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Nutrient and enzymatic adaptations of stream biofilms to changes in nitrogen and phosphorus supply

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ABSTRACT: We evaluated the effect of high and low nutrient nitrogen (N) and phosphorus (P) concentrations (Hn and Ln, respectively) and N:P supply (16:1 and 56:1, respectively) on the structure and activity of stream biofilms. Natural biofilms were exposed to 4 different nutrient conditions (16:1-Hn, 16:1-Ln, 56:1-Hn, 56:1-Ln) in a microcosm experiment in the laboratory over 35 d. Biofilm bacterial density and chlorophyll *a* (chl *a*) concentration decreased under lower N and P concentrations. Bacterial density was further sensitive to nutrient imbalance and decreased with decreasing P availability, while chl *a* concentration was not affected by P reduction. Greater P requirements and weaker ability to store large quantities of P in bacteria compared to algae may explain these differences. Biofilm responses to imbalanced N:P (56:1) were only observed under Hn conditions, and were expressed as an increase in the proportion of algal carbon with respect to bacterial carbon, and greater N and extracellular polymeric substance (EPS)-polysaccharide accumulation. Algae withstood N:P imbalance better than bacteria under Hn conditions, but this trend decreased at low water nutrient concentration (56:1-Ln condition). The cellobiohydrolase: phosphatase enzyme activity ratio was negatively correlated to the biofilm C:P molar ratio, evidencing the tight link between nutrient acquisition and storage in biofilms. Our experiment highlighted the rapid adaptation (3 to 28 d) of biofilm nutrient content and enzyme activities to changes in water nutrient availability.

KEY WORDS: Ecoenzymatic stoichiometry · Algae · Bacteria · N:P imbalance · Nutrient limitation

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INTRODUCTION

Nutrient dynamics in streams are regulated by nutrient exports between upland and riparian ecosystems, and by biogeochemical processes occurring in-stream (Mulholland 1992). Hydrology, soil characteristics, microorganisms, vegetation, and nutrient forms have the potential to regulate nutrient exports from terrestrial to aquatic ecosystems (McDowell & Wilcock 2004, Belnap et al. 2005). Nutrients vary seasonally, and larger variation ranges typically occur in streams in Mediterranean regions. The specificity of hydrological conditions of intermittent streams confers a characteristic temporal pattern in stream nutrient dynamics (Bernal et al. 2013). It has been described that more than half of the variance in nitrate and dissolved organic carbon (DOC) export in these systems takes place in the late summer to autumn period and is tightly linked to storm events and the antecedent soil moisture conditions (Bernal et al. 2002). Similarly, in rivers in desert regions, positive correlations between soluble reactive phosphorus (SRP), NO_3^-:SRP, and NO_3^-:NH^{4+} ratios, and rainfall have been observed (Harms & Grimm 2010). Varia-

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tions in nutrient export can also modify the ratios between dissolved inorganic nitrogen (DIN) and SRP, being much greater in intermittent (ranging from 11–2853) than in perennial (ranging from 6–171) Mediterranean streams (Von Schiller et al. 2008). It is also true that inter-annual nutrient-discharge relationships in Mediterranean stream watersheds are rather unpredictable (Butturini et al. 2008). Together, these variations in nutrient export highly modify nutrient availability for stream microbial communities in Mediterranean streams which face changes in both water nutrient concentration and N:P supply.

Stream biofilms associated with submerged rocky substrata are mainly composed of algal, bacterial, and cyanobacterial communities with the potential to uptake nutrients directly from water, but also to mineralize organic matter via extracellular enzyme activities (Romaní et al. 2013). Effects of nutrients in stream biofilm communities have been documented in the literature (Tank & Dodds 2003, Romaní et al. 2004, Artigas et al. 2013, Ribot et al. 2013). Total N and P water concentrations explained up to 40% of the variance of algal biomass in biofilms from US rivers (Dodds et al. 2002). Carr et al. (2005) estimated that NO_2^- + NO_3^- concentrations were the best predictors for chlorophyll variability in river biofilms. In the case of bacteria, DIN and SRP explained 10 to 20% of the variance of bacterial biomass in biofilms from Mediterranean, Andean, and Pampean streams (Artigas et al. 2013). Even though differential nutrient limitation between algal and bacterial communities in biofilms may exist (Lang et al. 2012), few studies have determined the combined effects of N and P concentrations and N:P supply on stream biofilm communities (Stelzer & Lamberti 2001, Luttenton & Lowe 2006). Some evidence exists that specific N:P ratios produce effects on algal communities (cell biovolume, community structure and composition) which slightly diverge from those produced by nutrient concentrations (Stelzer & Lamberti 2001).

The ecological stoichiometry theory (Sterner & Elser 2002) has permitted us to better comprehend the stoichiometric relationships between consumers and resources in aquatic food webs, but also between water N:P supply and primary producer stoichiometry (Hall et al. 2005). More specifically, the ecoenzymatic stoichiometry connects the elemental stoichiometry of microbial biomass to microbial nutrient acquisition capabilities (extracellular enzyme activities) and growth (Sinsabaugh & Follstad Shah 2012). In particular, the comparison between enzyme activity ratios (glycosidases:peptidases:phosphatases) and molar stoichiometry of C, N, and P has allowed researchers to determine the extent of nutrient limitation in biofilm and sediment microbial communities from US streams and rivers (Hill et al. 2012). In that study, the low scores of the peptidase:phosphatase ratio in biofilms indicated a strong allocation of microbial phosphatase activity. Romaní et al. (2013) showed that enzyme activity ratios in biofilms from Mediterranean rivers can drastically change between seasons and benthic substrata. During the dry period, C and P breakdown activities are maintained, but the degradation of N compounds (peptidase activity) sharply decreases (Timoner et al. 2012). Artigas et al. (2008) observed a close relationship between nutrient ratios (C:N and N:P) of water and biofilms in different substrate types (leaves, branches, sand, gravel), and they reported that extracellular enzyme activities were eventually stimulated when this correspondence diverged. It is expected, therefore, that the combined approach of biofilm enzyme activity ratios and biofilm nutrient ratios can shed further light on the complex relationship between stream biofilms (structure and activity) and water nutrient supply under varying N:P ratios.

The present study investigated the effect of contrasting water nutrient concentrations (N and P) and N:P supply in Mediterranean stream biofilms. With this purpose, natural biofilms colonized in an intermittent Mediterranean stream were subjected to high nutrient (Hn) and low nutrient (Ln) conditions at an N:P ratio of 16:1 or 56:1 in laboratory microcosms, resulting in 4 nutrient treatments (16:1-Hn, 16:1-Ln, 56:1-Hn, and 56:1-Ln). The nutrient conditions selected for this experiment reflected the natural nutrient variations observed in undisturbed forested streams. The ratios mimicked those observed in the Fuirosos stream (NE Spain), where the biofilms for the experiment were developed, which were equilibrated during low discharge (N:P of 16:1) and imbalanced (greater N export) during high discharge N:P of 56:1. Stream biofilm responses to nutrients were evaluated on microbial biomass (algae and bacteria), biofilm nutrient content and stoichiometry, content of extracellular polymeric substances (EPS), and extracellular enzyme activities over 35 d. We tested the hypothesis that biofilm communities will be more influenced by nutrient concentration than by nutrient molar ratios, and that effects will be reflected both by the biofilm nutrient content as well as by its nutrient acquisition mechanisms. N:P ratios are expected to influence biofilm nutrient content as well as the relationship between enzyme activities involved in N and P acquisition.

MATERIALS AND METHODS

Biofilm communities

Two-month old biofilm communities were obtained from the Fuirosos stream, a third-order forested Mediterranean stream located in NE Spain (41° 41′ N, 2° 34′ E). Biofilms were grown on sand-blasted glass tiles of 1 cm^2 glued onto 50 × 50 cm slabs (n = 6, with 120 tiles per slab) randomly submerged along a 100 m reach of the Fuirosos. During the 8 wk of biofilm colonization in the field, the average ±SE (from weekly measurements) water temperature was 18.0 ± 1.5°C, light irradiance was 100 ± 45 µmol photons m^{-2} s^{-1}, water discharge was 1.5 ± 2.0 l s^{-1}, and DOC was 2.5 ± 0.6 mg l^{-1}. Inorganic nutrient content during this period was 792 ± 236 µg $N\text{-}NO_3^-$ l^{-1} and 92 ± 38 µg $P\text{-}PO_4^{3-}$ l^{-1}, giving a water N:P molar ratio ranging between 16 and 19. Fig. 1 reflects the intra- and inter-annual variations in water N:P ratio supply at the Fuirosos stream. The stream reach was partly shaded by riparian vegetation mainly composed of *Alnus glutinosa* L. and *Platanus acerifolia* Mill. ex Münch. species. The streambed alternates riffles, composed of rocks and cobbles, and pools with mixed fine and coarse sand covered by fine detritus in the most littoral zone. At the end of the colonization period, biofilms were collected in clean plastic containers, filled with stream water, and transported cold (4°C) to the laboratory.

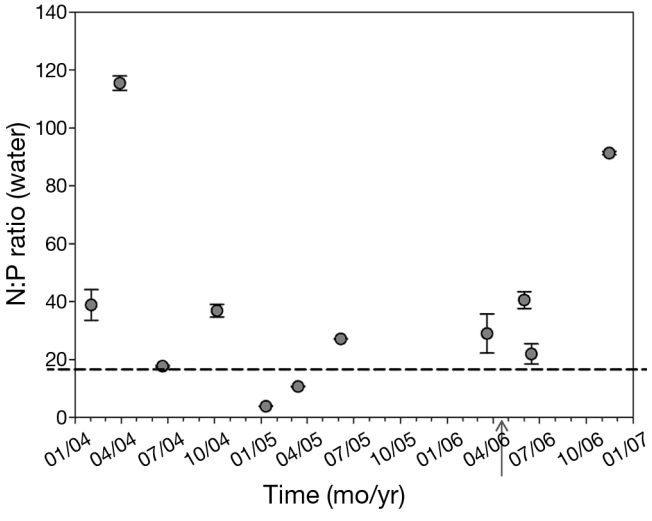

Fig. 1. N:P molar ratios of Fuirosos stream water (dissolved inorganic fraction) at the reach where biofilms were grown. Values (means ± SE, n = 3) correspond to monthly collected water samples during the period 2004 to 2006. N:P = 16 is indicated by the dashed line, while the arrow indicates the beginning of the biofilm colonization period

Microcosm experiment

Biofilms were placed in 12 clean circular glass microcosms (19 cm diameter and 2 l volume; Schott Duran) in the laboratory. Each microcosm contained a total of 60 biofilm-colonized tiles and was equipped with a water recirculating pump (Pico 300, Hydor) set up at 0.1 l s^{-1}. Water temperature and light conditions measured in the field were achieved by enclosing microcosms in an AGP-570 incubator (Radiber) set at 14:10 h day:night cycles (day: 100 µmol photons m^{-2} s^{-1}, 18.0 ± 0.5°C; night: 0 µmol photons m^{-2} s^{-1}, 16.0 ± 0.5°C).

Biofilms were exposed to 4 different nutrient conditions in triplicate: (1) N:P of 16:1 and high nutrient concentration (16:1-Hn; 750 µg l^{-1} $N\text{-}NO_3^-$, 101 µg l^{-1} $P\text{-}PO_4^{3-}$); (2) N:P of 16:1 and low nutrient concentration (16:1-Ln; 75 µg l^{-1} $N\text{-}NO_3^-$, 10.1 µg l^{-1} $P\text{-}PO_4^{3-}$); (3) N:P of 56:1 and high nutrient concentration (56:1-Hn; 750 µg l^{-1} $N\text{-}NO_3^-$, 27 µg l^{-1} $P\text{-}PO_4^{3-}$); and (4) N:P of 56:1 and low nutrient concentration (56:1-Ln; 75 µg l^{-1} $N\text{-}NO_3^-$, 2.7 µg l^{-1} $P\text{-}PO_4^{3-}$). The different nutrient conditions were achieved by using simulated sterile stream water (see Ylla et al. 2009 for details) with some modifications. These modifications consisted of the addition of DOC obtained from leaves collected in the same stream and lixiviated for 48 h, then supplied to each microcosm at a final concentration of 1.5 mg l^{-1}. The above-mentioned N and P concentrations were adjusted using ammonium nitrate (NH_4NO_3) and ammonium phosphate ($NH_4H_2PO_4$) pure salts (Sigma-Aldrich). Nutrient concentrations in each of the 4 nutrient conditions were checked at the beginning (Day 1) and in the middle (Day 21) of the experiment using standard methods (APHA 1989) (data not shown). These results confirmed that nutrient concentrations measured in the microcosms fitted to the nominal nutrient concentrations of the experiment. Every 3–4 d, water from each microcosm was replaced by fresh sterile water at the corresponding nutrient concentrations described above to avoid nutrient depletion and maintain each nutrient treatment condition.

Responses of biofilms to the different nutrient conditions were surveyed on Days 1, 3, 7, 14, 21, 28, and 35. On each sampling date, 8 biofilm samples (tiles) were randomly taken from each microcosm to measure chlorophyll *a* (chl *a*) concentration and bacterial density, biofilm nutrient content, and EPS-polysaccharide content. All samples were stored frozen (−20°C) until analyses, except bacteria, which were fixed with formalin (2%). Extracellular enzyme activ-

ity (EEA) measurements involved in the C (cellobiohydrolase activity), N (leucine-aminopeptidase activity), and P (alkaline phosphatase activity) organic compound decomposition were determined on fresh biofilm samples.

Biomass determination

Algal biomass was estimated from chl *a* concentration. Chlorophyll was extracted from the tiles with 90% acetone over a period of 12 h in the dark at 4°C. Samples were then sonicated (power: 40 W, frequency: 40 KHz; Selecta) for 2 min to ensure a complete chlorophyll extraction. Acetone extracts were filtered through 0.7 µm glass fiber filters (GF/F filters, Whatman International), and chl *a* concentration was determined spectrophotometrically (U-2000 Spectrophotometer; Hitachi) following the method described by Jeffrey & Humphrey (1975). Results are given as µg chl *a* per tile surface area.

Bacterial density was estimated after 2 min of sonicating the biofilm samples (as for chlorophyll). A sonication time of 2 min was considered sufficient to detach most of the attached bacteria without causing cell lysis (Romaní et al. 2004). Biofilm suspensions were diluted 10 times with sterilized water and stained for 5 min with DAPI (final concentration of 2 µg ml^{-1}; Porter & Feig 1980). The stained samples were then filtered through 0.2 µm irgalan black-stained polycarbonate filters (Nucleopore, Whatman International), and filters were mounted on microscope slides. Bacterial cells were counted using a fluorescence microscope (Eclipse 600, Nikon) at 1250× magnification. Fifteen fields were counted per filter, totaling ca. 400 to 600 cells per sample. Results are given as the number of bacterial cells per tile surface area.

The algal C:bacterial C ratio in biofilms was calculated from chl *a* concentration and bacterial density measurements. Algal biomass in C terms was calculated assuming the ratio C:chl *a* = 60 (Geider & MacIntyre 1996), whereas bacterial biomass calculation was based on the conversion factor 2.2×10^{-13} g C μm^{-3} (Bratbak & Dundas 1984) and considering an average bacterial cell biovolume of 0.1 μm^3 (Theil-Nielsen & Sondergaard 1998).

Biofilm nutrient content

C, N, and P content was analyzed in biofilms detached from glass tiles using a sterile cell scraper (Nunc). The biofilm was then suspended in 0.5 ml of de-ionized water and pipetted into pre-weighed tin crucibles. Oven-dried mass samples were assessed after drying at 60°C over a period of 48 h. Dried samples were then analyzed for total C and N content through a CN Elemental Analyzer (Carlo Erba 1500), using vanadium pentoxide as the oxidation catalyzer. The total P content was also determined on oven-dried biofilm samples after basic digestion (NaOH) in an autoclave (110°C for 90 min; Grasshoff et al. 1983). This basic digestion allowed the transformation of organic P from biofilms into inorganic P; total inorganic P was then quantified according to Murphy & Riley (1962). Results were expressed in µg C, N, and P per cm^2 of tile surface area. Calculations of N and P content per unit of biofilm microbial C (algal + bacterial) were also performed.

EPS analysis

EPS extraction was performed on detached biofilms and suspended in 1.5 ml of phosphate buffer (1.7 g l^{-1} KH_2PO_4; 4.5 g l^{-1} $Na_2HPO_4 \cdot 12\ H_2O$; pH 7.0) in Falcon vials (ca. 12 ml). The phosphate buffer was used to counteract the decrease in pH caused by the treatment with the cation exchange resin (DOWEX MARATHON C, Na^+-form, Sigma-Aldrich). Conditioned resin was added to each vial (0.5 g $vial^{-1}$), and EPS was extracted for 1 h in a shaking bath at 4°C and 300 rpm (Frølund et al. 1996). Crude EPS extracts were centrifuged at 12 000 × *g* for 15 min (Sorvall RC 5B Plus) to remove solid parts of the biofilm. Afterwards, the resulting clear EPS extracts concentrated in the supernatant were analyzed for polysaccharide content according to Dubois et al. (1956). The EPS-polysaccharide content determination was based on glucose standards (0–200 µg ml^{-1}). Results were expressed as glucose equivalents per cm^2 of tile surface area.

EEA assays

Cellobiohydrolase (CBH; EC 3.2.1.91), leucine-aminopeptidase (PEP; EC 3.4.11.1), and phosphatase (PHO; EC 3.1.3.1-2) enzyme activities were measured in biofilm samples following the protocols described by Romaní et al. (2004). All EEA assays were performed under substrate saturating conditions, determined from previously performed enzyme activity saturation curves. The CBH assay was conducted at a final substrate concentration of 1.5 mM of 4-methylumbelliferyl-β-D-cellobioside (Sigma-Aldrich

Chemie). PEP and PHO assays were conducted at 0.3 mM (L-leucine-7-amino-4-methylcoumarin hydrochloride and 4-methylumbelliferyl-phosphate, respectively; Sigma-Aldrich). The water used for EEA incubations was that from the microcosms (after being filtered through 0.2 µm Nylon filters), in order to keep water physico-chemical conditions, and especially the experimental nutrient conditions. EEA incubations were performed for 1 h in the dark in a shaking bath set at 18°C (equal to the temperature in the incubator). The activity of CBH and PHO was measured using methylumbelliferyl (MUF) fluorescent-linked substrates, while PEP activity was measured with aminomethyl-coumarin (AMC) fluorescent-linked substrate. EEA on biofilms and water (control measurement) was measured after the addition of 0.05 mol l^{-1} pH 10 glycine buffer (1:1 buffer:sample, by volume). Fluorescence was measured at 365–455 nm (excitation-emission) for MUF substrates and at 364–445 nm for AMC substrates, using a fluorometer (Kontron SFM 25). EEA was calculated as the amount of MUF and/or AMC substrate released per unit of tile surface area and time. The ratios between CBH, PEP, and PHO activities (ln CBH:ln PEP, ln PEP:ln PHO, and ln CBH: ln PHO) were calculated. These respectively accounted for C:N, N:P, and C:P acquisition ratios by the biofilms.

Data analyses

Effects of nutrient conditions on biofilm parameters (data log-transformed to achieve normality and homogeneity of variances) were analyzed by means of 2-way repeated-measures ANOVA. Sources of variation were tested for nutrient concentrations, nutrient ratios, as well as their interaction using the Greenhouse-Geisser statistic. Obtained p-values were adjusted by the number of ANOVA tested conditions using the Dunn-Sidak correction. Differences between the 4 nutrient conditions tested (16:1-Hn; 16:1-Ln, 56:1-Hn, 56:1-Ln) were assessed by Tukey's post-hoc pairwise comparisons ($p < 0.05$). All statistical analyses were performed using SPSS Statistics 17.0.

Relationships between EEA ratios (ln CBH:ln PEP, ln PEP:ln PHO, ln CBH:ln PHO) and nutrient molar ratios (ln C:N, ln N:P, ln C:P) in biofilms subjected to the 4 nutrient conditions were assessed by Pearson's (ρ) correlation analysis. Similarly, Pearson correlation analyses were run between PHO activity and biofilm P content, as well as between chl *a* concentration and bacterial density of biofilms, in the 4 nutrient treatments.

RESULTS

Algal and bacterial biomass

Average chl *a* concentration values (34.4 ± 2.0 µg cm^{-2}) and bacterial densities ($2.5 \pm 0.1 \times 10^8$ cells cm^{-2}) in biofilms were similar in the 4 nutrient treatments at the beginning of the experiment (Day 1, Fig. 2).

Chl *a* concentration significantly decreased under Ln conditions (N-NO_3^- = 75 µg l^{-1} and P-PO_4^{3-} ≤ 10 µg l^{-1}) compared to Hn conditions (N-NO_3^- = 750 µg l^{-1} and P-PO_4^{3-} ≥ 27 µg l^{-1}), but differences were not observed between N:P ratios (*C* effect, Table 1; Tukey's test, Fig. 2). Chl *a* concentration decrease between Days 1 and 35 was 69% for 16:1-Ln, 74%

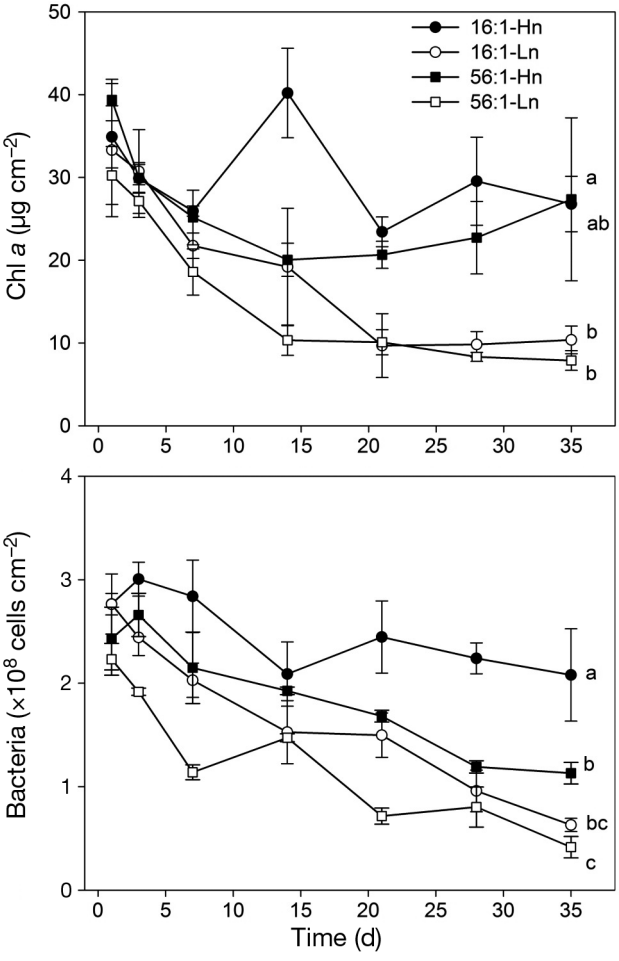

Fig. 2. Evolution of (A) chlorophyll *a* concentration and (B) bacterial density in biofilms subjected to 4 nutrient treatments (Hn: high nutrient and Ln: low nutrient conditions at an N:P ratio of 16:1 or 56:1). Values are means (n = 3) and SE of biomass results on each sampling date. Statistical differences between nutrient treatments for the whole study period are represented by the letters a > b > c (Tukey test, $p < 0.05$)

Table 1. Results of 2-way repeated-measures ANOVA on structure and activity parameters of biofilms subjected to 4 nutrient conditions. Probability values are supplied for the different sources of variation analyzed (nutrient concentration, *C*; N:P ratio, *R*; and their interaction, *C* × *R*) for each parameter. Interactions with time are not represented. Significant probability values are indicated in **bold**. EPS: extracellular polymeric substance

Parameter	Sources of variation	p
Algae	*C*	**<0.05**
	R	0.879
	C × *R*	1
Bacteria	*C*	**<0.001**
	R	**<0.001**
	C × *R*	0.467
Carbon content	*C*	0.064
	R	0.187
	C × *R*	0.336
Nitrogen content	*C*	0.069
	R	**<0.001**
	C × *R*	0.401
Phosphorus content	*C*	**<0.001**
	R	**<0.05**
	C × *R*	0.509
EPS-polysaccharide	*C*	0.094
	R	**<0.001**
	C × *R*	0.312
Cellobiohydrolase	*C*	1
	R	1
	C × *R*	1
Peptidase	*C*	**<0.001**
	R	0.830
	C × *R*	1
Phosphatase	*C*	1
	R	**<0.001**
	C × *R*	0.866

for 56:1-Ln, and rather negligible for the 16-1-Hn and 56:1-Hn conditions (<20%). Effects of Ln treatments on chl *a* concentration were observed after 3 wk of exposure (Day 21) and persisted until the end of the experiment (Time × Concentration effect, data not shown). Conversely, bacterial density was sensitive to both nutrient concentration and N:P ratio (Table 1). More precisely, bacteria showed different sensitivities to the 4 nutrient conditions tested (Fig. 2, Tukey's test) from the beginning of the experiment (Table 1). Bacterial density decreased in relation to a $P\text{-}PO_4^{3-}$ concentration decrease in water. The greatest bacterial density reduction between Days 1 and 35 was observed for 56:1-Ln (81%) and 16:1-Ln (81%), followed by 56-1-Hn (53%), and finally 16:1-Hn (25%).

Average algal C:bacterial C ratio in biofilms (including the 4 nutrient conditions) was 245 on Day 1. On Day 35, biomass ratios were more variable among nutrient conditions (ranging between 220 and 320 in 16:1-Hn, 16:1-Ln, and 56:1-Ln), and were highest under 56:1-Hn conditions (470).

Biofilm nutrient content

Average nutrient content in biofilms in the 4 nutrient conditions was 142.0 ± 9.7 µg C cm^{-2}, 15.5 ± 2.0 µg N cm^{-2}, and 5.3 ± 0.4 µg P cm^{-2} at the beginning of the experiment. C content in biofilms was not significantly modified by nutrient conditions, but N content varied depending on the N:P ratio (Table 1). The greatest nutrient effects were observed in the 56:1-Hn condition (Fig. 3, Tukey's test), where biofilms experienced a significant increase in N content at the end of experiment (Days 28 and 35). This trend was also observed in the 56:1-Ln condition on Day 35, although the effects were not statistically significant. P content in biofilms was sensitive to both nutrient concentration and N:P ratio from Day 7 of the experiment (Table 1, Fig. 3). Biofilm P content between Days 1 and 35 increased in 16:1-Hn (38%), but decreased in 16:1-Ln (8%), 56:1-Hn (45%), and 56:1-Ln (55%) following the decrease in $P\text{-}PO_4^{3-}$ concentration in water. However, this trend was not observed for 56:1-Hn biofilms ($P\text{-}PO_4^{3-}$ = 27 µg l^{-1}, biofilm P $content_{Day35}$ = 3.0 ± 0.3 µg P cm^{-2}), which had significantly lower P contents than 16:1-Ln biofilms ($P\text{-}PO_4^{3-}$ = 10 µg l^{-1}, biofilm P $content_{Day35}$ = 4.5 ± 0.5 µg P cm^{-2}; Fig. 3, Tukey's test).

Calculations of N and P content per unit of microbial C in biofilms largely coincided with those obtained with respect to the substratum surface area. Briefly, biofilm N content on Day 35 was significantly higher in the 56:1-Hn biofilms (83.3 ± 22.4 µg N mg C^{-1}) than in the rest of treatments (57.5 ± 5.6 for 16:1-Hn, 59.0 ± 18.8 for 16:1-Ln, and 42.4 ± 12.9 for 56:1-Ln). In the case of P content, the greatest variations were observed between Days 1 and 35 for the 56:1-Hn (from 30.1 ± 9.8 to 5.5 ± 0.5 µg P mg C^{-1}) and 56:1-Ln (from 22.1 ± 1.1 to 4.1 ± 0.6 µg P mg C^{-1}) biofilms.

N:P molar ratio in biofilms was different between 16:1 and 56:1 conditions, but not between Ln and Hn conditions at the end of the experiment (Tukey's test, $p < 0.05$). Specifically, N:P ratios on Day 35 were 3.0 ± 0.5 in 16:1-Hn, 4.2 ± 0.3 in 16:1-Ln, 33.0 ± 7.2 in 56:1-Hn, and 24.4 ± 8.0 in 56:1-Ln.

EPS-polysaccharide content

The content of polysaccharides in biofilm EPS was increased 3-fold between Day 1 (113 ± 19 µg glucose

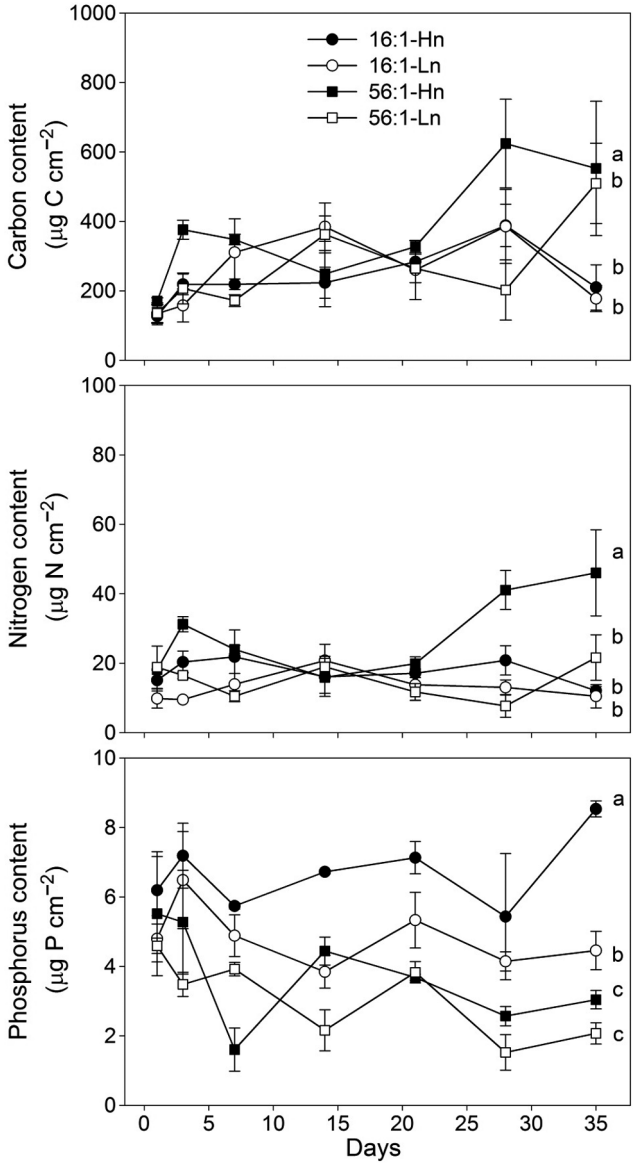

Fig. 3. Carbon, nitrogen, and phosphorus content per unit of biofilm surface area in 4 nutrient treatments (Hn: high nutrient and Ln: low nutrient conditions at an N:P ratio of 16:1 or 56:1). Values are means ± SE for each treatment and sampling time. Statistical differences between nutrient treatments for the whole study period are represented by the letters a > b > c (Tukey test, $p < 0.05$)

equivalents cm^{-2}) and Day 35 (324 ± 33 µg cm^{-2}) under the 4 nutrient conditions tested (Time effect, data not shown). Differences in the EPS-polysaccharide were observed between 56:1 and 16:1 conditions (*R* effect, Table 1), whereby the former condition accumulated the most (Fig. 4, Tukey's test). The greatest EPS-polysaccharide content was quantified in the 56:1-Hn condition and after 21 d of exposure (average Day 21 to Day 35 = 498 ± 34 µg glucose equivalents cm^{-2}, Fig. 4).

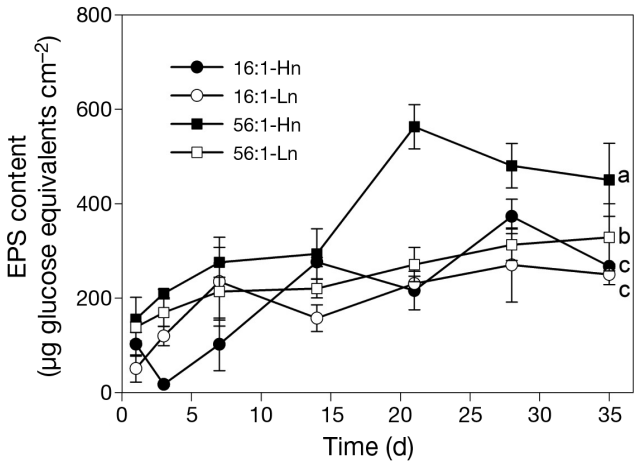

Fig. 4. Extracellular polymeric substance (EPS)-polysaccharide content, expressed in terms of the amount of glucose equivalents, in biofilms subjected to 4 treatments (Hn: high nutrient and Ln: low nutrient conditions at an N:P ratio of 16:1 or 56:1). Values are means ± SE for each treatment and sampling time. Statistical differences between nutrient treatments for the whole study period are represented by the letters a > b > c (Tukey test, $p < 0.05$)

EEA

The 3 measured EEA varied during the 35 d of the experiment. CBH activity was not affected by nutrients, but PEP and PHO activities were (Fig. 5). PEP activity was significantly higher under Hn than under Ln conditions from the beginning of the experiment, Day 7 (*C* effect, Table 1). Such differences, however, were more evident for 16:1-Ln than for 56:1-Ln, the latter experiencing more variable PEP activity values (Fig. 5). Conversely, PHO activity showed a greater increase under 56:1 (activity multiplied by 3.8) than under 16:1 (multiplied by 2.7) conditions between Days 1 and 35 (*R* effect, Table 1). Specifically for PHO, the increase in activity was not linked to water P-PO_4^{3-} concentrations, since PHO activity values were greater under 56:1-Hn (P-PO_4^{3-} = 27 µg l^{-1}) than under 16:1-Ln (P-PO_4^{3-} = 10 µg l^{-1}; Fig. 5) conditions.

Ratios between CBH, PEP, and PHO activities showed coefficients ranging between 0.3 and 1.3. Ln CBH:ln PEP and ln CBH:ln PHO ratios ranged between 0.3 and 0.5, while the ln PEP:ln PHO ratio ranged between 0.9 and 1.3 (Fig. 6). While differences in EEA ratios between nutrient conditions did not exist, time-specific changes were observed in ln PEP:ln PHO and ln CBH:ln PHO ratios. For instance, these EEA ratios tended to decrease between Days 1 and 35 because of the increase in PHO activity.

Relationships between EEA ratios and nutrient molar ratios in biofilms

Strong (negative) correlations between ln CBH:ln PHO and ln (biofilm C:P) existed in the 4 nutrient conditions tested (Fig. 6), the strongest being that of the 56:1-Ln condition ($\rho = -0.95$, $p < 0.005$). The ln CBH:ln PEP was not related to the ln (biofilm C:N), whereas only weak correlations were found between ln PEP:ln

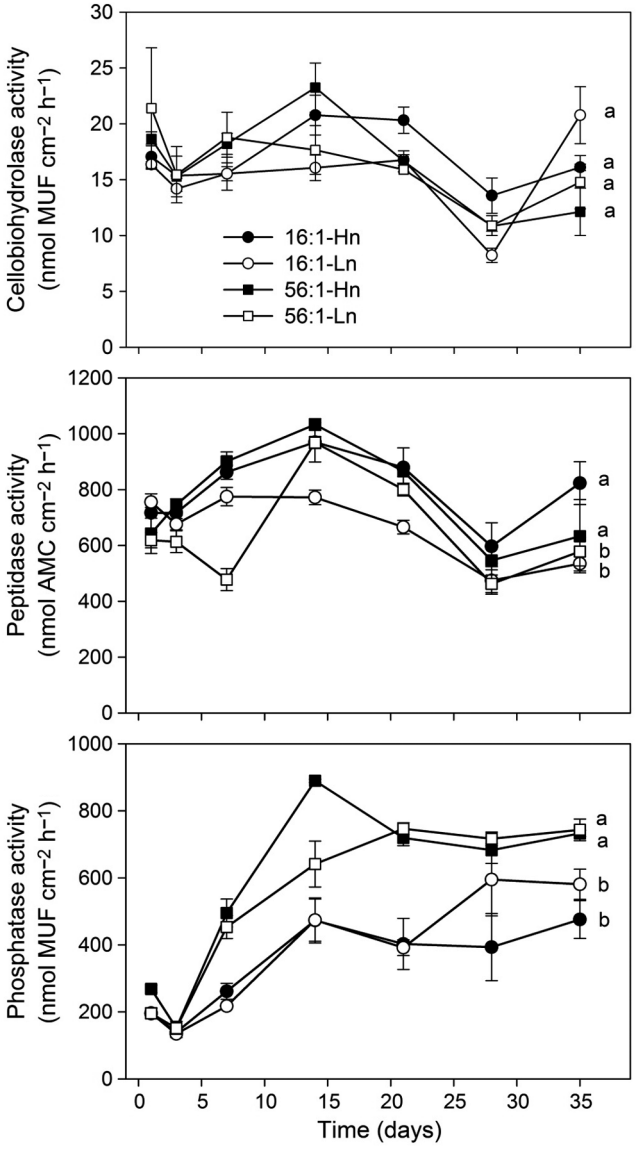

Fig. 5. Activity of cellobiohydrolase, peptidase, and phosphatase extracellular enzymes measured in biofilms subjected to 4 treatments (Hn: high nutrient and Ln: low nutrient conditions at an N:P ratio of 16:1 or 56:1). Cellobiohydrolase and phosphatase activities are expressed as the amount of methylumbelliferone (MUF) compounds released per unit of time and tile surface area. Units of leucine-aminopeptidase were expressed as the amount of aminomethyl-coumarin (AMC) released. Values are means ±SE for each treatment and sampling time. Statistical differences between nutrient treatments for the whole study period are represented by the letters a > b > c (Tukey test, $p < 0.05$)

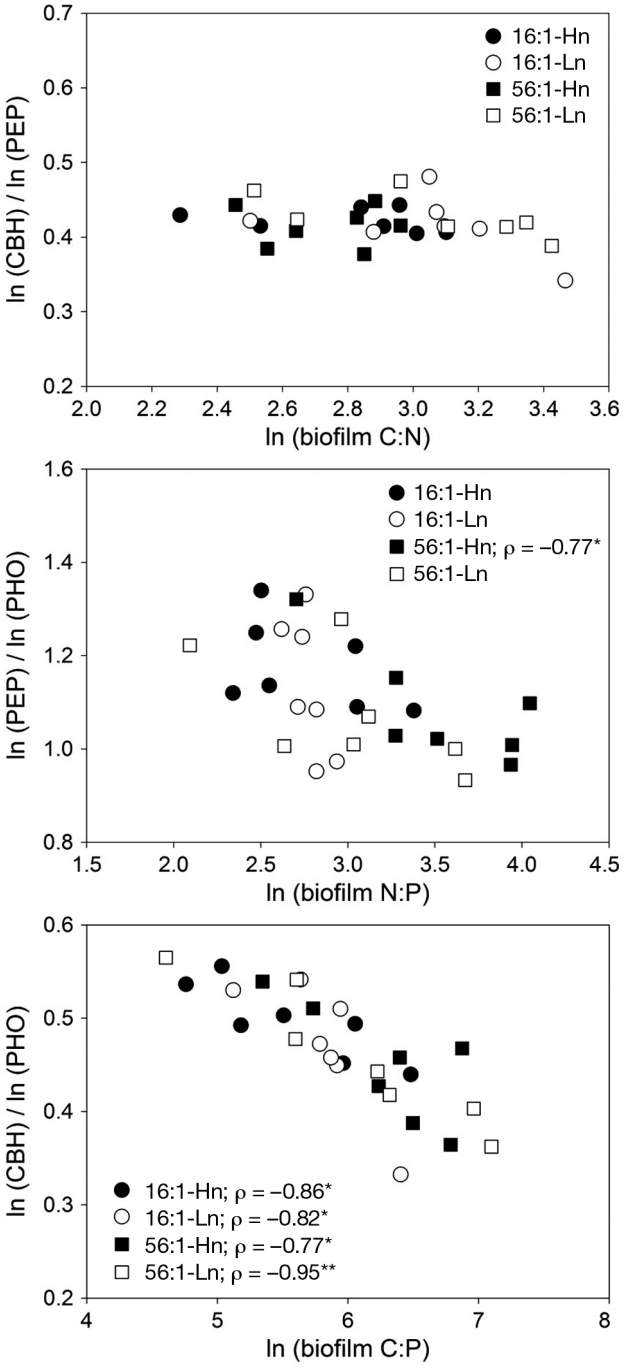

Fig. 6. Relationship between extracellular enzyme activity (EEA) and nutrient molar ratios in biofilms subject to 4 treatments (Hn: high nutrient and Ln: low nutrient conditions at an N:P ratio of 16:1 or 56:1). Significant Pearson's correlation coefficients (ρ) between EEA and nutrient ratios, and their corresponding probability values (**$p < 0.005$, *$p < 0.05$), are shown for each nutrient condition. CBH: cellobiohydrolase; PEP: peptidase; PHO: phosphatase

PHO and ln (biofilm N:P) under the 56:1-Hn condition ($\rho = -0.77$, $p < 0.05$, Fig. 6). Specifically for PHO, this activity was negatively correlated to biofilm P content in 56:1 treatments ($\rho = 0.80$ in Hn and $\rho = 0.91$ in Ln, $p < 0.005$) but not in 16:1 treatments (not significant). Relationships between chl *a* concentration and bacterial densities in biofilms were also observed under Ln conditions ($\rho = 0.93$ for 16:1-Ln and $\rho = 0.81$ for 56:1-Ln, $p < 0.05$), but not under Hn conditions.

DISCUSSION

Stream biofilms exposed to contrasting N and P concentrations and N:P supply rapidly adapted their structure and activity to the new water nutrient conditions. Biofilms modified their C, N, and P content and nutrient acquisition enzyme activities according to the inorganic N and P available in the flowing water. Algal chl *a* concentration responded to both N and P concentrations, although chl *a* responses to low P concentration at steady N levels were not observed. Bacterial density responded to N and P concentrations but showed greater sensitivity to a decrease in P. This differential sensitivity of algae and bacteria resulted in enhanced algal biomass with respect to that of bacteria under N:P imbalance (N:P = 56) in the water. These imbalance conditions also promoted EPS-polysaccharide accumulation in biofilms, especially at high N and P concentrations. According to these observations (Fig. 7), we cannot conclude that effects of nutrient concentration in biofilms prevail over those of nutrient molar ratios, but rather that this depends on the microbial group. In the case of algae, we can accept our hypothesis, but in the case of bacteria, both nutrient content and nutrient molar ratios determine changes in bacterial densities in an additive manner.

Biofilm algae and bacteria showed different sensitivities to changes in water N and P concentrations. These differences with respect to N and/or P have been described in planktonic (Currie et al. 1986) and benthic (Rier & Stevenson 2002) aquatic microbial communities. Higher limitation by N in photoautotrophs than in heterotrophs was detected in a nutrient-enrichment experiment (Lang et al. 2012), although P can also be limiting for autotroph growth (cf. Bothwell 1989). Carr et al. (2005) observed that mostly N concentration (NO_3^- and NO_2^- forms) explained the variance of chlorophyll concentration in river biofilms. This observation has been confirmed at the ecosystem level after the differential N and P spiraling dynamics in autotrophic and heterotrophic streams (Schade et al. 2011). At the same time, a positive effect of nutrients on algae may have a cascade effect on bacteria. The nitrogenized compounds released by algae during photosynthesis are used by bacteria, and this is reflected in the increase of PEP activity (Francoeur & Wetzel 2003). The PEP activity in our study was stimulated by Hn conditions in water (Fig. 7), suggesting that organic N recycling in biofilms is favored under eutrophic conditions, and that the algal photosynthates reinforce the microbial loop.

Biofilm responses to N and P supply

16:1-Hn

Biomass	↑ algae,bacteria
Nutrient content	↑P (↓N:P)
EEA	↑PEP ↑PHO

56:1-Hn

Biomass	↑↑algae ↑bacteria ↑ EPS
Nutrient content	↓P ↑ N (↑N:P)
EEA	↑PEP ↑↑PHO

16:1-Ln

Biomass	↓ algae, bacteria
Nutrient content	↓N (↓N:P)
EEA	↑PHO

56:1-Ln

Biomass	↓algae, bacteria
Nutrient content	↓P ↑ N (↑N:P)
EEA	↑↑PHO

Fig. 7. Conceptual model summarizing the influence of N and P supply (2 nutrient concentrations: Hn, high nutrient and Ln, low nutrient; 2 N:P ratios tested: 16:1, 56:1) on algal and bacterial biomass, nutrient content, and extracellular enzyme activities (EEA) in biofilms (PEP: peptidase; PHO: phosphatase). Arrows indicate either increase (↑) or decrease (↓) of each parameter as the observed biofilm responses (after 3 to 28 d) to the imposed changes in nutrient content and imbalances

Bacteria were more sensitive to water P concentration than to that of N. The high sensitivity of bacteria to P has been already described in streams (Hepinstall & Fuller 1994, Mohamed et al. 1998), and could be explained by their weak ability to store large quantities of P (Morris & Lewis 1992) and their greater P demands (Rhee 1978) compared to algae. Growth rates of bacteria are higher than those of algae (Rhee 1978), and according to the growth rate hypothesis (Elser et al. 2000), the faster the growth rate of a microorganism the greater the cellular P demands (increasing allocation of P to RNA). This indicates that bacteria may eventually be more P-limited than algae in stream biofilms. In our experiment, the reduced bacterial density

as a response to decreasing P concentration in water was accompanied by lower biofilm P content and higher PHO activity. Over the 35 d experiment, biofilm P content was modulated according to water P concentration, and this was probably mediated by the action of PHO activity (Bothwell 1985, Scott et al. 2007). We observed that PHO activity was negatively correlated to biofilm P content, and higher PHO occurred under the imbalanced 56:1 treatments (Fig. 7), indicating that it is not only the absolute content but also the stoichiometry between N and P availability in water which may determine lower P accumulation under the most limiting conditions (56:1) for the growing biofilm. The PHO activity results evidenced the link between water nutrient supply and biofilm nutrient content (Fig. 7), although this assumption cannot be validated in the case of PEP activity.

The major structural changes in the biofilm occurred in the 56:1-Hn treatment. The 56:1-Hn condition involved greater N and EPS-polysaccharide accumulation. Although N:P imbalance did not specifically affect algal biomass in our study, this has been observed in other studies (Francoeur et al. 1999, Stelzer & Lamberti 2001). At the end of the experiment, the algal C:bacterial C ratio increased under 56:1-Hn conditions. Presumably, algae withstand N:P imbalances better than bacteria at Hn concentration, though the biomass ratio decreased under Ln conditions. Contributing to this global biofilm biomass, the extracellular release of carbohydrates by algae has been also observed to increase under imbalanced N:P supply in plankton communities (Obernosterer & Herndl 1995). Bacteria, which readily utilize algal-produced carbohydrates, tend to reduce their metabolism when they become strongly limited by P (N:P = 100), resulting in greater carbohydrate accumulation and mucilage formation in the biofilm (Obernosterer & Herndl 1995). Responses observed in plankton communities are similar to those observed in our biofilms, except when N:P imbalance occurs at low N (75 µg $N\text{-}NO_3^-$ l^{-1}) and P (2.7 µg $P\text{-}PO_4^{3-}$ l^{-1}) concentrations, and allow us to assume that the EPS-polysaccharide production by algae in biofilms is reduced under Ln conditions (Fig. 7).

Relationships between EEA ratios and nutrient molar ratios in biofilms were negative between CBH/PHO and biofilm C:P, indicating the strong allocation of PHO activity in biofilms with low P content, while the decomposition of C compounds (CBH) was not modified (Fig. 5). This observation confirms the reliability of the ecoenzymatic stoichiometry in determining the link between the elemental stoichiometry of microbial biomass and the microbial nutrient acquisition (Sinsabaugh & Follstad Shah 2012). On the other hand, relationships between PEP/PHO and N:P molar ratios were also negative but only significant for the 56:1-Hn treatment, which makes it difficult to confirm our second hypothesis. This result may indicate that enzymatic stoichiometry for N and P organic compounds is especially sensitive only under nutrient-imbalanced conditions.

Our study confirms that stream biofilms adapt to changes in water nutrient availability relatively quickly (from 3 to 28 d), suggesting a high plasticity towards nutrient stress. Changes in water N and P availability affect biofilm communities in different ways, probably because each microbial group experiences different nutrient limitations. However, the ecoenzymatic stoichiometry approach that we used allowed us to link water nutrient availability with biofilm nutrient content and nutrient acquisition activities. The nutrient conditions tested in our experiment mimicked the eventual changes in nutrient concentration and N:P supply occurring in Mediterranean intermittent streams, although biofilms did not show strong resistance to this stress. Overall, the present study highlights the importance of taking into account temporal variations in water nutrient supply (daily, weekly, monthly) when studying the functioning of biofilm communities in stream ecosystems.

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