

Biofilm Structure and Function and Possible Implications for Riverine DOC Dynamics

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Abstract

Biofilms are major sites of carbon cycling in streams and rivers. Here we elucidate the relationship between biofilm structure and function and river DOC dynamics. Metabolism (extracellular enzymatic activity) and structure (algae, bacteria, C/N content) of light-grown (in an open channel) and dark-grown (in a dark pipe) biofilms were studied over a year, and variations in dissolved organic carbon (DOC) and biodegradable DOC (BDOC) were also recorded. A laboratory experiment on ¹⁴C-glucose uptake and DOC dynamics was also performed by incubating natural biofilms in microcosms. On the basis of our field (annual DOC budget) and laboratory results, we conclude that light-grown biofilm is, on annual average, a net DOC consumer. This biofilm showed a high monthly variability in DOC uptake/release rates, but, on average, the annual uptake rate was greater than that of the dark-grown biofilm. The higher algal biomass and greater structure of the light-grown biofilm may enhance the development of the bacterial community (bacterial biomass and activity) and microbial heterotrophic activity. In addition, the light-grown biofilm may promote abiotic adsorption because of the development of a polysaccharide matrix. In contrast, the dark-grown biofilm is highly dependent on the amount and quality of organic matter that enters the system and is more efficient in the uptake of labile molecules (higher ¹⁴C-glucose uptake rate per mgC). The positive relationships between the extracellular enzymatic activity of biofilm and DOC and BDOC content in flowing water indicate that biofilm metabolism contributes to DOC dynamics in fluvial systems. Our results show that short-term fluvial DOC dynamics

is mainly due to the use and recycling of the more labile molecules. At the river ecosystem level, the potential surface area for biofilm formation and the quantity and quality of available organic carbon might determine the effects of biofilm function on DOC dynamics.

Introduction

Dissolved organic matter (DOM) comprises most of the reduced C in streams and rivers [48] and is removed from or added to water through biotic and abiotic pathways [35]. The microorganisms in the water column or within attached biofilms in the river feed on organic matter transported by the water [23]. The uptake rate of organic compounds is heavily related to their lability, and microorganisms show a faster and preferential use of the most labile and fresh molecules [5, 32]. Bacterial utilization of DOM is determined by its size and diagenetic state [1]. However, the availability of dissolved organic carbon (DOC) for the epilithic bacterial community may be more dependent on the composition of organic compounds rather than molecular weight [12]. Bioavailability of DOC for bacteria in fluvial ecosystems is related to variations in the composition of DOC (H/C, O/C ratios [27]). However, a large part of DOC that enters the system is made up of humic substances and polymeric molecules [48], which require extracellular enzyme activity for heterotrophic uptake [6]. It has been suggested that, in fluvial ecosystems, labile compounds are quickly recycled, mainly within the benthic biofilm, while more refractory substances may be transported further before uptake [21].

In river ecosystems, the benthic community processes organic matter [26, 30]. Surfaces of the streambed are covered by structured autotrophic–heterotrophic as-

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semblages embedded within polysaccharidic material, thereby forming a biofilm [24]. These biofilms are major sites for the uptake and storage of fluvial DOC [3, 19] and contribute significantly to C cycling in rivers and streams. Epilithic microbial communities feed on allochthonous and/or autochthonous organic matter.

Carbon cycling also occurs within the biofilm [49]. The close contact between the algal and the heterotrophic community in attached biofilms favors the use of algal material by microorganisms within the biofilm [16, 31]. Algal accumulation and activity enhance the heterotrophic community's use of organic matter by increasing the amount of substrate available for bacteria [8, 38]. Labile DOC in flowing water could be the primary control factor of epilithic bacteria, while epilithic algae could stimulate bacterial growth in later phases [43]. The release of photosynthetically produced organic C and its use by bacteria in the microphytobenthos has also been observed in sediment [15].

Although a few studies have examined biofilm DOC uptake rates in rivers either *in situ* or by laboratory experiments [11, 19, 23], little information is available on the effect of biofilm structure and metabolism on uptake. Here we elucidate whether biofilms act as sinks or sources of fluvial DOC, depending on their structure and biomass accumulation. In particular, we hypothesized that (i) light-grown biofilms have a low DOC consumption rate because of high internal recycling (high-quality algal-released DOC available for heterotrophs within the biofilm); (ii) dark grown biofilms are net consumers of DOC; and (iii) the DOC budget is affected not only by community composition (algae, bacteria, microfauna) but also by the structural components of the biofilm (C and N content), which may directly affect DOC recycling. To contrast these hypotheses, a field study was performed along an open channel and also a dark pipe directly derived from the river. Whereas the former was subjected to light irradiation, thereby providing the conditions for the development of autotrophic organisms, the dark pipe allowed growth of only heterotrophic organisms. The use of these two systems allowed us to circumscribe the effect of DOC transport and storage in the water mass and the biofilm, and we avoided the influence of the hyporheic zone and groundwater.

Methodology

Sampling Area. The Ebro River, with a mean discharge of $500 \text{ m}^3 \text{ s}^{-1}$, drains $85\,550 \text{ km}^2$ of the NE Iberian Peninsula (Fig. 1). Because of the location of its headwaters and tributaries, this river has a nivo-pluvial regime with a low flow during the summer. The sampling area was located in the last 50 km of the river, where water is diverted through two main irrigation channels at a rate of $20 \text{ m}^3 \text{ s}^{-1}$. Approximately 15 km

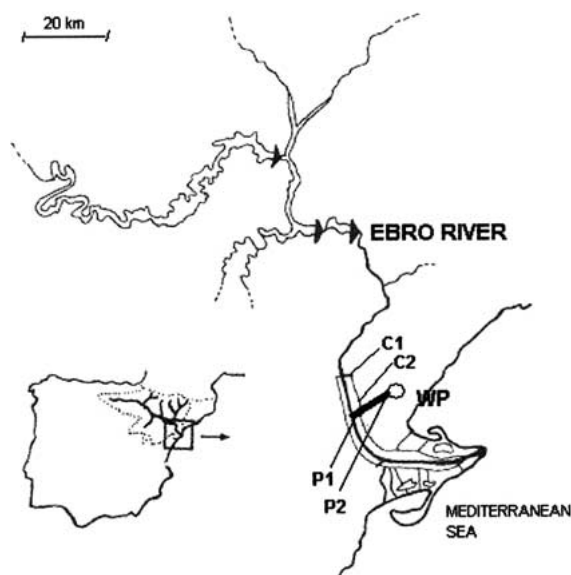


Figure 1. Sampling sites in the River Ebro. C1: channel 1; C2: channel 2; P1: pipe 1; P2: pipe 2; WP: water plant.

downstream from the beginning of the open channels, a pipe, 15 km long, transports water from one of the channel to a treatment plant, “Consorti d’Aigües de Tarragona” (CAT), which provides drinking water to ~ 1 million people. The sampling sites were located at the beginning (channel 1, C1) and at the end of the open channel (channel 2, C2), and at the beginning (pipe 1, P1) and at the end of the pipe (pipe 2, P2) (Fig. 1). The water channel is 10 m wide and 2.65 m deep. The pipe measures 1.6 m in diameter. Water flow was on average 1 m s^{-1} in both systems. Mean residence time in the channel and the pipe was 4.2 h. Mean incident light at midday at 20–40 cm depth was $845.9 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (SD = 493.3, $n = 16$).

Sampling Strategy

Annual Assessment. Etched 1 cm^2 glass substrata (400) were immersed at points C1, C2, P1, and P2 for 2 months before sampling to allow biofilm colonization. Special structures were designed to immerse the substrata within the channel and pipe. Substrata were inserted in Plexiglas racks (100 substrata per rack) which, in turn, were attached to a metal plate fixed to the sides of the channel (sites C1 and C2). They were then immersed at a depth of 20–40 cm. A metal box ($\sim 1 \text{ m}^3$) was used to immerse racks at P1. At P2, a specific holder 1.6 m long (the whole pipe diameter) was used. All substrata were placed parallel to water flow. Substrata were collected in September 2000, November 2000, May 2001, and July 2001. Samples were analyzed for biofilm structure (bacterial density, algal density and composition, chlorophyll, C and N biofilm content) and function (extracellular enzyme activity).

Water samples were collected monthly from September 2000 to November 2001 to analyze physical and chemical parameters, DOC and biodegradable DOC (BDOC).

In November 2001 and April 2002 water samples from C2 and P2 were also collected for the analysis of potential extracellular enzyme activity and algal density in water.

Experimental Approach. In April 2002, glass substrata were collected from C2 and P2. These samples were taken directly to the laboratory to measure ^{14}C -glucose uptake and DOC uptake and/or release rates in microcosm incubations. Samples were also analyzed for biofilm structure and function. Furthermore, denaturing gradient gel electrophoresis (DGGE) analysis was performed. Water samples from the four sampling sites were also collected for analysis of DOC, BDOC, and physical and chemical parameters.

Physical and Chemical Parameters. Water samples from the four sampling sites were analyzed monthly for conductivity, pH, ammonium, nitrite, nitrate, dissolved inorganic phosphorus, and carbonates, following standard methods [2]. DOC was measured using a total organic carbon analyzer (TOC-5000, Shimadzu). Particulate organic carbon (POC) was measured at three sampling times (in triplicate) by filtering 1 L of water on precombusted and preweighed filters (Whatman GF/F). The filters were dried (48 h at 110°C) and combusted (4 h at 450°C). The ash-free dry weight was calculated and transformed to organic C by the constant 2.4 [25].

BDOC was measured following the procedure described by Servais et al. [42]. Once taken, samples (five replicates for each sampling site) were incubated immediately for 28 days at room temperature (20–24°C) and in the dark. The glass flasks and ampoules used were previously heated for 4 h at 550°C to prevent the release of organic C. All DOC samples were fixed with sodium azide (2.7 mM) and preserved at 4°C until analysis.

Biofilm Structure

Bacterial Density. Bacterial density was estimated in triplicate after sonication (90 s, 40 W power, 40 kHz frequency). After appropriate dilution, fixed samples were stained for 5 min with DAPI (4,6-diamidino-2-phenylindole; 2 $\mu\text{g mL}^{-1}$ final concentration) and passed through 0.2 μm irgalan black-stained polycarbonate filters (Nuclepore). Bacteria were then counted under a fluorescence microscope (Nikon) at 1250 \times magnification [36]. Fifteen fields were counted per filter for a total of 400–800 organisms.

Chlorophyll Density. Chlorophyll density on the glass substrata (three replicates for each sampling site) was measured after extraction in 90% acetone and so-

nication (4 min). A second and third extraction was necessary in some biofilms until all chlorophyll had been extracted. Chlorophyll concentration was measured spectrophotometrically (PerkinElmer, Lambda UV/VIS spectrophotometer) after filtration (Whatman GF/F) of the extract, following the method described by Jeffrey and Humphrey [17]. The ratio of chlorophyll to carotenoids and/or chlorophyll degradation products (OD430/OD665, [25]) was also calculated.

Algal Composition and Abundance. Glass substrata (three replicates for each sampling site) were fixed with formalin to study algal composition and abundance. Substrata were sonicated (3 min) to extract all algae from the biofilm, and samples were then observed under a light microscope.

C and N Biofilm Content. Five mL of distilled water was added to the glass substrata, which were then sonicated (4 min) and scraped with a spatula. The extract was then filtered (precombusted filters Whatman GF/F). Successive extractions (2–3) were performed to ensure total extraction of the colonized biofilm. Filters were dried (2 days at 110°C) and analyzed for C and N with a 1500 Carlo Erba C/N Analyzer using vanadium pentoxide as the oxidation catalyzer.

Denaturing Gradient Gel Electrophoresis (DGGE). Samples from C2 and P2 (five replicates) collected in April 2002 were analyzed by DGGE. Universal PCR primers, GC27F and 517R, which target the regions (V1-3) of 16S rDNA gene of Eubacteria were used for amplification [22, 29]. A “touch-down” PCR program designed by Kilb et al. [22] was run with 10 μL of the biofilm suspension as target. DGGE analysis of the PCR products (~527 bp) by means of the D Code System (Bio-Rad Laboratories GmbH, Germany) was performed using gels with a gradient of 40 to 70% denaturant (formamide, urea). The DNA bands in the gels were subsequently stained with SYBR Green I (Molecular Probes). Pairwise community similarities were quantified using the Sørensen index [29].

Extracellular Enzyme Activity. The extracellular enzymes lipase (EC 3.1.1.3), leucine-aminopeptidase (EC 3.4.11.1), β -glucosidase (EC 3.2.1.21), β -xylosidase (EC 3.2.1.37), and phosphatase (EC 3.1.3.1-2) were measured using fluorescent-linked substrates (methylumbelliferyl, MUF, but aminomethylcoumarin, AMC, for the peptidase). Immediately after sampling, glass substrata were incubated at river temperature for 1 h in the dark in a shaking bath. Incubations were performed at a range of substrate concentrations—0.1, 10, 300, and 600 μM —in order to calculate saturation curves. Blanks and standards of MUF and AMC (0–100 μM) were also incubated. At

Table 1. Physical and chemical characteristics at the four sampling sites of the channel-pipe system in the River Ebro during the study period (September 2000–November 2001)

	Channel 1	Channel 2	Pipe 1	Pipe 2
Conductivity ($\mu\text{S cm}^{-1}$)	832.7 (199.5)	829.6 (199.8)	833.3 (199.1)	833.5 (195.3)
PH	8.2 (0.1)	8.2 (0.1)	8.2 (0.1)	8.1 (0.1)
NO ₃ -N (mg L ⁻¹)	2.07 (0.51)	2.12 (0.64)	2.13 (0.62)	2.14 (0.51)
NO ₂ -N (mg L ⁻¹)	0.018 (0.010)	0.019 (0.009)	0.018 (0.011)	0.014 (0.011)
NH ₄ -N (mg L ⁻¹)	0.047 (0.045)	0.037 (0.033)	0.027 (0.019)	0.026 (0.024)
PO ₄ -P (mg L ⁻¹)	0.095 (0.078)	0.091 (0.073)	0.079 (0.069)	0.086 (0.083)
CaCO ₃ (mg L ⁻¹)	161.3 (11.4)	161.2 (10.8)	161.0 (10.4)	159.9 (11.1)
POC (mg L ⁻¹)	0.90 (0.34)	0.83 (0.25)	0.97 (0.48)	0.90 (0.49)
DOC (mg L ⁻¹)	2.34 (0.45)	2.31 (0.42)	2.26 (0.63)	2.20 (0.39)
BDOC (mg L ⁻¹)	0.57 (0.67)	0.42 (0.37)	0.57 (0.57)	0.36 (0.25)

Values are means of monthly values and standard deviations in parentheses ($n = 14$). POC values are averages of three sampling times.

the end of the incubation, glycine buffer at pH 10.4 was added (1/1, v/v) and fluorescence was measured at 365/455 nm excitation/emission for MUF and 364/445 nm excitation/emission for AMC (Kontron, SFM25). All substrata and standards were prepared with filter-sterilized river water (0.2- μm pore-size cellulose nitrate membrane filters). Enzymatic kinetic parameters, V_{max} (maximal reaction velocity), and K_m (apparent Michaelis constant), were calculated by nonlinear regression analysis [40]. The turnover time (K_m/V_{max} ratio) was also calculated after transformation of the parameters to the same units. Potential enzymatic activity in the water was measured in a similar way by incubating 10 mL of water with 100 μL of concentrated artificial substrate to a final concentration of 600 μM .

¹⁴C-Glucose Assimilation and DOC Dynamics.

The uptake of ¹⁴C-glucose and DOC uptake or release rates by the biofilms from C2 and P2 were measured by incubation of colonized glass substrata in microcosms at the same temperature as found in the field (16.5°C). Each microcosm consisted of a glass jar (19 cm in diameter, 9 cm high) and 18 colonized glass substrata attached to the bottom by a plastic rod. Water (1.5 L) from the corresponding sampling site was continuously recirculated with a pump at a current of 0.3 m s⁻¹ on average. To measure ¹⁴C-glucose uptake, four microcosms plus a control were studied for each sampling site. The control microcosm contained uncolonized glass substrata and 0.2- μm filtered river water. For each microcosm, 5 μCi (1 ml) of ¹⁴C-glucose (specific activity 303 mCi mmol⁻¹, PerkinElmer) was added to a final concentration of 3.3 $\mu\text{Ci L}^{-1}$. Similar ¹⁴C-glucose concentrations were used for incubations of epilithic and wood biofilms [34, 44]. Three replicate water samples (1 ml) were taken from each microcosm at 0, 15, 30, 45, 60, 90, and 120 min. 15 ml of scintillation liquid (Biogreen 1, Scharlau) was then added to each sample. At the end of the experiment, three glass substrata were collected from each microcosm to measure ¹⁴C-glucose uptake by the biofilm. Each sub-

strate was gently rinsed with distilled water, sonicated for 2–3 min (with 5 mL of distilled water), and then 15 mL of scintillation liquid was added. ¹⁴C-Glucose abiotic adsorption was measured by using the glass substrata incubated in the control microcosm. Radioactivity was measured in a Packard Tri-Carb 1500 liquid scintillation analyzer (3 min per vial).

In a separate experiment, DOC uptake or release rates by the biofilms in the channel and pipe were tested by incubating colonized glass substrata in microcosms (same design as above) and changes in DOC and BDOC concentrations were recorded. Samples for DOC analyses (two 15-mL replicates) were taken at 0, 1, 2, 4, 6, and 8 h. They were collected with a syringe, filtered with precombusted Whatman GF/F filters, and preserved with sodium azide (2.7 mM) in the refrigerator until analysis. For the pipe biofilms, four microcosms (colonized biofilm plus water: P-BW) and two water controls (microcosms with pipe water and uncolonized glass substrata: P-WC) were considered. Incubation was performed in dark conditions (wrapped in aluminium foil). For the channel biofilms, light and dark conditions were simulated. Four microcosms (CD-BW) and two water controls (CD-WC) were incubated in the dark, and four microcosms (CL-BW) and two water controls (CL-WC) in the light (under artificial light, providing incident light of 180 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). A blank microcosm with uncolonized glass substrata and 0.2 μm filtered water was also analyzed. At the beginning of the experiment, we analyzed the DOC and BDOC concentrations of the four sampling points. At the end, the BDOC content of each microcosm (two replicates) was measured.

Statistical Analyses. The differences in the physical and chemical parameters among C1, C2, P1, and P2 were analyzed by *t*-test for paired values. For the comparison of DOC and BDOC between C1 and C2, and P1 and P2, from the same sampling time, a two-tailed *t*-test was used. The Pearson correlation coefficient was calculated for the physical and chemical parameters in the four

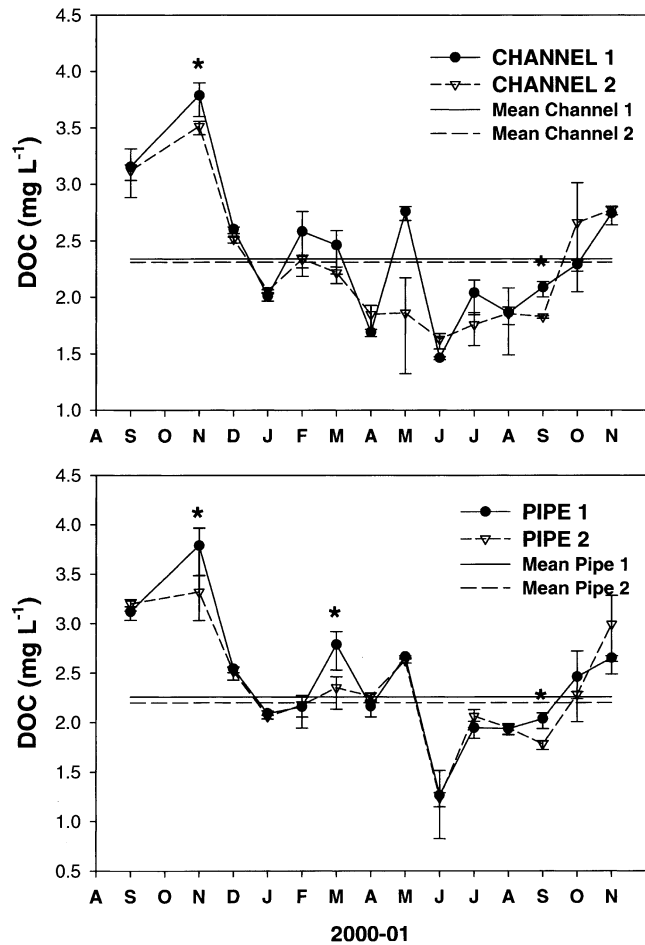


Figure 2. DOC dynamics along the channel and the pipe during the study period (September 2000–November 2001). Monthly values are means \pm standard errors ($n = 3$). Significant differences between sites 1 and 2 are indicated by asterisks (t -test, $P < 0.05$). Horizontal lines indicate annual means.

sampling sites and also for extracellular enzyme activity and turnover times. Differences in extracellular enzymatic activity between sites were analyzed by one-way analysis of variance followed by a Tukey's multiple comparison test for each activity.

Analyses of variance (ANOVA) were performed for the DOC and BDOC variations in the microcosms after 8 h of incubation.

River DOC Budget. The DOC and BDOC uptake or release rates of the channel and pipe were calculated by means of the DOC and BDOC balance between sites C1 and C2, and P1 and P2, respectively, at the sampling times when differences between sites were significant. The DOC and BDOC balance was transformed to $\mu\text{g cm}^{-2} \text{h}^{-1}$ by knowing the total water volume and surface area potentially covered by biofilm in each system (357,750 m^3 and 204,900 m^2 for the channel, 30,159 m^3 and 75,398 m^2 for the pipe), and the travel time of certain water

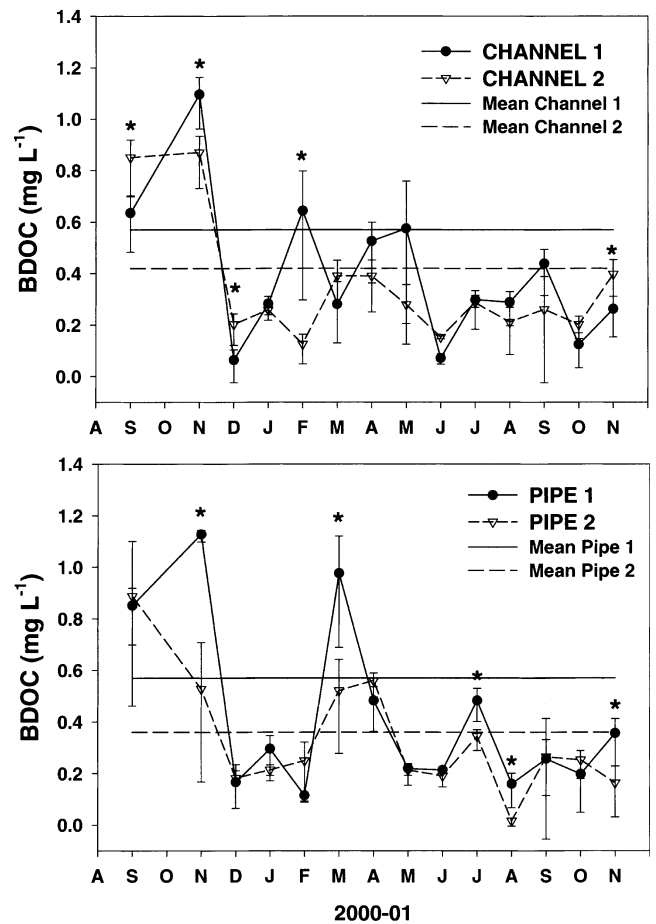


Figure 3. BDOC dynamics along the channel and the pipe during the study period (September 2000–November 2001). Monthly values are means \pm standard errors ($n = 5$). Significant differences between sites 1 and 2 are indicated by asterisks (t -test, $P < 0.05$). Horizontal lines indicate annual means.

parcel from C1 to C2 and P1 to P2, respectively. Mean annual uptake or release rates were calculated from monthly results (November 2000–October 2001), and a value of zero was assigned when nonsignificant differences were obtained between sampling sites.

Results

Physical, Chemical, and Biological Parameters of the Flowing Water. The Ebro River water has a high nutrient content, especially for dissolved N (Table 1). Mean water pH is 8.2 and temperatures range between 8.9°C in winter and 25°C in summer (annual mean 18.1, SD 5.7). The chemical characteristics of the river water (except BDOC) showed no significant differences between the four sampling points (t -test, $P > 0.2$).

The DOC and BDOC concentrations were higher at the beginning than at the end of the channel and pipe (Figs. 2 and 3), although significant differences were

Table 2. Composition and structure of the biofilms grown on the glass substrata in the channel and the pipe

	Channel 1	Channel 2	Pipe 1	Pipe 2
Chlorophyll ($\mu\text{g cm}^{-2}$)	3.96 (2.51)	6.07 (4.40)	0.46 (0.37)	0.18 (0.16)
OD430/665	2.92 (1.12)	2.59 (0.80)	5.08 (4.24)	4.54 (2.54)
Algae cell $\times 10^5 \text{ cm}^{-2}$	45.64 (71.83)	319.59 (339.09)	1.00 (1.25)	0.74 (1.18)
Bact. cell $\times 10^7 \text{ cm}^{-2}$	1.07 (0.55)	0.53 (0.32)	0.32 (0.15)	0.28 (0.10)
C ($\mu\text{g cm}^{-2}$)	600.44 (310.04)	675.55 (638.94)	69.46 (34.00)	228.47 (293.53)
N ($\mu\text{g cm}^{-2}$)	52.84 (36.15)	48.06 (36.51)	6.65 (5.18)	76.80 (108.66)
C/N	14.09 (3.33)	14.18 (4.43)	13.26 (2.92)	9.53 (5.23)

Values are means of the four sampling times and standard deviations in parentheses ($n = 12$).

found only for BDOC in the latter (t -test, $P = 0.05$, Fig. 3). Monthly results for DOC and BDOC showed significant differences between C1 and C2, and between P1 and P2 on several occasions (Figs. 2 and 3). The production of BDOC along the channel was observed in September and December 2000 and in November 2001, and consumption was observed in November 2000 and February 2001 (Fig. 3). Significant consumption of BDOC at five sampling times along the pipe was observed (Fig. 3).

Forty percent of the total cell density in the water column samples corresponded to cells of benthic origin. Algal cell densities in the samples from the channel and pipe ranged from 127 to 494 cell mL^{-1} and from 67 to 154 cell mL^{-1} , respectively. Extracellular enzymatic activity measured in the water column was clearly higher in April 2002 than in November 2001. Respectively for the two periods, β -glucosidase and β -xylosidase activities ranged between 0.95 and 36.56 $\text{nmol L}^{-1} \text{h}^{-1}$, phosphatase between 31.1 and 232.5 $\text{nmol L}^{-1} \text{h}^{-1}$, peptidase between 1619 and 8194 $\text{nmol L}^{-1} \text{h}^{-1}$, and lipase between 0.77 and 19.81 $\text{nmol L}^{-1} \text{h}^{-1}$. For most enzymatic activities, the pipe samples showed lower activity than those from the channel.

Biofilm Structure. The biofilms in C1 and C2 were characterized by a high C and N content (C/N ratio of 14.1, Table 2). The chlorophyll content did not significantly differ between these two sites (t -test, $P = 0.17$), reaching 2.9 $\mu\text{g cm}^{-2}$ in autumn–winter and 7.1 $\mu\text{g cm}^{-2}$ in spring–summer. The biofilms were mainly composed of benthic cyanobacteria (especially in autumn–winter), and diatoms and green algae (especially in spring–summer). Some of the taxa were of planktonic origin. Bacterial density ranged between 0.21 and 1.84×10^7 cell cm^{-2} , values being significantly higher in C1 than in C2 (t -test, $P = 0.02$).

Although the biofilms at P1 and P2 had a significantly lower C content than C1 and C2, they showed a similar N content, which resulted in a lower C/N ratio, especially for the P2 samples (Table 2). Chlorophyll and algal density was very low and had a high OD430/OD665 ratio. A few diatoms and green algae, mainly planktonic, were detected in the pipe biofilm. Bacterial cell density was on average $0.29 \times 10^7 \text{ cm}^{-2}$ (differences between P1

and P2 were not significant, t -test, $P = 0.52$), and significantly lower than C1 and C2 (t -test, $P = 0.02$). Ciliates, fungi, and nematodes were observed in P1 and P2.

DGGE analysis showed more bands in the microbial communities in the channel than in the pipe (Fig. 4).

Control P2a P2b C2a C2b

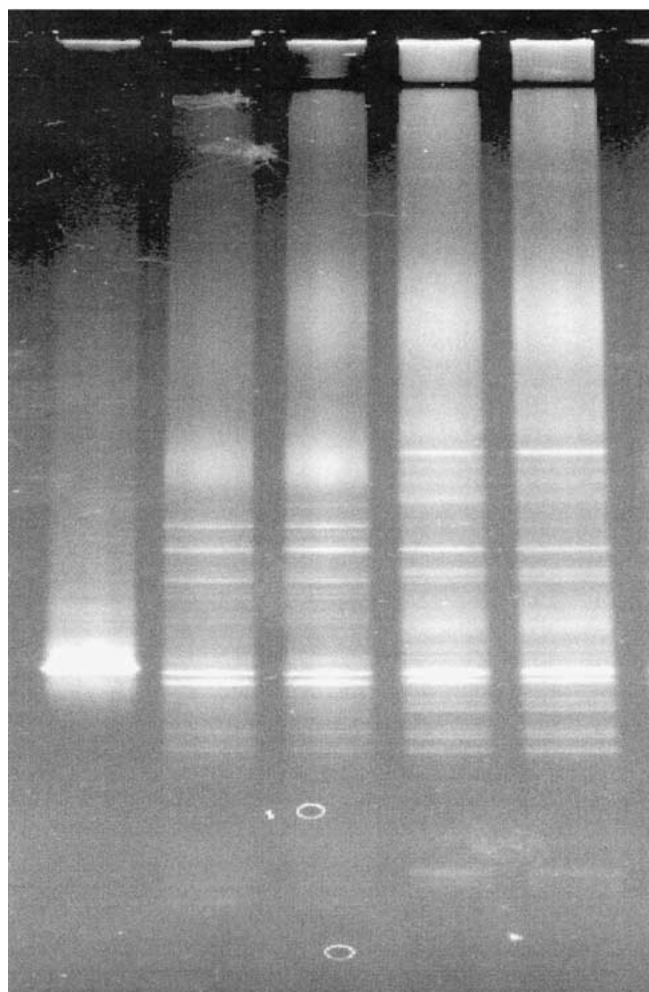


Figure 4. DGGE population analysis of bacteria in biofilms from the pipe (P2) and the channel (C2). The positive control is *Escherichia coli*. In each slot, 7 μL of PCR-products was applied.

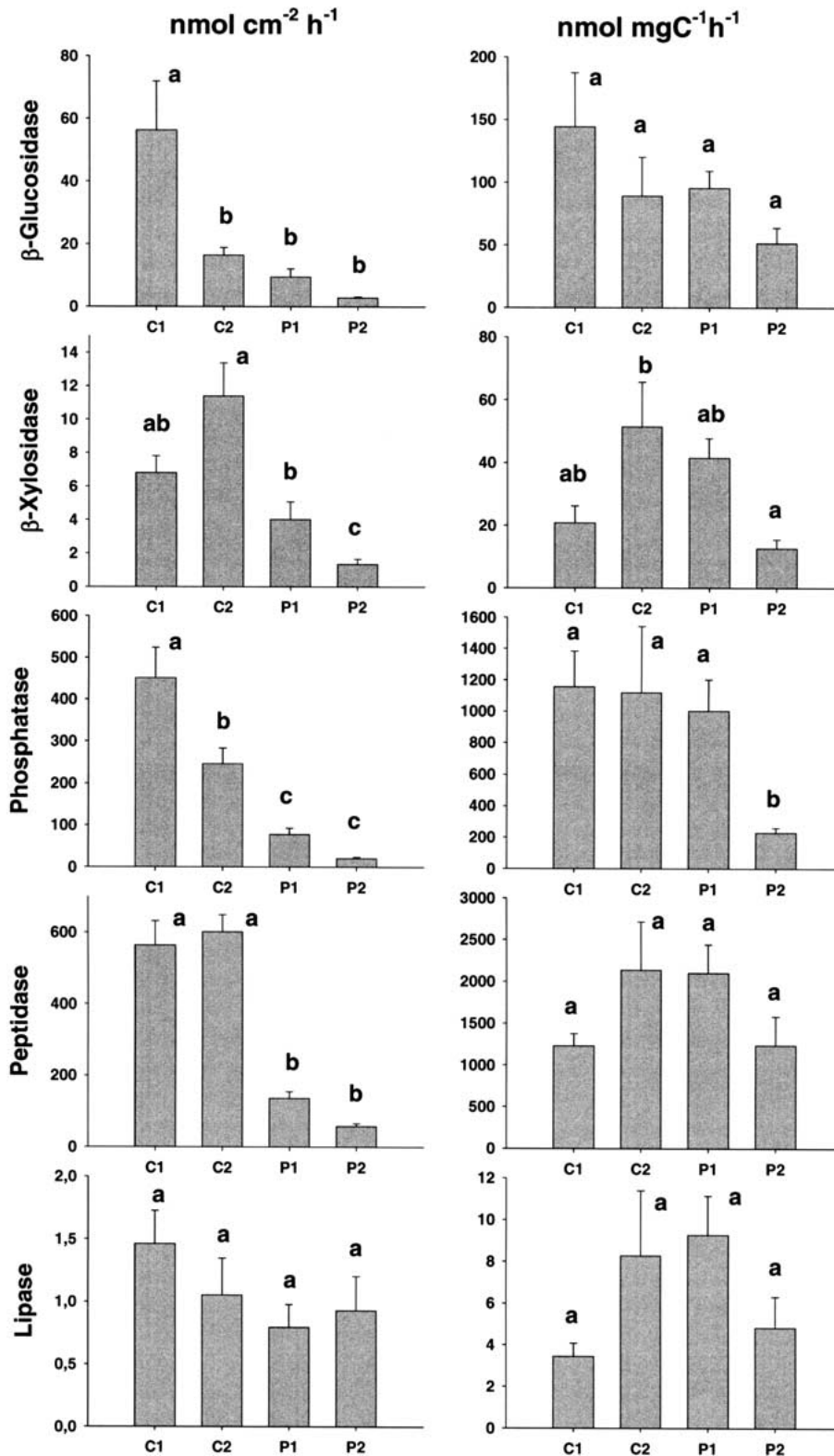


Figure 5. Extracellular enzymatic activities in biofilms from the channel and the pipe. In the left column results are expressed as nmol per square centimeter of biofilm per hour, and in the right as nmol per mg C in the biofilm per hour. Values are means of monthly results (September 2000, November 2000, May 2001, July 2001) + standard errors ($n = 16$). Lowercase letters indicate homogeneous subgroups after Tukey's test, $P < 0.05$.

Comparison between the bands of the two systems resulted in similarity index of 0.56. Similarity between replicates was ~ 0.96 –1.

Biofilm Function. The extracellular enzymatic activities per cm² of biofilm were lower in the pipe than in the channel, except for lipase (Fig. 5). However, when

Table 3. Turnover time of substrate hydrolysis (Tt) of the extracellular enzyme activities in the biofilms of the channel and the pipe

	Channel 1	Channel 2	Pipe 1	Pipe 2
β -Glucosidase Tt (h)	10.5 (13.8)	10.9 (14.3)	4.7 (5.4)	17.8 (21.9)
β -Xylosidase Tt (h)	18.3 (33.0)	31.1 (55.2)	4.2 (5.8)	25.3 (24.3)
Phosphatase Tt (h)	2.3 (3.5)	2.2 (2.3)	2.9 (4.2)	5.1 (6.0)
Peptidase Tt (h)	1.2 (1.1)	0.6 (0.3)	0.8 (0.5)	3.3 (4.3)
Lipase Tt (h)	7.0 (5.4)	19.1 (26.7)	20.2 (24.4)	20.4 (18.5)

Values (in hours) are means for the four sampling periods and standard deviations (in parentheses).

enzymatic activities were calculated per mg of C, differences were less obvious. The ANOVA analysis and the Tukey's test indicated significant differences between sites for β -xylosidase and phosphatase (Fig. 5).

Turnover times of substrate hydrolysis for the distinct enzyme activities (except lipase) were higher for P2 than for P1 (Table 3). The channel communities showed a high turnover time for the β -xylosidase activity, especially at C2.

Several enzymatic activities (considered per mg of C) were significantly correlated with BDOC and DOC concentrations, while turnover times were negatively correlated with these two parameters, especially for the pipe (Table 4). Pipe biofilms showed a positive correlation between most of the enzymatic activities and accumulated chlorophyll (Table 4).

¹⁴C-Glucose Assimilation and DOC Dynamics.

The decrease in water radioactivity during the 120 min biofilm incubation in the microcosms followed a linear model ($r^2 = 0.98$ and $r^2 = 0.99$ for the pipe and the channel microcosms, respectively). The slopes of the linear regressions were $-16.59 \text{ DPM min}^{-1}$ (SD = 4.20) and $-9.37 \text{ DPM min}^{-1}$ (SD = 2.46), for the channel and the pipe microcosms, respectively, leading to incorporation values of the whole system slightly higher than those measured specifically for the biofilm. Although respiration of incorporated ¹⁴C-glucose could occur during the

incubation, thereby leading to an underestimation of the uptake rate, this is reported to be <1% of incorporated ¹⁴C-glucose for epilithic biofilms [34]. The biofilm uptake rate of ¹⁴C-glucose was higher for the channel (Fig. 6). However, when considering the uptake of ¹⁴C-glucose per mg C of biofilm, values were higher in the pipe biofilms (Fig. 6).

DOC dynamics in the microcosms is summarized in Table 5. The channel (CL) microcosms showed a significant increase in DOC and BDOC after 8 h in the light. This increase was higher for those with water but no biofilm than for those with biofilm plus water (Table 5). The budget between the two revealed a net consumption of 0.26 mg L^{-1} of DOC and 0.34 mg L^{-1} of BDOC by the biofilms in the channel. Under dark conditions, the channel microcosms did not show significant differences between DOC after 8 h, but there was a significant increase of 0.18 mg L^{-1} in BDOC. The increase in BDOC in the pipe microcosms was similar to that observed for those of the channel in the dark, and the biofilm did not cause a significant variation (Table 5). Finally, in the blank microcosm, no significant differences in DOC and BDOC concentrations were observed after 8 h (ANOVA, $P = 0.7$). The uptake rates expressed in $\mu\text{g per cm}^2$ per hour are indicated in Table 6.

River DOC Budget. An increase in BDOC between 5.7 and $9 \mu\text{g per cm}^2$ of channel surface per hour

Table 4. Significant Pearson correlations between the extracellular enzymatic activities per mg C and turnover times (T_t), to chlorophyll biofilm content and DOC, BDOC water content for the channel and the pipe

	Channel			Pipe		
	Chl	DOC	BDOC	Chl	DOC	BDOC
β -Glucosidase		0.76*	0.78*	0.80**		
β -Xylosidase				0.75*	0.53	0.51
Phosphatase		0.75*	0.69	0.72*	0.71*	0.89**
Peptidase						
Lipase				0.68*	0.72*	0.90***
β -Glucosidase Tt		-0.82*	-0.78*		-0.56	
β -Xylosidase Tt					-0.50	
Phosphatase Tt		-0.76	-0.71	-0.64	-0.68	-0.68
Peptidase Tt						
Lipase Tt	0.84*	-0.68	-0.61	-0.63	-0.68	-0.71*

Significance is indicated by asterisks, ***: $P < 0.001$, **: $P < 0.01$, *: $P < 0.05$, no asterisk: $P < 0.1$.

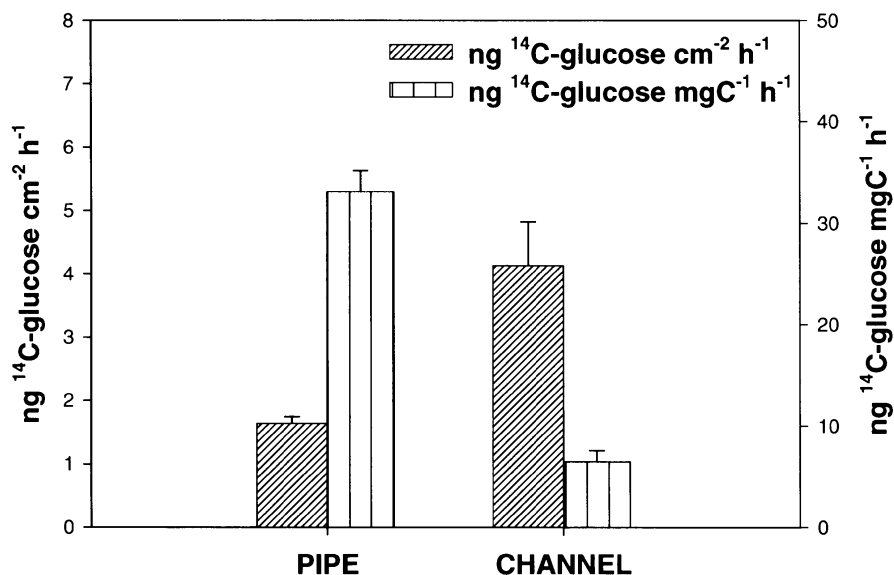


Figure 6. ¹⁴C-Glucose incorporated by the channel and the pipe biofilms expressed as ng per cm² per hour, and ng per mgC per hour. Values are means ± standard errors (*n* = 4).

was estimated in the channel on four occasions. However, a decrease occurred in two other periods, thereby producing a consumption of 9.4–21.7 μg BDOC cm⁻² h⁻¹. Therefore, the mean annual DOC uptake rate was 1.86 μg DOC cm⁻² h⁻¹ and 2.06 μg BDOC cm⁻² h⁻¹ (Table 6).

The pipe showed a net decrease of BDOC in five sampling times. The uptake rate ranged between 1.4 and 11.7 μg cm⁻² h⁻¹, while the mean annual DOC uptake rate for this system was 1.24 μg DOC cm⁻² h⁻¹ and 1.52 μg BDOC cm⁻² h⁻¹ (Table 6).

Discussion

The parallel analysis of biofilm structure and function, and river DOC dynamics in a controlled system, make feasible to elucidate the possible implications of biofilm structure and function for the C cycling in the fluvial ecosystem. The major autotrophic biomass, C content,

bacterial richness, and extracellular enzyme activities at the light-grown biofilm leads to contrasting implications for fluvial C cycling: the biofilm exerts a high DOC uptake from the flowing water but, at the same time, there exists an internal recycling of high-quality compounds within the biofilm and a release of algal exudates to the flowing water (especially in high primary production period). In contrast, the mainly heterotrophic biomass accumulated at the dark-grown biofilm, the minor bacterial richness and lower extracellular enzyme activities make this biofilm highly dependent on the DOM input from the flowing water yet showing a constant (but lower than the light-grown) DOC uptake rate and the preferential and efficient use of the more labile molecules.

The higher DOC uptake rate for the light-grown biofilm may be due to several processes that act simultaneously: (1) biotic factors, that is, major heterotrophic activity, and (2) abiotic factors, that is, biofilm DOM

Table 5. DOC and BDOC balance (mg L⁻¹) in the microcosms

	DOC in	DOC fin	ΔDOC	BDOC in	BDOC fin	ΔBDOC
CL-BW	2.73 (0.14)	3.88 (0.42)	1.15*	0.48 (0.11)	1.54 (0.31)	1.06*
CL-WC	2.73 (0.14)	4.14 (4.09–4.18)	1.41*	0.48 (0.11)	1.88 (1.92–1.85)	1.40*
CL-Biofilm			-0.26			-0.34
CD-BW	3.10 (0.52)	3.19 (0.42)	0.09	0.48 (0.11)	0.99 (0.42)	0.51*
CD-WC	3.10 (0.52)	3.17 (2.66–3.67)	0.07	0.48 (0.11)	0.81 (0.80–0.82)	0.33*
CD-Biofilm			0.02			0.18
P-BW	2.83 (0.26)	2.96 (0.23)	0.13	0.36 (0.09)	0.80 (0.12)	0.44*
P-WC	2.83 (0.26)	2.98 (2.96–3.01)	0.15	0.36 (0.09)	0.79 (0.76–0.82)	0.43*
P-Biofilm			-0.02			0.01

Values (in mg L⁻¹) indicate initial DOC and BDOC (DOC in, BDOC in, mean and SD or range for the WC) and final DOC and BDOC at the end of the 8 h incubation in the microcosms (DOC fin, BDOC fin, mean and SD or range for the WC). The balance is also indicated (ΔDOC). Significant differences between initial and final values are indicated by the asterisk (ANOVA, *P* < 0.01). The balance due to the biofilm is also expressed as the difference between that in the BW and WC microcosms. CL: Channel light; CD: channel dark; P: pipe; BW: biofilm and water; WC: water control (water and noncolonized substrata).

Table 6. DOC/BDOC biofilm uptake rates calculated from the field results and the microcosm experiment expressed in $\mu\text{g cm}^{-2} \text{h}^{-1}$

Biofilm C uptake rate ($\mu\text{g cm}^{-2} \text{h}^{-1}$)	Field annual C-balance		Microcosm C-balance	
	DOC	BDOC	DOC	BDOC
Channel	-1.86 (4.34)	-2.06 (7.07)	-2.71	-3.54
Pipe	-1.24 (2.64)	-1.52 (3.39)	ns	ns

The uptake rates from the field annual C balance express the annual average uptake rate in the whole channel and pipe systems (mean values and standard deviations, $n = 12$). The results from the microcosms C balance are specifically the uptake rates of biofilm grown at sites C2 and P2 in April 2002 (see Table 5). ns: Not significant.

adsorption and photochemical transformation of DOM. The development of an active autotrophic community in a light biofilm might provide appropriate colonization sites for heterotrophs and may also increase the amount of organic C available. Although light-grown biofilms show a slightly, but significantly, higher bacterial density (18 for an oligotrophic stream [38]), other studies report that differences in density between biofilms incubated in the light and dark are not significant [8, 43]. Finally, a higher bacterial density was observed in biofilms incubated in the dark in a eutrophic river [18]. The higher extracellular enzymatic activity observed in the light-grown biofilms indicates that heterotrophic degradation of polymeric organic matter occurs. However, this degradation activity may be caused by the use of allochthonous DOC sources taken directly from the flowing water or through the use of organic compounds from the biofilm. Changes in extracellular enzyme activities when changing organic carbon supply were observed especially when communities were exposed to labile DOM [9]. Moreover, a further biotic mechanism could be enhancement of DOM uptake or photoheterotrophy by exposure to light [33]. The photochemical degradation of DOM [28] may also contribute to the higher DOC uptake rate of the autotrophic biofilm, although Wiegner and Seitzinger [50] conclude that microbial degradation is more important than photochemical processes in degrading river dissolved organic matter. Finally, biofilm adsorption may produce an abiotic retention of organic matter [10]. However, although all of these mechanisms are possible, in our study the structure of the light-grown biofilm indicates that abiotic DOM adsorption and microbial heterotrophic activity may be responsible for the DOC uptake rate. In the Ebro River, the light-grown biofilm had a significant contribution of filamentous algal species with epiphytic diatoms and cyanobacteria which conferred a complicated biofilm structure, as shown by the high C content and C/N ratio and the DGGE analysis.

The structural and metabolic characteristics of the biofilms grown in light suggest internal recycling of DOM. Although ^{14}C -glucose uptake was higher for the light-grown biofilm, its efficiency of use (^{14}C -glucose uptake per mgC in the biofilm) was lower than for the

dark-grown biofilm. This observation indicates that the heterotrophic community may use a pool of easily degradable organic matter from the neighboring autotrophic component (algal exudates and/or excretion products [16]). Therefore, in the light-grown biofilm, the microbial community may not be limited by available organic C. A similar behavior was observed at the end of a biofilm colonization experiment, confirming the role of epilithic algae as a source of organic C for heterotrophs [43]. Several studies have reported the positive effect of algal biomass on biofilm metabolism [18, 38, 39]. Moreover, the development of a polysaccharide matrix (expressed by the high C/N ratio) could act as a reserve of C and matrix enzymes [13, 45], thereby enhancing internal DOM recycling.

In contrast, the dark-grown biofilm (characterized by a lower C/N content and lower bacterial richness) might use mainly allochthonous DOM sources. Changes in bacterial community structure (band patterns in the DGGE) have been observed in aquatic systems and related to changes in quality and quantity of available organic carbon [7, 47]. The lower bacterial richness measured for the biofilms grown in the pipe (site P2) might indicate the use of a lower quality organic matter source [47]. The quite constant DOC uptake rate throughout the year of the dark-grown biofilm suggests a limited availability of labile DOC molecules. The rapid response of the dark-grown biofilm to the input of organic compounds is expressed by the positive correlation between the extracellular enzyme activities with chlorophyll accumulated within the biofilm. Although chlorophyll accumulated on the pipe biofilms is highly degraded because of senescent algal cells, it may still be a crucial source of DOM. Also, a higher efficiency in the uptake of labile compounds for the dark-grown biofilm suggests a C limitation. The development of a thinner, less complex biofilm in the dark leads to a minor effect of abiotic factors on DOC uptake.

Biofilm metabolism, both in light and dark conditions, is highly related to DOC dynamics, which especially affects the "quality" of organic molecules. This is expressed by the positive correlations of most biofilm extracellular enzyme activities with DOC and BDOC. Fluvial DOC content was related to a lower turnover time

for substrate hydrolysis in the biofilm for most of the enzymes analyzed, indicating that DOC availability enhanced its recycling. The preferential heterotrophic use of the more labile molecules could explain the differences in DOC lability and utilization between sites P1 and P2 and indicates a reactivity decrease from P1 to P2.

The annual average rates of DOC uptake in the light-grown and dark-grown biofilms (2.1 and $1.5 \mu\text{g cm}^{-2} \text{h}^{-1}$ respectively) observed in our study are consistent with the range of values reported for other river systems [11, 19, 23]. The riverine DOC dynamics analyzed by the field results (DOC budget) and laboratory experiments (microcosm incubations) lead to analogous conclusions, but several points must be taken into consideration. The net uptake rate calculated from the DOC budget includes not only the effect of the biofilm but also the variations derived from the flowing water. In the light, BDOC might increase in several periods of the year because of phytoplankton activity, especially in spring. This effect was confirmed through the microcosm experiment. In the case of large rivers, such as the Ebro, the phytoplankton community is well developed [41]. However, the high current velocity, such as that in the Ebro River channel, produces the least favorable conditions for phytoplankton development [14]. Phytoplankton density in the channel was relatively high, especially in April, but there was a high proportion of benthic algae, suggesting that drift could account for a large proportion of the phytoplankton community. Therefore the uptake rate calculated from the DOC balance could underestimate the true DOC uptake rate of the channel biofilm, especially in periods of increased phytoplankton production. Furthermore, this real uptake rate value is complicated by the DOC released by the biofilm [23]. The joint variation of the two sources of DOC, i.e., fluvial DOC and that released from the biofilm, could explain the large seasonal variability observed in the DOC budget in the channel. In the dark pipe, the more constant DOC balance throughout the study period could be related to the less significant effect of the variations in the flowing water and the lower effect of the molecules released by algae from the biofilm.

Both types of biofilms are highly related to fluvial DOC and, especially, BDOC concentrations, which summarize the use of the more labile molecules, which consist of a low (but highly variable) proportion of all DOM entering the system. The function of the dark-grown biofilm (efficient uptake of labile molecules and constant DOC consumption throughout the year) permits the maintenance of low DOC levels. This is important for water management since the presence of DOC in drinking water allows noxious bacterial regrowth in water pipes [20]. From these results we may suggest that use of covered conduits may be advisable to substantially diminish the DOC concentration in the water.

At the river ecosystem level, these results underlie the importance of streambed heterogeneity for the whole C cycling [4]. In a highly heterogeneous stream, where cobbles, rocks, and sand cover the streambed area, there is a higher surface area available for biofilm formation at different physical conditions (light, flow velocity), which might improve microorganism interactions and, therefore, improve C-cycling processes. Apart from biofilm formation, the second key parameter is DOC concentration and composition: low availability of organic carbon for the microbial community might reinforce the relationships between the biofilm and the flowing water. The relationships found between biofilm structure and function and river DOC dynamics of the light- and dark-grown biofilms in the River Ebro channel-pipe system suggest that there is a low availability of fluvial C for the microbial community which can be C limited. This behavior could be related to the low DOC content in the River Ebro water in comparison to other river systems [46]. Finally, it is fair to say that this study offers an insight into a compartment affecting riverine C cycling, but others are substantially important (i.e., subsurface-sand, hyporheic, groundwater, and riparian habitats) and should be accounted for to achieve complete understanding of organic carbon in rivers (i.e., [37]).

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References

1. Amon, RMW, Benner, R (1996) Bacterial utilization of different size classes of dissolved organic matter. *Limnol Oceanogr* 41: 41–51
2. APHA, American Public Health Association (1989) Standard Methods for the Examination of Water and Wastewater, 17th ed.
3. Battin, TJ, Butturini, A, Sabater, F (1999) Immobilization and metabolism of dissolved organic carbon by natural sediment biofilms in a Mediterranean and temperate stream. *Aquat Microb Ecol* 19: 297–305
4. Cardinale, BJ, Palmer, MA, Swan, CM, Brooks, S, LeRoy Poff, N (2002) The influence of substrate heterogeneity on biofilm metabolism in a stream ecosystem. *Ecology* 83: 412–422
5. Cherrier, J, Bauer, JE, Druffel, ERM, Coffin, RB, Chanton, JP (1999) Radiocarbon in marine bacteria: evidence for the ages of assimilated carbon. *Limnol Oceanogr* 44: 730–736
6. Chróst, RJ (1990) Microbial ectoenzymes in aquatic environments. In: Overbeck, J, Chróst, RJ (Eds.) *Aquatic Microbial Ecology: Bi-*

- ochemical and Molecular Approaches. Springer-Verlag, New York, pp 47–78
7. Eiler, A, Langenheder, S, Bertilsson, S, Tranvik, LJ (2003) Heterotrophic bacterial growth efficiency and community structure at different natural organic carbon concentrations. *Appl Environ Microbiol* 69: 3701–3709
 8. Espeland, EM, Francoeur, SN, Wetzel, RG (2001) Influence of algal photosynthesis on biofilm bacterial production and associated glucosidase and xylosidase activities. *Microb Ecol* 42: 524–530
 9. Findlay, SEG, Sinsabaugh, RL, Sobczak, WV, Hoostal, M (2003) Metabolic and structural response of hyporheic microbial communities to variations in supply of dissolved organic matter. *Limnol Oceanogr* 48: 1608–1617
 10. Fischer, H (2002) The role of biofilms in the uptake and transformation of dissolved organic matter. In: Findlay, SEG, Sinsabaugh, RL (Eds.) *Aquatic Ecosystems: Interactivity of Dissolved Organic Matter*. Academic Press, San Diego, pp 285–313
 11. Fischer, H, Sachse, A, Steinberg, CEW, Pusch, M (2002) Differential retention of dissolved organic carbon by bacteria in river sediments. *Limnol Oceanogr* 47: 1702–1711
 12. Ford, TE, Lock, MA (1985) A temporal study of colloidal and dissolved organic carbon in rivers: apparent molecular weight spectra and their relationship to bacterial activity. *Oikos* 45: 71–78
 13. Freeman, C, Lock, MA (1995) The biofilm polysaccharide matrix: A buffer against changing organic substrate supply? *Limnol Oceanogr* 40: 273–278
 14. Friedrich, G, Viehweg, M (1984) Recent developments of the phytoplankton and its activity in the Lower Rhine. *Verh Int Ver Limnol* 22: 2029–2035
 15. Goto, N, Mitamura, O, Hisayoshi, T (2001) Biodegradation of photosynthetically produced extracellular organic carbon from intertidal benthic algae. *J Exp Mar Biol Ecol* 257: 73–86
 16. Haack, TK, McFeters, GA (1982) Nutritional relationships among microorganisms in an epilithic biofilm community. *Microb Ecol* 8: 115–126
 17. Jeffrey, SW, Humphrey, GF (1975) New spectrophotometric equations for determining chlorophylls *a*, *b*, and *c* in higher plants, algae and natural phytoplankton. *Biochem Physiol Pflanz* 167: 191–194
 18. Jones, SE, Lock, MA (1993) Seasonal determinations of extracellular hydrolytic activities in heterotrophic and mixed heterotrophic/autotrophic biofilms from two contrasting rivers. *Hydrobiologia* 257: 1–16
 19. Kaplan, LA, Bott, TL (1983) Microbial heterotrophic utilization of dissolved organic matter in a piedmont stream. *Freshwat Biol* 13: 363–377
 20. Kaplan, LA, Newbold, JD (1995) Measurement of stream-water biodegradable dissolved organic carbon with a plug-flow bioreactor. *Wat Res* 29: 2696–2706
 21. Kaplan, LA, Newbold, JD (2002) The role of monomers in stream ecosystem metabolism. In: Findlay, SE, Sinsabaugh, RL (Eds.) *Aquatic Ecosystems, Interactivity of Dissolved Organic Matter*. Academic Press, San Diego, pp 97–119
 22. Kilb, B, Kuhlmann, B, Eschweiler, B, Preuß, G, Ziemann, E, Schöttler, U (1998) Community structures of different ground-water habitats investigated using methods of molecular biology. *Acta Hydrochim Hydrobiol* 26: 349–354
 23. Kuserk, FT, Kaplan, LA, Bott, TL (1984) In situ measures of dissolved organic carbon flux in a rural stream. *Can J Fish Aquat Sci* 41: 964–973
 24. Lock, MA (1993) Attached microbial communities in rivers. In: Ford, TE (Ed.) *Aquatic Microbiology: An Ecological Approach*. Blackwell Scientific Publications, Oxford, pp 113–138
 25. Margalef, R (1983) *Limnología*. Ediciones Omega SA, Barcelona
 26. Meyer, JL (1988) Benthic bacterial biomass and production in a blackwater river. *Verh Int Ver Limnol* 23: 1832–1838
 27. Meyer, JL (1994) The microbial loop in flowing waters. *Microb Ecol* 28: 195–199
 28. Moran, MA, Zepp, RG (1997) Role of photoreactions in the formation of biologically labile compounds from dissolved organic matter. *Limnol Oceanogr* 42: 1307–1316
 29. Murray, AE, Hollibaugh, JT, Orrego, C (1996) Phylogenetic composition of bacterioplankton from two different California estuaries compared by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Appl Environ Microbiol* 62: 2676–2680
 30. Naiman, RJ, Melillo, JM, Lock, MA, Ford, TE, Reice, SR (1987) Longitudinal patterns of ecosystem processes and community structure in a subarctic river continuum. *Ecology* 68: 1139–1156
 31. Nakano, S (1996) Bacterial response to extracellular dissolved organic carbon released from healthy and senescent *Fragilaria crotonensis* (Bacillariophyceae) in experimental systems. *Hydrobiol* 339: 47–55
 32. Norrman, B, Zweifel, UL, Hopkinson, CS, Fry, B (1995) Production and utilization of dissolved organic carbon during an experimental diatom bloom. *Limnol Oceanogr* 40: 898–907
 33. Paerl, HW, Bebout, BM, Joye, SB, DesMarais, DJ (1993) Microscale characterization of dissolved organic matter production and uptake in marine microbial mat communities. *Limnol Oceanogr* 38: 1150–1161
 34. Paul, BJ, Corning, KE, Duthie, HC (1989) An evaluation of the metabolism of sestonic and epilithic communities in running waters using an improved chamber technique. *Freshwat Biol* 21: 207–215
 35. Petersen, RC, Cummins, KW, Ward, GM (1989) Microbial and animal processing of detritus in a woodland stream. *Ecol Monogr* 59: 21–39
 36. Porter, KG, Feig, YS (1980) The use of DAPI for identifying and counting aquatic microflora. *Limnol Oceanogr* 25: 943–948
 37. Pusch, M, Fiebig, D, Brettar, I, Eisenmann, H, Ellis, BK, Kaplan, LA, Lock, MA, Naegeli, MW, Traunsperger, W (1998) The role of micro-organisms in the ecological connectivity of running waters. *Freshwat Biol* 40: 453–495
 38. Romani, AM, Sabater, S (1999) Effect of primary producers on the heterotrophic metabolism of a stream biofilm. *Freshwat Biol* 41: 729–736
 39. Romani, AM, Sabater, S (2000) Influence of algal biomass on extracellular enzyme activity in river biofilms. *Microb Ecol* 41: 16–24
 40. Romani, AM (2000) Characterization of extracellular enzyme kinetics in two Mediterranean streams. *Arch Hydrobiol* 148: 99–117
 41. Sabater, S, Muñoz, I (1990) Successional dynamics of the phytoplankton in the lower part of the river Ebro. *J Plank Res* 12: 573–592
 42. Servais, P, Anzil, A, Ventresque, C (1989) Simple method for determination of biodegradable dissolved organic carbon in water. *Appl Environ Microbiol* 55: 2732–2734
 43. Sobczak, WV (1996) Epilithic bacterial responses to variations in algal biomass and labile dissolved organic carbon during biofilm colonization. *J N Am Benthol Soc* 15: 143–154
 44. Tank, JL, Winterbourn, MJ (1995) Biofilm development and invertebrate colonization of wood in four New Zealand streams of contrasting pH. *Freshwat Biol* 34: 303–315
 45. Thompson, AJ, Sinsabaugh, RL (2000) Matric and particulate phosphatase and aminopeptidase activity in limnetic biofilms. *Aquat Microb Ecol* 21: 151–159
 46. Thurman, EM (1985) *Organic Geochemistry of Natural Water*. Nijhoff, Dordrecht
 47. van Hannen, EJ, Mooij, W, van Agterveld, MP, Gons, HJ, Laanbroek, HJ (1999) Detritus-dependent development of the mi-

- icrobial community in an experimental system: qualitative analysis by denaturing gradient gel electrophoresis. *Appl Environ Microbiol* 65: 2478–2484
48. Volk, CJ, Volk, CB, Kaplan, LA (1997) Chemical composition of biodegradable dissolved organic matter in streamwater. *Limnol Oceanogr* 42: 39–44
49. Wetzel, RG (1993) Microcommunities and microgradients: linking nutrient regeneration, microbial mutualism, and high sustained aquatic primary production. *Neth J Aquat Ecol* 27: 3–9
50. Wiegner, TN, Seitzinger, SP (2001) Photochemical and microbial degradation of external dissolved organic matter inputs to rivers. *Aquat Microb Ecol* 24: 27–40