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Variability of heterotrophic activity in Mediterranean stream biofilms: A multivariate analysis of physical-chemical and biological factors

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ABSTRACT

Environmental factors responsible for inter-site and temporal variations in extracellular enzymatic activities in benthic biofilms were studied in three Mediterranean streams. Results from canonical correlation analysis suggest that ionic content of the water and chlorophyll *a* content of biofilms (algal biomass) were the main factors accounting for the variability in extracellular enzyme activity of the biofilms in these streams. Water temperature was not an important factor accounting for differences among streams, while nutrient concentration played a role only in special situations such as periods when flow stops. Episodes of flow cessation and high discharge greatly affected the extracellular enzymatic activities. Biofilms differed in their efficiency in the use of polysaccharides (defined as the activity of β-glucosidase and β-xylosidase per bacterial cell). Biofilms at the site showing high nutrient concentrations (Ter River) were the least efficient, while those occurring at lower nutrient concentrations (La Solana and sandy sediments of Riera Major) were the most efficient. The highest efficiency was associated with stromatolitic communities in La Solana. The epilithic biofilms of Riera Major showed a lower efficiency than biofilms on sand in the same stream, suggesting that biofilm type may be another factor inducing variation in biofilm activity.

Introduction

Stream biofilms are autotrophic-heterotrophic assemblages of living organisms and organic material that colonize streambed surfaces (Lock, 1993). Biofilms are composed of algae, bacteria, protozoa and fungi, which are embedded in a matrix of polysaccharides and other polymers (Lock et al., 1984). In the biofilm, most bacteria (and cyanobacteria), as well as some algae, are able to degrade macromolecules using extracellular enzymes (Sinsabaugh et al., 1992; Claret and Fontvieille, 1997). Extracellular enzymes are those enzymes bound to the cell surface, or dissolved freely in the environment (or associated with the polymer matrix). They convert high molecular weight compounds to low molecular weight ones, which are then

available for bacterial uptake (Chróst, 1994). Extracellular enzyme activity can be an expression of the heterotrophic activity of microbial consortia (Somville, 1984; Chróst and Overbeck, 1990; Chróst, 1991; Admiraal and Tubbing, 1991). Thus, extracellular enzymes may be useful indicators of bacterial activity in aquatic environments (Gajewski and Chróst, 1995), although a direct relationship between the two does not always exist (Karner et al., 1992).

Several studies have shown that extracellular enzyme activities are mainly related to substrate availability (e.g. Meyer-Reil, 1987). In streams, they may also be affected by environmental factors such as temperature (Sinsabaugh and Linkins, 1988; Chappell and Goulder, 1994), dissolved nutrient concentrations and discharge (Romaní and Sabater, 1999a), light (Sinsabaugh and Linkins, 1988; Sabater et al., 1998) and the presence of humic material (Freeman et al., 1990). Biological parameters such as algal and bacterial abundance can also account for variations in heterotrophic activities (Chappell and Goulder, 1994; Sabater and Romaní, 1996).

Many Mediterranean streams show a pronounced seasonality in environmental conditions: rainfall is irregularly distributed, with heavy rainfalls occurring in the autumn and spring, and drier conditions prevailing in winter and especially summer. Temperature and light intensity are highest in summer. These seasonal changes are likely to influence the heterotrophic activities in streams under Mediterranean climate. Moreover, other factors, such as streambed lithology, riparian vegetation type and cover, and nutrient content of the stream water could influence the activities of heterotrophs in biofilms (Sabater et al., 1992).

The aim of this study was to gain insight into which environmental factors may account for inter-site and temporal variations in the extracellular enzyme activities in Mediterranean stream biofilms. We report biofilm and environmental data from three Mediterranean streams with different streambed lithology, nutrient content, and type and density of riparian vegetation. Their patterns and relationships are analyzed by multivariate analyses.

Materials and Methods

Study sites

Three different Mediterranean streams (located in NE Spain) were selected for this study (Table 1). The first site, in the Ter River, was strongly influenced by human activities such as agriculture, industry, and water regulation. The selected site was located in a riffle zone in the middle stretch of the river (Ter at Montesquiu, 4th order) (Romaní and Sabater, 1999a). The benthic algal community was mainly composed of diatoms, and filaments of *Cladophora glomerata* develop in spring and summer.

The second site, Riera Major, was located in a siliceous watershed; it was a forested, undisturbed second-order tributary of the Ter River. Its bed was characterized by a sequence of rapids and runs. Rapids were dominated by boulders (>256 mm diameter) and cobbles (64–256 mm), while coarse sand (0.5–1 mm diameter) was abundant in runs. On average, 40% of the stream was covered by sand and 60% by coarser substrata (Romaní et al., 1998).

Table 1. Physiographic features of Riera Major, La Solana, and Ter River (4th order stretch) watersheds. Values of discharge, temperature, conductivity, DOC, Ca^{+} and Mg^{+} content are annual means with ranges in parentheses (n=12)

	Riera Major	La Solana	Ter River
Location	41°56′N 2°25′E	42°70′N 2°13′E	41°58′N 2°9′E
Order	2	2	4
Watershed area (km^2)	15.9	16.1	3010
Altitude range (m above sea level)	960–460	980–500	680–460
Stream length (km)	6	8.2	208
Lithology	granodiorite	calcite and dolomite	calcareous conglomerates
Composition of riparian vegetation	*Alnus glutinosa*	*Salix elaeagnos, Salix purpurea, Corylus avellana*	*Fraxinus excelsior, Betula pendula*
Cover (%)	10–90	5–25	5–20
Discharge ($L\ s^{-1}$)	57.8 (15.0–89.9)	15.7 (0–42.5)	9600 (932–14350)
Temperature (ºC)	12.4 (1.9–17.1)	9.2 (0.7–24.8)	11.6 (5.9–21.1)
Conductivity ($\mu S\ cm^{-1}$)	206 (126–254)	413 (352–470)	310 (291–333)
DOC ($mg\ L^{-1}$)	2.7 (1.1–5.9)	3.8 (1.8–15.1)	6.3 (2.5–9.7)
Ca^{2+} content ($mg\ L^{-1}$)	22.8 (12.0–28.9)	59.2 (40.7–77.7)	46.1 (38.2–64.3)
Mg^{2+} content ($mg\ L^{-1}$)	4.1 (2.2–5.0)	20.7 (18.2–27.2)	5.8 (3.4–10.3)
pH	7.9 (7.0–8.5)	8.2 (7.6–8.9)	8.2 (7.9–8.5)
Alkalinity (HCO_3^- $mg\ L^{-1}$)	79.5 (48.8–115.9)	213.0 (189.1–237.9)	122.6 (90.3–183.2)
Main benthic algae and cyanobacteria	*Phormidium autumnale,* diatoms, *Hildenbrandia rivularis, Cladophora glomerata*	*Rivularia biasolettiana, Schizothrix penicillata,* diatoms, *Mougeotia* spp., *Zygnema* spp.	Diatoms, *Cladophora glomerata*

The third site, La Solana, was located in a calcareous watershed; it was also an undisturbed second-order stream and tributary of the Ter River. The streambed was covered by carbonate-encrusting cyanobacteria, which formed thick stromatolitic crusts (Romaní and Sabater, 1998).

Data collection

Monthly measurements between 1994 and 1995 provided data for the present analysis. Parameters include extracellular enzymatic activities (β-glucosidase, β-xylosidase, and phosphatase), respiratory activity (ETS = Electron Transport System activity), and bacterial abundance and chlorophyll content, from different streambed substrata (Table 2). The enzymatic activities were measured fluorometrically following the procedure of Hoppe (1983). The fluorometric procedure of Blenkinsopp and Lock (1990) was used to measure respiratory activity. Bacterial

Table 2. Extracellular enzyme activities, respiratory activity (ETS), bacterial density and chlorophyll *a* content in stream biofilms from the three study sites (Riera Major, La Solana, Ter River). Values are means of monthly values from the study period in each stream (Riera Major mid-channel sand: January 1994–August 1995, stream-edge sand and tiles: January 1994–February 1995, subsurface sand: October 1994–August 1995, La Solana: January 1994–February 1995, Ter River: February 1994–February 1995). The standard deviation of the mean is also shown except for the diatom bloom (n = 2) where the range is shown in parentheses

		β-glucosidase activity nmol cm^{-2} h^{-1}		*β*-xylosidase activity nmol cm^{-2} h^{-1}		Phosphatase activity nmol cm^{-2} h^{-1}		Respiratory activity (ETS) µg cm^{-2} h^{-1}		Bacterial cell density cells 10^{10} cm^{-2}		Chlorophyll *a* content µg cm^{-2}	
Site and substrate	n	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
Riera Major													
Mid-channel sand	21	14.6	7.2	6.5	3.6	20	15	0.81	0.61	0.97	0.89	1.8	2.1
Stream-edge sand	10	15.6	9.4	7.2	7.2	15	9	0.74	0.67	0.87	0.63	2.0	1.7
Subsurface sand	9	6.6	1.4	2.5	0.7	15	8	0.35	0.07	0.20	0.14	0.3	0.2
Artificial substrata (tiles)	10	5.7	3.6	2.4	1.3	39	32	1.55	1.70	2.41	0.62	4.1	2.2
La Solana													
Patches of the cyanobacterial crust:													
Mixed community	11	64.8	20.6	44.5	22.2	137	76	3.59	1.91	0.46	0.24	13.3	4.4
Rivularia community	6	49.3	38.0	22.3	19.8	384	457	8.53	5.92	0.98	0.42	4.4	14.4
Zygnema-Spirogyra community	6	50.3	28.8	20.9	9.5	163	101	3.89	0.45	0.60	0.23	20.0	9.2
Diatom bloom	2	15.7	(4.2–34.3)	6.7	(3.1–12.4)	66	(28–90)	12.95	(3.42–34.14)	0.41	(0.33–0.61)	32.7	(19.4–43.2)
Ter River													
Artificial substrata (tiles)	8	39.7	31.7	10.0	7.0	49	42	4.95	2.58	2.47	1.92	31.1	19.8

abundance was determined by counting under the microscope after DAPI staining (Porter and Feig, 1980). The chlorophyll *a* content of biofilms was measured spectrophotometrically after extraction with acetone (Jeffrey and Humphrey, 1975). Environmental parameters included concentrations of dissolved organic carbon (DOC), dissolved inorganic carbon (DIC), soluble reactive phosphorus (SRP), ammonium, nitrate, as well as pH, water temperature, conductivity, and ambient light intensity. The collected data have been used in previous papers (Romaní and Sabater 1998, 1999a, 2000b) where details on measurement methods can be found.

Statistical analyses

Extracellular enzymatic activities, ETS activity, chlorophyll *a* content and bacterial cell abundance at all sites and sampling times (75 cases) were used in a Principal Components Analysis (PCA). Data were logarithmically transformed in the case of the light, bacteria/algae biomass ratio and ammonium data, which did not follow normal distribution. Normality was tested with the Kolmogorov test, using the Lilliefors table ($p < 0.01$).

Canonical Correlation Analysis (CCA) was performed between the heterotrophic activities (β-glucosidase, β-xylosidase, phosphatase and ETS) and the physical, chemical and biological variables (bacterial abundance, chlorophyll *a*, bacteria/algae biomass ratio, DOC, DIC, SRP, nitrate, ammonium, pH, temperature, conductivity and light). CCA was designed to elucidate which physical, chemical and biological variables most influenced the variation of heterotrophic activities in the investigated streams. CCA determines linear combinations within the variables of each set to obtain the canonical variables (Manly, 1995). These linear combinations are removed from each set in order to obtain the maximum correlation (canonical R) between them. The canonical roots are pairs of variables with the maximal correlation between them under the condition that the roots removed are not correlated between them. To interpret each canonical variable, we used the correlation between the variables and the canonical roots in each set (Manly, 1995).

Results

The first component of the PCA (57.1% of the variance) grouped β-glucosidase, β-xylosidase, phosphatase and ETS activities, as well as chlorophyll *a* content (Table 3). This component thus accounts for a high proportion of variability of both heterotrophic (enzymatic and ETS activities), and autotrophic (algal biomass) parameters. The second component (19.1% of the variance) separated bacterial cell abundance from β-xylosidase and β-glucosidase activities. This component could be interpreted in terms of the efficiency in polysaccharide degradation, which is defined here as the enzymatic activity per bacterial cell. Therefore, the highest efficiency is reached when a low bacterial density coincides with high β-xylosidase and β-glucosidase activities.

The first component separates the siliceous site, Riera Major (sand and tiles), from the River Ter and La Solana (Fig. 1), reflecting the fact that on average the

Table 3. Results of the PCA performed with data from Riera Major, La Solana, and the Ter River. Loadings for the factors 1 and 2 (PC I and PC I I) and the eigenvalues and percentage of total variance explained by each factor are also shown

Variable	PC I	PC I I
β-glucosidase	0.84	– 0.36
β-xylosidase	0.78	– 0.49
Phosphatase	0.77	0.22
ETS	0.85	0.15
Chlorophyll *a*	0.84	0.27
Bacterial density	0.28	0.81
Eigenvalue	3.42	1.14
% variance	57.1	19.1

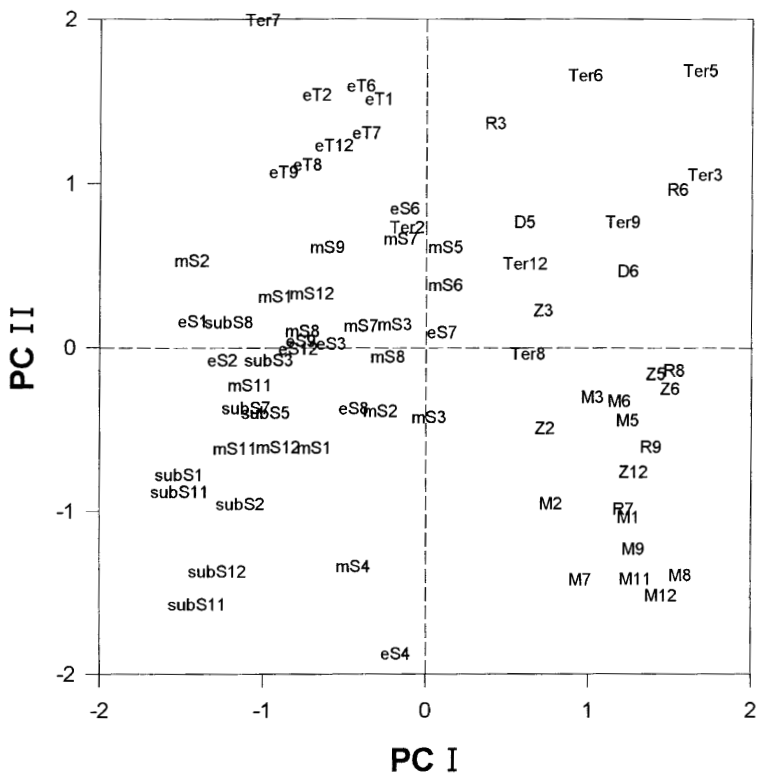

Figure 1. Scores of a PCA performed with Riera Major mid-channel sand (mS), stream-edge sand (eS), subsurface sand (subS) and epilithic biofilm (eT), the algal patches of La Solana cyanobacterial crust including a Mixed community (M), *Rivularia* community (R), *Zygnema-Spirogyra* community (Z) and Diatom bloom (D), the Ter River epilithic biofilm (Ter). The numbers indicate the sampling month

Table 4. Chi-Square tests with successive roots removed by the CCA. The canonical correlation (R), Chi-square, degrees of freedom (df) and probability (p) are shown for each root. All roots removed are significant ($p < 0.5$)

Root	R canonical	Chi^2	df	p
1	0.901	185.8	48	< 0.0001
2	0.744	80.0	33	< 0.0001
3	0.506	28.7	20	0.093
4	0.380	9.9	9	0.357

biofilms from Riera Major were characterized by lower heterotrophic activity and algal biomass (lower values of the PC I) than the biofilms from La Solana and the Ter River (higher values of the PC I). These two latter sites are arranged separately by the second component. The second component also separates the epilithic from the epipsammic (sand-colonizing) biofilms of Riera Major.

The results of the CCA show that the first three canonical roots removed were highly significant (Table 4). Algal biomass (chlorophyll *a* content), conductivity and DIC content (i.e., ionic content of the stream water) were the main parameters accounting for the variability in heterotrophic activity within the three study streams (root 1, Table 5). Dissolved nutrient concentration was not significantly cor-

Table 5. Correlation of each variable (from the left and right set) to the canonical roots removed. The most indicative variables for each canonical variable are those which have the highest correlation ($> +0.5, < -0.5$)

Left set	Root 1 U1	Root 2 U2	Root 3 U3	Root 4 U4
ETS	– 0.838	0.106	– 0.455	0.282
β-glucosidase	– 0.760	– 0.292	0.207	– 0.43
β-xylosidase	– 0.486	– 0.720	– 0.186	– 0.458
Phosphatase	– 0.693	– 0.354	0.195	0.597
Right set	Root 1 V1	Root 2 V2	Root 3 V3	Root 4 V4
Bacteria	– 0.220	0.436	0.117	– 0.104
Chlorophyll	– 0.765	0.357	– 0.001	– 0.016
Bacteria/algae	0.287	0.071	0.064	0.010
DOC	– 0.308	– 0.298	0.733	– 0.257
DIC	– 0.774	– 0.432	– 0.092	0.277
SRP	– 0.247	0.280	0.164	– 0.366
Nitrate	0.467	0.138	0.126	0.022
Ammonium	– 0.141	– 0.234	0.581	– 0.303
pH	– 0.251	0.015	– 0.070	0.118
Temperature	– 0.332	0.098	0.167	– 0.254
Conductivity	– 0.838	– 0.457	0.095	– 0.114
Light	– 0.492	0.259	– 0.199	0.335

related with this root. Low ETS and extracellular enzyme activities are consistent with the typical late-autumn/winter conditions in Mediterranean streams, when discharge is high (floods usually take place in autumn) and concentrations of dissolved nutrients and other ions are low. The reverse situation (high conductivity, DIC concentration and chlorophyll *a* content) was reached in late spring and summer, when the highest values of extracellular enzyme and ETS activities were found. Root 2 of the CCA suggests a similar explanation with xylosidase activity, which is again related to DIC concentration and conductivity (Table 5). Moreover, root 3 relates high concentrations of DOC and ammonium in stream water with low ETS values (Table 5), a common situation during periods in summer when flow ceases.

Discussion

Results of Canonical Correlation Analysis suggest that the ionic content of the water (DIC and conductivity) and the algal biomass in biofilms were the major factors accounting for the variability of heterotrophic activity in the Mediterranean stream biofilms examined in the present study (Table 5). Streamwater ionic content may have an effect on the seasonal variation of extracellular enzyme activity and respiration since this physical-chemical factor is directly related to discharge, which typically follows a strong seasonal pattern in many Mediterranean streams (Sabater, 1988; Armengol et al., 1991). Discharge usually increases during snow melt in spring and following heavy rainfall in autumn, and greatly decreases in summer (Sabater et al. 1992). As a result, water flow can almost completely cease during late spring and summer, when ionic content is high (Table 5) and biofilm heterotrophic activity low. During such low-flow conditions, low values of respiratory activity (ETS) were related with high concentrations of DOC and ammonium in the stream water (root 3, Table 5), especially in La Solana (Romaní and Sabater, 1998). Depletion of nutrients (e.g. SRP) during these dry periods might limit bacterial and algal metabolic activity, in spite of high concentrations of DOC, which may serve as a substrate for heterotrophs.

Ionic content of the stream water appeared to account also for the variability in heterotrophic biofilm activity among streams. Specifically, the high ionic content at the calcareous sites (Ter River and La Solana, Table 1) may have been responsible for the higher extracellular enzyme activities (Table 2, Fig. 1), because these sites exhibit high concentrations of calcium and magnesium (Sabater, 1988; Martí and Sabater, 1996), both of which, but especially magnesium, can act as activating cations in enzyme reactions (Chróst, 1990). A positive response of β-glucosidase to added calcium and magnesium has also been observed in marine sediments (King, 1986). Likewise, greater extracellular enzyme activity was observed in several streams draining calcareous watersheds in N England (Chappell and Goulder, 1994).

Results of PCA and CCA suggest that algal biomass (chlorophyll *a* content) was the most important biological factor accounting for the observed variations in heterotrophic biofilm activities (Table 3, Fig. 1, PC I; Table 5, root 1). Algae are an important source of high quality organic matter for heterotrophs and a suitable site for bacterial attachment (Sabater and Romaní, 1996). In autotrophic biofilms, algae

and bacteria are intimately associated in a common matrix (Lock, 1993), facilitating the use of algal excretion products or other autochthonous materials (algal lysis products) by bacteria (Marxsen and Witzel, 1991). Stimulating effects of algal biomass on heterotrophic activity in biofilms have indeed been reported in several studies (Murray et al., 1986; Chapell and Goulder, 1994; Romaní and Sabater, 1999b).

Although temperature regulates cell metabolism (e.g. Kaplan and Bott, 1989), including extracellular enzyme (Münster et al., 1992; Wiebe et al., 1992) and respiratory activities (Peters et al., 1987), and can contribute to variation in extracellular enzyme activities among streams of different biomes (Romaní and Sabater, 2000a), temperature did not account for the between-system variability in heterotrophic activities in the present study (Table 5). This is not surprising in view of the proximity of the three streams examined here, which resulted in similar temperature regimes (Sabater, 1988; Martí and Sabater, 1996). Other studies also failed to correlate extracellular enzyme activity and temperature on an ecosystem scale (Jones and Lock, 1993; Hoch et al., 1996). In correlation analyses, relationships between temperature and extracellular enzyme activity might be masked if the response of enzyme activity to changing water temperature is delayed (Hoppe et al., 1988). Other causes cannot be ruled out either. For example, under culture conditions, substrate concentration was more important for controlling bacterial growth rate than temperature, which was varied from 8–25°C (Barillier and Garnier, 1993), and both substrate availability and nutrient content have been found to influence extracellular enzyme activities in temperate streams (Sinsabaugh and Linkins, 1988; Jones and Lock, 1993).

In the present study, the biofilms in the three Mediterranean streams differed in their efficiency in the use of polysaccharides (defined as enzymatic activity per bacterial cell) as revealed by the second component of the PCA (Fig. 1). Biofilms at the site with the highest nutrient concentration (Ter River) were the least efficient, while those experiencing lower nutrient concentrations (La Solana and sandy sediments of Riera Major) were the most efficient. Biofilms of the two calcareous sites also showed different efficiencies in polysaccharide use, as revealed by the second component of the PCA (Fig. 1), where spring and mid-summer samples from the Ter River (Ter5, Ter6, Ter7) are apart from the mixed community in La Solana in summer and autumn (M7, M8, M9, M11, M12). The high efficiency associated with the mixed community in La Solana (Romaní and Sabater, 1998) is a special feature of the stromatolitic crust in this stream (Sabater et al., 2000). In Riera Major, the epilithic biofilms showed a lower efficiency than the sandy biofilms (Fig. 1), a finding that may be related to the higher surface area and greater accumulation of organic matter of the latter (Romaní and Sabater, 2000b).

In conclusion, several factors appear to co-act in the regulation of heterotrophic activities in Mediterranean stream ecosystems. Their relative importance is likely to depend on the temporal and spatial scales considered. Among streams in a given biome that contrast in regard to water chemistry, ionic content of the water and algal biomass appear to be important determinants of biofilm heterotrophic activity, while nutrient availability can play a role in special situations such as periods when flow stops. Since the efficiency in the use of polyssaccharides may vary among biofilm types (epilithic, stromatolitic, epipsammic), biofilm type also needs to be considered as a critical regulation factor of heterotrophic activity.

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