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Periphyton Responses to non-point Pollution in Eutrophic-Humic Environments: An Experimental Study

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ABSTRACT: We proposed to use artificial channels in laboratory assays to expose periphyton to substances released in rural environments in order to test the following hypotheses 1) a high concentration of humic substances decreases the biofilm biomass and alters its metabolism; 2) periphyton biomass and metabolism increase in response to nutrient addition in spite of the previous high nutrient concentration; 3) periphyton response to nutrient addition is smaller in the presence of humic acids. Nutrient loading associated with non-point pollution often occurs both during and after signiûcant precipitation events. Humic acids also increase their levels after rain. This may limit the availability of light and thus, the development of the autotrophic community. However, the influence of these pollutants on periphyton in eutrophic environments may be either modest or too difficult to detect using traditional endpoints. We found that in short exposures: 1) humic substances do not decrease the biomass of periphyton nor alter its metabolism; 2) periphyton biomass and metabolism increase in response to the addition of fertilizer but not to the addition of a single nutrient; 3) periphyton response to nutrient addition is smaller in the presence of humic acids. These finding have implications for river ecosystems as they suggest that changes produced by nutrient inputs into the eutrophic stream could be fast and clearly affect periphyton algae and other related organisms such as grazers and decomposers. In addition, the presence of humic acids decreases these responses.

Key words: Non-point pollution, Eutrophic environment, Nutrient, Humic acid, Artificial channels, Algae

INTRODUCTION

In the last 50 years, agriculture in the Pampean region of Argentina has expanded and produced modifications of natural landscapes and large areas of pasture with little tillage. The use of fertilizers and pesticides has increased and thus the need to study their impact (Viglizzo *et al*., 2011). This kind of exploitation generates various types of pollutants, whose impact is difficult to assess because they constitute non-point source pollution. Across the world, intensive agricultural practices and outdoor cattle farming are generally regarded as high risk for phosphorus and nitrogen losses to rivers, because soils are either regularly over-fertilized, recycle large amounts of manure, or are highly vulnerable to soil erosion (Jarvie *et al*., 2010).

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Periphyton is a relevant indicator of perturbation because of its major functional role in the lotic environments particularly those of Pampean biome (Vilches & Giorgi, 2010) and could help to make predictions of the effects on the whole ecosystem (Sabater *et al*., 2007). In addition, the periphyton community is especially responsive to non-point pollution and, as a consequence, can be used to help establish nutrient criteria. However, the influence of these pollutants on periphyton may be either modest or too difficult to detect using traditional endpoints (Steinman *et al*., 2011).

Numerous studies have examined the relationship between periphyton and nutrients. In this sense, Francoeur (2001) established that the co-limitation of periphyton by nitrogen and phosphorus is more

common than its limitation by a single nutrient. Periphyton biomass (chlorophyll-*a*) is also expected to increase when nutrients increase (Chessman *et al*., 1992; Delong & Bruswen, 1992). Abiotic factors, such as irradiance, can influence the degree to which nutrients affect periphyton (Biggs, 1996). Also, increased nutrient loading from agricultural land uses results in differences in community composition and metabolism (Allan, 2004). Nutrient loading associated with non-point pollution often occurs during and after signiûcant precipitation events and its effect usually occurs throughout the entire watershed and not in a localized area. Nutrient delivery associated with stormwater runoff is usually an immediate nutrient concentration spike associated with the ûrst ûush, followed by an exponential decline in concentration (Jarvie *et al*., 2008). On the other hand, the potentially stimulatory effect of increased nutrients on periphyton may be offset by the scour associated with increased ûow (Boisson & Perrodin, 2006). Humic substances may constitute up to 60% of the organic matter dissolved in water. These substances may come from runoff of soils adjacent to water bodies and are characterized by lack of biodegradability. The Argialbol soils, and especially the Natraqualf soils, that develop near Pampean streams are characterized by little polymerized organic matter, which results in a significant loss of humic substances through flood water and/or runoff (Taboada *et al*., 1987). The concentration of humic acids increases in the water bodies after the rains by the drag from the surrounding fields (Serrano, 1992) and decreases in dry periods by the photo-oxidation of organic matter (Serrano, 1994). Steinberg (2003) asserts that humic substances modify the underwater light climate and consequently change the living conditions for autotrophic organisms. This lower light availability has important consequences for production and community composition, and thus affects photochemical processes, nutrient uptake and growth of periphyton (Julian *et al*., 2008). Previous studies in Pampean streams have shown eutrophic characteristics in their waters and that the community development is heterotrophic at sites where there are also high concentrations of humic acids (Vilches, 2012). Consequently, it is possible that high concentrations of humic substances limit the availability of light and thus, the development of the autotrophic community. Therefore, we proposed to use artificial channels in laboratory assays to expose periphyton to the action of these substances in order to test the following hypotheses: 1) a high concentration of humic substances decreases the biofilm biomass and alters its metabolism; 2) periphyton biomass and metabolism increase in response to nutrient addition in spite of the previous high nutrient concentration; 3) periphyton response to nutrient addition is smaller in the presence of humic acids.

MATERIAL & METHODS

Two independent experiments were conducted with periphyton exposed to substances produced by rural activities. Each experiment was performed using twelve recirculating Perspex channels (90 cm long x 10 cm wide) (Fig. 1). Each channel unit was affixed with a Perspex piece to keep the water column height at 8 cm. Water input at the head of the channel unit was provided by a pump connected with silicone tubes (Atman® AT-301). The system was supplied with water from La Choza stream (an eutrophic Pampean stream situated in Buenos Aires province, Argentina; Vilches *et al*., 2011); the water was filtered through a plankton net (50 µ pore) to eliminate macrophyte pieces, aquatic larvae and gross particles. Water in the channels was renewed twice a week to avoid nutrient depletion during the colonization period. General conditions of the experiments were set at a temperature of 20.1 ± 0.1 °C, pH of 8.5 ± 0.1, conductivity of 1662.2 ± 15 µS cm^{-1}, and dissolved oxygen of 8.5 ± 0.3 mg L^{-1} (n = 48). Fluorescent tubes were used to provide natural light between 450-660 nm at an intensity of 80 µE m^{-2} s^{-1} following a 12 h/12 h light/dark cycle. Fifteen sandblasted glass substrates (10 x 4 cm) were placed vertically at the bottom of each channel and parallel to the flow direction. Biofilm colonization was achieved by introducing twice a week during the first three weeks of colonization (total time of colonization: 45 days) aliquots of a scrapped and resuspended natural periphyton community obtained from La Choza stream macrophytes.

After colonization, four different conditions were assessed for their respective effects on biofilm structure and function: a) control (stream water); b) simulation of increased concentration of humic acids (stream water with a concentration of 50 ppm sodium salt of humic acid, Aldrich ®); c) simulation of increased nutrients (stream water with high nutrient concentration); and d) simulation of increased concentration of humic acids + increased nutrients. Biofilms were exposed to each of the four conditions for a week in each experiment. The water was not renewed during exposure as it tried to simulate a pulse of non-point pollution, as it would be in a rain event. The two experiments carried out with this design were as follows: *Experiment 1)* phosphorus was used as the only nutrient in the simulation of increased nutrients (0.5 mgP-PO_4^{-3} L^{-1}). *Experiment 2)* a commercial liquid fertilizer (Nitrofoska ®) was used in the simulation of increased nutrients. This fertilizer contains 10% total nitrogen, 2% assimilable phosphorus, 6% of water

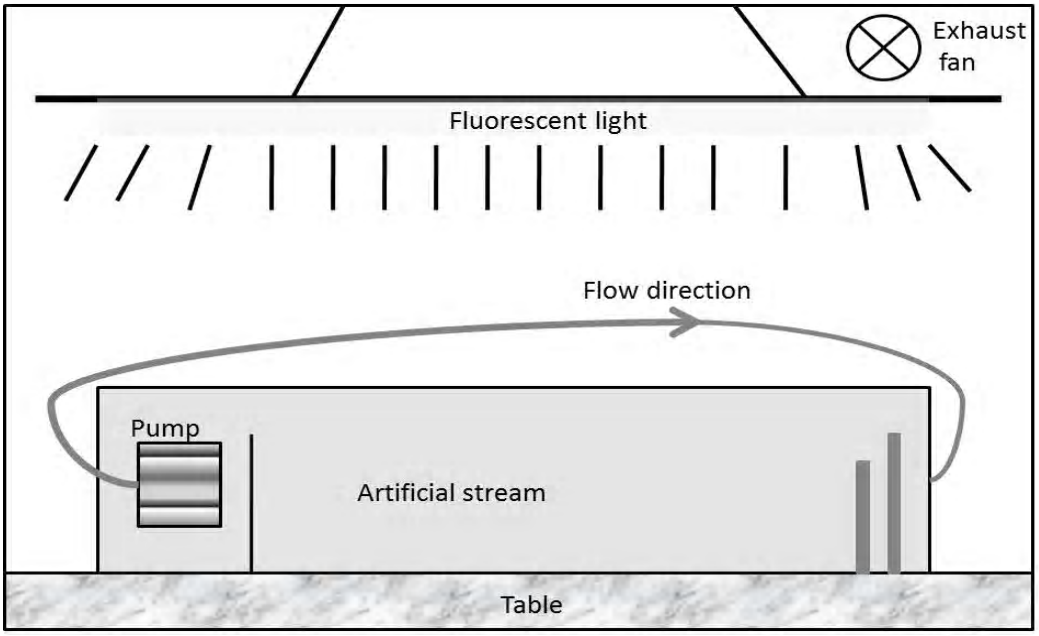

Fig. 1. Diagram of artificial stream system

soluble potassium and 0.31% magnesium as main compounds. The concentration selected was: 5 ml fertilizer L^{-1}, which is similar to the concentration of phosphorus and nitrogen in the basin after rainfall (Vilches *et al*., 2011). Each of the four established conditions was tested in triplicate (three independent channels per treatment).

Biofilms were sampled before (t_0) the establishment of the experimental conditions and after one week of exposure (t_f) to ascertain its structural and functional responses to each condition as well as to any possible combined effect. On each sampling day, seven colonized glass substrates were randomly collected from each channel and analyzed for biofilm biomass (ash free dry mass, chlorophyll-*a*), total polysaccharides, total nitrogen, total phosphorus, metabolism (net production, respiration), and extracellular-enzyme activity.

The water concentration of humic acids and nutrients was also estimated at the beginning of the experimental conditions (t_0) and after one week of exposure (t_f). Samples were collected in each channel and immediately filtered through glass fiber filters (pore size = 0.7 mm; Whatman GF/F filters, Maidstone, UK). Humic acids concentration was estimated according to Lavado *et al*. (1982). Soluble reactive phosphorus (SRP) was measured with the ascorbic acid method, nitrites and nitrates by reaction with sulfanilamide (with a previous Cd reduction in the case of nitrate), and ammonium with the phenol-hypochlorite method. All nutrients concentrations were measured with a Hitachi U-2001 spectrophotometer (Hitachi Ltd, Tokyo, Japan). Analyses were conducted according to APHA (2005). The following physicochemical parameters of the water were measured on each sampling time: dissolved oxygen (with a handheld HQ40d oxygen meter; HACH Company, Loveland, Colorado), conductivity, temperature and pH (with Portable Hanna instruments; Woonsocket, USA). In addition, the organic and inorganic particulate matter concentration were estimated by drying, chlorides with the silver nitrate method, chemical oxygen demand (COD) with an SQ 118 Spectroquant® kit (Merck) and biological oxygen demand after 5 days incubation at 20°C (BOD_5).

With the aim of assess structural parameters of biofilm, one colonized glass substrate (40 cm^2, for each determination) was collected from each channel. The algal film was removed by scraping the surface with a soft bristle brush and immediately filtered through glass fiber filters and stored at -20 °C in order to measure biofilm biomass (ash free dry weight (AFDW) and chlorophyll-*a*). Total biomass was measured as dry weight (DW) at 60°C for 48 h and AFDW as the difference in weight between the DW and the weight combusted at 450 °C for 4 h. For chlorophyll-*a* analysis, a 50-ml aliquot was filtered using Whatman GF/F glass fiber filters and immersed in 90% acetone for 24 h in the dark at 4°C. The extract was read with a spectrophotometer and chlorophyll-*a* concentration was obtained according to APHA (2005). One colonized glass substrate from each channel for each

determination was stored at -20 ºC in order to measure nitrogen, phosphorus, and polysaccharides in the biofilm. Total nitrogen was estimated by Kjeldahl method (APHA, 2005), total phosphorus by the peroxydisulfate digestion method (APHA, 2005) and total polysaccharides by phenol/sulfuric acid (DuBois *et al*., 1956).

The following functional parameters were evaluated on periphyton on the same sampling day: metabolism (production and respiration) (Bott *et al*., 1997) and activity of exoenzymes (β-glucosidase, alkaline phosphatase and cellobiohydrolase) (Romaní & Marxen, 2002). Oxygen production and consumption were measured in the laboratory putting colonized glass substrate into transparent, plastic and hermetic boxes at constant temperature (20 °C). Production was determined by the variation in oxygen concentration in the boxes after 1 h of incubation in the light, whereas respiration was determined by the variation in oxygen concentration after 2 h of incubation in the dark. Dissolved oxygen levels in each box were measured with an oxygen meter. To quantify β-glucosidase (EC 3.2.1.21), cellobiohydrolase (EC 3.2.1.91) and alkaline phosphatase (EC 3.1.3.1–2), exoenzymatic activities were measured using fluorochrome-linked substrates (methylumbelliferyl (MUF)). Biofilm from the glass substrates was sonicated during three 3-min sessions separated by 1-min intervals, in a glass container with 20 mL of tap water. Samples at a final concentration of 300 mmol/L of MUF (saturation concentration determined for these communities), MUF calibration solutions (0–100 µmol/L) and water controls were incubated in a shaker in the dark for 1 h in a water bath adjusted to the stream water temperature. After incubation, enzymatic cleavage was inhibited by adding 5 mL of 0.05 M glycine buffer (pH 10.4). Activity was determined by fluorescence measurement at 365/455 nm (excitation/emission for MUF). Exoenzymatic activities were expressed as nmol MUF/h.

To study the effects of increased humic acids and increased nutrients on periphyton, the mean of each biological variable measured for each channel on each sampling date was used as an independent replicate. This dataset included the extracellular enzymatic activities (alkaline phosphatase, cellobiohydrolase, β-glucosidase), biomass (DW, AFDW, chlorophyll-*a*), content of biofilm (total nitrogen, total phosphorus, total polysaccharide) and metabolism (net production, respiration). A single matrix for each experiment was used for statistical treatment. This matrix was obtained by the datum-to-datum difference of the final value minus the initial one. Thus, possible variations within each channel and the possible variations in the controls in each experiment were excluded from the analysis. Therefore, the matrix with which we worked was composed of the value of the variation of each channel, and allowed comparing the variation between treatments. Statistical analysis was performed using the statistical package Statistica 6.0 ®. Normality of the variables was checked with the Kolmogorov-Smirnov test, and the variables were transformed when necessary. We used two-way analysis of variance (ANOVA) to test for the effect of simulation of increased concentration of humic acids, simulation of increased nutrients and the combined effects of the two factors. Statistical significance was set at $p < 0.05$.

RESULTS & DISCUSSION

No significant differences were evidenced in the physicochemical parameters between channels during the colonization period ($p > 0.05$; $n = 48$) in the two experiments. Concentrations of SRP and humic acids at t_0 in *experiment 1* were 10 X greater than controls (Vilches *et al*., 2011). Concentrations of SRP and humic acids as well as those of ammonium, nitrite and nitrate were 10 X greater than controls in *experiment 2*.

Biofilm was exposed to four treatments in *experiment 1*: control (C), humic acid increase (H), simulation of increased phosphorus (P) and humic acids + phosphorus (H+P). The manipulated variables (SRP and humic acids) decreased in all treatments after one week. SRP and humic acid decreased significantly compared to the control in the treatments where these parameters were added. The chemical parameters estimated in water (nutrients, chloride, organic and inorganic particulate matter, COD, BOD_5) showed no significant differences.

The simulation of increased phosphorus caused a significant increase ($F=21.3$, $p<0.01$) in the total phosphorus content in the biofilm and a significant decrease ($F=97.1$, $p<0.001$) in alkaline phosphatase activity (APA) (Table 1). Total phosphorus increased significantly after one week of exposure in the channels for treatments P and H + P (Fig. 2a), whereas APA was greater in treatments without added phosphorus and significantly lower in the treatments P and H + P (Fig. 2b). These two variables showed no significant differences between treatments P and H + P.

On the other hand, Biofilm was exposed to four treatments in *experiment 2*: control (C), humic acid increase (H), simulation of input of fertilizer (F) and humic acids + fertilizer (H+F). The concentration of the manipulated variables remained higher than controls at T_f: SRP and nitrates were significantly greater than the control in treatment H + F; nitrite and chloride in treatments F and H + F. Humic acids and ammonium showed no significant differences. The

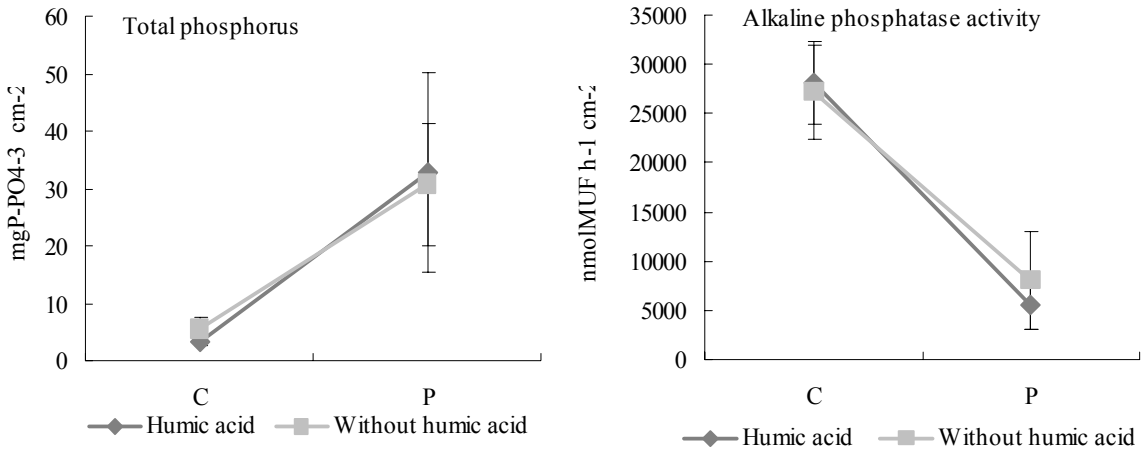

Fig. 2. Means with standard errors (n=3) at final time in a) total phosphorus and b) alkaline phosphatase activity of the biofilm in *experiment 1*. C=control, P= increase in phosphorus

other estimated chemical parameters in water showed no significant differences compared to the control. Total phosphorus ($F=6.6$, $p<0.05$), chlorophyll-*a* ($F=6.4$, $p<0.05$) and net production ($F=7.3$, $p<0.05$) in the biofilm showed significant differences (Table 2). Total phosphorus was significantly increased in treatment F, whereas this increase was not significant in treatment H + F (Fig. 3a). The concentration of chlorophyll-*a* increased in all treatments at t_f (Table 2), but this increase was significantly higher in treatment F (Fig. 3b). The net production was greater after exposure to the fertilizer only in the treatment without addition of humic acids (Fig. 3c).

The hypothesis that a higher concentration of humic substances decreases the biomass of periphyton and alters its metabolism must be rejected. This hypothesis was based on the possible decrease in biomass due to the limitation of available light, among other factors, exerted by humic acids (Julian *et al*., 2008). However, no significant differences were found in terms of periphyton structure and function in treatments with only addition of humic acids in the two experiments. This may be because the periphyton community was obtained from a stream that there are usually humic acid and they increase regularly, probably algae are adapted their photosynthetic process to a lower irradiance. For that reason periphyton could withstand stress periods accumulating nutrients when there is excess and perform the photosynthetic process when is possible or in suboptimal conditions (Graham & Wilcox, 2000). In our experiments, the net production was calculated in chambers outside the artificial channels and all the colonized substrates were exposed to similar conditions of temperature, water and light to detect only the differences produced at the community after one week of treatment. Chlorophyll-*a* could have the highest development in fertilized conditions but in conditions of light restriction the cells also tend to produce more chlorophyll-*a*, (Graham & Wilcox, 2000) so there were no significant differences between treatments, except with the control where any factor promote the increase in chlorophyll-*a*. This situation also contributes to reducing the differences observed in net production estimations.

The concentration of chlorophyll-*a* and net production were significantly higher in the treatment of increased nutrients through fertilizer than in the control and are consistent with that observed by McCormick *et al*. (2001). On the other hand, in *experiment 1*, where the increase in nutrients was only an increase in P, we found no effects on periphyton biomass and metabolism. This absence of significant differences between control and treated communities is consistent with the fact that the periphyton community is not P-limited. This could be attributed to the fact that the community was nutrient-replete and further addition of a single nutrient may not stimulate periphyton growth (Steinman *et al*., 2011). However, total phosphorus increments in periphyton of both experiment was observed. This suggests that there is a luxury consumption of phosphorus by algae but not growth (Feijoó *et al*., 2011; Payne *et al*., 1988). We also measured the alkaline phosphatase activity (APA) as an indicator of phosphorus limitation (Bothwell, 1985; Labry *et al*., 2005). *Experiment 1* (only phosphorus) showed lower APA in the treatments with addition of phosphorus and greater APA in the other treatments. No significant differences were found in *experiment 2* (fertilizer). These results agree with those by Newman

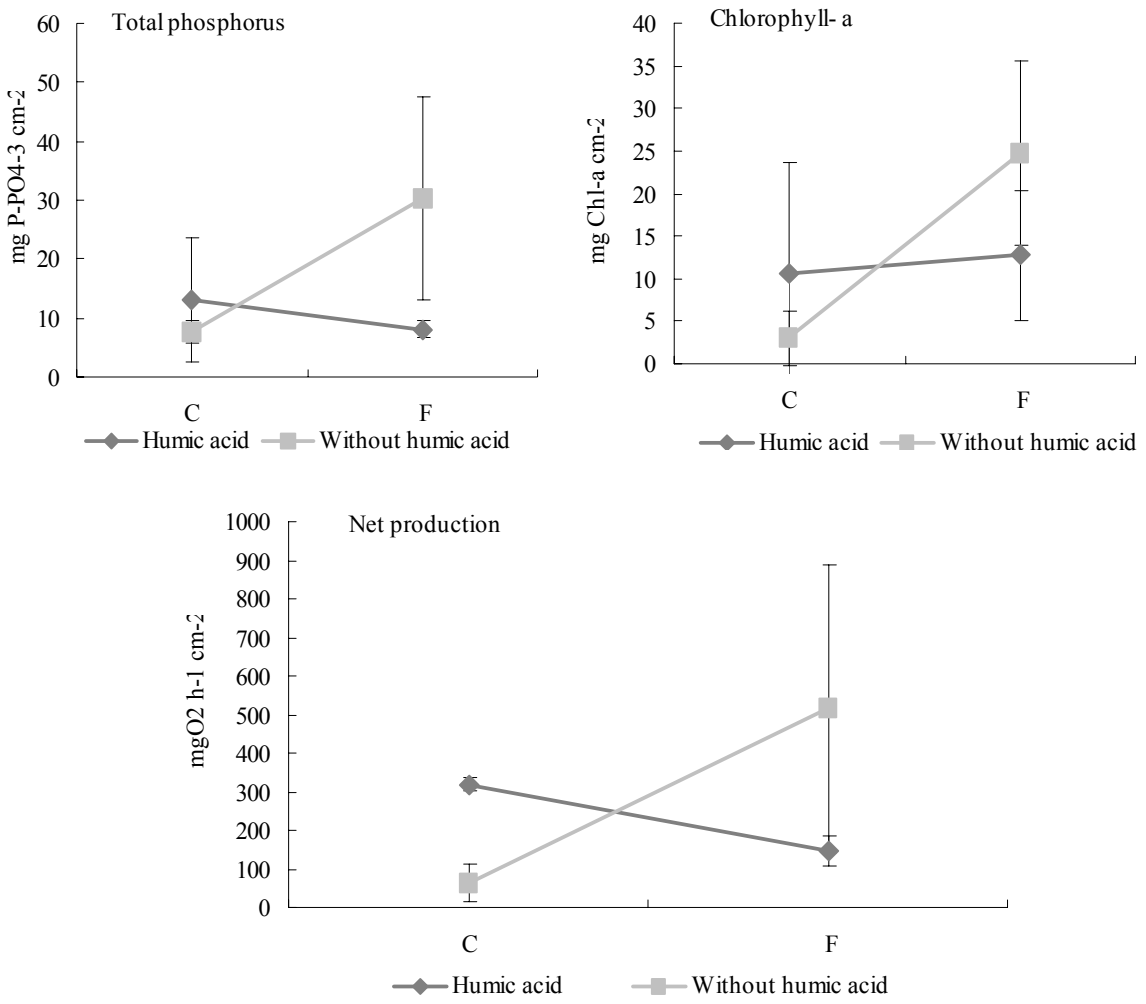

Fig. 3. Means with standard errors (n=3) at final time in a) the total phosphorus, b) chlorophyll-*a* and c) net production of the biofilm in *experiment 2*. C=control, F= fertilizer addition

et al. (2003), who suggested that APA would be a useful indicator only in the case that phosphorus is the only parameter changed.

Then, we confirmed our second hypothesis (periphyton biomass and metabolism increase in response to nutrient addition in spite of the previous high nutrient concentration) only when several nutrients (fertilizer) are added together. The relevance of our findings resides in the fact that the non-point pollution from runoff of agricultural land is due to fertilizer application and not to a single nutrient. Thus, the effect on periphyton communities in the natural environment would be similar to that recorded in the second experiment.

Natural humic acids of Pampean streams come through runoff as nutrients generated by agricultural activities. Our data, in agreement with previous studies (Blank, 2002; Vinebrooke *et al*., 2004), suggests that an increase of humic acids produce anticipated structural changes. These changes would give an increased tolerance of the periphyton community and may also induce tolerance to other stressor agents. These findings contribute to supporting the third hypothesis. In *Experiment 1*, humic acids did not affect the incorporation of P to algae. However, in *Experiment2*, P uptake in the treatment of the interaction (H + F) was not significant, although phosphorus concentration in periphyton of the fertilizer treatment (F) was significantly higher. In addition, this experiment showed a significant increase in net production in spite of previous high concentration of nutrients, only in the fertilizer treatment but not in the treatment of the interaction (H + F). The nutrient uptake was variable depending on the nutrient type

(phosphorus or fertilizer) but also because of the effect of humic acids.

This could mean that the input of different substances from agricultural activities (humic acids and nutrients) into the water bodies causes effects which would produce changes in periphyton communities. The mixture of different substances could lead to situations in which some of them, such as humic substances, totally or partially inhibit others such as nutrients (by complexing humic-iron-phosphate or by coagulation associated with co-precipitation of complexed metals (Steinberg, 2003)).

CONCLUSION

This study demonstrates that the changes produced by the nutrient input into the hypothetical eutrophic stream could be fast and clearly affect the periphyton community. Periphyton biomass and metabolism increase in response to nutrient addition, but only when several nutrients are added together because periphyton community is not P-limited. On the other hand, contrary to our hypothesis higher concentration of humic acid no decreases the biomass of periphyton and no alters its metabolism. However, an increase of humic substances produce anticipated structural change by major tolerance of the perifiton community and this change may also induce tolerance to other stressor agents like nutrients. Then, the mixture of substances generated by agriculture could generate different situations as that humic acids could inhibit fully or partially the uptake of nutrients by algae, affecting the efficiency of this community in debugging of streams.

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