ENVIRONMENTAL MICROBIOLOGY

Effects of Warming on Stream Biofilm Organic Matter Use Capabilities

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Abstract The understanding of ecosystem responses to changing environmental conditions is becoming increasingly relevant in the context of global warming. Microbial biofilm communities in streams play a key role in organic matter cycling which might be modulated by shifts in flowing water temperature. In this study, we performed an experiment at the Candal stream (Portugal) longitudinally divided into two reaches: a control half and an experimental half where water temperature was 3 °C above that of the basal stream water. Biofilm colonization was monitored during 42 days in the two stream halves. Changes in biofilm function (extracellular enzyme activities and carbon substrate utilization profiles) as well as chlorophyll *a* and prokaryote densities were analyzed. The biofilm in the experimental half showed a higher capacity to decompose cellulose, hemicellulose, lignin, and peptidic compounds. Total leucine-aminopeptidase, cellobiohydrolase and β-xylosidase showed a respective 93, 66, and 61 % increase in activity over the control; much higher than would be predicted by only the direct temperature physical effect. In contrast, phosphatase and lipase activity showed the lowest sensitivity to temperature. The biofilms from the experimental half also showed a distinct functional fingerprint and higher carbon usage diversity and richness, especially due to a wider use of polymers and carbohydrates. The changes in the biofilm functional capabilities might be indirectly affected by the higher prokaryote and chlorophyll density measured in the biofilm of the experimental half. The present study provides evidence that a realistic stream temperature increase by 3 °C changes the biofilm metabolism to a greater decomposition of polymeric complex compounds and peptides but lower decomposition of lipids. This might affect stream organic matter cycling and the transfer of carbon to higher trophic levels.

Introduction

The predicted increase in global temperature includes main air temperature increase and a rise in river and stream temperatures [1, 2]. The expected increase in river and stream waters is deduced from the correlation between air and water temperature [3]. Whichever the extent of the change, today it is widely accepted that climate change affects ecosystems [4]. In river and stream ecosystems, warming may affect key processes, such as microbial leaf litter decomposition [5] and benthic community respiration [6–8]. At the organism level, temperature is one of the major factors affecting different key physiological mechanisms such as respiration, growth, metabolic rate, and feeding [9, 10]. In stream benthic biofilms, due to the tight coupling between microorganisms, temperature might not only be affecting individuals but also the interaction between organisms [11].

Biofilms developing on the streambed are microbial assemblages that are highly dependent on environmental changes and at the same time are responsible for organic matter dynamics [12, 13]. Stream biofilms play a key role in river biogeochemistry and are responsible for organic matter mineralization and the transfer of carbon to higher trophic levels. Therefore, any change in biofilm functioning due to warming may determine a change in the stream ecosystem services in terms of organic carbon cycling. The extracellular enzymes released from biofilm microbes play a key role in organic carbon transformation since they transform the polymeric material into soluble monomers that can be assimilated; their action represents a limiting step for the organic matter to enter

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the food web [14, 15]. In this regard, soil enzyme activities were found to be sensitive to temperature [16]. German et al. [17] also demonstrate the temperature effect on extracellular enzyme kinetics and a greater sensitivity of soils from cooler climates indicating the adaptation of soil microorganisms.

At microbial river biofilm level, an increase of 4 °C determined increases in the use of recalcitrant organic matter sources [18]. Experiments including a wider range of temperatures indicate a significant and non linear effect of temperature on biofilm metabolism such as denitrification, primary production and community respiration [19, 20]. The warming of flowing water is known to increase biofilm bacterial respiration; however, predictions should include hydrology and organic matter availability since they modulate the biofilm respiration response [21]. Furthermore, these metabolic changes should be linked to the expected structural changes in biofilm due to warming. Díaz Villanueva et al. [11] found that when increasing the flowing water temperature by 3 °C, the biofilm showed faster growth, although attaining similar maximal biomasses both of algae and bacteria, while the effect on extracellular enzyme activities was less evident. Similarly, Davis et al. [22] showed that warming scenarios combined with hydrological shifts may lead to earlier initiation of biofilm growth. Changes in prokaryote community composition due to a minor increase of 2 °C of the flowing water temperature were also observed but mainly when the biofilm developed in the dark (Romaní et al. pers. com.). Specifically for aquatic bacteria, temperature effects have been found on bacterial production but also on rapid changes of community composition leading to complex temperature responses [23]. Therefore, the effect of warming on biofilm organic matter use capabilities is difficult to predict. Also, the biofilm organic matter use capacity should not be seen as a sum of specific enzyme activities, but the combination of diverse decomposition capacities (i.e. functional diversity) may provide the biofilm with a greater capacity to cope with different available organic matter of different complexity. At the same time, it has been shown that the capacity of biofilms to use a wide range of organic substances is linked to their community diversity [24]. A further complementary approach to the heterotrophic capabilities of the biofilm community is the analysis of community-level substrate utilization where functional diversity can be measured [25].

Most experiments on the effect of temperature on biofilms have been performed in the laboratory and/or include a collection of several samples from different climatic regions, while no studies exist in the field where the results can be much more realistic. In the present research, a longitudinal reach of a low-order stream was longitudinally divided in halves; one half was provided by water directly warmed in the field about 3 °C above the basal stream water flowing in the other half. The experimental design at the Candal stream allows a real ecosystem context where multiple interactions can occur [26].

The main objective of this study was to determine the effect of a 3 °C increase in stream water temperature on biofilm organic matter degradation capacity in the context of global warming. For this purpose, we followed intensively biofilm development in the control and experimental (+3 °C) halves of the Candal stream. We analyzed the biofilm activity of seven extracellular enzymes involved in the degradation of cellulose, hemicellulose, lignin, peptides, lipids and organic phosphorus compounds in order to include a wide range of organic matter substrate quality available in flowing waters. We further analyzed the carbon substrate utilization profile of the developing biofilm by community-level physiological profiling (Biolog EcoPlates incubation) in order to assess biofilm functional diversity. We hypothesize that biofilm heterotrophic organic matter use capacity will be globally increased by the increased temperature but also that not all decomposing activities will show the same effect and thus the specific biofilm performance will be modulated.

Methods

Study Area and Experimental Setup

The study took place in Ribeira do Candal, Lousã mountain, Central Portugal (40° 04′ 48.10″ N, 8° 12′ 11.16″W, 634 m a.s.l.). Ribeira do Candal is a second-order stream that runs through a schist substratum, and is bordered by native deciduous forest, dominated by chestnut (*Castanea sativa* Mill.) and oak (*Quercus robur* L.) trees. The study section (~1 m wide; <10 cm deep) was divided longitudinally into halves along 22 m with schist stones. During the study period, mean ambient water temperature was 9.4 °C (5.9–12.1 °C range from hourly measurements, see below). One side of the study section (corresponding to the experimental half) was warmed up by 3 °C above the ambient temperature registered in the other side (corresponding to the control half). A 3 °C increase in water temperature is realistic considering the predictions of Miranda et al. [27] for increases in air temperature in central Portugal. Warming was initiated 10 months before the experiment started and continued throughout the study period (November 2011–January 2012). Stream water was derived from upstream into two 260-L tanks; one of the tanks was equipped with 30 electrical resistors (2,000 W each), supplied with a power of 42 kW, that continuously warmed the water by 3 °C and discharged it into the experimental half of the study reach. During the study period both halves received water at a constant flow of 2.0 L s^{-1}±0.2 measured volumetrically and granted by the outlet valves of the tanks (see [26] for further details).

Biofilm Colonization

To run the biofilm colonization, flat schist stones (12×9 cm) with glued sand-blasted glass tiles of $1 \times 1 cm^2$ were placed in each stream side on November 1, 2011 to follow the biofilm colonization in the control and experimental halves. Each stone was considered as a replicate, and for each stream half, five stones were established (total of 10 stones). Glass tiles were used as substrata for epilithic biofilm development. After 1 week of submerging the stones in the corresponding stream half, glass tiles were sampled at random from each stone on days 7, 14, 21, 28, 35, and 42 for the analysis of a range of extracellular enzyme activities (β-glucosidase, β-xylosidase, cellobiohydrolase, leucine-aminopeptidase, lipase, phosphatase, phenol oxidase), chlorophyll *a* and prokaryote density. On days 14, 21, and 28, carbon substrate utilization of the biofilm community was measured by Biolog EcoPlates [25]. Enzymatic activities, Biolog EcoPlate incubations, and prokaryote density were measured immediately after collection. Samples for chlorophyll *a* determination were frozen (−20 °C) until analysis.

Physical and Chemical Conditions in the Stream Halves

Water temperatures in both stream halves were assessed hourly with a temperature data logger (Hobo Pendant UA-001-08, Onset Computer Corp., MA, 106 USA) during the experiment. Chemical conditions in the stream water were also monitored during the experiment. Water samples from each half were filtered through a 0.2 μm pore diameter nylon filter to analyze inorganic nutrients and dissolved organic carbon. Soluble reactive phosphorus concentration was determined by the ascorbic acid method [28]. Nitrate, nitrite, and ammonium concentrations were determined by ion chromatography (Dionex DX-120, Sunnyvale, CA, USA). Dissolved organic carbon was analyzed by a TOC analyzer (Elementar Analysensysteme Gmbh LiquiTOC, Hanau, Germany). The stream's oxygen concentration (Oxi 3210, WTW, Weilheim, 132 Germany), pH (pH 3110, WTW, Weilheim, Germany) and conductivity (conductivity meter, LF 330, WTW, Weilheim, Germany) were also analyzed.

Biofilm Microbial Biomass

Chlorophyll *a* concentration on the glass tiles (five replicates for each stream half, for each sampling day) were measured after extraction in 90 % acetone for 12 h in the dark at 4 °C. To ensure complete extraction of chlorophyll *a*, samples were further sonicated for 2 min in a sonication bath (Selecta) previously protected from light. Extracts were passed through 1.4 μm mesh size fiberglass filters (GF/C Whatman) and chlorophyll *a* concentration was further determined

spectrophotometrically using a Jenway 6505 UV/VIS spectrophotometer, following Jeffrey and Humphrey [29].

Prokaryote cell abundance on the glass tiles (five replicates for each stream half, for each sampling day) were estimated via direct counts using an epifluorescence microscope (Nikon Optiphot) after 4-6-diamidino-2-phenylindole hydrochloride (DAPI, Sigma) staining [30]. In the field, 5 ml of autoclaved Ringer solution was added to each vial with a glass tile. Sodium pyrophosphate was added to a final concentration of 0.05 mmol L^{-1} to help sample dispersion. After 30 min, the samples were homogenized (2 min) by using a sonication bath (Selecta). After appropriate dilution (10 times) with filtered (0.2 μm pore size, nylon filter) and autoclaved stream water, the samples were stained with DAPI for 10 min and collected in black 0.2 μm polycarbonate filters (Nucleopore, Whatman). Bacterial slide counting was carried out at ×1,000 magnification with a Nikon E600 epifluorescence microscope. At least 20 random fields were counted for each slide.

Biomass of primary producers was transformed from chlorophyll *a* density using the C:Chl conversion factor of 60 (used for diatom-dominated communities, as those observed in the experiment), and prokaryote biomass was calculated using the conversion factor of 2.2×10^{-13} g C μm^{-3} assuming a mean bacterial cell volume of 0.1 μm^3 [31]. The ratio of % C of prokaryotes/primary producers during the biofilm development at the two stream halves was calculated.

Biofilm Function

Extracellular Enzyme Activities

Seven extracellular enzyme activities were measured on each sampling day on the collected colonized glass tiles (five replicates for each stream half, for each sampling day). The hydrolytic enzymes cellobiohydrolase (EC 3.2.1.91), β-glucosidase (EC 3.2.1.21), β-xylosidase (EC 3.2.1.37), leucine-aminopeptidase (EC 3.4.11.1), lipase (EC 3.1.1.3), and phosphatase (EC 3.1.3.1-2) were measured spectrofluorometrically by using fluorescent-linked artificial substrates (MUF-cellobioside, MUF-β-D-glucoside, MUF-β-D-xyloside, L-leucine-7-amido-4-methylcoumarin hydrochloride (Leu-AMC), MUF-palmitate, and MUF-phosphate, respectively, Sigma-Aldrich). Phenol oxidase (EC 1.10.3) was measured using L-3,4 dihydroxyphenylalanine (L-DOPA, Sigma-Aldrich) as the substrate. All enzymatic activities were measured under saturating conditions; we used 0.3 mmol L^{-1} final substrate concentration for β-glucosidase, β-xylosidase, lipase, phosphatase and leucine-aminopeptidase, 0.8 mmol L^{-1} final substrate concentration for cellobiohydrolase and 5 mmol L^{-1} final substrate concentration for the measurement of phenol oxidase activity. The freshly collected glass tiles were

placed in glass tubes with filtered (0.2 μm pore size, nylon filter) and autoclaved stream water, and the respective artificial substrates were added. Glass tubes were placed in test-tube racks that were immersed at the corresponding stream half in the dark (covered with black plastic) for their incubation. All incubations lasted 1 h except for phenol oxidase, which lasted 2 h. Blanks and standards of MUF and AMC were included. At the end of the incubation period, glycine buffer (pH 10.4) was added (1:1 vol:vol), and fluorescence was measured at 360/465 nm excitation/emission for MUF and AMC using a plate reader (Ultra 384, Tecan, Switzerland). Blanks were subtracted from the samples to correct for abiotic hydrolysis of the substrate or fluorescent substances in the medium. Phenol oxidase activity was measured following the method outlined by Sinsabaugh et al. [32]. At the end of incubations, absorbance was measured at 460 nm (Jenway 6505 UV/VIS spectrophotometer). Extracellular enzyme activities were expressed as nanomoles MUF (for β-glucosidase, β-xylosidase, cellobiohydrolase, lipase, phosphatase), AMC (for leucine-aminopeptidase) or DIQC (2,3-dihydroindole-5,6-quinone-2-carboxilate, for phenol oxidase) released per square centimeter and per hour. All enzyme activities except phosphatase were also calculated in a cell basis by dividing the activity by the number of prokaryote cells. This calculation was not done for phosphatase since eukaryotes can also significantly contribute to this activity [33].

Carbon Substrate Utilization Profiles

Biolog Ecoplates™ microplates (Biolog Inc., Hayward, CA, USA) were used in order to determine differences in the metabolic fingerprint of the biofilm communities from the control and the experimental stream halves. Each microplate contains three replicate wells of 31 carbon sources and a blank with no substrate. The substrates were assigned to chemical guilds as polymers, carbohydrates, carboxylic acids, amino acids and amines [34]. For this analysis, three replicates were used and thus three schist stones were selected from each stream half. At days 14, 21, and 28 of biofilm colonization, six (two from each schist stone) glass tiles from each stream half were collected. Fifteen milliliters of Ringer solution was added to each vial (containing 2 glass tiles). Biofilm from the glass tiles was extracted by sonicating the samples (2 min, Selecta sonication bath) and by scraping the tiles using sterile cell scrappers. Microplates were inoculated under sterile conditions with 130 μl of the biofilm extract to each well and incubated at each respective temperature (the same as the control and the experimental halves) in dark conditions for 8 days (192 h). Every 24 h, the color development in each well was measured through optical density at 590 nm (microplate reader BioTek, Synergy™ 4). The color measured in each well was corrected by the color measured in the respective blank well from each microplate.

Data Analysis

The effects of warming on chlorophyll a concentration, prokaryote density, the ratio of %C from prokaryote versus primary producers, and the extracellular enzyme activities throughout biofilm formation (both in activity per cm^2 and activity per cell) were analyzed by repeated measures analysis of variance (one-way RM-ANOVA, treatment as a fixed factor) using the Statistical Package for the Social Sciences (SPSS v. 15.0). All variables included in the analyses were log- (log (x)) transformed, except for chlorophyll a, which was log- $(x+1)$ transformed. Pearson correlation between prokaryote density and chlorophyll a was further calculated for both control and experimental biofilms.

Integrated enzyme activity for the whole experiment was calculated by integrating the activity for the whole time period (considering the time between each sampling day and the temporal development of each enzyme) to obtain the total activities in nanomoles per square centimeter. Nonmetric multidimensional scaling (NMDS) was performed to visualize the spatial distribution pattern of the samples due to their extracellular enzyme activities. This was performed for all enzyme measurements throughout the study ($n=60$), and for the integrated enzyme activity values ($n=10$). Analysis of similarity (ANOSIM) was further applied in order to test whether the control and experimental samples were significantly separated. These analyses were performed by the software R [35] based on Bray–Curtis distances.

Analysis of differences in biofilm carbon use capabilities between treatments was performed with data obtained after color saturation in the Biolog Ecoplates. A similarity percentages (SIMPER) test was used to highlight and detect the characteristic substrates used in the control and experimental halves. The test performed was a one-way SIMPER analysis with the normalized absorbance (divided by plate average well color development) measured for each substrate in the EcoPlate for each replicate per treatment and per sampling date. Previous ANOVA analyses showed no significant differences between sampling dates (14, 21, 28) in the use of the 31 carbon substrates and thus the one-way SIMPER analysis focused on treatment differences. The distance matrix was built with Bray–Curtis similarity. This analysis was performed using PRIMER v.6.0 for Windows (Primer-E Ltd., Plymouth, UK). Based on these data, NMDS and ANOSIM were performed to visualize the spatial distribution pattern of the samples and test treatment differences by using the software R [35]. Data from each microplate after color saturation were further analyzed based on the number of positive wells (functional richness), Shannon's diversity index (H'), and Evenness

(J') to evaluate microbial community functional diversity. Shannon's diversity index is defined as $H=-\sum$ pi (ln pi) where pi is the ratio of the corrected absorbance value of each well to the sum of absorbance value of all wells of each plate.

Color development in the Biolog EcoPlates was analyzed after grouping the five types of substrates included in the plate (amines, amino acids, carbohydrates, carboxylic acids, and polymers). The color development on plates was fitted to sigmoid equation and the parameters a (maximum absorbance in the event of color saturation), $1/b$ (slope, maximum rate of color development per incubation time), and X_0 (time when maximum color development rate is achieved) were estimated using SigmaPlot 2000 (Systat Software, Inc., San Jose, CA, USA). These parameters were calculated for each sampling site and substrate guild. The percentage of the different types of substrates for each day and treatment were also analyzed after color saturation. Differences between treatments for the parameters of the sigmoid curves and percentage of types of substrates were analyzed by ANOVA.

The relationship between extracellular enzyme activities and carbon substrate utilization profile was analyzed by Mantel tests and Pearson correlation (function mantel.rtest, software R [35]). Three matrices (Bray–Curtis distances) were considered: extracellular enzyme activities matrix (7 activities), Biolog results (31 substrates), Biolog results as substrate groups (5 groups).

The physical and chemical characteristics of the stream water in the two stream halves were compared by a paired t test. Differences between observed and predicted integrated enzyme activities and enzyme ratios were analyzed by t tests. All ANOVA and the t test analyses were performed using the SPSS software package for Windows, v. 14.0.1.

Results

Physical and Chemical Parameters

Physical and chemical parameters in the control and experimental halves during the whole study remained nearly constant and no significant differences between halves were found (t test, $p>0.05$). Mean conductivity was 26.6 ± 0.2 μS/cm, dissolved oxygen 9.7 ± 0.2 mg/L, pH 7.19 ± 0.05, and discharge 2.41 ± 0.1 L/s. Inorganic nutrients were 0.25 ± 0.02 mg/L of nitrate and 8.7 ± 2.2 μg/L of soluble reactive phosphorus, and dissolved organic carbon was 1.37 ± 0.07 mg/L. Mean water temperatures in the control and experimental halves are summarized in Table 1. Difference in water temperature between control and experimental halves was significant (t test, $p<0.001$). The water in the experimental half had a mean of 2.8 ± 0.04 °C ($n=1379$) more than the control half during the entire experiment. During the study period, the differences in temperature upstream to downstream were

0.07 ± 0.003 °C for the control half, and 0.039 ± 0.003 °C for the experimental half (see [26] for further details).

Biofilm Microbial Biomass

Chlorophyll a density in biofilms, used as an indicator of biomass of primary producers, was significantly higher in the experimental half (Fig. 1b, Table 2). The density of prokaryotes increased in the two treatments and was also significantly higher at the experimental half (Fig. 1a, Table 2). From days 7 to 14, there was a greater increase of bacterial density in the experimental than in the control half, determining a different time evolution between treatments (Fig. 1a, Table 2, Day× Treatment effect). A significant correlation between bacterial density and chlorophyll was found for the control half biofilms ($r=0.49$, $p=0.006$, $n=30$), but not for the experimental half biofilms ($r=0.23$, $p=0.22$, $n=30$). The ratio between biomass (in terms of carbon content) of prokaryotes versus primary producers was 1.35 ± 0.15 % on average and it was not significantly different through time and treatment ($p>0.05$, Fig. 1c). However, when considering the two treatments separately, this ratio changed significantly with time for the biofilms of the experimental half ($p=0.004$), being highest on day 21 and lowest on days 7 and 34 (Tukey's test, $p<0.05$).

Extracellular Enzyme Activities

Most extracellular enzyme activities in the developing stream biofilms were changing through time but changes were not significantly affected by the treatment (Fig. 2, Table 2, Day and Day×Treatment effect). However, the activities of leucine-aminopeptidase, β-xylosidase, cellobiohydrolase, and phenol oxidase were significantly higher in the biofilms of the experimental half (Fig. 2, Table 2).

When the extracellular enzymes where expressed per number of prokaryote cells, the activity of leucine-aminopeptidase per cell was significantly higher in the biofilms of the experimental half (ANOVA, $p=0.027$) while the activity of lipase per cell was significantly lower at the biofilms of the experimental half (ANOVA, $p=0.003$, Fig. 3). The time evolution of β-glucosidase, β-xylosidase, cellobiohydrolase, and leucine-aminopeptidase was affected by the treatment due to a significant higher activity per cell in the experimental half at the beginning of the colonization (day 7) (ANOVA, Day×treatment effects, $p<0.05$, Fig. 3).

Multivariate analysis of the extracellular enzyme activities revealed a significant separation of biofilms from the control and experimental halves when the total integrated enzyme activities were considered (Fig. 4, ANOSIM $r=0.49$, $p=0.015$). The enzymes lipase (positively related with the control biofilms) and leucine-aminopeptidase, cellobiohydrolase, β-xylosidase, and phenol oxidase (positively related with the experimental biofilms) were significantly correlated with the

Table 1 Summary of the water temperature which was continuously measured in the control and experimental halves, upstream and downstream of the studied reach

Date	Control half temperatures		Experimental half temperatures	
	Upstream	Downstream	Upstream	Downstream
1–6 Nov. 2011	10.93±0.06	10.98±0.06	12.83±0.11	12.41±0.10
7–13 Nov. 2011	10.70±0.06	10.77±0.06	12.71±0.09	12.32±0.08
14–20 Nov. 2011	10.38±0.030	10.46±0.031	13.45±0.056	13.02±0.053
21–27 Nov. 2011	9.73±0.032	9.79±0.034	13.34±0.040	12.89±0.038
28 Nov.–4 Dec. 2011	9.19±0.036	9.32±0.045	12.73±0.036	12.34±0.036
5–11 Dec. 2011	9.27±0.054	9.36±0.057	11.94±0.141	11.56±0.137
12–18 Dec. 2011	9.25±0.064	9.33±0.067	12.006±0.165	11.63±0.161
19–25 Dec. 2011	8.13±0.054	8.18±0.056	9.69±0.175	9.35±0.171
26 Dec.–1 Jan. 2012	7.31±0.057	7.37±0.060	9.97±0.137	9.55±0.133
2–3 Jan. 2012	8.42±0.107	7.57±0.109	10.87±0.103	10.42±0.104

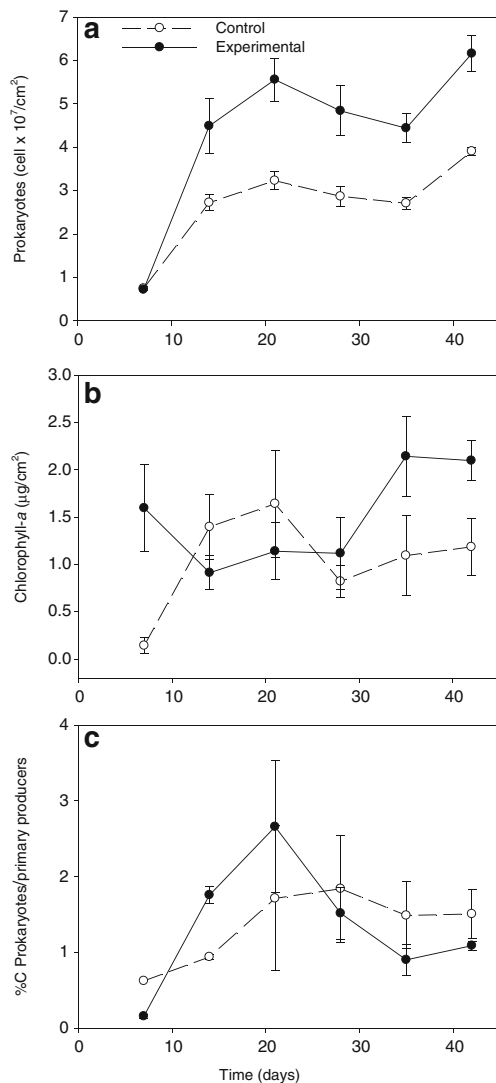Values are reported as mean weekly water temperatures±SE

Fig. 1 Development of **a** prokaryote density, **b** chlorophyll *a*, and **c** the ratio of %C of prokaryotes versus primary producers, at the biofilms grown in the control and experimental halves. Values are means±SE ($n=5$)

sample ordination (Fig. 4). However, the multivariate analysis of the enzyme activity results when considering all sampling dates showed no significant differences between control and experimental biofilms (ANOSIM, $r=0.005$, $p=0.31$).

The sensitivity of the different extracellular enzymes to the 3 °C increase was measured as the ratio between the values in the experimental versus the control halves (Table 3). The most sensitive enzyme was leucine-aminopeptidase which showed a 93 % increase in activity over the control. The increase of the leucine-aminopeptidase, β-xylosidase and cellobiohydrolase enzyme activities was higher than would be predicted by the physical effect of temperature applying the Arrhenius equation (Table 3), and observed values at the experimental half were significantly higher than predicted (*t* test, $p<0.02$). Lipase was the least sensitive to temperature increase even showing a 14 % mean reduction in activity. Lipase activity at the experimental half was significantly lower than predicted (*t* test, $p=0.02$). This differential sensitivity of enzymes to temperature determined a significantly higher leu-aminopeptidase/phosphatase enzyme ratio in the experimental half ($p=0.031$), and higher lipase/peptidase, lipase/phosphatase, lipase/phenol oxidase, lipase/(β-glucosidase+β-xylosidase+cellobiohydrolase) in the control half ($p<0.05$). The higher leu-aminopeptidase/phosphatase and lower lipase/phenol oxidase and lipase/(β-glucosidase+β-xylosidase+cellobiohydrolase) enzyme ratios in the experimental half were significantly different than expected (*t* test, $p<0.04$).

Carbon Substrate Utilization Profiles

The capabilities for using the 31 organic substrates available in the Biolog EcoPlate were different for the biofilm communities grown in the control or in the experimental temperature conditions as shown by the clear separation of samples in the NMDS plot after the similarity analysis (Fig. 5). The polymers (positively related with the experimental biofilms) and amines

Table 2 Results of a repeated measures analysis of variance with one factor (treatment (T): control vs. experimental halves) for extracellular enzyme activities and microbial biomasses measured throughout the experiment (days 7, 14, 21, 28, 34, and 42)

Source of variation	Chl	Prok	β-glu	β-xyl	Cbh	Pep	Lip	Phos	Ph-ox
Treatment	*0.038*	*0.002*	0.143	*0.002*	*0.006*	*0.003*	0.056	0.082	*0.002*
Day	0.214	*0.002*	0.087	*0.002*	*0.002*	*0.002*	*0.002*	0.079	*0.021*
Day×T	0.070	*0.033*	0.670	0.101	0.107	0.377	0.266	0.112	0.090

Probability within groups (Day and interaction Day×Treatment) are corrected for sphericity by the Greenhouse–Geisser correction. All probabilities are adjusted by the Dunn-Sidak correction. Values below 0.05 are indicated in italics. The variables considered are the following: β-glucosidase (β-glu), β-xylosidase (β-xy), cellobiohydrolase (Cbh), lipase (Lip), leucine-aminopeptidase (Pep), phosphatase (Phos), phenol oxidase (Ph-ox), chlorophyll (Chl), prokaryote density (Prok)

and carboxylic acids (positively related with the control biofilms) were significantly correlated with the sample ordination (Fig. 5). Accordingly, the percentage of the different groups of substrates used in the two treatments indicates a significantly greater use of amines and carboxylic acids in the control half but a significantly greater use of polymers in the experimental half (Table 4). The temporal color development for the different groups of substrates fitted significantly to sigmoid curves and the parameters a, b, and X_0 were estimated. The parameter X_0 (time when maximum color development rate is achieved) was significantly lower in the experimental treatment for amines, amino acids and carbohydrates indicating an earlier increase of the color development for these substrates (Table 5). The parameter a (absorbance after color saturation) was significantly lower at day 14 than at days 21 and 28 ($p=0.004$, Tukey's test, $p<0.05$).

The characteristic substrates for each treatment identified after the SIMPER analysis as those that mostly contribute to sample similarity, indicated that in the biofilms of the control half, the heterotrophic community was characterized by the use of the carbohydrates D-mannitol, D-cellobiose and α-D-lactose and the amino acids L-asparagine, L-arginine, L-serine, while polymers were less relevant. L-Asparagine, especially, contributed to the dissimilarity between control and experimentally grown biofilms (Table 6). In the experimental half, the biofilm community was characterized by the use of polymers and carbohydrates which show a longer list of characteristic substrates than those used at the biofilms of the control half. The carbohydrate i-erythritol and the polymer α-cyclodextrin were specially used in the biofilms of the experimental half and were relevant contributors to dissimilarity between treatments (Table 6). Functional diversity and richness were significantly higher in the biofilm communities of the experimental half (Table 6).

Relationship Between Extracellular Enzyme Activities and Carbon Substrate Utilization Profiles

The results of the Mantel test with data from days 14, 21, and 28 revealed no significant relationship between distance matrices obtained from the biofilm extracellular enzyme activities and from the biofilm carbon substrate utilization profiles indicating that both parameters determine a different distance pattern between samples ($r=0.04$, $p=0.31/0.34$; $r=-0.19$, $p=0.87/0.87$; considering all 31 substrates or the 5 groups of substrates, respectively, $n=16$). However, carbon substrate utilization profiles either from day 21 or from day 28 were correlated with the integrated extracellular enzyme activities (Mantel test, $r=0.69$, $p=0.021/0.014$; $r=0.52$, $p=0.035/0.059$, for 31 carbon substrate utilization data at days 21 and 28 respectively, $n=6$). Although the integrated extracellular enzyme activities showed no correlation with the groups of substrates from the Biolog (Mantel test, $r=0.28$, $p=0.21/0.15$; $r=0.46$, $p=0.09/0.09$, for data at days 21 and 28, respectively, $n=6$), a significant Pearson correlation was found between phenol oxidase activity and the use of polymers ($r=0.86$, $p=0.027$).

Discussion

Recent research indicates that community respiration in aquatic ecosystems, including stream benthic communities, is enhanced at increased temperatures [6, 7]. Boulêtreau et al. [19] reported a Q_{10} value of 2.6 for respiration activity in stream biofilms. Thus, a consequent increase in extracellular enzyme activities and organic matter decomposition in stream biofilms would be expected. Studies in soils reported Q_{10} values for hydrolytic extracellular enzyme maximum reaction velocities (Vmax) in the range of 1.64 to 2.27 [16]. Also, in alpine soils, Kock et al. [39] reported greater temperature sensitivity for carbon degrading enzymes (average Q_{10} of 2) than for nitrogen degrading enzymes (average Q_{10} of 1.7) and both were negatively related to soil organic matter quality. In microbial planktonic communities, a realistic temperature increase may affect the carbon cycling and stimulate the polysaccharide degrading enzymes [40, 41]. Degerman et al. [42] reported a synergistic effect of 4 °C increase and nutrient availability on planktonic bacterial growth and a much lower response of bacteria to temperature at low nutrient conditions. In river and

Fig. 2 Development of extracellular enzyme activities in the biofilms grown in the control and experimental halves. Values are means±SE (*n*=5). Results for lipase at day 34 are not included due to some problems with the fluorescence measurements
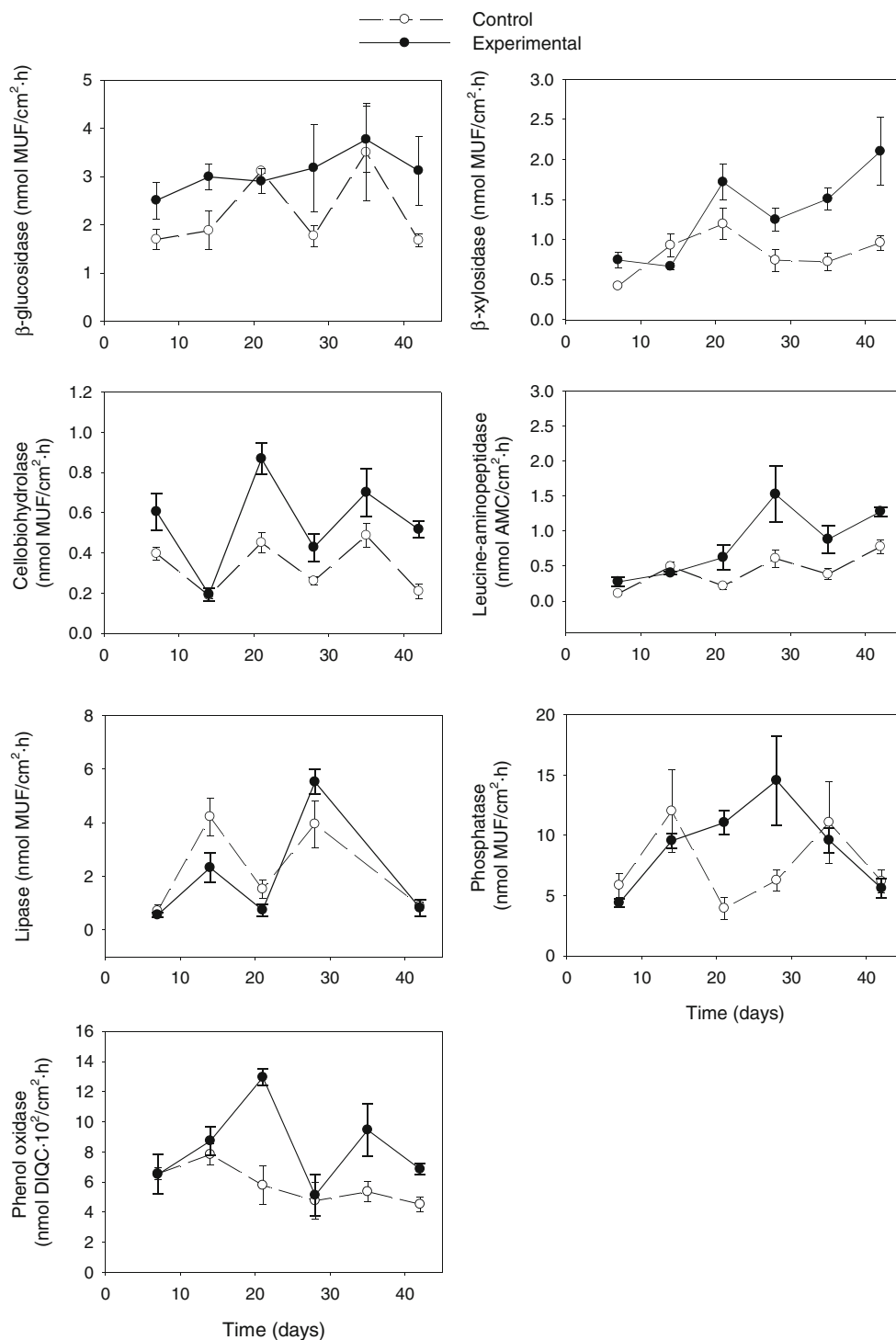
stream biofilms, some temperature effects might be similar than those reported for soils or for planktonic communities but the biofilm spatial structure and the microbial interactions may modulate its response [21]. Due to the key role of stream biofilms on organic carbon and energy transduction [11], it is relevant to know not only the degree of increase in enzyme activities but the possible changes in the organic matter use capabilities. Our results show that the increase in organic matter decomposition capacity is not equal for all enzymes, indicating a different pattern of biofilm degrading capacities in the biofilm grown at +3 °C. At the same time, the organic carbon use fingerprint also appears to be different in the biofilm developed at

Fig. 3 Development of extracellular enzyme activities per prokaryote cell in the biofilms grown in the control and experimental halves. Values are means±SE (*n*=5)
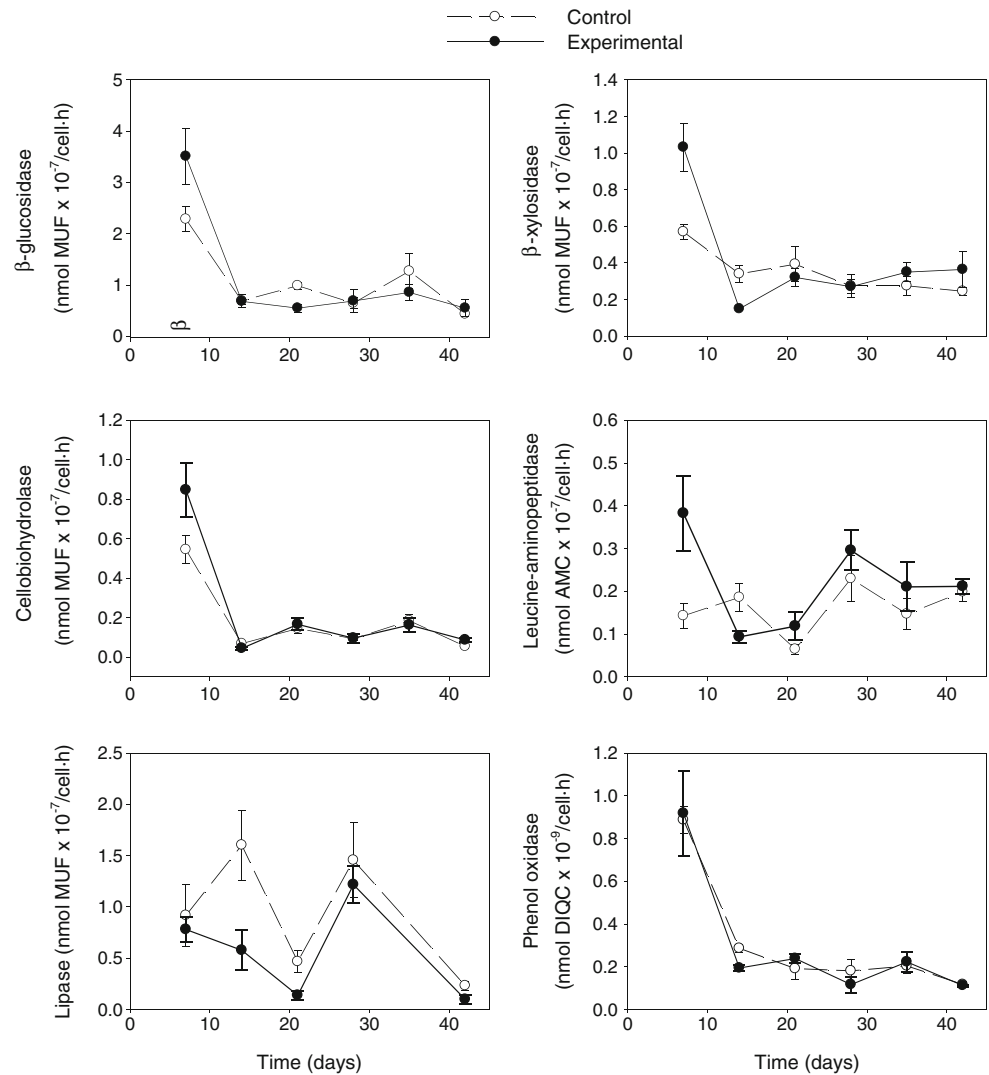
warming temperature (Fig. 5), also suggesting changes in the functional performance of the biofilm microbial community.

The extracellular enzymes measured showed a distinct sensitivity to temperature, and while the capacity to decompose peptides was nearly doubled (93 % increase), the decomposition of cellulose and hemicellulose (cellobiohydrolase, β-xylosidase) increased by 60 %, and the decomposition of phosphorus compounds and especially of lipids were hardly affected by the 3 °C increase (Table 3). From reported values of activation energy and applying the Arrhenius equation, hydrolytic enzymes might increase by about 20 % when increasing the temperature from 9.4 to 12.4 °C (the mean temperatures in our experiment). However, leu-aminopeptidase and also cellobiohydrolase and β-xylosidase, showed much higher values indicating these activities were further enhanced in the biofilms of the experimental half. Phenol oxidase activity, since it requires higher activation energy, shows higher temperature

dependency [43], and the observed activity increase (about 40 %) falls within what would be expected from Arrhenius equation (Table 3). Since the available dissolved organic carbon from the stream was exactly the same in both halves, the enhanced potential enzyme capabilities might be related to increased organic matter availability in biofilms of the experimental half as an indirect response to warming. The higher prokaryote density and the higher decomposition of organic matter due to increased extracellular enzyme activities can determine a production and release of more refractory dissolved organic compounds [44]. This would determine the higher capacity to decompose polymeric carbon compounds such as cellulose and hemicellulose. At the same time, it has been reported that temperature enhances the production of algal extracellular polymeric substances [45] and thus providing an extra source of carbohydrates. The higher sensitivity of phenol oxidase to temperature might further enhance the decomposition of complex compounds in the biofilms of the
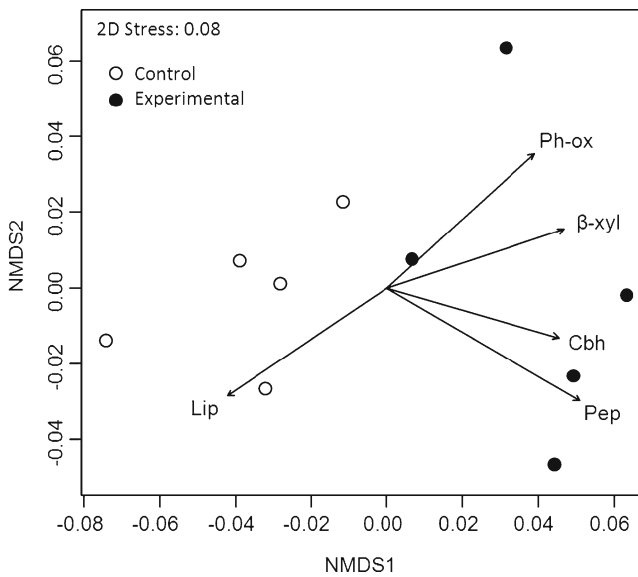
Fig. 4 Nonmetric multidimensional scaling (NMDS) ordination plot of integrated activities of seven extracellular enzymes for the whole biofilm colonization process in the control and experimental halves in the Candal stream. Kruskal 2D stress is shown. The two groups of samples (control vs. experimental) were significantly separated (ANOSIM analysis, $r=$ 0.49, $p=0.015$). The enzymes mostly correlated with the spatial distribution of samples ($p<0.05$) are also indicated

Fig. 5 NMDS ordination plot of carbon substrate use (from the 31 substrate use in the Biolog EcoPlate) of samples from control and experimental biofilms from days 14, 21, and 28 ($n=8$ since one replicate from day 14 was lost). Kruskal 2D stress is shown. The two groups of samples (control vs. experimental) were significantly separated (ANOSIM analysis, $r=0.89$, $p=0.001$). The groups of substrates mostly correlated with the spatial distribution of samples ($p<0.05$) are also indicated

experimental half. Similarly, in a laboratory experiment, a greater effect of increasing temperature (+4 °C) to the biofilm use of recalcitrant than labile compounds was observed [17]. The enhanced cellulose and hemicellulose degrading enzyme activities in the experimental half were also observed when

Table 3 Sensitivity to the 3 °C increase of the seven extracellular enzymes expressed as the ratio between experimental and control results (enzymes are ordered in decreasing sensitivity). Theoretical expected values from reported activation energy values after applying the Arrhenius equation are also indicated

	Observed experimental/ control	Expected[a] experimental/ control
Leucine-aminopeptidase	1.93	1.19–1.22
Cellobiohydrolase	1.66	1.19
β-xylosidase	1.61	1.22
Phenol oxidase	1.42	1.14–1.58
β-glucosidase	1.35	1.19–1.22
Phosphatase	1.21	1.19–1.22
Lipase	0.86	1.06–1.19

[a] The expected experimental/control enzyme activity ratios were calculated from activation energy values in the literature for extracellular enzymes (Steinweg et al. [36] and references therein for hydrolytic and phenol oxidase activity (Bhatti and Amin [37], Fischer and Kleber [38]), for lipase activity). The temperatures used for the calculation were 9.4 °C and 12.4 °C (the mean values at the control and experimental stream halves during the study)
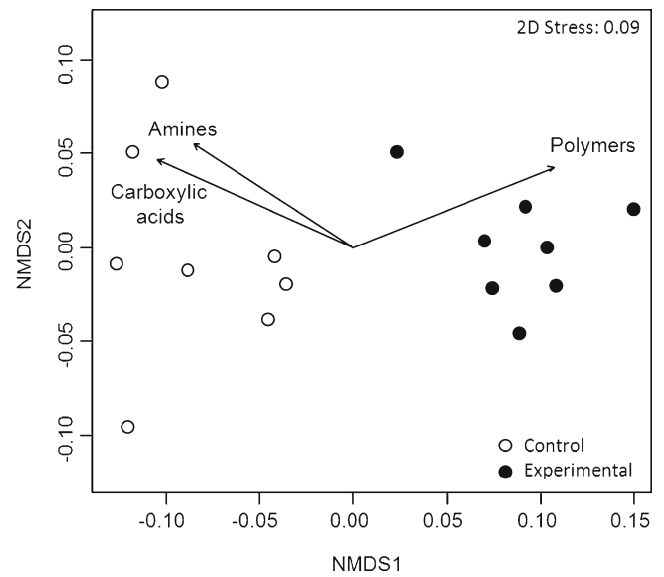
activities were standardized by prokaryote cells. The higher values of activity per cell in the experimental half, but especially at the beginning of the biofilm colonization (Fig. 3), may indicate greater enzyme efficiency for cellulose and hemicellulose degradation occurring in the young biofilm.

A significant functional change occurring in the biofilms of the experimental half was the largest increase in leu-aminopeptidase activity, biasing the nutrient acquiring enzymes to a greater use of nitrogen than of phosphorus compounds (significant higher leu-aminopeptidase/phosphatase enzyme ratio in the experimental half). The higher leu-aminopeptidase enzyme activity may indicate that either (1) more substrate is available for this enzyme in the experimental half than in the control half and/or (2) there are major cell needs for nitrogen compounds. We suggest that the two processes are co-occurring but mainly the first one is responsible for the imbalance between nitrogen and phosphorus acquiring enzymes. In the biofilms of the experimental half, a higher algal biomass was measured (Fig. 2, Table 2), which could be responsible for a major release of peptides by the biofilm primary producers increasing substrate availability for leu-aminopeptidase [21, 46]. At the same time, the higher leu-aminopeptidase activity per cell may indicate a faster turnover for peptides and probably also for prokaryotic cells and thus a greater need for organic nitrogen per cell. Considering the whole colonization sequence, prokaryotes are enhanced in the experimental warming conditions. The growth of prokaryotes could explain the needs for nitrogen but this should also enhance the needs for phosphorus.

Table 4 Results from the ANOVA analysis of the differences between groups of substrates (in percentage) being used in the biofilms of the control and experimental halves

	Amines	Amino acids	Carbohydrates	Carboxylic acids	Polymers
Treatment	*0.003*	0.080	0.936	*0.036*	*0.013*
Day	0.719	0.562	0.08	0.940	0.579
Day × Treatment	0.095	0.257	0.413	0.614	0.513

The probability due to factors Treatment (control versus experimental), Day (sampling days 14, 21 and 28), and interaction are shown

Values below 0.05 are indicated in italics

Phosphorus is necessary for the synthesis of phospholipids, nucleic acids and ATP in microorganisms and is therefore a key element for microbial growth [47]. However, in the studied stream, there is no nitrogen limitation in relation to phosphorus (molar N:P ratio of 49). Thus, the further enhanced leu-aminopeptidase activity with respect to phosphatase in the biofilms of the experimental half may not be directly linked to nitrogen acquisition for prokaryote growth but as a response to greater availability of peptides.

In contrast, the warming conditions reduce the activity and the efficiency (activity per cell) of the biofilm prokaryote cells to decompose lipid compounds (Figs. 3 and 4). Lipases are carboxyl esterases that catalyze the hydrolysis of long-chain triacylglycerol to liberate fatty acids and glycerol [48]. The changes in the use of lipidic compounds could be related to potential changes in the composition of extracellular polymeric substances in the biofilms of the experimental half [49, 50] and/or the preference of the prokaryote cells to use other carbon sources such as peptides (providing both carbon and nitrogen) or polysaccharides. At the same time, reported values from activation energy for lipases are lower than those found for carbon hydrolytic enzymes [37, 38], indicating a lower sensitivity of lipases to temperature.

Table 5 Results of the parameter X_0 (time when maximum color development rate is achieved) estimated from the sigmoid curve of the color development at the Biolog Ecoplates for the different groups of substrates (data from biofilms collected on days 14, 21 and 28)

	Control X_0 (lag time/h)	Experimental X_0 (lag time/h)
Amines	140.22 (12.17)	94.90[a] (4.95)
Amino acids	127.37 (9.66)	90.73[a] (3.01)
Carbohydrates	146.68 (8.86)	96.91[a] (4.68)
Carboxylic acids	120.55 (13.50)	89.72 (2.00)
Polymers	162.74 (23.11)	110.09 (7.15)

The estimated values and standard error (in parenthesis) are shown

[a] The significant lower values (shorter lag times) in the experimental half after a one-way analysis of variance

The relationship between the results obtained from the extracellular enzyme patterns and the carbon substrate utilization profiles was not tight. The samples from the two stream halves can be clearly separated by the carbon substrate utilization profiles while the separation between control and experimental was less evident when considering the enzyme activity patterns (Figs. 4 and 5). The carbon substrate use profiles inform us of the capacity of the incubated community to grow with specific substrates (from a range of 31 carbon substrates), giving us a kind of functional fingerprint of the community. This method, although limited by being a culture method [51], gives us the potential use of diverse substrates by a community, and a measurement of functional richness and diversity can be obtained. On the other hand, the extracellular enzymes are measured in the short term with fresh samples and they indicate the potential capacity to decompose certain bonds (linked to the decomposition of several organic compounds), and absolute potential enzyme activities can be calculated. Enzyme activities are less specific than the 31 Biolog substrates but they have a major link with carbon cycling and biogeochemical processes at the ecosystem level. Similarly, Sinsabaugh and Foreman [52] did not find a systematic correlation between extracellular enzymes and carbon substrate use profiles in river bacterioplankton, suggesting that carbon substrate use profiles are more indicative of potential diversity while extracellular enzymes are more linked to in situ functional diversity and the mechanics of organic matter processing. However, in our study, there was some agreement between extracellular enzymes and functional fingerprint. The functional fingerprint indicates a characteristic greater use of polymers in the experimental half and this was correlated with the phenol oxidase activity. Also, the results from the carbon substrate utilization profiles indicate a use of a broader range of carbohydrates in the biofilms of the experimental half which might be linked to the increased cellobiohydrolase and β-xylosidase. The functional fingerprint also indicated that a greater use of amines and carboxylic acids occurred in the control half which might be related to the greater use of simpler molecules. However, the fate of the amino acids probably available after the enhanced leu-aminopeptidase activity is still a question. One would expect that provided amino acids would be used by prokaryotes but, in the carbon substrate utilization profiles, there was not a clear increase in the use of amino acids at the experimental site but only an earlier color development in the Biolog Ecoplates for amines and amino acids (Table 5).

Considering the whole functional fingerprints, our results showed an increase in functional richness and diversity in the biofilms of the experimental half. This could be a result of changes at the prokaryote community composition level or only at the functional level, and thus the community expressing its plasticity to a use of a wider range of available substrates, especially from the polymers and carbohydrates guilds [53]. The changes in the functional fingerprint could be related

Table 6 Results from the SIMPER analysis of the 31 carbon substrates utilization in the biofilms grown in the control and experimental stream halves (data from biofilms collected on days 14, 21 and 28)

C substrates	Substrate type	Contribution to av. sim. (%)		Contribution to av. diss. (%)
		Control 91.15 % av. sim.	Experimental 91.76 % av. sim.	Control vs. experimental 14.18 % average diss.
D-Mannitol	Carbohydrate	7.58	6.10	
D-Cellobiose	Carbohydrate	5.80	5.56	
α-D-Lactose	Carbohydrate	4.74	5.86	
D-Xylose	Carbohydrate		3.98	
i-Erythritol	Carbohydrate	(0.94)	(3.47)	8.63
L-Asparagine	Amino acid	7.41	5.88	5.93
L-Arginine	Amino acid	5.90	5.01	
L-Serine	Amino acid	4.33		
L-Phenylalanine	Amino acid	(2.51)	(0.09)	9.09
Phenylethylamine	Amine	5.44	4.70	
Tween 80	Polymer	4.46	5.50	
α-Cyclodextrin	Polymer	(2.26)	4.24	6.81
Glycogen	Polymer		3.90	
4-Hydroxy benzoic acid	Carboxylic acid	4.30		
2-Hydroxy benzoic acid	Carboxylic acid	(1.18)	(1.98)	5.26
Functional diversity (Shannon)		3.13 (0.06)	3.27[a] (0.03)	
Richness (positive wells)		27.12 (1.73)	29.87[a] (0.83)	
Evenness		0.95 (0.01)	0.96 (0.01)	

The average similarity (av. sim.) between the samples from each treatment is shown as well as the average dissimilarity (av. diss.) between treatments. The organic substrates contributing up to 50 % of total average similarity and until 5 % of average dissimilarity are listed. The similarity values in italics and parentheses correspond to those substrates not contributing to the first 50 % similarity but included for better comparing the two treatments. Functional diversity, richness and evenness are also indicated for each treatment.

[a] The significant higher values at the experimental half

to changes in the interaction of the biofilm microbial groups as indicated by the greater growth of prokaryotes, the lack of correlation between chlorophyll *a* and prokaryotes, and the highly variable prokaryote/primary producer biomass ratio in the biofilms of the experimental half.

In summary, if stream waters were to warm by 3 °C, the biofilm performance would change in a differential capacity for the use of organic compounds. There would be a general increase in the capacities to use organic compounds but this would not be equal for all molecules. The biofilm under warmer conditions have a much higher capacity to use cellulose, hemicellulose and peptides than that expected by the only direct physical effect of temperature indicating an indirect enhance decomposition of these compounds. The greater use of polymeric and complex compounds is also underlined by the results from the functional fingerprint and the enhanced phenol oxidase activity. Also, changes at the community composition level and/or high functional plasticity of the community are suggested by the clear distinct functional fingerprint obtained for the biofilms grown at the control and experimental conditions. These functional performance changes are

occurring in a realistic field experiment and thus they are expected to also occur in similar low-order streams. The greater decomposition of polymeric complex compounds and peptides but lower decomposition of lipids may have two effects at the ecosystem level: (1) affecting biofilm quality and thus its role as a carbon source to the stream food web (i.e. grazers and collectors), and (2) affecting on the biogeochemical cycling of carbon, nitrogen, and phosphorus compounds from the flowing dissolved organic matter, enhancing the cycling of organic nitrogen and cellulose/hemicellulose carbon compounds.

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