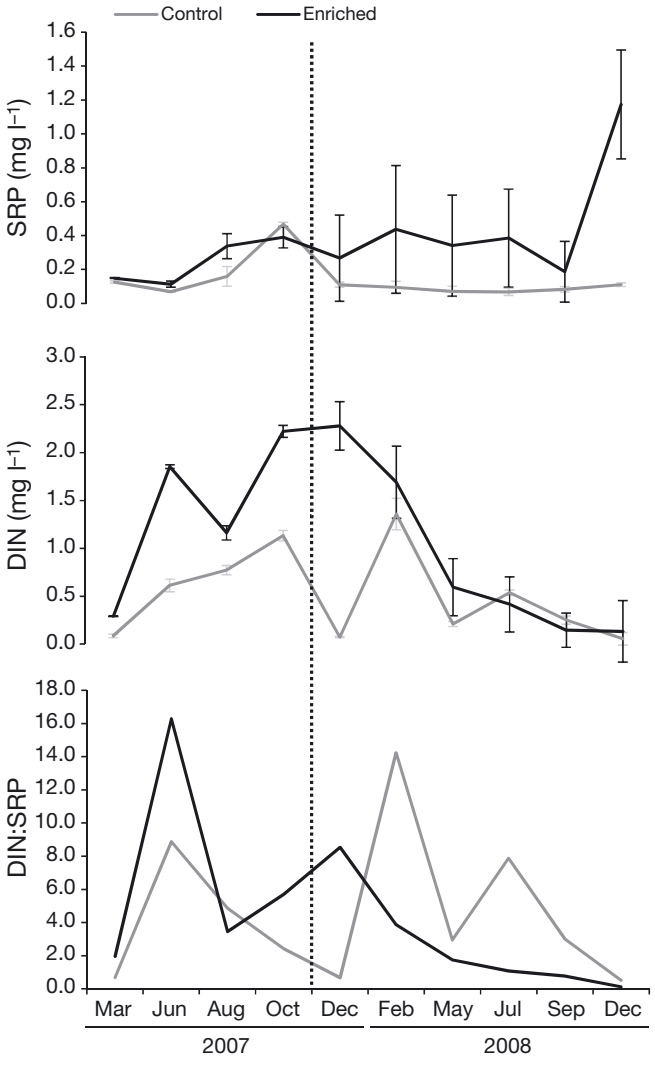

Fig. 3. Contents of soluble reactive phosphorus (SRP) and dissolved inorganic nitrogen (DIN) in the control and enriched reaches of the La Choza stream during the study period, and the DIN:SRP ratio. Means (±SD, n = 3) for the SRP and DIN values are shown, and means for the DIN:SRP ratio. Dotted line marks the beginning of the fertilization period

reaches, but after fertilization had started, it decreased significantly to 8.5 ± 2.2 mg l^{-1} in the E reach (Table 1). Similarly, pH values were nearly the same in both reaches throughout the study period, but, at the end of the experiment, pH tended to decrease in the E reach (Fig. 2, Table 1).

Nutrient addition increased average SRP concentrations by about 5-fold in the E reach (Fig. 3) when compared to the C reach, and the DIN concentration by about 2-fold. However, with regards to DIN, before the fertilization period the C reach had an average concentration of 0.65 ± 0.43 mg l^{-1}, and the E reach, of 1.38 ± 0.84 mg l^{-1}. During the fertilized period the average concentration of DIN in both reaches decreased: to 0.41 ± 0.49 mg l^{-1} in the C reach and to 0.87 ± 0.89 mg l^{-1} in the E reach. Although the effect of fertilization was significant (Table 1), this was mainly due to the first 2 mo after fertilization began; after that time the DIN remained similar in both reaches.

Table 1. Results obtained for the BACIPS (before–after/control–impact paired series) ANOVA for physical–chemical variables and their partial η^2 values. Significant ($p < 0.05$) values are marked in **bold**. Control–enriched (CE), before–after (BA) and time (TIME) sources of variation are considered. SRP: soluble reactive phosphorus; DIN: dissolved inorganic nitrogen

	CE	BA	TIME(BA)	CE × BA	CE × TIME(BA)
Conductivity (µS cm^{-1})					
p	0.000	0.079	0.000	0.644	**0.000**
Partial η^2	0.876	0.336	0.973	0.028	0.713
Dissolved oxygen (mg l^{-1})					
p	0.922	0.866	0.002	0.564	**0.003**
Partial η^2	0.001	0.004	0.903	0.043	0.421
pH					
p	0.918	0.022	0.113	0.227	**0.001**
Partial η^2	0.001	0.500	0.710	0.177	0.465
SRP (mg l^{-1})					
p	0.004	0.748	0.318	**0.027**	**0.005**
Partial η^2	0.387	0.007	0.586	0.241	0.094
DIN (mg l^{-1})					
p	0.026	0.639	0.001	0.093	**0.000**
Partial η^2	0.484	0.029	0.933	0.312	0.558

The DIN:SRP relationship changed from 3.2 to 4.6 in the C reach and from 5.6 to 1.9 in the E reach, indicating an imbalance leading to nitrogen limitation in the E reach.

Granulometry analyses showed that >90% of the sediment in the samples was composed of particles with a diameter <62 µm. The specific sand surface area for biofilm colonization was 106 cm^2 g^{-1} in the E reach and 132 cm^2 g^{-1} in the C reach.

Biological variables

The biological parameters obtained from both stream reaches throughout the sampling periods (*before* and *after* fertilization) are shown in Figs. 4 & 5.

The bacterial density was similar in both reaches in the *before* period ($3.4 \times 10^8 \pm 5.2 \times 10^8$ cells cm^{-2} in the C reach and $2.9 \times 10^8 \pm 3.3 \times 10^8$ cells cm^{-2} in the E reach); after fertilization the difference between the reaches was greater ($2.8 \times 10^8 \pm 2.3 \times 10^8$ cells cm^{-2} in the C reach and $5.0 \times 10^8 \pm 5.3 \times 10^8$ cells cm^{-2} in the E reach). The BACIPS ANOVA results showed a significant *pulse* effect in bacterial density, increasing in the E reach about 10 mo after fertilization began (Table 2, Fig. 4).

The chl *a* content was similar in both reaches in the period *before* fertilization, while during fertilization there was an increase in the E reach (2.43 ± 1.83 µg cm^{-2} in the C reach and 3.63 ± 3.74 µg cm^{-2} in the E reach), mainly due to increases in January 2008 and November 2009. The BACIPS ANOVA results evidenced a significant immediate effect in the chl *a* concentration in the E reach (Table 2).

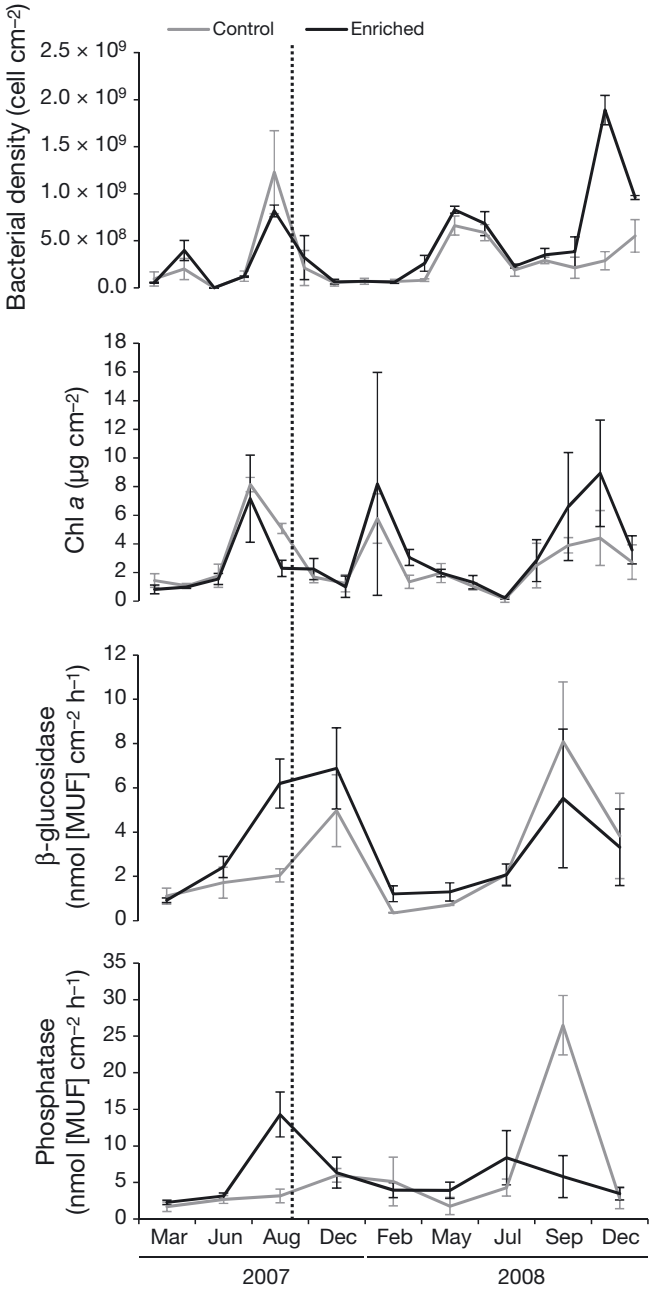

Fig. 4. Time evolution of bacterial biomass, chlorophyll *a* and the extracellular enzymes β-glucosidase and phosphatase in the control and enriched reaches of the La Choza stream during the study period. Means (±SD, n = 3 for bacteria and chlorophyll *a*, n = 5 for extracellular enzymes) are shown. Dotted line marks the beginning of the fertilization period

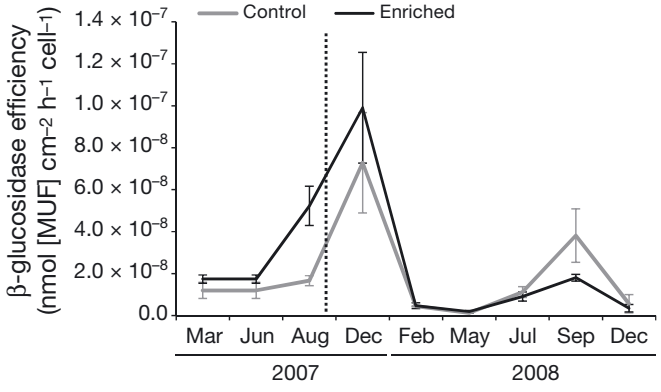

Fig. 5. β-glucosidase efficiency (further explanations see, 'Materials and methods; Extracellular enzyme activities' in the control and enriched reaches of the La Choza stream during the study period. Means (±SD, n = 3) are shown. Dotted line marks the beginning of the fertilization period

With regards to enzymatic activity, the β-GLU in the period *before* fertilization was higher in the E reach (1.63 ± 0.60 nmol [MUF] cm^{-2} h^{-1} in the C reach and 2.97 ± 2.30 nmol [MUF] cm^{-2} h^{-1} in the E reach). After fertilization had begun, the concentration of β-GLU remained higher in the E reach at first, but by the end of the sampling period its activity was higher in the C reach (Table 2).

Phosphatase, on the other hand, was lower in the C reach in the period *before* fertilization (2.50 ± 0.92 nmol [MUF] cm^{-2} h^{-1} in the C reach and 6.02 ± 5.64 nmol [MUF] cm^{-2} h^{-1} in the E reach). During the fertilization period, the average concentration of phosphatase increased in the C reach and decreased in the E reach (7.45 ± 8.48 and 5.07 ± 2.56 nmol [MUF] cm^{-2} h^{-1}, respectively). The significant differences found in the phosphatase concentration were a result of the increase in the C reach after fertilization had begun (Table 2).

Table 2. Results obtained for the BACIPS ANOVA for biological variables and their partial η^2 values. Significant interaction values ($p < 0.05$) are marked in **bold**. Control–enriched (CE), before–after (BA) and time (TIME) sources of variation are considered. MUF: methylumbelliferyl

	CE	BA	TIME(BA)	CE × BA	CE × TIME(BA)
Bacterial density (cell cm^{-2})					
p	0.369	0.664	0.017	0.210	**0.000**
Partial η^2	0.054	0.013	0.759	0.103	0.795
Chlorophyll *a* (μg cm^{-2})					
p	0.896	0.983	0.000	**0.003**	0.546
Partial η^2	0.001	0.000	0.961	0.469	0.167
β-glucosidase (nmol [MUF] cm^{-2} h^{-1})					
p	0.189	0.691	0.007	0.356	**0.002**
Partial η^2	0.234	0.024	0.886	0.124	0.282
Phosphatase (nmol [MUF] cm^{-2} h^{-1})					
p	0.309	0.398	0.191	0.251	**0.000**
Partial η^2	0.148	0.104	0.666	0.184	0.556
β-glucosidase efficiency (nmol [MUF] cm^{-2} h^{-1} $cell^{-1}$)					
p	0.202	0.184	0.187	0.184	**0.000**
Partial η^2	0.220	0.237	0.669	0.237	0.863

When analyzing the β-GLU efficiency, the C reach had lower values in the period *before* fertilization ($2.5 \times 10^{-6} \pm 3.9 \times 10^{-6}$ nmol [MUF] cm^{-2} h^{-1} $cell^{-1}$ in the C reach and $1.5 \times 10^{-5} \pm 2.1 \times 10^{-5}$ nmol [MUF] cm^{-2} h^{-1} $cell^{-1}$ in the E reach). In the period *after* fertilization, the efficiency of the enzyme decreased in both reaches, and the differences between the reaches diminished (Table 2).

The relationship among the biological variables showed significant positive correlations ($p < 0.05$) between the activities of phosphatase and β-GLU.

Table 3. Pearson's product moment correlation values obtained between biological variables (BACT: bacterial biomass; CHL *a*: chlorophyll *a*; β-GLU: β-glucosidase; PHO: phosphatase) and the physical–chemical variables (COND: conductivity; TEMP: temperature; DO: dissolved oxygen; SRP: soluble reactive phosphorus; DIN: dissolved inorganic nitrogen; PAR: photosynthetically active radiation). Significant correlations ($p < 0.05$) are marked in **bold**

	BACT	CHL *a*	β-GLU	PHO	COND	TEMP	DO	pH	SRP	DIN	PAR
BACT	–										
CHL *a*	−0.26	–									
β-GLU	−0.08	**0.78**	–								
PHO	−0.20	**0.55**	**0.62**	–							
COND	0.40	0.33	0.30	0.47	–						
TEMP	0.39	0.08	0.25	−0.16	−0.02	–					
DO	0.03	**0.52**	0.40	0.22	0.30	−0.45	–				
pH	**0.52**	−0.06	−0.03	−0.08	**0.62**	0.14	0.12	–			
SRP	**0.65**	0.12	0.18	0.16	0.46	0.40	−0.03	0.18	–		
DIN	−0.41	0.31	0.27	0.28	0.39	−0.07	0.08	−0.09	−0.05	–	
PAR	0.19	0.33	0.44	−0.04	−0.01	**0.94**	−0.31	0.01	0.31	0.08	–

Also, the activities of both enzymes were significantly correlated to the chl *a* content; at the same time, the bacterial biomass was correlated to the SRP concentration (Table 3).

DISCUSSION

The La Choza stream is a characteristic fluvial ecosystem in the Pampean grassland. These streams lack riparian vegetation, have a very low slope, and a high nutrient content. The metabolism in the stream is mainly fueled by autochthonous primary production developing in either the epipelon, plankton, or macrophytes (Feijoó et al. 1999, Acuña et al. 2011). These characteristics affected the nutrient addition experiment itself and the delayed and buffered response of the epipelic biofilm microbial biomass and heterotrophic metabolism.

The addition of phosphorus and nitrogen to the La Choza stream caused the SRP concentration to be significantly higher in the E reach, as expected, but this was not observed for DIN. The dissolved nitrogen that was added to the stream may have either been rapidly assimilated by algae, macrophytes, or bacteria, or may have left the system by denitrification processes caused by anaerobic bacteria in anoxic conditions. Assimilation of nitrogen by the biological communities may have taken place as the low DIN:SRP ratios (3.2 to 5.6) suggested nitrogen limitation (Stelzer & Lamberti 2001). However, relevant denitrification was also suggested as anoxic conditions were measured both in the sediment and in the water at night (Acuña et al. 2011), which would indicate a plausible scenario for denitrification processes taking place. Even when subsurface sediments are well-oxygenated and have a relatively low nitrogen concentration, the denitrification potential can be substantial, as has been observed in a desert stream (Holmes et al. 1996).

Even with the high nutrient contents of the La Choza stream, the addition of nutrients led to a delayed but significant increase in algal biomass at the epipelon, which showed a first peak in summer (2 to 3 mo after fertilization began) and a second and more significant peak in spring (about 10 mo after fertilization began). When analyzing the effects of both light and nutrients on the periphytic community, Schiller et al. (2007) concluded that light was the main factor affecting algal biomass in a Mediterranean stream, and that an increase in nutrient availability did not enhance algal biomass accrual, at either low or high light intensities. However, our study suggests that in a stream where light availability is not a limiting factor, a rise in nutrient concentration still increases the algal biomass significantly. A positive interaction between nutrient and light availability on primary producers was also described in Ylla et al. (2007) for streambed algae and mosses. In autotrophic biofilms, the relationship between primary producers and potential phosphorus limitation can be deduced from changes in phosphatase activity, since algae are highly responsible for the variations of this activity. In this experiment, phosphatase activity was maintained at values between 1.67 and 26.50 nmol [MUF] cm^{-2} h^{-1}, with >90% of all values <10 nmol [MUF] cm^{-2} h^{-1}. When compared to the activity measurements in other studies, these values are within the lower range for river biofilms (Hill et al. 2010, 2012, Romaní et al. 2012). In the experiments conducted by Rier et al. (2007), for instance, such low values were only measured in biofilms at low light intensities.

Although a decrease in phosphatase activity would be expected in the E reach, due to greater phosphorus availability (Romaní et al. 2004a, Hill et al. 2006), this was not observed in our research. It could be that the phosphatase activities measured were basal activities uninhibited by enrichment. However, in the C reach, a peak of phosphatase increase was measured in spring, coinciding with the increase in algal biomass which could indicate phosphorus demand during algal development. This demand probably did not occur in the E reach, since sufficient inorganic phosphorus for algal growth was being provided.

The addition of nutrients in the La Choza epipelon caused bacteria to have a delayed effect, which did not show their largest effect until spring (about 10 mo after fertilization began). The bacterial response seems to be highly linked to the autotrophic response, since the bacterial biomass was slightly increasing after the first peak of algal biomass (summer) and more significantly after the second peak of algal biomass (in spring, 10 mo after fertilization began). In contrast, in a Mediterranean low-order stream, long-term fertilization caused a first and immediate response of bacteria, independent of any increase in algal biomass (Sabater et al. 2011), which would be expected in a detritus-base stream (Chadwick & Huryn 2003). The increases in bacterial biomass in the La Choza epipelon, which always occurred after a chlorophyll peak, suggest that bacteria in this microhabitat are mainly using autotrophic organic compounds. The bacterial metabolism of the epipelon of such a low water velocity and open prairie stream is more similar to a typical epilithic biofilm — highly linked to algal biomass and

activity — than to sediment biofilm in which microbial heterotrophs efficiently use allochthonous-available organic matter (Romaní & Sabater 2001). Several studies of epilithic biofilms concluded that algal exudates are a major carbon source for bacteria (such as Espeland et al. 2001, Romaní et al. 2004b, Francoeur et al. 2006, Rier et al. 2007), and Romaní & Sabater (1999) concluded that the algal accumulation in an epilithic biofilm influences the use of organic matter by increasing the amount of organic substrate available for bacteria, although it confers a slower response to the microbial community in relation to its own accrual. This is consistent with our findings, which show that algal biomass significantly increases with the addition of nutrients and that bacterial biomass also increases, but in a more gradual manner.

Stimulating effects of algal biomass on heterotrophic activity in epilithic biofilms have indeed been reported in several studies (Murray et al. 1986, Chappell & Goulder 1995, Romaní & Sabater 1999). Carr et al. (2005), using path analysis, rejected the idea that bacteria in biofilms compete with algae for nutrients and suggested instead that bacteria and algae in biofilms coexist in an association that offers space and resources to sustain the production of both groups of organisms.

Despite the positive correlation found between β-GLU and the algal biomass, their variations were not as tightly coupled as in other studies (Somville 1984, Jones & Lock 1993), which concluded that the polysaccharides released from the autotrophic component of the biofilm enhanced the activity of the enzyme.

β-GLU and phosphatase values have not been previously reported for the sediments of Pampean streams, but are relatively low when compared to other streams (Romaní et al. 2004a, Rulík & Spá il 2004). Other studies show that phosphatase concentrations are inversely correlated with water and sediment P concentrations (Findlay et al. 2001, Harbott & Grace 2005, Sinsabaugh et al. 2010, Hill et al. 2012), and Hill et al. (2012) concluded that nutrient-poor runoff from forested catchments stimulates extracellular enzyme production for the acquisition of N and P. Hence, in a phosphorus-rich stream such as La Choza, it is consistent that the extracellular enzyme concentrations would be lower than in other streams with lower nutrient concentrations.

We hypothesized that the β-GLU would increase with the addition of nutrients, since the stoichiometrical C:N:P balance would be disrupted when both nitrogen and phosphorus were added, which would result in carbon deficiency for bacteria. However, β-GLU only increased at the end of the experiment and after the increase in algal biomass, in both C and E reaches. Epipelic β-GLU activity seems to be more regulated by substrate availability than by any nutrient imbalance. However, variation in β-GLU efficiency with the addition of nutrients translates into a weaker need for the bacterial cells to produce the enzyme, which is consistent with an increase in the algal biomass in the E reach which serves them with a labile carbon source. This is consistent with previous findings (Romaní & Sabater 2000) reporting that the efficiency of the biofilms in producing enzymes was lower at sites with higher nutrient concentrations.

Overall, the response of the epipelon in such a high-nutrient Pampean stream produces a slow and delayed effect on the increase of algal biomass, which might determine a delayed effect on the increase of bacterial densities. These changes might be responsible for the slight but significant decrease in dissolved oxygen and pH in the E reach due to enhanced respiration activity. However, it is noteable that the effects on the heterotrophic use of organic matter were punctual and appeared only when changes in microbial biomass were recorded.

According to Artigas et al. (2013) the effect of nutrient enrichment may be less predictable in systems that have high basal nutrient concentrations, such as naturally enriched Pampean streams, which could be close to saturation. Although changes are slow, should some of these changes become chronic, they would affect the functioning and services of the entire stream ecosystem.

Although nutrient enrichment of aquatic systems due to the increase in runoff from cultivated lands is a major concern, the effects that other variables related to runoff might have on the biofilm are also of interest, since they might alter the way these communities respond to enrichment. Manipulative studies may provide insight into what impact other variables, such as the concentration of suspended solids or water velocity, might have on epipelic biofilms. A combination of both *in situ* and *ex situ* approaches will facilitate a more accurate understanding of the factors that determine the development and dynamics of the communities in biofilms.

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