Universitat de Girona

THE ROLE OF FUNGI AND BACTERIA ON THE ORGANIC MATTER DECOMPOSITION PROCESS IN STREAMS: INTERACTION AND RELEVANCE IN BIOFILMS

Joan ARTIGAS ALEJO

ISBN: 978-84-692-2181-5
Dipòsit legal: GI-355-2009

Universitat de Girona
Facultat de Ciències
Institut d'Ecologia Aquàtica

THE ROLE OF FUNGI AND BACTERIA ON THE ORGANIC MATTER DECOMPOSITION PROCESS IN STREAMS: INTERACTION AND RELEVANCE IN BIOFILMS

Memòria presentada per Joan Artigas i Alejo per optar al grau de Doctor per la Universitat de Girona

Vist-i-plau dels directors: El doctorand:

Dr. Sergi Sabater Cortés
Institut d'Ecologia Aquàtica
Universitat de Girona

Dra. Anna M. Romaní Cornet
Institut d'Ecologia Aquàtica
Universitat de Girona

Joan Artigas Alejo

Girona, Novembre 2008

AGRAÏMENTS

És molta la gent que m'ha ajudat a tirar endavant aquest treball i espero no oblidar-me'n de cap! En primer lloc, gràcies Anna i Sergi per haver-me obert aquesta porta, haver-me ensenyat el que sabeu i haver-me contagiat el vostre entusiasme. Gràcies a la meva família (la mare, el pare i en Narcís) perquè sempre m'heu donat suport i cregut amb el que estava fent, tot i que a vegades us ho expliqués fatal. I sobretot, gràcies Carlitus per ser-hi tu i la resta de la família (el Paco, la Lluna i la Nelly), per interessar-te i preguntar-me coses de la feina i sobretot per escoltar-me quan tenia un problema. Gràcies a la meva medicina particular, el patinatge, perquè m'ha ajudat a fer complets resets dels problemassos i anades d'olla que es tenen en el "dia a dia" de la tesi. Sense l'ajuda de tots vosaltres, aquest treball no hagués estat possible.

Floretes Sandra i Bet, vam començar fa molts anys! No diré quan… Hem viscut moltes coses junts! Gràcies per haver-hi sigut, sense vosaltres la tesi no hagués tingut el mateix gust. Irene veïna de taula, hem rigut molt i hem pencat molt! Un xin-xin per l'Ebre! Podria seguir fent comentaris de tots i cadascun perquè ja sabeu que quan m'hi poso puc batejar de nou "la Verge de la pota arrossegada". Però no seria el cas, perquè quan obro la boca "puja el preu del pa". Gràcies companys del FLUECO team (Helena, Marta, Berta, Natàlia, Chloe, Leslie, Lorenzo, Anita, Francesco, Xisca, Susanna, Gemma U i Gemma V). Ups, me n'oblidava dels companys de la UB (Ainoha, Rut, Isabel, Isis, Julio)! Gent, ens les hem vist i desitjat quan la Tordera anava com les cabres i quan Fuirosos estava fred com el glaç. Lo millor de tot el dia "inuncat", amb la Clara, l'Anna i el del tractor remolcador… encara al.lucino de la situació. No menys important ha sigut el suport i "bon rotllo" que he rebut de la resta de gent de l'Àrea d'Ecologia. Aiguamollerus i peixòlegs sou uns cracks tots plegats. Diuen que la recerca ha de ser "interdisciplinar", doncs merci genètics, micros, bio cel.lulars, zoòlegs per aquells favorets i per les bones estones del dinar.

Aquesta tesi ha estat finançada pels següents projectes: Patterns of biofilm formation: changes in community structure and metabolism under different light and temperature conditions (HA2004-0020) del MEC, Efectos de episodios de interrupción del flujo sobre la continuidad estructural y funcional de sistemas fluviales (CGL2007-65549/BOS) del CICYT i Cambios globales en sistemas fluviales: efectos sobre la biodiversidad, la red trófica y el funcionamiento del sistema (GLOBRIO) de la FBBVA. També he gaudit d'una beca predoctoral BR concedida per la Universitat de Girona.

Això de fer la tesi ha sigut molt "xulo"!

INDEX

SUMMARY

The microbial structure and metabolism of benthic microbial communities was analyzed over a hydrological year in a Mediterranean forested stream (the Fuirosos). Microbial heterotrophic biomass (bacteria plus fungi) was generally higher than autotrophic biomass (algae), except during short periods of high light availability in the spring and winter. During these periods, algal biomass and peptidase activity increased in benthic communities. Heterotrophic biomass and activity (polysaccharide and lignin degrading activities) showed a peak in autumn. The Mediterranean summer drought provoked an earlier leaf fall. The resumption of the water flow caused the weathering of riparian soils and subsequently a large increase in dissolved organic carbon and nitrate, which led to growth of bacteria and fungi.

The aquatic microfungi in the Fuirosos mainly developed in leaves, but were also relevant in sand and gravel habitats. In general, the enzymatic activities of microbial communities were linked to their nutrient molar ratios (biofilm C:N and N:P) and, at the same time, these ratios might be modulated by the different nature of available organic matter in each substratum and by the water nutrient concentrations. According to this, microbial communities associated to leaves and branches showed higher C: N and lower N: P molar ratios and higher polysaccharide degrading activity. Instead, biofilms on sand and gravel, where algae accumulated and fine detritic materials were more available, showed lower C: N and higher N: P molar ratios, as well as higher ligninolytic and peptidase activity.

Leaf litter colonization and breakdown in streams is mostly carried out by fungi. A three-month litter breakdown experiment using two different leaf species (*Platanus acerifolia* and *Populus nigra*) revealed different patterns of fungal and bacterial biomass accumulation. Bacteria increased earlier than fungi in leaves, though fungi prevailed in terms of biomass and activity. The widest and fastest leaf structural changes and the maximum enzyme activities per unit of microbial carbon took place between the second and third week of experiment. There was an exponential relationship between bacterial biomass and most of the enzyme activities measured in the two leaf species. However, this relationship was consistent between fungal biomass and activities only in the *Platanus* leaves, which has the lowest breakdown rate. These results suggest that fungi probably required of a higher stability than bacteria to develop and to degrade substrates.

In contrast to leaf communities, stream epilithic biofilms (communities growing on rocky substrata) are mainly colonized by algae, bacteria and protozoa. Patterns on epilithic biofilm formation in two streams of different bioclimatic regions (a Central

European, the Walzbach and a Mediterranean, the Fuirosos) were related to environmental characteristics. Flooding episodes produced a drastic reset in diatom populations in the Fuirosos. Overall, the higher structural complexity of the epilithic biofilms in the Mediterranean stream (higher bacterial biodiversity and biomass and extracellular polysaccharide content) did not result on major enzyme activity efficiency per bacterial cell. Conversely, the environmental stability in the Central European stream played an important role in shaping biofilm structure and in regulating a more efficient use of the organic matter resources. Deriving from this experiment, an extraction analysis using a cation exchange resin was optimized to measure matrix-enzymes (those included in the extrapolymeric substances matrix) in freshwater biofilms. Analysis of extracellular enzyme activity distinguishing enzyme activity in EPS matrix (matrix-enzymes) and total biofilm extracellular enzyme activity (undisrupted biofilm) revealed that matrix-enzymes accounted for 65-81% of total biofilm enzyme activity, though matrix-enzyme activity decreased as biofilm matured.

Nutrient enrichment in streams often results in enhanced decomposition rates and chemical conditions that affect the suitability of the habitat for benthic communities. With this purpose, two nutrient enrichment experiments (nitrate and phosphate addition) were conducted on benthic microbial communities to determine: i) whether long- and short-term (1.5 years and 48h, respectively) nutrient enrichment affected sporulation and diversity of aquatic hyphomycetes, and ii) whether changes in total water nutrient concentration and N:P molar ratios affected epilithic biofilms integrity. Long-term nutrient additions did not affect potential sporulation rates, though short-term nutrient additions produced insignificant increases in these rates. However, long-term nutrient enrichment produced significant changes in the hyphomycetes community composition. Further, biofilms exposed to high water nutrient concentration showed higher N recycling (increase of peptidase activity) within the biofilm, and strong P limitation (N:P ratio of 56:1) mostly affected bacteria and stimulated EPS accumulation and phosphatase activity. Results from both experiments revealed that stream benthic communities are dynamic and sensitive to the changes in the water nutrient availability.

RESUM (*in Catalan*)

L'estructura i metabolisme de les comunitats bentòniques microbianes foren analitzades durant un any hidrològic en una riera mediterrània forestada (la riera de Fuirosos). La biomassa microbiana heterotròfica (bacteris i fongs) era, en general, superior a la biomassa dels autòtrofs (algues), excepte en períodes curts en els quals la disponibilitat de llum al riu era més gran (primavera i hivern). Durant aquests períodes la biomassa algal i l'activitat peptidasa va incrementar en les comunitats bentòniques. La biomassa i activitat dels heteròtrofs (activitats de descomposició de polisacàrids i lignina) mostraven un pic a la tardor. La sequera estival dels sistemes mediterranis va avançar la caiguda de fulles. El restabliment del flux d'aigua va provocar el rentat dels sòls de la conca i el conseqüent increment de carboni orgànic dissolt i nitrats, els quals permetien el creixement de fongs i bacteris.

Els fongs aquàtics microscòpics a la riera de Fuirosos es desenvolupen principalment a les fulles, però també en els hàbitats sorrecs i a la grava. En general, les activitats enzimàtiques de la comunitat microbiana es relacionen amb la respectiva relació molar de nutrients del biofilm (p. ex. quocients C:N i N:P dels biofilms), i al mateix temps, aquests quocients podrien ser modulats per les característiques del material orgànic disponible a cada substrat i per les concentracions de nutrients a l'aigua. D'acord amb aquesta premissa, les comunitats microbianes associades a fulles i branques mostraren un major quocient C:N i un menor N:P, així com una superior descomposició de polisacàrids. En canvi, les comunitats a la sorra i a la grava tenien un menor quocient C:N i un major N:P i una major activitat ligninolítica i peptidasa, ja que en aquests hàbitats les algues s'hi acumulen i hi ha una major disponibilitat de materials detrítics fins.

Els fongs són els principals responsables de la colonització i descomposició de fullaraca als rius. Un experiment de descomposició de fullaraca, en el qual es van utilitzar fulles de plàtan (*Platanus acerifolia*) i pollancre (*Populus nigra*), va revelar un patró d'acumulació diferent entre la biomassa fúngica i bacteriana. Els bacteris van créixer més ràpid que els fongs a les fulles, encara que els fongs foren predominants tant en termes de biomassa com d'activitat durant tot l'experiment. Els canvis més ràpids i bruscs en l'estructura de les fulles es van detectar entre la segona i tercera setmana d'experiment, coincidint amb la màxima activitat enzimàtica per unitat de carboni microbià. Hi havia una relació exponencial entre la biomassa bacteriana i la majoria d'activitats enzimàtiques mesurades a les dues fulles. En canvi, la relació entre biomassa fúngica i activitats només fou consistent en les fulles de *Platanus* (aquelles amb menor taxa de descomposició). Aquests resultats suggereixen que els fongs

probablement requereixen d'una major estabilitat, per tal de desenvolupar-se i degradar els substrats, que no pas els bacteris.

Al contrari que en les comunitats de fulles, les algues, els bacteris i els protozous són els principals colonitzadors dels biofilms epilítics fluvials (comunitats que creixen a les pedres). Els patrons de formació de bioflms epilítics foren analitzats en dos rius que pertanyen a diferents regions bioclimàtiques: un riu Centre Europeu (Walzbach) i un riu Mediterrani (Fuirosos). Els episodis d'inundació a Fuirosos van produir un dràstic declivi en les poblacions de diatomees. En general, la major complexitat estructural dels biofilms epilítics en el riu Mediterrani (major biodiversitat bacteriana, biomassa i contingut de polisacàrids extracel·lulars) no fou reflectit en una major eficiència de l'activitat enzimàtica per unitat de cèl·lula bacteriana. En canvi, la major estabilitat ambiental en el riu Centre Europeu va jugar un paper molt important en la fisiognomia dels biofilms i en la regulació d'un ús més eficient dels recursos.

Sorgit d'aquest anterior experiment, es va optimitzar un protocol d'extracció utilitzant una reïna d'intercanvi catiònic apropiat per a la mesura d'enzims lligats a la matriu extracel·lular en biofilms d'aigua dolça. L'anàlisi de l'activitat enzimàtica extracel·lular, distingint l'activitat enzimàtica a la matriu (enzims de la matriu) i l'activitat total del biofilm, va revelar que els enzims de la matriu explicaven entre un 65-81% de l'activitat enzimàtica total del biofilm al principi del procés de colonització. No obstant, l'activitat dels enzims de la matriu disminueix a mesura que el biofilm madura.

L'enriquiment per nutrients en els rius normalment augmenta les taxes de descomposició i les condicions químiques alterant la idoneïtat de l'hàbitat per a moltes espècies que viuen a les comunitats bentòniques. Dos experiments d'enriquiment (nitrat i fosfat) foren duts a terme en comunitats bentòniques per tal de determinar: i) si els enriquiments a curt (48 hores) i llarg termini (1.5 anys) afectaven les taxes d'esporulació i la diversitat dels hifomicets aquàtics, i ii) si els canvis en la concentració total de nutrients i en la relació molar N:P a l'aigua afecten l'integritat dels biofilms. En el primer experiment, l'enriquiment a llarg termini no va afectar les taxes d'esporulació potencials d'hifomicets, mentre que, els enriquiments a curt termini van produir un increment insignificant en les taxes. Malgrat això, l'enriquiment amb nutrients a llarg termini va provocar canvis significatius en la composició de la comunitat fúngica. En el segon experiment, els biofilms exposats a elevades concentracions de nutrients van augmentar el reciclatge de nitrogen (activitat peptidasa), dins el biofilm. Mentre que una forta limitació per fòsfor en els biofilms (N:P ratio de 56:1) sobretot afectava als bacteris, i estimulava l'acumulació de polisacàrids extracel·lulars i l'activitat fosfatasa. Els resultats d'ambdós experiments van revelar que les comunitats bentòniques fluvials són dinàmiques i sensibles als canvis en la disponibilitat de nutrients a l'aigua.

GENERAL INTRODUCTION

The stream ecosystem

Running waters are enormously diverse; they range from small streams to large rivers and occur under widely differing conditions of climate, vegetation, topography and geology (Allan, 1995). Several studies pointed that rivers belonging to different biomes and ecorregions show consistent differences in biogeochemical characteristics (Meybeck & Helmer, 1989), hydrological conditions (Gasith & Resh, 1999) and biological structure and functioning (Margalef, 1983). Specifically, the chemical nature of materials in the watershed may be one of the major determinants of river water chemistry (Berner & Berner, 1987). However, when streams flow through a region of relatively insoluble rocks, the stream water can be chemically very similar to the rain in its composition (Allan, 1995). Precipitation regimes also regulate the water flow in streams and are the main vehicle for material transport through riparian zones and from surface water to groundwater (Bernal et al., 2003; Vázquez et al., 2007).

Figure 1.1. Middle courses of the Ebro river (left side) and of the Fuirosos stream (right side), both in the Mediterranean region.

The dimensions of the river (size of the channel, water flow, shape of the banks) largely determine the development of biological communities and its functioning (Allan, 1995; Figure 1.1). According to the river continuum concept (Vannote et al., 1980), the connection between the terrestrial and aquatic environments is greater in low order streams than in larger rivers, where the planktonic compartment prevails. Specifically, the coarse particulate organic matter (CPOM) input from the riparian forest becomes the main energy source for stream biological communities in headwater streams. Microbial benthic communities play a key role in these systems, enhancing respiration processes over autotrophic production (production: respiration ratio < 1, Vannote et al., 1980). Nevertheless, short light pulses in forested streams may rapidly stimulate

autotrophic production in benthic communities (Mallory & Richardson, 2005; Taulbee et al., 2005; Ylla et al., 2007), thus providing stream consumers with high-quality authochthonous OM sources.

The microbial benthic community

Microbial communities may develop in organic (leaves and wood) and inorganic (sand, gravel, cobbles, rocks) stream benthic substrata (Figure 1.2). In general, biofilms are composed by algae, bacteria, fungi and protozoa embedded in an extrapolymeric substances matrix (Lock et al., 1984). Depending on the prevalence of different microbial groups in the substratum, the community structure and function may also vary (Romaní & Sabater, 2000). Each microbial group shows preferences for the colonization and development of the substrata in the stream. Previous studies have reported that heterotrophic microorganisms, especially fungi, mainly colonize the submerged CPOM such as leaves and wood (Diez et al., 2002; Gulis & Suberkropp, 2003a), while bacteria prevail on mineral surfaces (fine sediment) and small OM particles (fine detritic materials; Pusch et al., 1998; Findlay et al., 2002) as well as protozoa (Perlmutter & Meyer, 1991). Conversely, algae prefer the hard and relatively inert substrata such as rocks and cobbles, but may also grow on plants and larger algae (Stevenson, 1996).

A range of ecological studies have focused on microbial benthic communities and their role on nutrient recycling in streams, and as sources of nutrients to the higher trophic levels in the stream food web (Lamberti, 1996). Fungi are key factors in plant litter decomposition and dissolved organic matter (DOM) production in streams (Gessner et al., 1997), while bacteria play an important role as decomposers in the interstitial benthic sediments and in the overlying water (Findlay et al., 1993; Gessey et al., 1978). In contrast, benthic algae provide oxygen for aerobic organisms through photosynthesis, and the fixed carbon provides food for algivores (Lamberti, 1996). According to this description, dissolved nutrient concentrations, particulate and dissolved organic matter availability and light incidence in the stream water will influence development of fungi, bacteria and algae in benthic surfaces. But also, water temperature and water flow dynamics are relevant in the development of benthic biological communities (Hauer & Lamberti, 1996).

Figure 1.2. Benthic substrata composition of a Mediterranean forested stream (the Fuirosos). Thick biofilms colonize the coarse (cobbles) and fine sediments (gravel and sand), especially under high light conditions. Source: Irene Ylla.

CPOM breakdown in streams

Main input of CPOM in temperate streams takes place during the fall season. Leaves of different plant species are known to lose mass at different rates during breakdown process (Webster & Benfield, 1986). The physical and chemical characteristics of materials (i. e. nutrients, lignin and tannins content and durability of material) determine their breakdown rates (Melillo et al., 1983; Taylor et al., 1989; Gallardo & Merino, 1993; Suberkropp & Chauvet, 1995). But also, environmental conditions such as water temperature, nutrient concentration and current are relevant in this process (Couteaux et al., 1995; Suberkropp & Chauvet, 1995).

Most studies describe leaf breakdown process in three distinct stages separated in a temporal scale: i) leaching of leaf soluble compounds, ii) conditioning by microbial heterotrophs and iii) fragmentation by macroinvertebrates (Abelho et al., 2001). Nevertheless, recent studies propose new conceptual models in which the coincidence and interplay of various subprocesses during litter breakdown are more strongly recognized (Gessner et al., 1999). According to this, six primary breakdown products are considered: bacterial, fungal and shredder biomass; dissolved organic

matter; fine-particulate organic matter; and inorganic mineralization products such as CO_2, NH_4^+ and PO_4^{3-} (Hieber & Gessner, 2002, see also Figure 1.3).

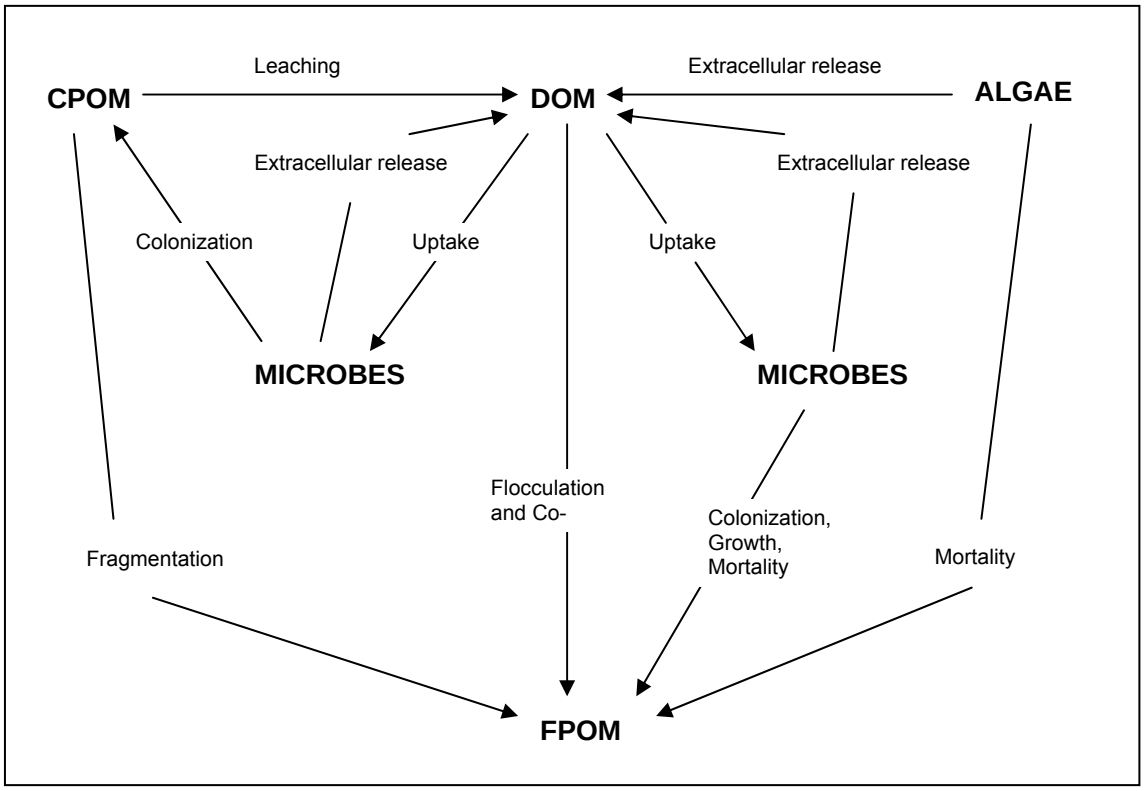


Figure 1.3. Simplified diagram of nutrient resource pools and microbe state variables and the transfer functions between them. CPOM denotes coarse particulate organic matter (< 1mm); FPOM fine particulate organic matter (1 mm – 0.45 μm); DOM dissolved organic matter (< 0.45 μm; after Cummins, 1992).

Use of POM and DOM in microbial benthic communities

In aquatic environments, bacteria have been mostly related to the uptake and degradation of DOM (Kaplan & Newbold, 2003) while net carbon transformations in CPOM is mainly a reflection of fungus degradative ability (Baldy et al., 1995; Gessner, 1997). DOM is the largest pool of organic matter in the aquatic ecosystem (Wetzel, 1992). Monomers such as amino acids and glucose can be uptaken directly by heterotrophs, although they appear in very low concentrations (Banoub & Williams, 1972; Wright & Hobbie, 1965). It has been widely demonstrated that respiration of these monomers (Weiss & Simon, 1999) is important in the cycle of carbon and other elements (Kirchman, 2003). However, DOM is mainly composed by high molecular weight organic matter compounds which must be hydrolized and/or oxidized outside the microbial cell to sizes small enough to permit transport across the outer membrane (Arnosti, 2003).

Fungi and bacteria are the most important producers of extracellular enzyme in the stream ecosystem and as such are responsible of the hydrolysis of polysaccharides (Mansfield, 2005), oxidation of lignin (Griffin, 1994) and breakdown of peptides (Francoeur & Wetzel, 2003) and organic phosphorus compounds (Romaní et al., 2004a). Epilithic and epipsammic biofilms mostly rely on DOM sources from the water column and fine detritic materials accumulated in the sediment. This may be reflected on their particular use of OM sources (Chapter 3). Conversely, microbial communities growing on leaves and wood may rely both on the water DOM and on the substrata itself. In any case, extracellular enzyme production is tightly controlled by the microbial cell. Some of these enzymes are constitutive, but many more are induced under specific circumstances (i. e. under nutrient limitation and/or substrate availability, Arnosti, 2003).

Microbial community composition and interaction between groups

The analysis of community composition is essential to determine colonization patterns of microbes (i. e. Gessner et al., 1993), to evaluate the ecological status of the stream ecosystem (i. e. Pan et al., 1996) and to determine the interactions between microbial groups and between species within groups (i. e. Rier & Stevenson, 2001; Gulis & Suberkropp, 2003a). Classical identification of algal and fungal (i. e. aquatic hyphomycetes) species in streams is easier than identification of bacterial diversity. However, the improved DNA sequencing techniques (the 16S rDNA gene with a length of about 1500 nucleotides) is a phylogenetic marker frequently used to demonstrate phylogenic kinships of prokaryota (Amann et al., 1995; Bavykin et al., 2004).

Several studies have been focused on the interaction between fungi and bacteria in the leaf substrata (Bengtsson, 1992; Moller et al., 1999; Gulis & Suberkropp, 2003a). Bengtsson (1992) suggested that fungi and bacteria showed synergistic relationships, so that each group grew significantly faster in presence of the other group. Nevertheless, more recent studies reveal that fungal biomass accumulation in leaves was approximately 12 times higher in the absence than in the presence of bacteria (Mille-Lindblom & Tranvik, 2003; Figure 1.4).

In the case of epilithic biofilms, most studies have been focused on the algal-bacterial interactions (Rier & Stevenson, 2001; Francoeur & Wetzel, 2003; Carr et al., 2005; Figure 1.4). An early study of Haack & McFeters (1982), demonstrated that algae may supply bacteria with dissolved organic carbon (DOC) resulting from excretion processes during photosynthesis (release of exudates). However, algae and bacteria may become strong competitors when water nutrient availability is scarce (Sobczak, 1996).

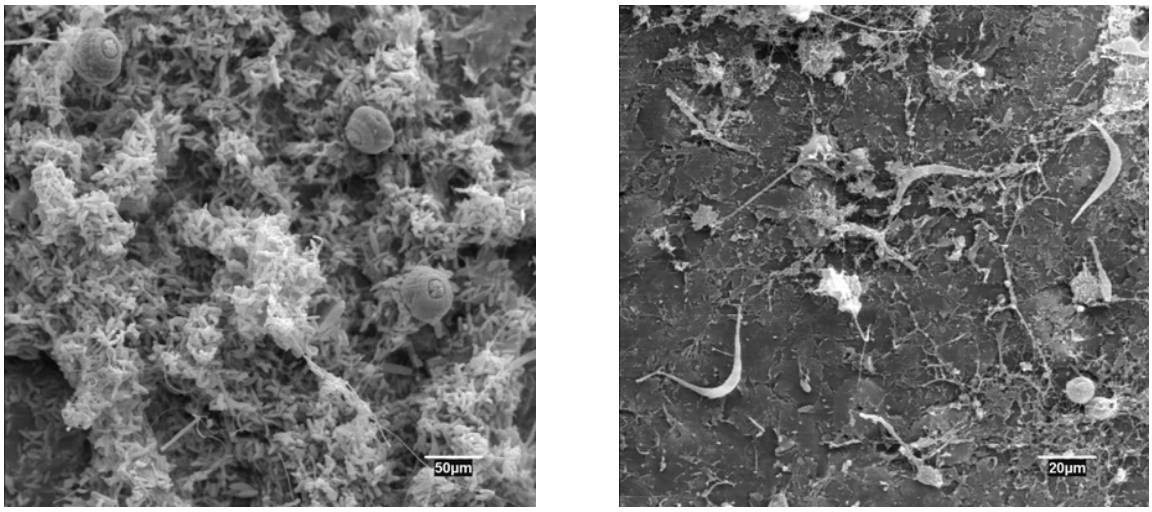


Figure 1.4. Scanning electron microscope (SEM) images of microbial communities colonizing rocks (left) and leaves (right). Diatoms and small bacterial cells embedded in a mucilaginous matrix shape the biofilm primary structure on rocks, but also, consumers (i. e. Vorticella cells) may develop in biofilms and interact with the rest of microbial components. Aquatic hyphomycetes conidia (Lunulospora curvula) dominated in leaves.

The Mediterranean-type stream ecosystem: the case study

Most of the experiments presented in this thesis have been performed in a Mediterranean stream, the Fuirosos. Mediterranean streams are characterized by seasonal events of flooding and drying over an annual cycle (Gasith & Resh, 1999; Figure 1.5), that exert both direct and indirect effects on stream ecosystems.

Figure 1.5. Wet and dry periods in the Fuirosos stream reach where most of this research was performed. Images correspond to the spring (left) summer (right) 2005.

Concerning droughts, direct effects include loss of water, loss of habitat for aquatic organisms and loss of stream connectivity. Indirect effects include the deterioration of water quality, alteration of food resources, and changes in the strength and structure of interspecific interactions (Lake, 2003). In contrast, flooding episodes drastically change flow-related parameters (i. e. current velocity, nutrient concentration,

conductivity) affecting the benthic substrata distribution and retention of DOM and POM in the stream (Bernal et al., 2002; Acuña et al., 2004).

Studies focusing on microbial benthic comunities suggested that droughts temporarily limit microbial mineralization and alter bacterial community structure (Amalfitano, 2008). When the stream starts to dry up, oxygen and nutrients are rapidly consumed, and therefore, strong competition between microbial groups takes place (Acuña et al. 2005; Beche & Resh 2007). In contrast, floods enhance dissolved oxygen availability and lixiviation of watershed soils, which increase nutrient content in the stream water (Bernal et al., 2003; Vázquez et al., 2007). Nevertheless, increases in water current velocity severely affect the structure of benthic microbial communities (Sabater et al., 2006), and indirectly affect in many habitat conditions for microbes development (Stevenson, 1996).

The Fuirosos is an intermittent third-order Mediterranean forested stream that drains a 18 Km^2 siliceous basin (90% granite) north-eastern Spain (Sala & Franch, 1980; Figure 1.6). Most water course of the Fuirosos is included in a Natural Park range (Parc del Montnegre-Corredor) in which agricultural and/or industrial impact is very low.

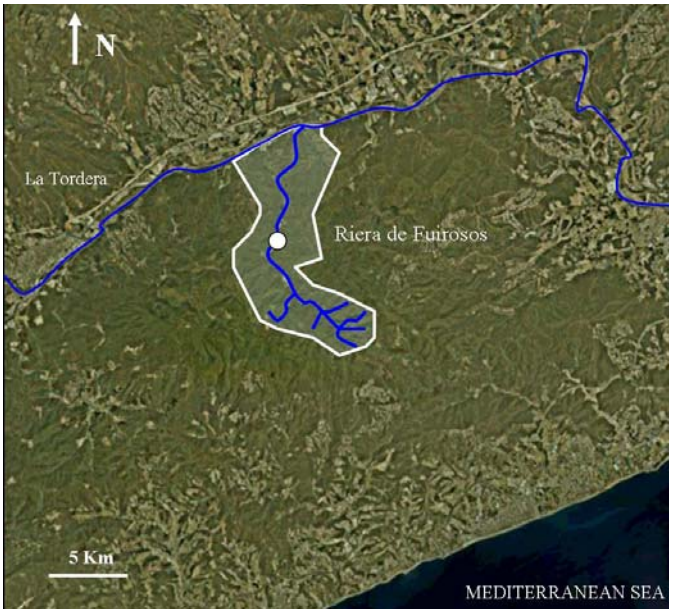


Figure 1.6. Map of the Fuirosos stream watershed. The Fuirosos is a tributary of La Tordera river which flows into the Mediterranean Sea. The stream wateshed area (in white) and the sampling site (white dot) are also represented. Source: Google Earth.

The hydrological features of the Fuirosos are largely determined by the Mediterranean climate. Baseline water flow ranges from 5 to 20 L s^{-1} but can increase by 100 times during autumn and spring spates, while in summer it can be reduced to

zero (Figure 1.5). Main terrestrial OM input from the riparian vegetation [*Platanus acerifolia* (Aiton-Willd.), *Populus nigra* (L.) and *Alnus glutinosa* (Gaertn.) dominated] occurs between summer (because of water stress in the riparian forest) and autumn. Light incidence is generally low except in spring and winter when the forestry canopy is open (Acuña et al., 2004). Most of the experiments of this study were carried out in a 50 m reach (latitude 41º 42'N, longitude 2º 34'), 3-4 m wide and *c*. 10-50 cm in water depth (Figure 1.5). Streambed morphology alternates between riffles and pools. Coarse substrata (rocks, boulders and cobbles) are found in the high-current areas of the channel while fine substrata (fine-to-medium gravel and sand) tend to accumulate in pools. Classification of mineral substrata in the reach was performed according to the Wentworth Scale (1922) and after Cummins (1962) and Minshall (1984) revisions. Leaves and branches tended to accumulate in the streambed, particularly near the banks and in slow-moving areas.

Objectives of this study

First studies at the Fuirosos stream date from the early 1980s and consisted on geomorphological descriptions and processes of lixiviation of the watershed soils (Sala & Franch, 1980; Sala, 1982). In the early 2000s up to nowadays, the Fuirosos stream has been widely studied considering both the processes occurring in the riparian zone (i. e. Butturini & Sabater, 2000; Sabater et al., 2001; Butturini et al., 2002; Bernal et al., 2002), as well as those occurring in-stream (Sabater et al., 2006; Sabater et al., 2005). The structure and metabolism of benthic communities has been well described in studies of Acuña et al. (2004 and 2005). Respiration rates in the Fuirosos were high when compared with analogous streams. Therefore, the Fuirosos can be considered as a heterotrophic stream, though with punctual periodic episodes of autotrophs metabolism (Ylla et al., 2007). Studies of Romaní et al. (2004) and Sabater et al. (2005) mainly focused on the organic matter use by benthic microbial heterotrophs (mainly bacteria) in rocky and sandy habitats. However, less attention has been paid to the role of aquatic fungi in these habitats, as well as to determine their contribution to OM recycling in the stream.

The main objective of this study is to investigate on the use of organic matter by fungi and bacteria inhabiting the different benthic substrata and to analyze the effect of environmental (floods and droughts) and human-induced (nutrient enrichment) perturbations on the structural and functional stability of these communities. The more specific objectives are summarized as follows:

1. To determine the seasonal and spatial variation of microbial communities over one hydrological year in a Mediterranean stream.
2. To evaluate the role of fungi on the decomposition of organic matter during autumn in a Mediterranean forested stream, and defining how this role was shared between the distinct benthic substrata (leaves, branches, sand and gravel).
3. To determine how the patterns of organic matter utilization in distinct microbial communities (leaves, branches, sand and gravel communities) are related to their nutrient molar ratios (C:N and N:P).
4. To define the potential relationships between microbial biomass (fungi and bacteria) and extracellular enzyme activities during leaf litter breakdown.
5. To analyse the patterns of epilithic biofilm formation in streams of different bioclimatic regions (Central European and Mediterranean).
6. To examine the activity of matrix related enzymes (including free and linked to extracellular polymeric substrances) and their relationships with total biofilm enzyme activity during biofilm formation.
7. To examine the effects of water nutrient concentration on the sporulation and diversity of aquatic hyphomycetes colonizing distinct stream substrata (leaves, sand and rocks).
8. To analyze the effect of water nutrient concentrations and N:P molar ratios on biofilm structure and metabolism.

Development of this thesis

To achieve these objectives, I have mostly used the measurement of several hydrolytic (β-glucosidase, β-xylosidase, β-glucosaminidase, cellobiohydrolase, phosphatase, leucine-aminopeptidase) and oxidative (phenol oxidase and peroxidase) extracellular enzymes involved in DOM and POM decomposition. These were assayed in microbial communities associated to organic (leaves and branches) and inorganic (rocks, gravel, sand) stream substrata. Moreover, each substratum was estimated for fungal, bacterial and algal biomass and for carbon, nitrogen and phosphorus content and extracellular polysaccharides in the EPS (extracellular polymeric substances). Then, the metabolic (enzyme activities) and structural (biomass and nutrient content) parameters obtained for each benthic community were used to describe the development and responses of microbial populations exposed to environmental and human-induced perturbations. In addition, the metabolic and structural parameters of each benthic community were contrasted to determine relationships between community structure and OM use. Community composition analysis was performed by

means of aquatic hyphomycete and diatom taxa identification, and through 16S rDNA gene analysis for bacteria.

The seasonal and spatial variation of benthic microbial communities in the Fuirosos stream was analyzed over a hydrological year (from March-2003 to February-2004). Major environmental perturbations of Mediterranean streams were analyzed in this study, since the stream drought in summer (July-August), and had flooding episodes in autumn (October). Benthic community structure and metabolism was approached by means of microbial biomass standing stock and extracellular enzyme activities. Special emphasis was given to upscale measures performed at the microhabitat scale (cm^2) up to the reach scale (m^2), offering a more realistic approach on the microbial benthic community functioning at the Mediterranean-type stream ecosystem.

Highest development of microbial heterotrophs (bacteria and fungi) in stream benthic communities took place in autumn. For this reason, an intensive experiment was conducted in the Fuirosos to determine the effect of litterfall and flooding episodes on benthic microbial communities. The specific role of aquatic fungi was determined at the different benthic substrata (leaves, branches, sand and gravel) by means of fungal biomass standing stock and OM decomposition activities (cellobiohydrolase, phenol oxidase and peroxidase) analysis. In parallel, the elemental composition (C, N, and P content) and the biomass standing stock of other microbial components of the microbial community (bacteria and algae) were analyzed at the different substrata to determine possible links between the OM use (enzymatic activities) and the nutrient molar ratios (C:N and N:P) of microbial attached communities.

Aquatic fungi mostly accumulate and degrade leaves and branches submerged in the stream (Findlay et al., 2002; Gessner, 1997), although bacteria are also relevant in litter decomposition process (Bengtsson, 1992; Mille-Lindblom & Tranvik 2003; Romaní et al., 2006). The relationship between fungal and bacterial biomass accumulation and extracellular enzyme activities in leaves was analyzed during a colonization experiment. The activity of a range of polysaccharide and lignin degrading enzymes was analyzed together with changes in the physical and chemical properties of leaves.

Algae and bacteria are key components of the epilithic metabolism. Colonization experiments of epilithic biofilms were performed in parallel in streams of different bioclimatic regions (Central European, Walzbach and Mediterranean, Fuirosos). The Walzbach is a forested stream located in the south west of Germany and very close to the Forschungszentrum Karlsruhe GmbH (FZK), where most of this experiment was performed. The two streams had different hydrological regimes and

water nutrient concentration (Figure 1.7), but similar order and benthic substrata composition. The bacterial intergenic sequence region (ISR) and 16S rDNA gene sequence amplification protocols were optimized by epilithic biofilm samples. This allowed the determination of successional changes in bacterial populations during biofilm formation in the two streams. All these analyses were accompanied of structural (microbial biomass, CNP and EPS content) and metabolic (extracellular enzyme activities) measurements in biofilms, in order to determine whether biofilm development was influenced by the sampling site. Resulting from this study and togther with the FZK, a protocol for extracellular polymeric substances (EPS) separation and polysaccharide quantification in biofilm samples was also optimized. Since the biofilm matrix is an effective store of exoenzymes (Sinsabaugh et al., 1991), an additional colonization experiment served to investigate on the relevance of matrix enzymes and their relationship with total biofilm enzyme activity during colonization process. Special emphasis was given on the first days of colonization, when biofilm microbes produce large amounts of EPS (Battin et al. 2003).

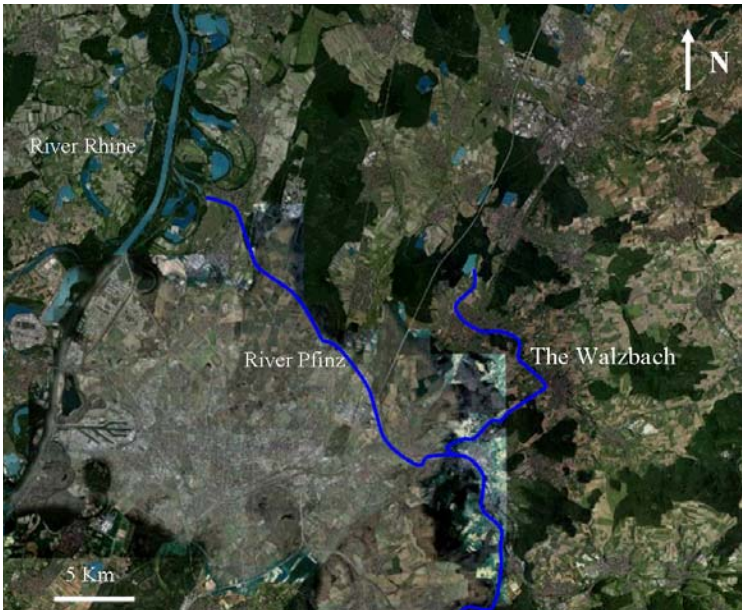


Figure 1.7. Map of the Walzbach stream. It is a tributary of the Pfinz river that flow into the river Rhine. Source: Google Earth.

Nutrient enrichment in streams has direct effects on benthic microbial community structure and metabolism (Carr et al., 2005). Two enrichment experiments were conducted by using biofilms growing on organic and inorganic substrata. The first experiment evaluated how nutrient concentrations would affect sporulation rates and species composition of aquatic hyphomycetes growing in leaves, sand and rocks. In the second experiment, biofilms grown at similar nutrient conditions were subjected to

changes in the water nutrient concentrations (high and low nutrient concentrations) and N:P molar ratios (from 16 to 56). The effect of nutrients was evaluated on biofilm CNP content and biomass standing stock as well as on extracellular enzyme activities.

MATERIALS AND METHODS

2.1 Sampling of benthic substrata

The stream bed is made up of different substrata of both inorganic (rocks, cobbles, sand) and organic (leaves, wood) nature, that are colonized by microbial communities (Figure 2.1). Although the substratum characteristics can influence on the microbial composition, variations of in-stream environmental conditions (i. e. current velocity, oxygen and light) may also shape microbial communities. Therefore, studies on microbial ecology in streams need to integrate all this variability by analyzing the different benthic substrata all over the studied zone (in pools, runs, riffles and in shaded and unshaded zones). The sampling of the different benthic substrata carried out in this thesis has been specified in the following table:

Substrata	Sampling
Rocks, cobbles and boulders	Glass and/or ceramic artificial substrata of 1-10 cm^2 of surface area were glued onto stream cobbles and immersed in the stream for colonization, at least 6-8 weeks, before sampling.
Gravel	Gravel samples were taken directly from the streambed as individual grains that measured from 1.5 to 2 cm in diameter.
Sand	Sand substratum was sampled by coring perspex cylinders (2-6 cm in diameter and 3 cm in depth). Then, sand was transferred into a plastic container and subsamples were later collected with an untapped syringe (*c.* 1 ml sand volume per sample)
Leaves	A metal borer (1.12 cm in diameter) was used to cut leaf discs from the entire leaves.
Branches	Long branches of 0.5 to 1.5 cm in diameter were cut in pieces of 1.5 cm in length.

In all cases, the surface area of the whole substratum was considered as potential surface area colonized by microbes. Surface area of leaf samples was 0.987 cm^2 in each side of the disk, while branches surface area was obtained after calculating the surface of the whole cylinder. Surface area of sand was calculated through a conversion factor obtained by granulometry, which related sand dry mass to its surface area (1.003 gDW cm^{-2} for the Fuirosos sand), while the surface area of gravel was

obtained by measuring the surface area of an aluminium foil paper used to wrap the grains.

In the case of leaves and branches substrata, dry weight (80ºC during 48h in the oven) and/or ash free dry weight (450ºC during 4h in the incinerator) of materials was also measured.

Figure 2.1. Images of the most common benthic substrata analyzed. On the left side, sanblasted glass tiles of 1 cm^2 used as rocky substrata surrogates, on the right side leaf and sand substrata.

2.2 Microbial biomass

2.2.1 Fungal biomass

Ergosterol concentration analysis can be used as a surrogate for fungal biomass in plant litter (Newell, 1992). Ergosterol is the major membrane constituent of fungal cells and is considered one of the best descriptors of fungal metabollicaly active biomass, rather than other fungal cell constituents (i. e. chitin, Newell, 1992; Charcosset & Chauvet, 2001; Gessner & Newell, 2002). Although it is widely understood that stream microfungi mainly develop on particulate organic matter (POM; especially in leaves, Findlay et al., 2002; Hieber & Gessner, 2002), this study also investigated the fungal biomass accrual in the submerged inorganic substrata (sand, gravel, rocks).

Sampling procedure

Ergosterol concentration was analyzed in leaves of distinct species (*Platanus acerifolia, Populus nigra, Alnus glutinosa*), fine substrata (sand and gravel) and coarse substrata (cobbles, boulders and rocks). The distinct substrata were analyzed considering the following amounts of sample: 1.5 g of leaf dry mass for leaves (*c*. 10 leaf disks), 4 g for sand (*c*. 10 ml sand volume), 4 grains for gravel, 6 g of wood dry mass for branches (*c*. 6 branch pieces) and 5 ceramic tiles for coarse substrata. Each substratum sample was taken in triplicate and cleaned with stream water to remove silt particles. Then samples were placed in polyethylene vials (30 ml capacity) and transported at 4ºC. Once in the laboratory, samples were frozen at -20ºC until analysis.

Ergosterol extraction, separation and quantification

Frozen samples were liophilyzed (Cryodos, Telstar, Spain), weighted (DW) and placed in screw-cap extraction tubes (40 ml capacity). Extraction of ergosterol was performed with KOH methanol (8 g L^{-1}) and incubating samples at 80ºC during 30 min in a shaking bath. The resultant extracts, especially those belonging to the fine and coarse substrata, were filtered through 0.7 μm (glass fiber filters, Whatmann GF/F) to reduce fine detritic particles. Clean extracts of lipids were passed through solid-phase extraction cartridges (Waters Sep-Pak®, Vac RC, tC18, 500 mg, Figure 2.2) in order to separate and concentrate the ergosterol (Gessner & Schmitt, 1996). Then, ergosterol was eluted using isopropanol.

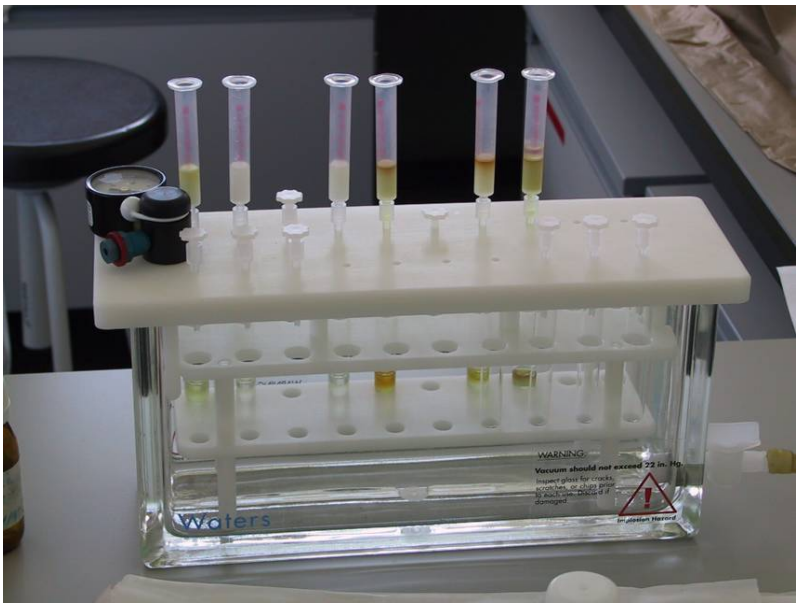


Figure 2.2. Vacuum manifold for solid phase extraction (Waters Extraction Manifold).

Detection of ergosterol was performed by high pressure liquid chromatography (HPLC, Waters Corporation, USA). HPLC system consisted of one pump, injector, column (Licrospher 100 RP-18, Merck, Germany), UV detector set to 282 nm and a recording unit. Chromatograms were set as follows: 100% mobile phase (100% methanol), flow rate of 1.4 ml min^{-1}, column temperature at 33 ºC, detection wavelength at 282 nm and injection volume of 10 μl (Gessner & Schmitt, 1996).

Quantification of ergosterol was performed by integrating and calculating peak areas (approx. 8 min. retention time) and then comparing to ergosterol pure standards (0-200 μg ergosterol ml^{-1}, Fluka Chemical Corp., Figure 2.3). Results were expressed in μg of ergosterol per unit of mass (for leaves and wood) and/or surface area. Fungal biomass in terms of carbon was estimated on the basis of an ergosterol content of 5.5 mg g^{-1} fungal biomass (Gessner & Chauvet, 1993), and considering a 43% carbon content in fungal dry mass (Baldy & Gessner, 1997).

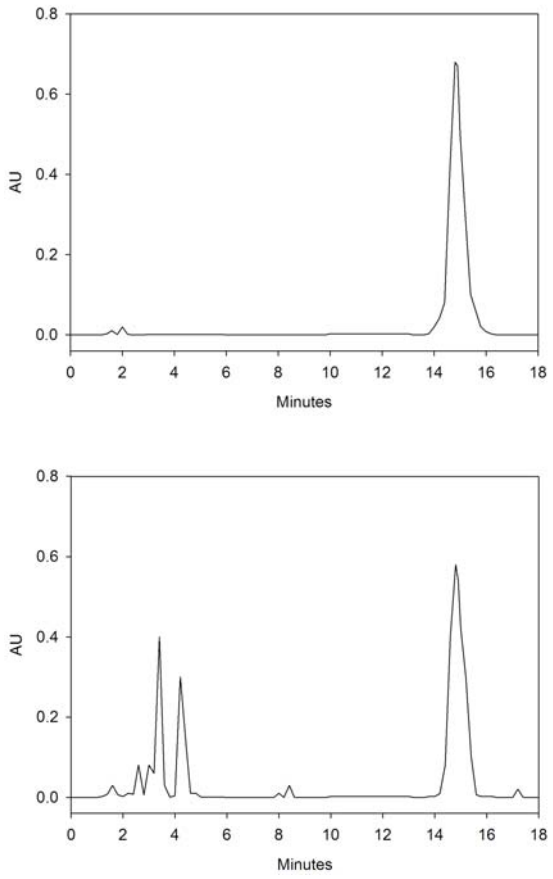


Figure 2.3. Chromatograms of an ergosterol standard (100 μg ergosterol ml^{-1}, upper graph) and a sample of Platanus acerifolia leaves collected in the Fuirosos stream (lower graph). The retention time of the ergosterol peak was delayed (up to min 15) because of a guard-column installation.

2.2.2 Bacterial biomass

Samples for bacterial biomass measurements consisted of one leaf disk, 1 ml of sand volume, one piece of wood, one gravel grain and one ceramic/glass tile. Substratum samples were placed in glass vials (30 ml capacity) filled with stream water and fixed with formalin (2%).

Bacterial density was estimated after the sonication of the samples. The 2 + 2 min sonication time (40 W power, 40 KHz frequency, Selecta, Spain) allowed to detach most of the bacteria attached on substrata and to avoid bacterial cell lysis (Romaní et al. 2004). After the appropriate dilution, bacterial suspensions were stained for 10 min with DAPI (4,6-diamidino-2-phenylindole) at a final concentration of 2 μg ml^{-1}, filtered through 0.2 μm irgalan black stained polycarbonate filters (Nuclepore), and bacteria were counted using a fluorescence microscope (Eclipse E600, Nikon, Japan) with × 1000 magnification (Porter & Feig, 1980). Fifteen fields were counted per filter, accounting for a total of 400-800 cells. Bacterial biomass in terms of carbon was estimated based on the conversion factor of 2.2 x 10^{-13} g C μm^3 (Bratbak & Dundas, 1984) and considering a bacterial biovolume of 0.1 μm^3 (Theil-Nielsen & Sondergaard, 1998).

2.2.3 Algal biomass

Algal biomass was analyzed by means of the chlorophyll-*a* concentration. Samples for chlorophyll-*a* analysis consisted of one leaf disk, 1 ml of sand volume, one piece of wood, one gravel grain and one ceramic/glass tile. Samples were placed in polyethylene vials (without water and preserving from light) and stored frozen (-20ºC) until analysis. The chlorophyll concentration in fine (sand and gravel) and coarse (tiles) substratum biofilms was measured after extraction in 90% acetone (during 24 h at 4ºC). To ensure complete extraction of the chlorophyll, samples were sonicated before and after addition of acetone (2+2 min).

In contrast, determination of chlorophyll concentration in leaves and wood communities required the previous detachment of the microbial community. With this purpose, each sample was thawed in 2 ml of purified water and then carefully sonicated with an ultrasonic probe (2 min, Labsonic 2000, Braun, Germany) to obtain the extract of the attached community. The detachment of the colonizing community on leaves was verified by microscopic observation before and after sonication. The 2-min sonication was chosen to detach the major part of the attached microbial community and, at the same time, to avoid the fragmentation of leaf material. The extract was

filtered (glass fiber filter, Whatman GF/F) and chlorophyll from the filter was further extracted with acetone (90% final concentration, during 24 h at 4ºC).

Chlorophyll concentration was determined spectrophotometrically (U-2000 Spectrophotometer, Hitachi, Japan) after filtration of the chlorophyll extracts (Whatman GF/F, glass fiber filter 1.4 um porus) and according to the method of Jeffrey & Humphrey (1975). Algal biomass in terms of carbon was calculated based on the relationship Algal Carbon: Chlorophyll-*a* = 60 (Geider & MacIntyre, 1996).

2.3 Microbial community composition

2.3.1 Aquatic hyphomycetes diversity

Aquatic hyphomycete species composition was measured in leaves of *Platanus acerifolia* and *Populus nigra* (8 leaf disks per sample), as well as in fine (5 ml of sand volume per sample) and coarse (5 ceramic tiles per sample) inorganic substrata (Figures 2.4 and 2.5).

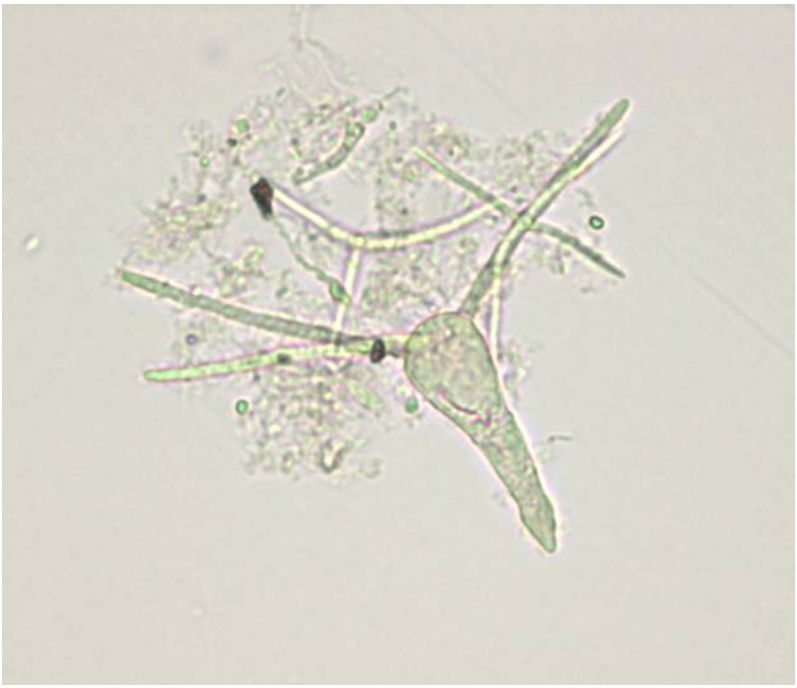


Figure 2.4. Clavariopsis aquatica and Alatospora acumiata conidia released from the microbial community attached on Platanus acerifolia leaves. Image from the optical microscope at 1000 × without Trypan blue staining.

Each substratum was incubated in 250 ml Pyrex flasks containing 100 ml of sterilized stream water (water filtered through 0.2 μm nylon filters, Whatman) to stimulate fungal sporulation (this technique is further explained in the "*Enzymatic activities and sporulation rates*" procedures). After 48h of incubation, fungal suspended

conidia were filtered through 5 μm-pore sized filters (cellulose nitrate filters, Whatman) and the retained conidia were stained with 0.1% Trypan blue in 60% lactic acid (Baldy et al., 2002). Then, conidia were counted and identified to determine the relative proportion of each species following the Ingold (1975) guide for aquatic and water-borne hyphomycetes and Roldan (1988). Ten microscopic fields were counted at × 400 (usually ×1000 were required for species identification) for the leaf samples, while the whole filter was counted for the fine and coarse substrata samples.

Scannig Electron Microscopy (SEM) observations

Incubated substratum samples (leaf disks, sand grains and ceramic tiles) were also prepared for SEM observation by fixing them with 2.5% glutaraldehyde in 0.1 M cacodilate buffer pH 7.2 to 7.4 to preserve the soft structures of the attached microbial community. Samples were first dehydrated by an alcoholic series (65 to 100%), then drought by the critical point of CO_2 (CPD) and finally gold coated with a sputtering diode. Observations were made using Zeiss DSM 960A electron microscope (× 10 to × 300000 magnification and 70 Å resolution).

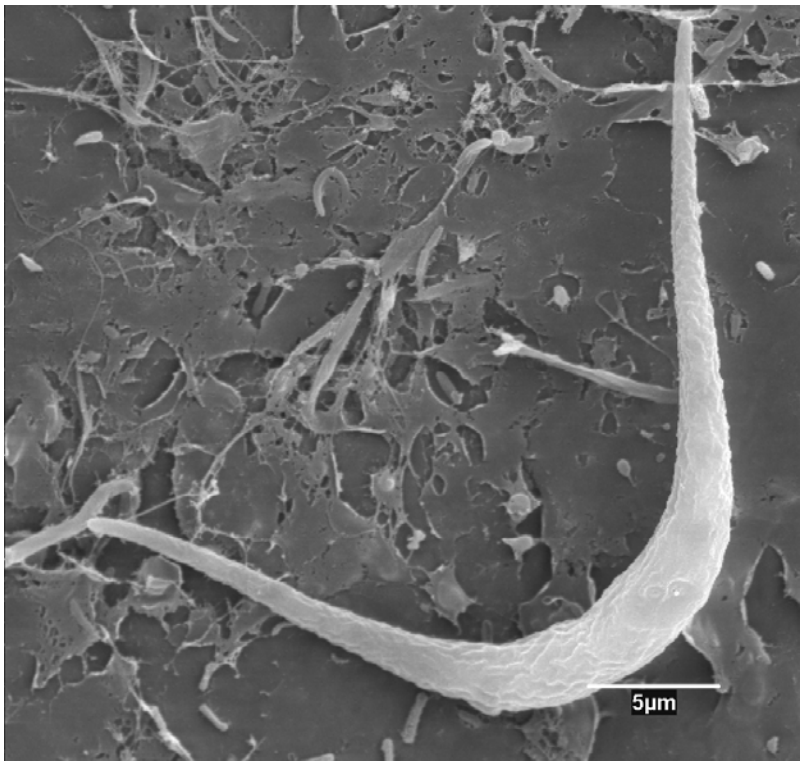


Figure 2.5. SEM image of Lunulospora curvula conidia attached on Populus nigra leaves.

2.3.2 Bacterial diversity: comparison of ribosomal DNA sequences

Molecular tools to monitor bacterial diversity in complex microbial assemblages have developed during the last decade using 16S ribosomal DNA-based approaches. One of the most used methods was the 16S rDNA based polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) technique described by Muyzer et al. (1993). An extension of this method has been recently used to analyze bacterial diversity in the stream epilihic biofilm (Jakson et al., 2001; Lyautey et al., 2003).

As evolutionary distances between bacterial species increase, the diversity found in the conservative bacterial 16S rDNA genes is often sufficient and, thus, the genetic relationships of related species can be defined accurately. However, very close taxonomic relationships limit the sequence differences and significance of gel-based community fingerprinting analysis (Rogall et al., 1990; Cilia et al., 1996). In order to overcome these limitations, some studies included a phylogeny analysis of the bacterial intergenic spacer region (ISR; the region between the 16S and 23S rDNA conservative regions; Leblond-Bourget et al., 1996; Mehta & Rosato, 2001). The amplification of ISR reveals a greater sequence diversity and length variation than the bacterial 16S rDNA, improving intra-specific relationships.

Sampling procedure

Sampling of epilithic biofilms was performed using sandblasted glass tiles of 9.6 cm^2 of surface area. Two tiles were scraped per replicate using a sterile cell scraper (Nunc, Wiesbaden, Germany) and suspended in 1 ml sterile stream water (filtered through 0.2 μm Whatmann nylon filters). Biofilm suspensions were kept cold (4 ºC) until arrival at the laboratory, where the amount of fine particulate organic matter (FPOM) in the samples was reduced by a resuspension-sedimentation cleaning step. Afterwards, a master biofilm suspension was generated for each sampling date by mixing the three replicates and diluting 10 times with ultra pure water. Lysis of microbial cells and total DNA release was accomplished by some freeze-thawing (-20ºC/37ºC) steps. The solutions were used as templates for subsequent molecular analysis.

Bacterial rDNA analysis, fingerprinting, and sequencing

The successional changes of bacterial populations during the epilithic biofilm formation were evaluated by amplification of the variable V3 and V5 regions of the bacterial 16S rDNA gene using the 341F-GC (including GC-clamp for DGGE) and 907R specific-primers. Also, bacterial ISR was amplified using the 16F and 23R forward and reverse primers, respectively (Table 2.1).

Primer	Sequence (5' to 3')	Reference
341F-GC*	CCT ACG GGA GGC AGC AG	Muyzer et al. (1997)
907R	CCG TCA ATT CCT TTG AGT TT	Muyzer et al. (1997)
16F	CTT GTA CAC ACC GCC CGT C	Garcia-Martínez et al. 1996)
23R	TGC CAG GGC ATC CAC CGT G	Garcia-Martínez et al. (1996)

Table 2.1. Sequences of the primers used in this study. (*) GC is a 40-nucleotide GC-rich sequence attached to the 5' end of the primer. The GC sequence is 5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3'

The PCR mixture for the 16S rDNA gene fragment amplification was composed of 5 μl of 10 × PCR buffer, 1 μl deoxyribonucleotide triphosphate (dNTPs, 200 μM), 0.3 μl of each primer (0.1 mM), 0.25 μl (1.25 U) of HotStar Taq-DNA polymerase (Quiagen, Germany), 3 μl biofilm template, and ultrapure water to give a final volume of 50 μl. The PCR temperature profile consisted of an initial Taq-polymerase activation step of 15 min at 95ºC, followed by 30 cycles of 1 min at 95ºC, 1 min at 54ºC, and 1.30 min at 72ºC. Then, the solution was kept for 10 min at 72ºC, and finally, held at 4ºC. All PCR assays were performed using a GeneAmp PCR System 9700 (Applied Biosystems, Germany).

The PCR reaction mixture for the amplification of the ISR was composed of 5 μl of 10 × PCR buffer, 1 μl dNTPs (200 μM), 1.5 μl of each primer (0.01 mM), 0.25 μl (1.25 U) of HotStar Taq-DNA polymerase (Quiagen Germany), 10 μl of biofilm templates, and ultrapure water to give a final volume of 50 μl. The PCR temperature profile consisted of 15 min at 95ºC, followed by 35 cycles of 30 s at 94ºC, 2 min at 54ºC, 3 min at 72ºC. Then, the solution was kept for 7 min at 72ºC, and finally, held at 4ºC.

The resultant amplicons of PCR assays were subjected to electrophoresis in 1% agarose gels in order to check PCR fitness. A volume of 10 μl of the 16S rDNA and ISR amplicons was loaded on agarose gels and stained with ethidium bromide. The sizes of the amplicons were defined by comparing samples to a DNA molecular weight marker of 100 base pairs (bp) ladder (XIV Roche Diagnostics GmbH, Germany; Figure 2.6).

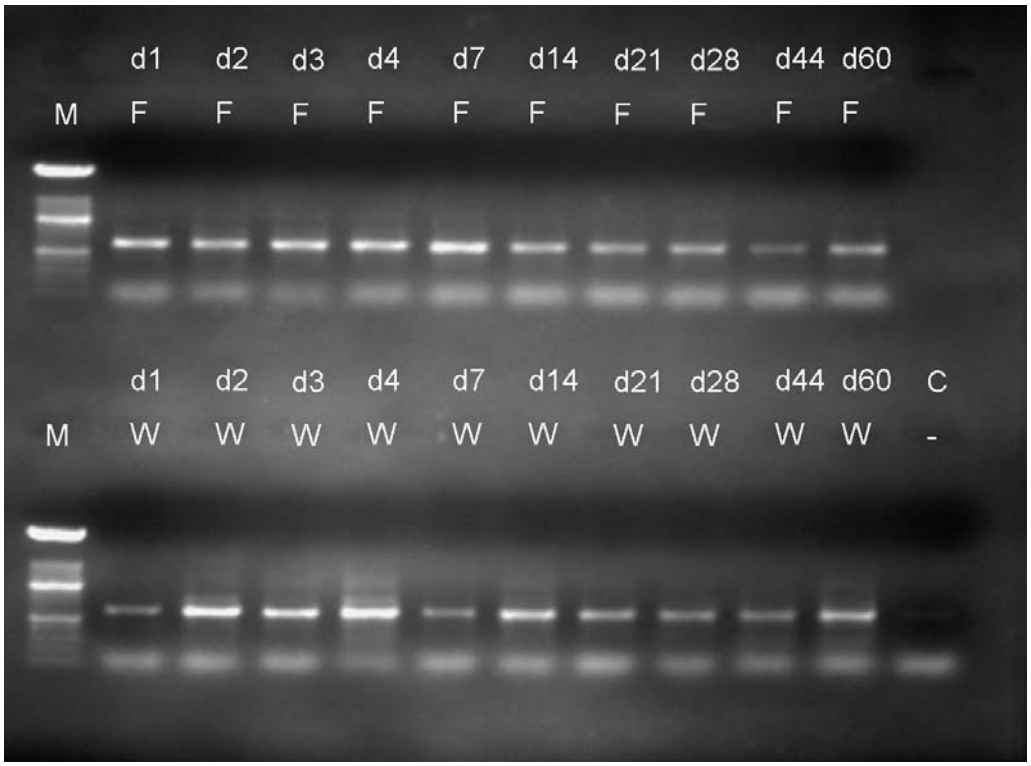


Figure 2.6. Agarose gel of PCR amplicons from the biofilm developed at the Fuirosos (upper row) and at the Walzbach (lower row). Each run correspond to one sampling date, 10 for each sampling site in total. On the left side of each row, a DNA molecular weight marker was loaded (M), and a negative control was assayed in the last run of the lower row (C-). Two bands were detected in each run, the upper belongs to the amplificated 16S rDNA fragment and the lower belongs to the DNA of specific primers used for the PCR.

Screening of 16S rDNA amplicons (volumes ranging from 15 to 24 μl) was performed through denaturing gradient gel electrophoresis (DGGE). Perpendicular DGGE was performed using the D-Code system following the procedures described elsewhere (Bio-Rad, Germany). The DGGE gels possessed a gradient ranging from 40 to 70% urea (where 100% denaturant was 7 M urea and 40% deionised formamide). PCR amplicon aliquots from 20 to 24 μl were loaded on gels. Electrophoresis conditions were 70V for 14 h at 56°C, the gels were run in 1 × TAE buffer (40 mM Tris, 20 mM actetate, 1 mM EDTA, pH 8.5).

ISR amplicons (volumes ranging from 10 to 17 μl) were subjected to perpendicular acrilamide gel electrophoresis (PAGE) in 6 % polyacrylamide gel in 1 × TAE buffer (40 mM Tris, 20 mM actetate, 1 mM EDTA, pH 8). Electrophoresis conditions were at room temperature and 250 V for 4 h using the D-Code system (Bio-Rad Laboratories GmbH, Germany).

After electrophoresis, both the DGGE and PAGE experiments were stained with SYBR™ Gold (Sigma-Aldrich, Germany) and immediately visualised by UV trans-

illumination in the LumiImager Working Station™ (Roche Diagnostics, Germany; Figure 2.7).

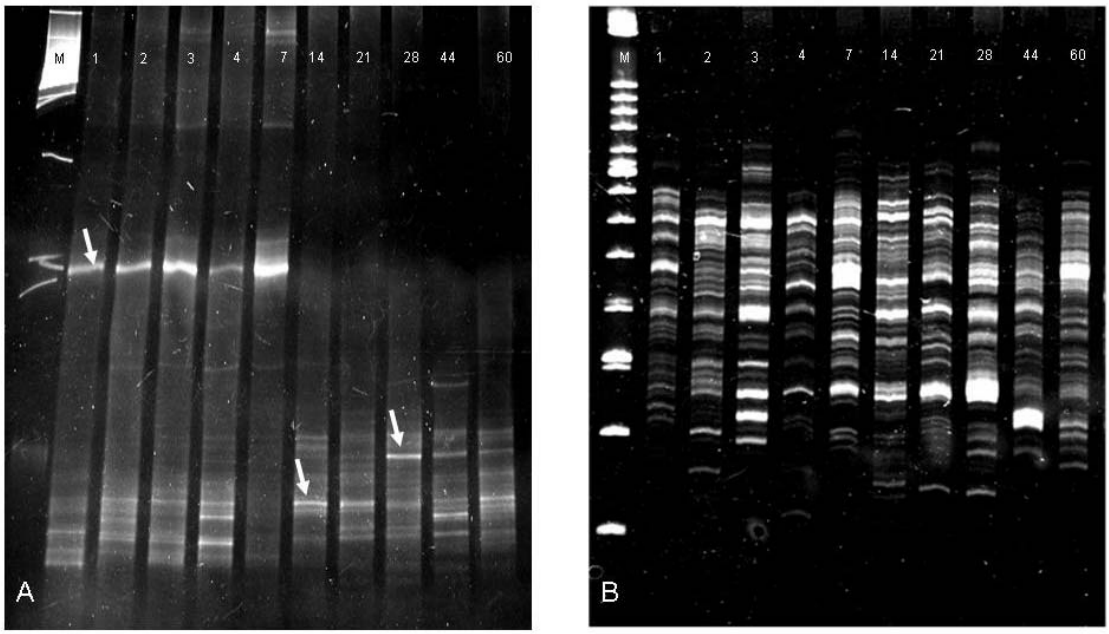


Figure 2.7. 16S rDNA-DGGE (A) and ISR-PAGE (B) experiments with biofilm samples from the Fuirosos stream are shown. The numbers on the top correspond to the age of the biofilm in days, while the white arrows point the bands that were excised for 16S rDNA sequence analysis. On the left side of each gel, a DNA molecular weight marker was loaded to determine the size, in base pairs, of the bands.

Selected dominant bands (based on band intensity, Figure 2.7A) were excised from DGGE gels and placed in 20 µL of sterile water, where DNA was eluted by diffusion over night at 4°C. Eluted DNA was re-amplified according to the protocol described above and a second round of DGGE (conditions identical to those described earlier) was used to check the purity of the excised bands. The amplified products from these bands were sequenced in a dedicated facility (ABI 310, Applied Biosystems, Germany). Sequence analysis and phylogenetic alignments were carried out using the ARB software package.

2.3.3 Diatom species diversity

Diatom species diversity was determined during colonization of sandblasted glass tiles. Tiles (4.2 cm^2 of surface area) were placed in glass vials filled with stream water and fixed with formalin (2%). Diatoms were detached from tiles by sonication (2 + 2 min) and kept in suspension. Then, samples were cleaned from the organic material using sulphuric acid, dichromate potassium and hydrogen peroxide following the protocol described by Barber & Haworth (1981). Clean diatom frustules were mounted

on permanent glass slides using Naphrax (1.74 refringence index, Brunel Microscopes Ltd.). A total of 400 valves were counted per sample by perfoming random transects under light microscopy using Nomarsky differential interference contrast optics at a magnification of ×1000 (Figure 2.8). Diatom taxa were identified mainly according to Krammer & Lange-Bertalot (1991-1997) and Lange-Bertalot (2001) guides.

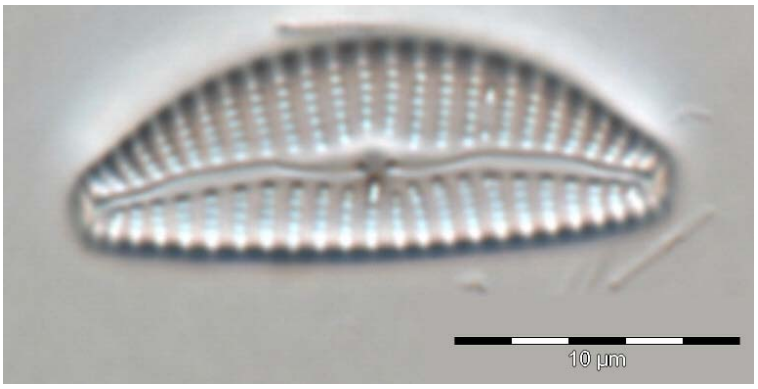


Figure 2.8. Cymbella affinis species present in epilithic biofilm samples developed in the Walzbach stream. Source: Elisabet Tornés.

2.4 Chemical composition of attached microbial communities

2.4.1 Carbon, nitrogen and phosphorus content

Carbon, nitrogen and phosphorus content was analyzed in microbial communities associated to leaves, branches and fine and coarse inorganic substrata. Sample collection and storage was identical to that described for the chlorophyll-*a* analysis.

For attached microbial communities in leaves and branches, each sample was thawed inside glass vials, and 5 ml of purified water were added. Biofilms on fine and coarse inorganic substrata were detached by sonication using an ultrasonic bath (2+2 min, 40 W power, 40 KHz frequency, Selecta) while an ultrasonic probe (2 min, Labsonic 2000) was used to remove the attached community in leaves and branches. The resultant suspension of the microbial community was filtered on pre-combusted and pre-weighed GF/F filters (0.7 µm, glass fiber filter Whatman). Then, filters were dried for 48h at 80 ºC, and later on, analyzed for CNP. C and N content of filters was determined by means of a CN Elemental Analyzer (Carlo Erba 1500, Italy) using vanadium pentoxide as the oxidation catalyzer. In contrast, P content was determined after the basic digestion (NaOH) of filters in an autoclave (110ºC for 90 min; Grasshoff et al., 1983). Digestion of filters transformed all organic phosphorus forms into inorganic forms, and then, total P content was determined according to APHA (1989).

Based on total C, N and P content of biofilms, C:N, C:P and N:P biofilm molar ratios were calculated.

The detachment of leaves and branches microbial communities was tested to rule out influences of plant material in the community nutrient analysis. Verifications were made by microscopic observations (before and after sonication), but also comparing C:N molar ratios of recently fallen leaves of *Platanus* and *Populus* species from the Fuirosos riparian forest and their corresponding microbial detached communities. Results showed that C:N molar ratio values of leaves (77.7 ± 0.8 and 91.5 ± 3.2 in *Platanus* and *Populus* n = 10, respectively) were higher than the measured for the microbial detached communities (22.5 ± 2.7 in average in the *Platanus* and *Populus* fresh and decaying leaves n = 10). These differences suggested that leaf particles were not influencing on the biofilm nutrient measurements. Moreover, the efficiency of the GF/F filters in retaining the detached microbial community during the filtration process was tested. Several samples (n = 30) of the filtered products were taken and analyzed for total C, N and P content and then compared with the total C, N and P of the filter. The results revealed that the 80% of microbial community materials were retained in the filters, while a 20% loss of microbial community must be assumed when using this methodology.

2.4.2 Extracellular polymeric substances (EPS) extraction and polysaccharide content analysis

For EPS extraction, complete biofilm from three tiles for each replicate was detached (sterile silicone scraper) and taken up in 1.5 ml of phosphate buffer (1.7 g/L KH_2PO_4; 4.5 g/L $Na_2HPO_4 \cdot 12\ H_2O$; pH-value 7.0). Phosphate buffer was used to counteract the decrease in pH-value caused by treatment with the cation exchange resin (DOWEX MARATHON C, Na^+-form, strongly acid, Sigma-Aldrich, Germany). The resin was conditioned before application, following the manufacturer's instructions. Conditioned resin was added to each vial (0.5 g per vial) and EPS extraction was performed for one hour on a shaker at 300 rpm and 4°C. The extraction time and resin concentration used, minimize microbial cell disruption and maximize EPS extraction (Frølund et al., 1996; Chapter 6). Crude EPS extracts were centrifuged at 12000*g* for 15 minutes (Sorvall RC 5B Plus, USA) to remove solid parts of the biofilm. The resulting clear EPS extracts were analyzed for polysaccharide content.

Polysaccharide content of the whole biofilm and EPS fraction was determined following the protocol described by Dubois et al. (1956). For the whole biofilm, biofilm from 1 tile was detached in 0.5 ml phosphate buffer (pH 7), while for EPS, 0.5 ml of EPS extract was used. Phenol-solution (80%) was added immediately (12.5 µL), and

then 1.25 ml of concentrated sulfuric acid was pipetted rapidly into the tubes to ensure sufficient mixing. The samples were allowed to stand for 10 minutes before mixing and were later incubated in a water bath at 30°C for 20 minutes. Absorbance was measured at 485 nm against a reagent blank (U-2000 Spectrophotometer, Hitachi). The polysaccharide content was calculated using glucose for the standard curve (0-200 $\mu g\ ml^{-1}$).

2.5 Physical and chemical leaf properties

Microbial communities developing in leaf substrata are not only attached but included in leaf tissues (especially fungi). Therefore, the physical and chemical properties of the leaves, which might be highly variable on time, may be influencing the development of the microbial community on these organic substrata.

2.5.1 Carbon, nitrogen and phosphorus content

C, N and P content was analyzed in leaves (including the CNP content from the leaf and the attached microbial community together). Sample collection and storage was identical to that described for the chlorophyll-*a* analysis. Leaf circles were dryed and subsamples of known weight were taken and placed in tin foil crucibles for C/N analysis through CN Elemental Analyzer (Carlo Erba 1500, Italy). P content was determined after the basic digestion (NaOH) of leaf subsamples in an autoclave (110ºC for 90 min; Grasshoff et al., 1983). Based on total C, N and P content of leaves, C:N, C:P and N:P biofilm molar ratios were calculated.

2.5.2 Lignin content and leaf toughness

Lignin content and toughness of leaves was determined in triplicate in *Platanus acerifolia* and *Populus nigra* species. Lignin content of leaf circles was measured spectrophotometrically at 280 nm after the digestion of samples with a mixture of 25% acetyl bromide in acetic acid and 4% perchloric acid at 70 ºC for 30 min (Iiyama & Wallis, 1990). The lignin content of the samples was determined using the equation of Morrison (1972). Results were expressed as the percent of lignin remaining in the leaf circle.

Leaf toughness was estimated as the force needed to tear apart leaf circles. A tearing device equipment was specially designed for this experiment, following the methodology described by Graça & Zimmer (2005). Leaf tear strength was expressed in KNewtons.

2.6 Extracellular enzyme activity and fungal sporulation rates

2.6.1 Extracellular enzyme activities

In this study, several hydrolytic and oxidative extracellular enzyme activities were assayed in microbial communities developing on distinct stream substrata. Samples consisted of 1 leaf disk, 1 piece of branch, 1 ml. of sand volume, 1 gravel grain and 1 tile placed in glass vials filled with stream water (5 ml) and kept cold (4ºC) until arrival at the laboratory. Enzyme assays were performed the same sampling date, or maximum the following day in order to not over- and/or infra-estimate microbial metabolism measurements. Extracellular enzyme activity was also measured in the EPS matrix (matrix-enzyme activity). In this case, 0.1 ml of EPS extract was diluted to 5 ml with stream water.

Prior to enzyme assays, several enzyme activities (β-glucosidase, leucine-aminopeptidase and phenoloxidase) were tested to determine whether analysis with 1) suspended detached microbial community, 2) sonicated community and, 3) intact community show differences on enzyme activity values (data not shown). For this exercise, leaf disks of *Platanus acerifolia* species were used. Results showed that some activity still remained in the sonicated leaves, but also that the activity of the suspended detached microbial community was higher than the activity in the intact community. This test indicated that the disruption of the attached microbial cells improved diffusion and nutrient availability, leading to an overestimation of the real activity. As a methodological compromise, enzyme activities were therefore analysed on intact community.

Hydrolytic enzyme activity assays

Measurements of the extracellular hydrolytic enzyme activity in microbial communities followed the methodology described by Romaní et al. (2001). A total of six different hydrolytic enzyme assays were performed in this study. Polysaccharide compounds degradation was determined by means of the β-glucosidase (EC 3.2.1.21), β-xylosidase (EC 3.2.1.37) and cellobiohydrolase (EC 3.2.1.91) activity. See mode of action of polysaccharide degrading enzmes in Figure 2.9. Organic phosphorus compounds and peptides decomposition was determined by means of the phosphatase (EC 3.1.3.1-2) and leucine-aminopeptidase (EC 3.4.11.1) activity, respectively. And, chitin decomposition was determined through the β-glucosaminidase activity (EC 3.2.1.30).

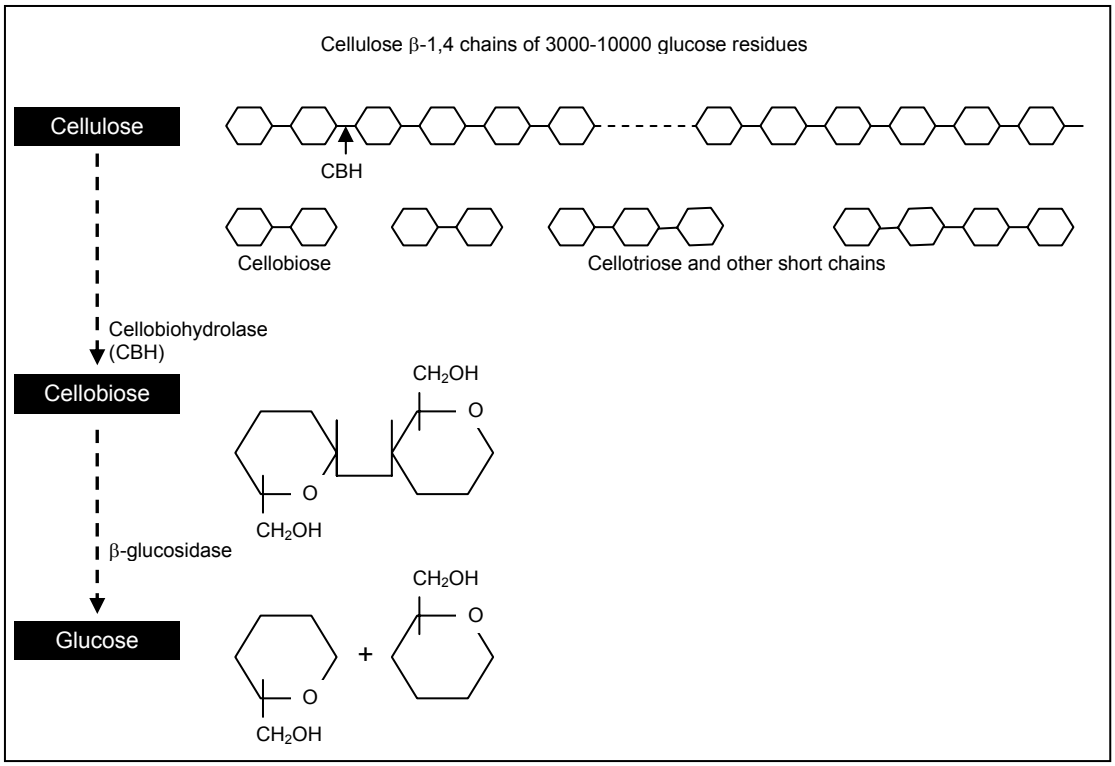


Figure 2.9. Breakdown of cellulose molecule by microbial enzymatic activity. Mode of action of the cellobiohydrolase and β-glucosidase degrading enzymes.

β-glucosidase (4-methylumbelliferyl-β-D-glucopyranoside), β-xylosidase (4-methylumbelliferyl-β-D-xyloside), cellobiohydrolase (4-methylumbelliferyl-β-D-cellobioside), phosphatase (4-methylumbelliferyl-phosphate) and β-glucosaminidase (4-methylumbelliferyl-β-D-glucosaminide) activities were measured using MUF (methylumbelliferyl) fluorescent-linked substrates while leucine-aminopeptidase activity was measured using AMC (aminomethyl-coumarin) fluorescent-linked substrate (L-Leucine-7-amido-4-methylcoumarin hydro-chloride). All enzyme assays were performed under substrate saturating conditions, which were determined from previously performed saturation curves (Figure 2.10). Thus, β-glucosidase, β-xylosidase, phosphatase, leucine-aminopeptidase and β-glucosaminidase activity assays were conducted at 0.3 mM final substrate concentration, while cellobiohydrolase activity was conducted at 1.5 mM.

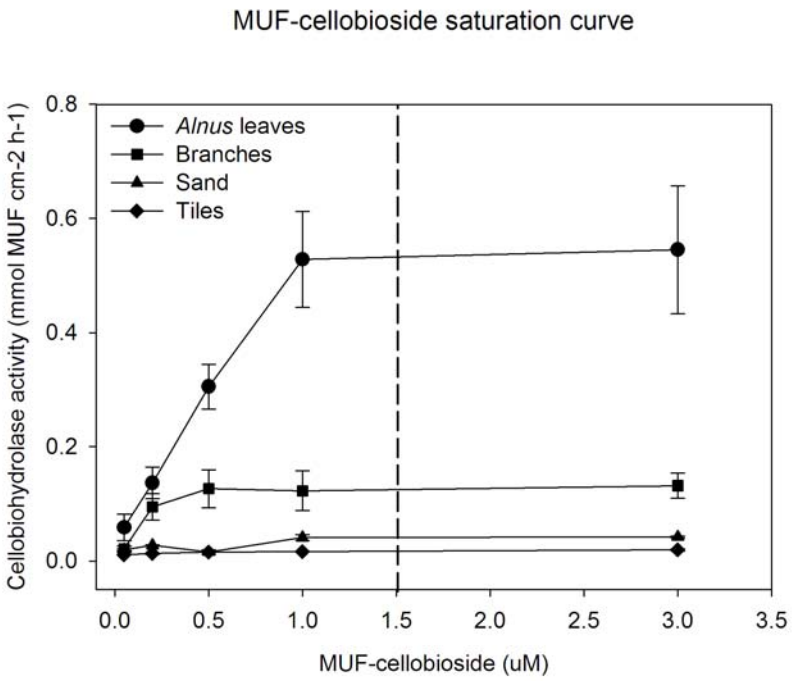


Figure 2.10. Cellobiohydrolase activity saturation curve in leaves of Alnus glutinosa. Dashed line represents current MUF-cellobioside concentration (1.5 mM) used for cellobiohydrolase activity determination.

After MUF and AMC-substrates addition, incubation of samples was performed during one hour in a shaking bath set at stream water temperature. Samples were preserved from the light and water controls (measure of enzyme activity in the water) were also run during each assay. After incubation, 5 ml of 0.05 mol/L pH 10.4 glycine buffer was added to samples in order to stop microbial metabolism. Then, fluorescence of samples was measured at 365-455 nm (excitation-emission wavelengths, respectively) for MUF-substrates and at 364-445 nm for AMC-substrate using a fluorimeter (Kontorn, SFM 25, Germany). Enzyme activity was calculated as the amount of MUF and/or AMC substrate released per unit of time and surface area (h and cm^2).

Oxidative enzymes

The phenol oxidase and peroxidase extracellular enzyme activities were also determined in microbial communities colonizing distinct stream substrata. Ligninolytic peroxidases and phenol oxidases (i. e. laccase) produced by fungi oxidize the lignin polymer, thereby generating aromatic radicals (Hammel, 1997; Figure 2.11). Assays of oxidative enzyme activity involve a substrate that serves as electron donnor, generating a product that can be quantified spectrophotometrically (Mason, 1984). In this study, L-3,4-dihydroxyphenylalanine (L-DOPA) was used as electron-donnor

substrate for detection of phenol oxidase activity. However, when hydrogen peroxide (H_2O_2) is added to the sample, the activity of peroxidases can also be estimated following the same methodological approach (Hendel et al., 2005). Phenol oxidase and peroxidase enzyme assays conducted in this study followed the methodology described in Sinsabaugh & Linkins (1990) and Sinsabaugh et al. (1994).

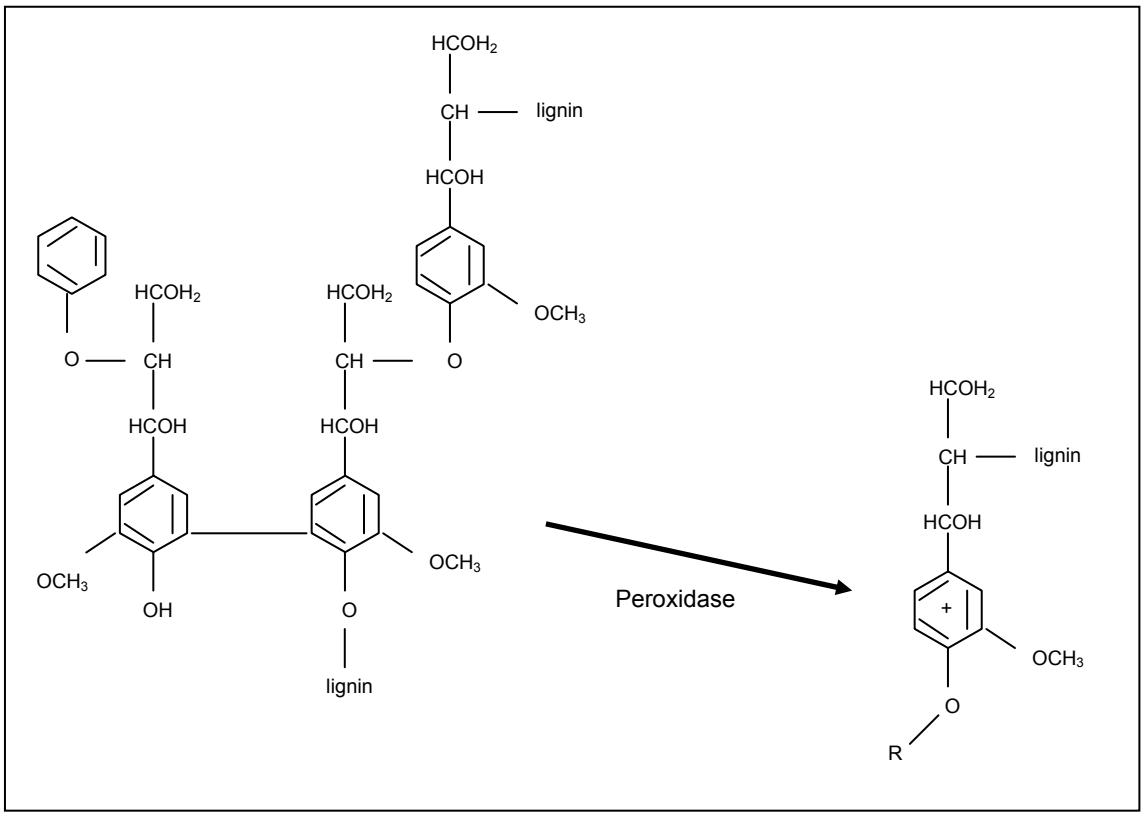


Figure 2.11. Lignin breakdown through peroxidase activity. Lignin peroxidase is an iron-containing enzyme which accepts two electrons from hydrogen peroxide, and then passes them as single electrons to the lignin molecule.

Enzyme incubations of stream substrata were performed in the dark, during one hour in a shaking bath set at stream water temperature. L-DOPA saturation curves determined a substrate saturating concentration of 1.5 mM L-DOPA (in pH 5 acetate buffer) for both the phenol oxidase and peroxidase enzyme assays. However, 0.2 ml of 0.3% H_2O_2 was added to samples for peroxidase activity determination. During incubation, L-DOPA molecule is converted into 2,3-dihydroindole-5,6-quinone-2-carboxilate (DIQC) which can be quantified spectrophotometrically at a wavelength of 460 nm. In contrast to the hydrolytic enzymes, oxidative enzyme activity must be immediately measured after incubation. L-DOPA can be degraded non-enzymatically under some conditions (i. e. light; Sinsabaugh & Linkins, 1990). Additional controls of

each stream substrata (without L-DOPA) and stream water (with and without L-DOPA) were performed for both enzymes and taken into account for final activity calculations.

Calculation of ligninolytic activity was performed according to the following equations:

$$\text{Phenol oxidase: } A_{phenoloxidase} = Abs_{460} / k$$

$$\text{Peroxidase: } A_{peroxidase} = Abs_{460} / k - A_{phenoloxidase}$$

where, (Abs_{460}) is the absorvance at 460 nm and (k) is the extinction coefficient, which is 1.66 mM for DOPA under the conditions of this assay. Phenol oxidase and peroxidase activity was expressed as mmol DIQC converted per unit of time and surface area.

2.6.2 Aquatic hyphomycete sporulation rates

Sporulation rates of aquatic hyphomycetes were measured in leaf disks (8 disks per sample) of *Platanus acerifolia* and *Populus nigra* species and in fine (*c*. 5 ml of sand volume per sample) and coarse (9 ceramic tiles per sample) inorganic substrata. Substratum samples were placed in 250 ml Pyrex flasks containing 100 ml of sterilized stream water (filtered through 0.2 μm Whatman Nylon filters). Tiles and sand samples, as well as leaves, were cleaned with sterilized water before the incubations in order to eliminate deposited particles from biofilm surfaces. All incubations were done in a shaking bath (80 rpm) set at 10 ºC for 48 h to induce the sporulation. After incubation, 250 μl of 0.5% (w/v) Triton X-100 solution was added and 20 ml of the conidial suspension was passed through 5 μm-pore sized filters (cellulose nitrate filters, Whatmann). The conidia retained were stained with 0.1% Trypan blue in 60% lactic acid (Baldy et al., 2002). Ten microscopic fields were counted at × 400 for the leaf samples, while the whole filter was counted for the fine and coarse substrata samples. Sporulation rates were expressed as number of conidia produced per unit of surface area and time (cm^2 and day).

2.7 Physical and chemical parameters of stream water and CPOM input and dynamics

2.7.1 Physical and chemical parameters

Dissolved oxygen (DO), pH, conductivity and temperature were measured in the field with portable meters (WTW 340i), as well as the incident light (underwater sensor, Li-Cor quantum sensor Li-192SB). Discharge was measured using the slug-injection method with sodium chloride as a tracer (Gordon et al., 1992) and calculated by integration of the concentration hydrograph (Triska et al., 1989).

Water samples for dissolved nutrient content were collected in triplicate (2L in plastic bottles) and filtered on pre-combusted glass fibre filters (GF/F, Whatman) prior to analysis. Ammonium and phosphate concentrations were analysed following standard methods (indophenol blue method and ascorbic acid method, respectively; APHA, 1989), while nitrate concentrations were determined through ionic chromatography (761 Compact IC, Metrohm, Switzerland). Three replicates were also used to determine dissolved organic carbon (DOC) and dissolved inorganic carbon (DIC) concentrations. DOC and DIC concentrations were analyzed through a total organic carbon analyzer (TOC-5000; Shimadzu) that analyzed both the non-purgeable organic carbon and the inorganic carbon concentrations in the water. Total dissolved C in the water was the sum of DOC and DIC fractions. Total dissolved N and P concentrations were determined after the basic digestion (NaOH) of water samples in an autoclave (110ºC for 90 min; Grasshoff et al., 1983). Digestion of water samples transformed all the dissolved organic N and P forms into inorganic forms, and then total nitrate and phosphate concentrations were determined according to the standard methods (APHA, 1989).

POM in transport was determined by collecting *c.* 2 L of stream water (in triplicate) and filtering in pre-combusted and pre-weighted glass fibre filters (GF/F, Whatman). Then, filters were dryed (80ºC for 48h) and determined for DW. Afterwards, the same filters were used to determine total C, N, P content of POM, following the same methodology described for microbial community C, N, P content analysis.

2.7.2 Coarse Particulate Organic Matter (CPOM)

During the period March 2003-February 2004, total CPOM input in the Fuirosos stream reach was analyzed. Vertical CPOM input in the stream channel was estimated by a monthly collection of leaf and plant material accumulated in five traps distributed along the reach and suspended 0.6 m above the water surface. The traps consisted of a square wooden frame (1 × 1 m) and a nylon net (mesh size = 1 mm; CPOM > 1 mm). Lateral CPOM input was determined by means of ten randomly-placed traps (five on each bank of the stream). Lateral traps consisted of a nylon net (mesh size = 1 mm; CPOM > 1 mm) contained in a wooden frame (0.3 × 0.55 m). Total CPOM input was considered as the sum of vertical and lateral inputs. During the same period, in-stream

CPOM standing stock was collected by triplicate coring samples (15 cm diameter) at random over the wetted channel. The water was removed from samples by filtering through a nylon net (mesh size = 1 mm). The materials collected for the analysis of CPOM input and CPOM standing were dried at 80ºC for 48 h and then weighed.

2.8 Statistical analyses

The variations in biofilm parameters (variables) provoked by the seasonality and by specific controlled factors (i. e. nutrient availability, sampling site) were analyzed thorugh the analysis of variance (ANOVA). The use of repeated measures ANOVA was carried out in experiments where repeated observations made on individual units were either sequential on time or under some treatment that is applied sequentially on time. Conversely, the multivariate analysis of variance (MANOVA) was used to compare the response of a group of variables (multiresponse variables) subjected to one or more factors.

Before ANOVA analysis, data obtained from biofilm parameters were first tested for normal distribution (Kolmogorov-Smirnov test), variance homogeneity (Levene's test) and independence between experimental units (Sokal & Rohlf, 1995). Almost, in all cases variables data required of a logarithmic transformation. Depending on the amount of ANOVA tests performed in multiple variables testing situation, significance of probability values was adjusted to control Type I error rates through the Dunn-Sidak procedure. This procedure is a modification of the Bonferroni procedure that improves power for each comparison which is tested.

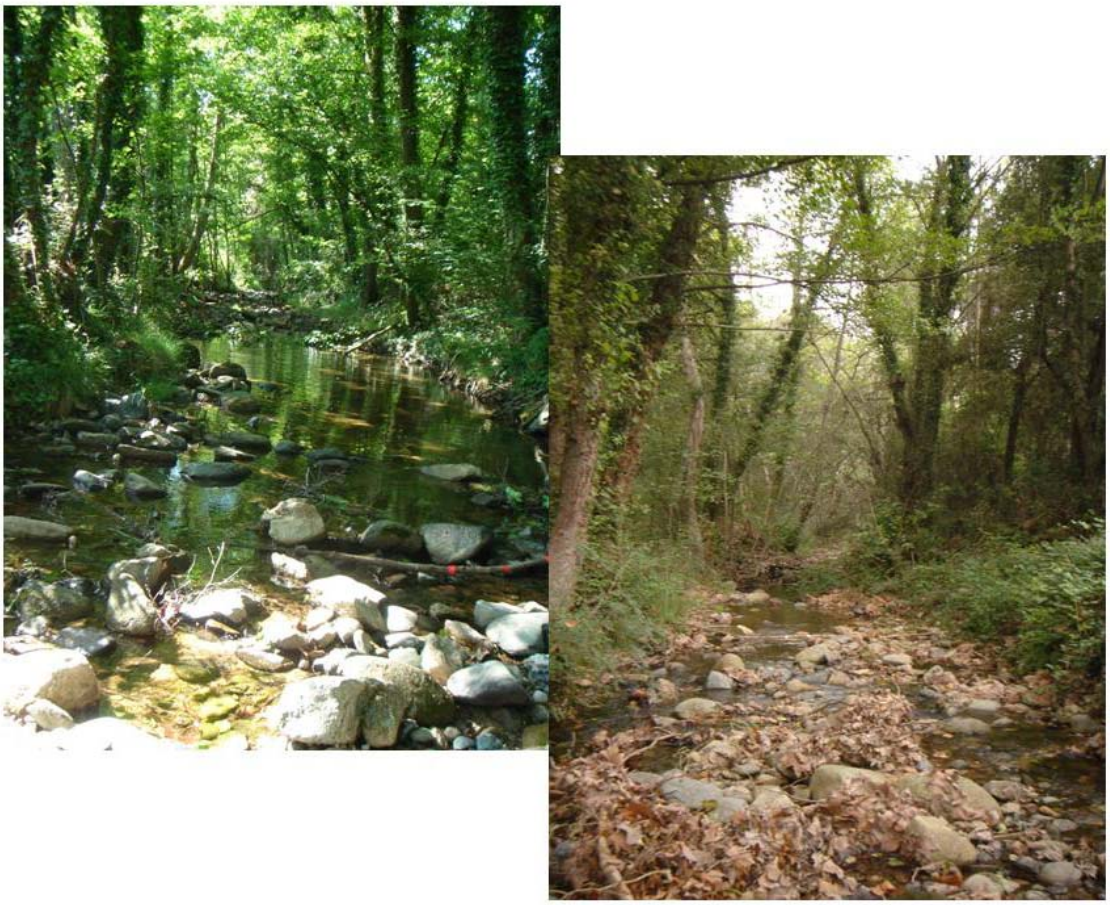
Multiple comparison tests were used to compare levels within one factor (i.e. low and high nutrient concentration, types of benthic substrata). These tests (i. e. Tukey's HSD test) compare each group mean with every other group mean in a pairwise manner and controls the family-wise Type I error rate to more than the nominal level (e. g. 0.05; Quinn & Keough 2002). In chapter 1, the Scheffé test (based on the F-ratio statistics) was used as multiple comparison test.

The linear relationship between random variables (i. e. biofilm biomass and water chemical characteristics) was analyzed through the Pearson's correlation coefficient. However, a sort of non linear regression models (exponential, logarithmic, inverse) were used when the relationship between two variables was not explained by linear regression (Chapter 3).

Ordination analyses were applied on aquatic hyphomycete, bacteria and diatom biodiversity data obtained during biofilm experiments. Principal component analysis (PCA) is a commonly used multivariate statistical technique that was applied on hyphomycete data to reduce many variables (hyphomycete species) to a smaller

number of new derived variables that adequately summarize the original information. Prior to PCA analysis fungal species data were square root transformed. Instead, the classification analysis (cluster analysis) was used to group bacterial and diatom populations of similar attributes (based on Jaccard and Bray-Curtis similarity indexes) during a sequence of biofilm colonization. This analysis consisted in produce groups of objects where each object within a group is more similar to other objects in that group than objects in other groups. The dissimilarties between clusters and between clusters and objects (i. e. samples) were recalculated by the group mean linkage strategy (unweighted pair-groups method using arithmetic averages; UPGMA method).

Chapter 1



Organic matter availability structures microbial biomass and activity in a Mediterranean stream

Abstract

We compared microbial biomass (bacteria, fungi, algae) and the activity of extracellular enzymes used in the decomposition of organic matter among different benthic substrata (leaves, coarse and fine substrata) over one hydrological year in a Mediterranean stream. Microbial heterotrophic biomass (bacteria plus fungi) was generally higher than autotrophic biomass (algae), except during short periods of high light availability in the spring and winter. During these periods, sources of organic matter (OM) shifted towards autochthonous sources derived mainly from algae, which was demonstrated by high algal biomass and peptidase activity in benthic communities. Conversely, heterotrophic activity peaked in the autumn. Bacterial and fungal biomass increased with the decomposition of cellulose and hemicellulose compounds from leaf material. Later, lignin decomposition was stimulated in fine (sand, gravel) and coarse (rocks, boulders and cobbles) substrata by the accumulation of fine detritus. The Mediterranean summer drought provoked an earlier leaf fall. The resumption of the water flow caused the weathering of riparian soils and subsequently a large increase in dissolved organic carbon and nitrate, which led to growth of bacteria and fungi.

Introduction

Most energy in headwater stream ecosystems flows through the microbial benthic community (Gessner et al., 1999; Hieber & Gessner, 2002; Graça & Canhoto, 2006). Respiration usually exceeds primary production (Acuña et al., 2004) because of ample allochthonous organic matter and low light (Hill et al., 1995). However, when canopy shading is reduced, short pulses of algal production may provide autochthonous energy source for microbial heterotrophs (Sundh, 1992).

Microbial heterotrophs (bacteria and fungi) can produce a broad range of substrate-specific extracellular enzymes that enable allochthonous and autochthonous organic matter (OM) mineralisation (Arnosti, 2003). The correlation between such enzyme activity and POM decomposition rates is consistent in freshwater environments (Sinsabaugh et al., 1992; Sinsabaugh et al., 1994), so extracellular enzyme activity is a reliable indicator of microbial decomposition (Jackson et al., 1995). Extracellular enzyme production depends on substrate availability, but also on nutrient demands of microbial cells. However, measurements of enzyme activity at the community level reflect the nutrient demands of all coexisting microbial groups (bacteria, fungi, algae). Microbial interactions may also influence the regulation of microbial enzyme production (Francoeur & Wetzel, 2003; Romaní et al., 2006).

Microbial community structure in streams depends on the type of stream substrata. Decomposing submerged leaves are mainly colonised by aquatic hyphomycetes (Hieber & Gessner, 2002); fine detritus is colonised by bacteria (Lock et al., 1984; Findlay et al., 2002), and hard stream-bed substrata (i.e. rocks, cobbles, gravel) are colonised by algae (Stevenson, 1996). Benthic microbial density and biomass are also affected by physical, chemical and biological conditions in the stream ecosystem (Dodds et al., 1996; Hill et al., 1995). Nevertheless, the heterogeneity and relative contribution of the different substrata in the stream largely determine the structure and functioning of microorganisms in the community (Tank et al., 1998; Findlay et al., 2002; Cardinale et al., 2002). Streams are dynamic systems, especially in regions with variable hydrology (Gasith & Resh, 1999), such as the Mediterranean region. In such hydrological conditions the structure of benthic substrata changes with episodes of high and low water, so the respective contribution of the microbial community to OM cycling also varies.

This study aimed to determine how climatic and hydrological dynamics affect biomass standing stock and the use of organic matter by the benthic microbial community over one hydrological year. Microbial biomass (bacteria, fungi, algae) and the activity of extracellular enzymes were compared in communities that colonise different benthic substrata (leaves, coarse and fine substrata). These data were related to a wide range of environmental variables (i.e., water chemistry, flow, organic matter input) to determine the major factors affecting the structure and function of benthic microbial communities. Specifically, the following two questions were considered: 1) How do episodes of maximum and minimum organic matter input (allochthonous and/or autochthonous) influence microbial biomass and enzyme activity in the stream? 2) Are changes in benthic microbial biomass related to the specific use (determined by enzyme activity) of the organic matter present in the stream?

Materials and methods

Sampling

Monthly samples were taken (from March 2003 until February 2004) at different scales in the Fuirosos stream reach. At the stream reach scale, we measured the input and standing stock of coarse particulate organic matter (CPOM), POM transported by the water, and water nutrient concentration (section 2.7). We visually estimated the relative cover (%) of coarse substrata (cobbles, boulders and rocks), fine substrata (gravel and sand), leaves (distinguishing the relative cover of *Platanus acerifolia*, *Populus nigra* and *Alnus glutinosa*), branches and fine detritus every 20 cm along five

transverse transects spaced every 8 m through the reach. At each sampling time, percentages of each substratum along all transects were averaged and related to the total wetted surface area of the reach. Dissolved oxygen (DO), pH, conductivity, temperature, incident light and discharge were measured in the field at each sampling time (section 2.7.1).

At the habitat scale, we collected distinct benthic substrata (leaves, sand and tiles) according to the section 2.1 and analysed them for microbial biomass (algae, bacteria and fungi; section 2.2) and extracellular enzyme activity (β-glucosidase, β-xylosidase, leucine-aminopeptidase, phosphatase, cellobiohydrolase and phenoloxidase activity; section 2.6.1). Benthic substrata were classified as coarse (rocks, boulders and cobbles), fine (sand) or leaves (the three dominant species in the reach; *Platanus acerifolia*, *Populus nigra* and *Alnus glutinosa*).

Scaling up structural and functional data of benthic communities

To convert structural and functional measurements from the microhabitat scale (cm^{-2}) to the reach scale (m^{-2}), surface area of each substratum sample (clay tile, sand core and leaf disk) was multiplied by the fraction cover of the corresponding substrata (coarse substrata, fine substrata and leaves) in the reach at each sampling time and then divided by the wetted surface area of the stream reach. Total biomass and enzymatic activity in the reach is the sum of the reach values for biomass and activity in the three main substrata.

Statistical analysis

Differences in biomass and enzyme activity both between the different benthic substrata (coarse substrata, fine substrata and leaves; S) and over time (March 2003 to February 2004; T) were analysed at the reach scale using a repeated measures analysis of variance. All data were log-transformed ($x = \log_{10}(x + 1)$) prior to analysis and the Dunn-Sidak correction was applied to the resultant *p*-values. Temporal variations in biomass and enzyme activity in each substratum were analysed separately using a multiple comparison test (Scheffé, α=0.05). The statistical analyses were carried out using SPSS for Windows (Ver. 12.0, SPSS Inc. 2003).

Results

Environmental variables and stream characteristics

Long-term patterns in rainfall and air temperature demonstrate the high seasonal and interannual variability of the Fuirosos stream (Figure 3.1). Rainfall is

generally highest in spring and autumn, however total rainfall can vary from year to year (e.g. precipitation in 2002 was 13 times higher than the total precipitation in 1999).

During the study period temperature varied between 6ºC and 26 ºC. Stream discharge was highest in the autumn (118 L s^{-1} in October) and dramatically decreased in early summer (1 L s^{-1} in June) to zero flow in July and August. The first rainfall in September restored the stream's water flow and increased its dissolved nutrient content (nitrate, phosphate and DOC) (Figures 3.2a & b). Stream water was low in P and high in N (N:P = 63) most of the year, but N:P ratio increased up to 543 after autumn leaching.

The majority of CPOM input occurred during autumn (especially in October), but there was a secondary peak in spring (March, Figure 3.2c). In both cases, the largest input of CPOM came from *Platanus acerifolia*. CPOM standing stock and POM transport were particularly high in September, shortly after water flow resumed. However, spates in October rapidly washed the accumulated CPOM downstream (Figure 3.2c).

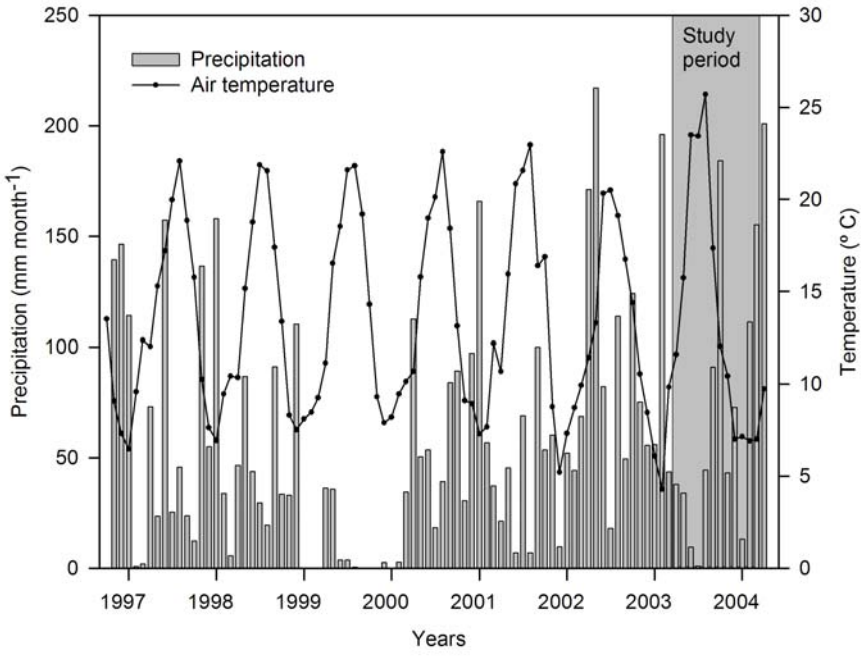


Figure 3.1. Long-term data of monthly rainfall and air temperature (1997 to 2004) from a weather station in the Fuirosos stream basin. The period of study (from March 2003 until February 2004) is marked in the graph.

Mean wetted surface area was 69.6 ± 4.3 m^2. Coarse substrata covered *c.* 70% of this area, while 30% was covered by fine substrata (Figure 3.3). Coarse substrata dominated in the centre of the channel, while gravel and sand accumulated in pools and in the depositional zones near the banks. October rainfall increased the fine

substrata cover to 52% of the total surface area of the reach. The decrease in water level from June onwards narrowed the wetted channel and resulted in the loss of depositional zones and in the subsequent predominance of coarse substrata (up to 75% of reach surface). Leaf accumulation in the autumn covered as much as 80% of the total wetted surface area, while fine detritus were more abundant in spring and winter (up to 28% of reach area). The highest accumulation of branches occurred in autumn (7% of reach area; Figure 3.3).

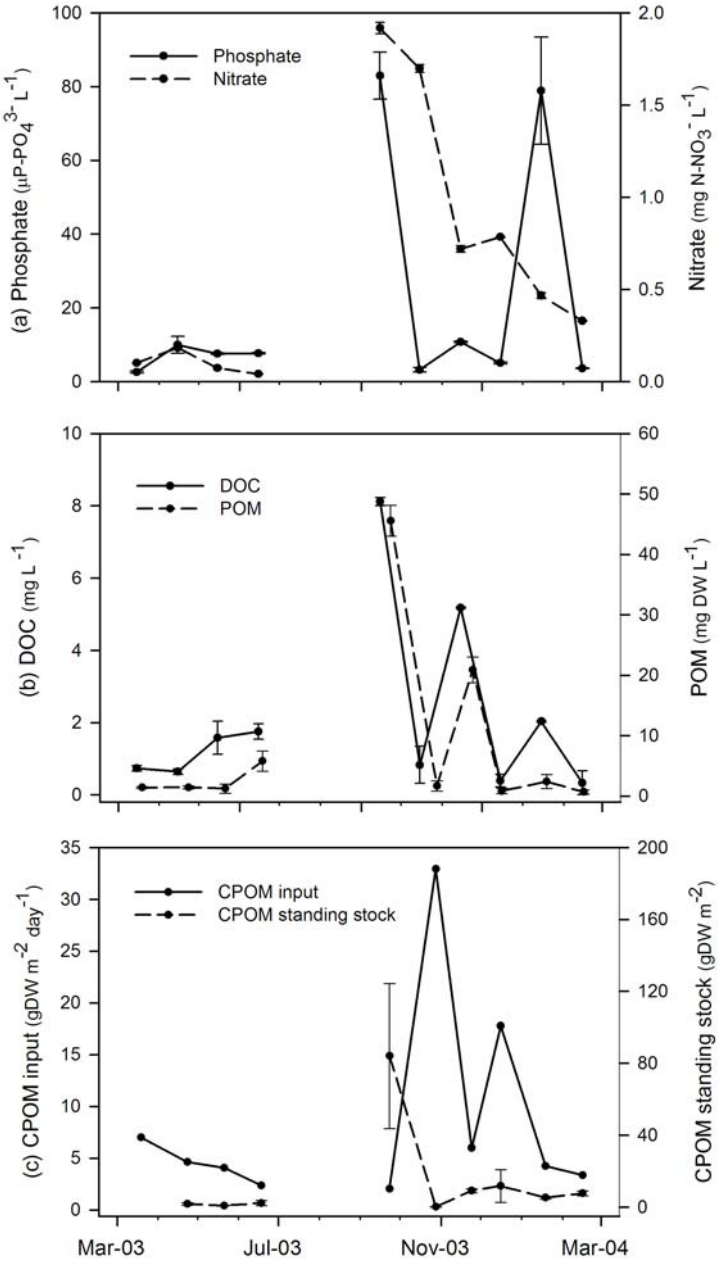


*Figure 3.2. Physical and chemical characteristics of the Fuirosos stream water during the study period. Values of phosphate (μg P-PO_4^{3-} L^{-1}) and nitrate (mg N-NO_3^- L^{-1}) (**a**), dissolved organic carbon (mg DOC L^{-1}) and particulate organic matter (POM) in transport (**b**) and CPOM input (Σ vertical and lateral inputs) and standing stock (**c**) are*

shown. March-03 data are missing for the CPOM standing stock. Low or zero flow occurred from the end of June to early September 2003.

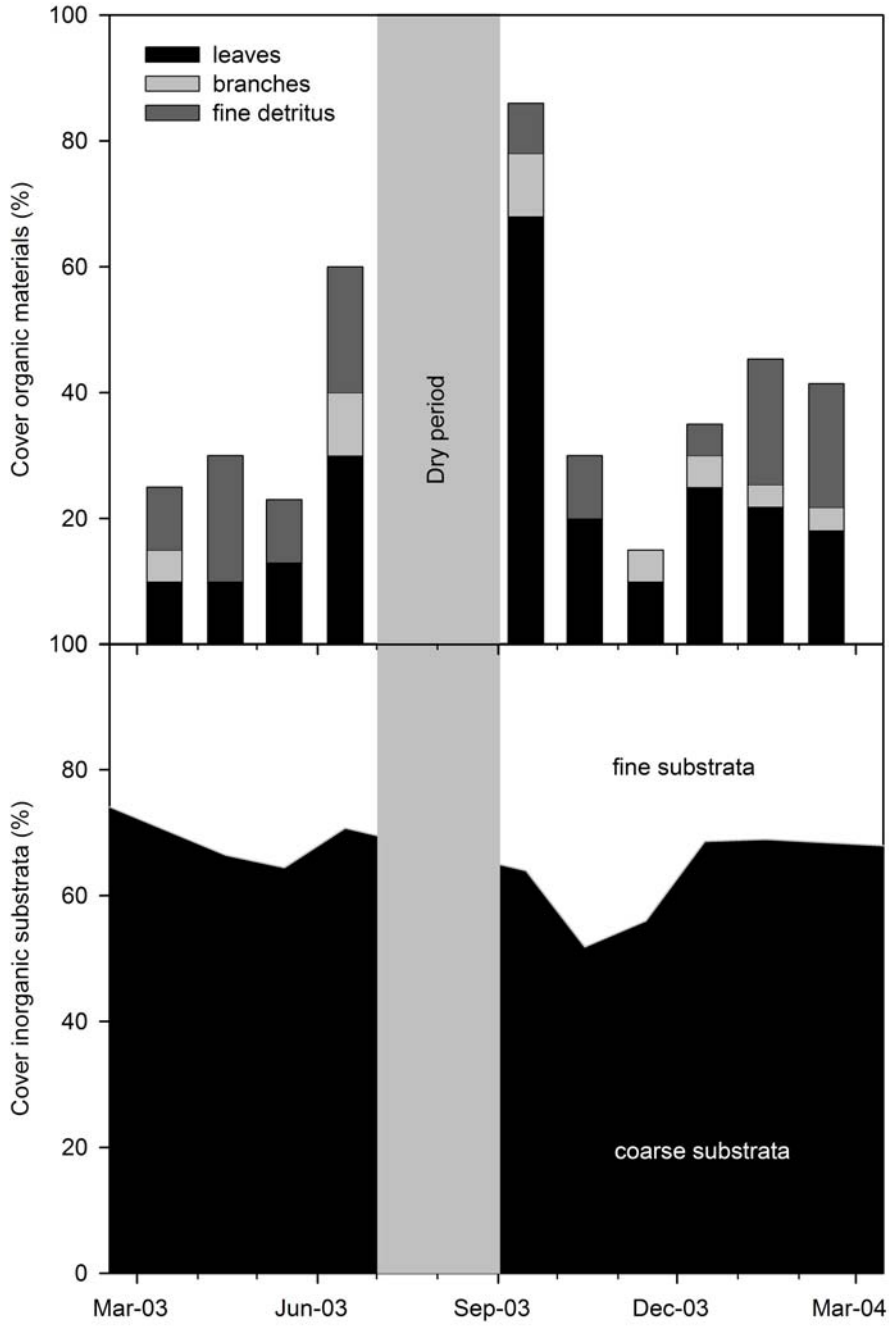


Figure 3.3. Relative coverage (%) at each sampling time of the inorganic (coarse and fine substrata) and organic (leaves, branches, fine detritic materials) benthic substrata in the studied reach.

Comparison of microbial biomass among substrata

Reach-scale values of bacterial, fungal and algal biomass in the different benthic substrata varied seasonally (ANOVA, T effect, $P < 0.005$; Figure 3.4). Bacterial

and fungal biomass peaked in September, although their temporal variations also depended on the substratum type (ANOVA, S × T effect, $P < 0.05$). Bacterial standing stock on fine substrata and tiles was highest during spring, which coincides with the higher algal biomass episode (Scheffé's test, $P < 0.05$, Figures 3.4 a & c).

Each microbial group showed certain preferences in substratum colonisation (ANOVA, S effect $P < 0.005$; Figure 3.4). In general, bacteria mainly accumulated on tiles, except in September when submerged leaves were preferred, fungi colonised leaves while algae accumulated on tiles. Fungi dominated the biomass of the microbial community (up to 10 gC m^{-2}, in autumn), followed by algae (up to 8.5 gC m^{-2} in spring) and bacteria (up to 0.16 gC m^{-2} in autumn; Figure 3.4). The microbial autotrophic: heterotrophic C ratio shifted from being highly autotrophic in spring and winter (ratio of 3 and 2.1, respectively) to highly heterotrophic in autumn (ratio of 0.1).

Extracellular enzyme activity

Enzyme activity in different substrata demonstrated a marked seasonality (ANOVA, T effect, $P < 0.005$; Figure 3.5). The activity of three enzymes involved in polysaccharide degradation (β-glucosidase, β-xylosidase and cellobiohydrolase) peaked in early autumn (September), coinciding with the rise in benthic heterotrophic biomass (Figures 3.5 a, b & c). However, β-glucosidase activity was highest between June and October, while β-xylosidase activity peaked between May and November. There was a sharp increase in cellobiohydrolase activity between June and September and again in February the following year. Phosphatase and phenol oxidase activity steadily increased in spring and peaked in September (Figures 3.5 d & e), activity then decreased during the autumn and recovered in winter. Trends in peptidase activity contrasted with that of other enzymes (Figure 3.5 f).

The temporal dynamics of each enzyme activity varied with benthic substratum type (ANOVA, S × T effect, $P < 0.005$), except in the case of the phenol oxidase activity ($P = 0.33$). In general, polysaccharide-degrading enzymes were most active (β-glucosidase and β-xylosidase) in leaf material, while phosphatase and peptidase prevailed on tiles. However, cellobiohydrolase activity did not differ between substrata (ANOVA, S effect, $P = 1$). The decomposition of lignocellulosic compounds was most common on fine substrata (ANOVA, S effect, $P < 0.005$). In general, the capacity of benthic communities in the decomposition of simple C molecules (β-glucosidase, Figure 3.5 a) was 100 times greater than their capacity to decompose hemicelluloses and celluloses (β-xylosidase and cellobiohydrolase, Figure 3.5 b & c). The greatest

capacity was that measured for organic phosphorus compounds and peptide decomposition (Figures 3.5 d & f)

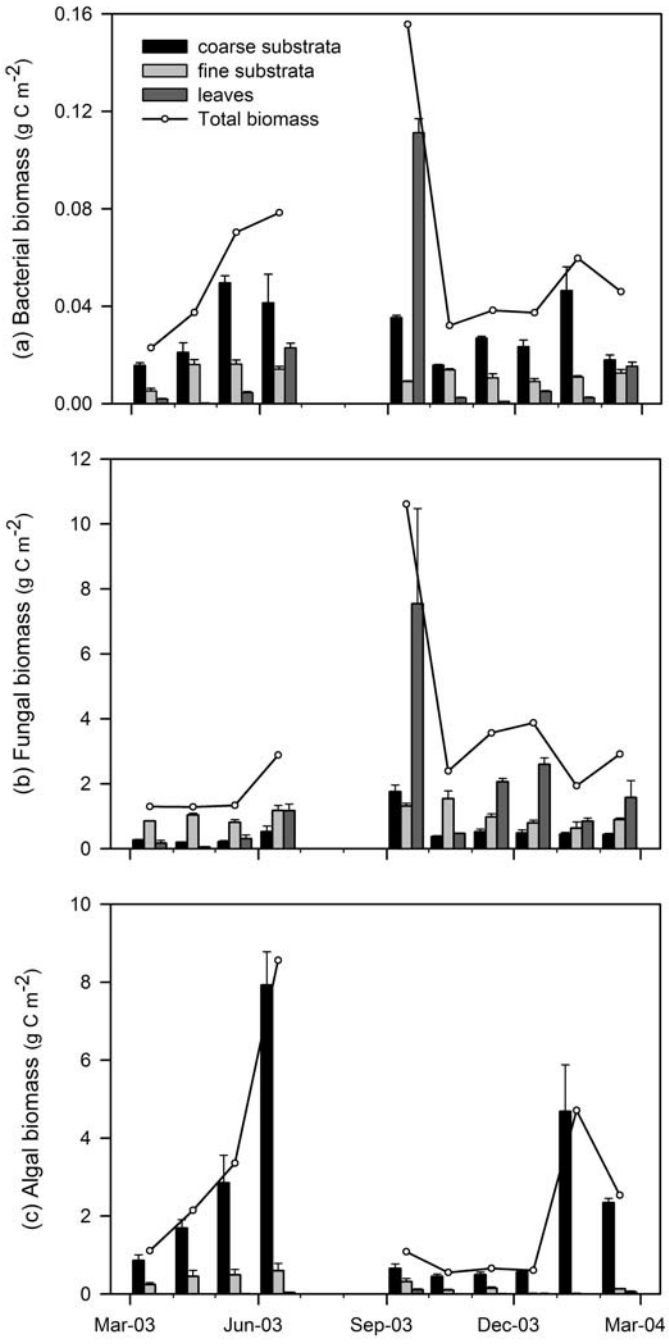


*Figure 3.4. Biomass of bacteria (**a**), fungi (**b**) and algae (**c**) expressed in terms of carbon per unit of reach surface area (m^{-2}) on different benthic substrata (leaves, coarse and fine substrata) in the study reach. Total values of benthic microbial biomass accumulated in the reach (Σ biomass in coarse and fine substrata and leaves) are also shown.*

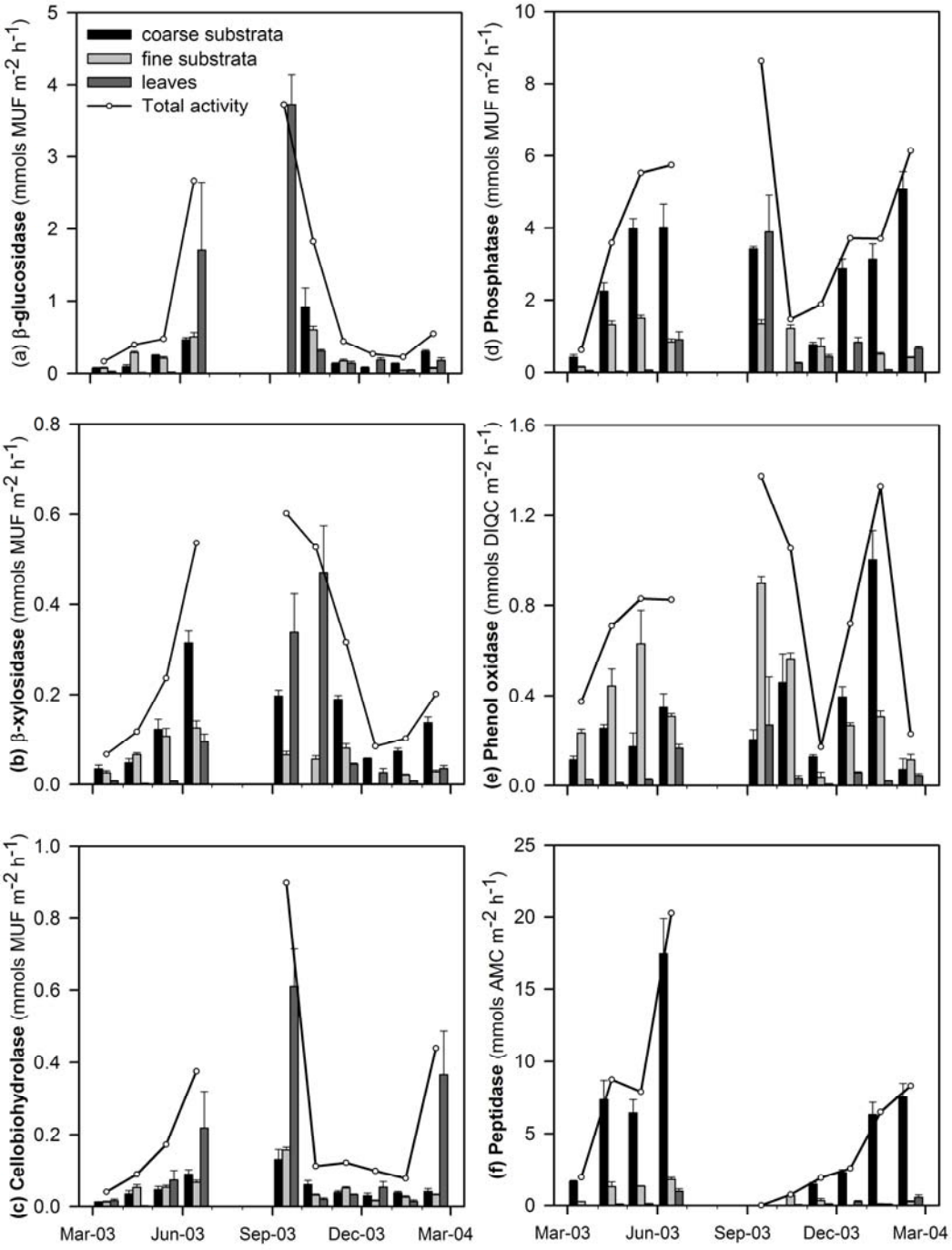


*Figure 3.5. Dynamics of extracellular enzyme activity [β-glucosidase (**a**), β-xylosidase (**b**), cellobiohydrolase (**c**), phosphatase (**d**), peptidase (**e**) and phenol oxidase (**f**)] measured on coarse and fine substrata and leaves in the study reach. Units of enzyme activity are expressed as the amount of methylumbelliferone (MUF for β-glucosidase, β-xylosidase, cellobiohydrolase and phosphatase), aminomethyl-coumarin (AMC for peptidase) and 2,3-dihydroindole-5,6-quinone-2-carboxilate (DIQC for phenol oxidase) released per unit of reach surface area per time (m^{-2} h^{-1}). Total values of different enzyme activity (Σ activity in coarse and fine substrata and leaves) in the reach are also shown.*

Discussion

Microbial benthic biomass and the activity of both autotrophic and heterotrophic microbes were highly dynamic in time and space. The structure of microbial communities was related not only to substrata availability but also to seasonal changes in the physical (i.e. flow, temperature, light) and chemical (nutrients) characteristics of the stream, confirming earlier observations (Townsend, 1989; Cox, 1990; Biggs, 1999; Methvin & Suberkropp, 2003; Sabater et al., 2006).

Our observations on enzyme activity and microbial biomass reveal two distinct periods of high microbial enzyme activity: the first is in late winter-early spring, and the second in late summer-autumn. In late winter-spring, light reaching the stream bed was not restricted by canopy cover, which caused an increase in water temperature. For a short period, there was a sharp increase in autotrophic biomass, particularly on coarse substrata, resulting in a dense autotrophic population (high autotrophic: heterotrophic C ratio). The highest algal peak occurred in periods of relatively low dissolved inorganic nutrient concentration (June 2003), which is consistent with theories of light-dependant algal development in forested streams (Mallory & Richardson, 2005; Taulbee et al., 2005; Ylla et al., 2007). During this period, it is likely that algae outcompeted bacteria for dissolved inorganic nutrients (Rier & Stevenson, 2002). Peptidase activity was highest during periods of greatest algal biomass development in coarse substrata. This relationship may indicate the use of algal exudates (mainly peptidic molecules) by bacteria (Cole, 1982; Romaní et al., 2004), since bacterial enzyme production (especially peptidases) can be stimulated by active algal photosynthesis (Espeland et al., 2001). During this period, heterotrophic microbial activity may depend largely on the use of autochthonous sources.

The second episode of high microbial enzyme activity is based on allochthonous OM sources, especially leaf litter from the riparian forest. The activity of polysaccharide-degrading enzymes (involved in the degradation of cellulose and hemicellulose compounds) was associated mainly with the CPOM standing stock in the reach. The magnitude and duration of high enzyme activity can be explained by temporal shifts in organic matter supply, as well as by changes in the storage capacity of sediments (Wilczeck et al., 2005). Although this heterotrophic episode is correlated with leaf litter input and accumulation in the stream, microbial benthic communities also showed active decomposition activity during the winter. At this time, the decomposition of lignocellulose (phenol oxidase activity) and organic phosphorus compounds (phosphatase) was greatest in coarse substrata where fine detritus accumulated (Chapter 2).

Particulate organic matter inputs in Mediterranean streams are prolonged and intense, especially during summer drought episodes (Acuña et al., 2007). Water stress during the summer accelerates leaf fall in the riparian forest (Bernal et al., 2003); this results in the accumulation of CPOM (mainly leaves) in the dry stream channel, which is where decomposition begins. After stream flow resumes, the first large storms in autumn leach the organic-rich surface horizons from the riparian soils causing an increase in DOC and nitrate concentrations in the stream water (Bernal et al., 2002). The consequent peaks in fungal and bacterial biomass initiate a long period of a low autotrophic: heterotrophic carbon ratio. Moderate increases in the dissolved inorganic nutrients are then sufficient to enhance bacterial and fungal biomass (Gulis & Suberkropp 2003a; Gulis et al., 2004; Baldy et al., 2007).

Mediterranean systems also exhibit intense and frequent spates and drought (Gasith & Resh, 1999) that interfere with the dynamics of dissolved and particulate OM. Interruptions in streamflow can increase total dissolved organic carbon in transport in the subsequent rewetting periods; this increase has not been noticed during periods of constant streamflow (Vázquez et al., 2007). Such changes in stream hydrology are fundamental in regulating the availability of particulate OM (Acuña et al., 2007), which determines the structural and functional parameters of microbial benthic communities. Climate change projections (IPCC, 2007) predict that heat waves and heavy precipitation events will become more frequent, particularly in the Mediterranean drainage basin, which would alter organic matter accumulation and the nutrient retention ability of soils (Caruso, 2002). Subsequent impacts on the biomass and activity of heterotrophic and autotrophic organisms in streams might also result. A comparison between the present results and other studies conducted under wet conditions (permanent streamflow in summer; Chapter 2) revealed that the autumn peaks of microbial biomass and activity were two to three times lower in years of permanent streamflow than in years which streamflow is interrupted by the summer drought. These differences indicate that the contrast between autotrophic and heterotrophic peaks could be enhanced by dry conditions, and diminished by wetter conditions.

Chapter 2

Organic matter decomposition by fungi in a Mediterranean forested stream: contribution of streambed substrata

Abstract

Aquatic microfungi play a fundamental role in organic matter decomposition in fluvial ecosystems. These micro-organisms degrade recalcitrant compounds like lignin, thereby enhancing the utilization of organic material by the microbial community. The main input of allochthonous organic matter in Mediterranean streams occurs during the autumn. In-stream breakdown processes can be affected by high physical abrasion during floods but changes in stream water chemistry may also affect decomposition enzymatic activities of stream microorganisms. Two ligninolytic activities (phenol oxidase and peroxidase) and a cellulolytic activity (cellobiohydrolase) were measured in leaves, branches, sand and gravel substrata in a reach of a Mediterranean stream. Highest ligninolytic activities were measured in biofilm developed on inorganic substrata (sand and gravel) were also accumulated the highest fungal biomass (ranging from 3.3×10^{-4} to 7.22 mg ergosterol $gAFDM^{-1}$). Conversely, cellulolytic activities were significantly higher in biofilm on organic substrata (leaves and branches). Physical and chemical factors, such as discharge and stream water nutrient concentration (Dissolved Inorganic Nitrogen, DIN) were affecting enzymatic activities, particularly enhancing phenol oxidase. Moreover, the chemical composition of the available OM (high cellulose in leaves, high lignin in fine detritic materials) strongly influenced the decomposition activity in each biofilm. A precise description and quantification of the benthic substrata was used to obtain enzymatic activity values in terms of stream reach. These results showed a temporal pattern in the decomposition activities in the reach, beginning with the decomposition of cellulose (October) followed by lignin compounds (November and December).

Introduction

In forested streams where light is a limiting factor for primary production, energy sources are mainly allochthonous (leaf litter), the metabolic processes being typically heterotrophic (Fisher & Likens, 1973; Minshall et al., 1983). In Mediterranean streams, the greatest input of plant material occurs in autumn, thereby providing the potential for decomposition processes. The initial breakdown of leaves in the stream is mainly carried out by aquatic hyphomycetes (Suberkropp & Klug, 1976; Findlay & Arsuffi, 1989; Gessner & Chauvet, 1994: Mathuriau & Chauvet, 2002), while the contribution of bacteria is lower (Hieber & Gessner, 2002). This group of fungi is crucial in decomposition process as it breaks down lignified carbohydrates, which constitute a natural protection of polysaccharide components against enzymatic attack (Griffin, 1994). After cellulose, lignin is the most abundant form of aromatic carbon in the world. Lignin degradation does not provide a primary source of carbon and energy for fungal

growth, but decay processes and utilization of carbohydrates for fungal growth can occur only with the coordinate degradation of this carbon (Griffin, 1994).

Decomposition process may be related to variations in inorganic nutrient availability. It has been observed that the addition of nitrogen (N) increases cellulolytic activity and decreases lignin-degrading phenol oxidase activity (Carreiro et al., 2000). Moreover, the chemical nature of streambed substrata, such as the tannin content or the physical properties of leaves (high waxy cuticles), has been proposed as a major determinant of breakdown rate (Barlöcher et al., 1995). Sinsabaugh et al. (1992) determined that phenol oxidase activity was substrate-related but not site-related.

Studies on the decomposition of organic matter (OM) have mainly focused on organic substrata (leaves ad wood; e. g. Gessner & Chauvet 1994; Pozo et al., 1998; Diez et al., 2002). However, other riverbed compartments recieve significant accumulations of allocthonous material during autumn. The accumulation of decomposing material occurs in pools or litoral zones and in debris dam. Specifically, sand sediments accumulate large amounts of fine detritic materials.

Here the role of fungi on the decomposition of OM during autumn in a forested Mediterranean stream was evaluated, and studied the way in which their role was shared between the distinct streambed substrata (leaves, sand, gravel and branches) where substantial OM decomposition may occur. For this purpose, ligninolytic and cellulolytic enzyme activities were measured on gravel and sand, on the leaves of several species [*Alnus glutinosa* (Gaertn.)*, Populus nigra* (L.) and *Platanus acerifolia* (Aiton-Willd.)] and on branches, to determine the most active site of OM decomposition among the substrata types. The relationships between enzymatic activities and fungal biomass (ergosterol content) of biofilms was studied in each substratum, but also, with the physical-chemical parameters of stream water to establish which variables were controlling the decomposition process in the different streambed substrata.

Materials and methods

Sampling

Sampling of leaves, branches, sand and gravel was performed in the Fuirosos stream reach at fortnight intervals from October 2002 to January 2003 (for a total of seven sampling dates; section 2.1). In total, 9 substrata types (defined as follows) were sampled. Leaves of three species were collected: *Alnus glutinosa*, *Platanus acerifolia,* and *Populus nigra*, considering separately the leaves just fallen into the stream (as fresh leaves) and those being already immersed (as decaying leaves). Branches, sand and gravel samples were collected as well.

At each sampling time, the different substrata were analyzed for ergosterol concentration and extracellular enzyme activity [cellobiohydrolase (CBH), phenol oxidase (PO) and peroxidase (P) activity] following the methodology described in the Methods (sections 2.2.1 and 2.6.1, respectively). Also, eleven transects (3 m apart each other) were assayed in the studied reach in order to determine the relative cover (%) of each substratum every 20 cm. The distinct substrata considered in transects were: fresh leaves, decaying leaves, branches, sand-gravel (which were classified together because of the high mixture and compaction of grains) and rocks (including rocks, cobbles and boulders). Accumulation of fine detritic materials was quantified visually. The coverage of the distinct substrata in the reach (in terms of surface area) was used to calculate enzymatic activity and ergosterol concentration in terms of stream reach.

Moreover, temperature, conductivity, pH, dissolved oxygen and light were measured in the field with portable meters and water samples for ammonia, nitrate, phosphate and dissolved organic and inorganic carbon (DOC and DIC) determination were also collected (section 2.7.1).

Statistical analyses

Differences between enzymatic activities and ergosterol concentration (fungal biomass) of the distinct streambed substrata and over time were analysed using a two-way analysis of variance (ANOVA). A Multiple Comparison test (Scheffé test) was used to observe the differences in activities between substrata. To detect the significance of the differences between organic and inorganic substrata, another ANOVA was performed after grouping leaves and branches versus sand and gravel, respectively. All variables were log transformed in order to assess the homogeneity of variances and the normal distribution of data before performing the ANOVA. Correlation analysis of environmental and biological variables was performed using the product-moment Pearson coefficient. The statistical analyses were carried out using SPSS for Windows (Ver. 12.0, SPSS Inc. 2003).

Results

Water physico-chemistry

The physico-chemical parameters varied considerably during the study period. There was a progressive decrease in stream water temperature, which reached the lowest values in January 2003 (Table 4.1). Discharge increased up to 10 times the basal flow throughout the study period, producing drastic increases in inorganic nutrients (especially nitrate) and to a lesser extent in DOC.

Discharge variations also caused changes in the proportions of substrata during autumn (Table 4.2). The leaf material during the fall peak in October and November covered nearly 50% of the streambed surface area. Accumulation of fine detritic materials was inversely correlated with current values (r= -0.904, $p < 0.01$).

Date	2-10 2002	16-10 2002	30-10 2002	13-11 2002	27-11 2002	17-12 2002	13-1 2003	Mean	SD
Temp (ºC)	14.5	16.0	14.8	12.8	8.8	8.2	2.7	11.1	4.7
Disch (L/s)	8.3	30.6	21.4	19.6	87.6	47.1	28.2	34.7	26.2
NO_3-N(μg/L)	11.7	516.4	105.6	30.7	762.4	842.3	564.5	404.8	351.5
SRP(μg/L)	2.5	12.6	4.6	3.6	12.6	14.6	3.9	7.8	5.2
DOC (mg/L)	2.6	4.9	4.1	3.6	4.4	1.5	3.3	3.5	1.1

Table 4.1. Physical and chemical characteristics of the Fuirosos stream water during the study period. Values for water nutrient concntrations are means (n=3) and those for temperature and discharge are individual values of each of the seven sampling dates. Means and standard deviations (SD) for all the study period are also shown.

Sampling date	Rock	Sand-gravel	Branches	Decaying leaves	Fresh leaves	Detritic material
2/10/02	63.4	36.6	1.14	6.43	29.11	42.31
16/10/02	63.4	36.6	0.85	2.77	21.24	12.38
30/10/02	63.4	36.6	0.87	4.35	47.65	12.23
13/11/02	63.4	36.6	1.59	15.42	44.09	12.25
27/11/02	66.0	34.0	2.23	20.5	12.61	2.27
17/12/02	59.0	41.0	1.93	26.62	0.37	5.15
13/01/03	65.0	35.0	3.08	10.19	No presence	7.75

Table 4.2. Benthic substrata description of the Fuirosos stream reach during autumn-winter 2002/03. Values are the percent of each substratum occupying the streambed.

Extracellular enzyme activities

There were significant differences in CBH and PO activities between biofilms developed on different streambed substrata and throughout the study period (ANOVA, Table 4.3, Figure 4.1). P activity showed significant differences among substrata but not over time (Table 4.3). CBH activity was significantly higher biofilms developed on organic substrata, while PO and P were significantly higher in those growing on inorganic substrata (Table 4.3). The highest CBH activities were detected in biofilms of

decaying *Populus nigra* leaves (Scheffé test; Figure 4.1), followed by *Alnus glutinosa*, *Platanus acerifolia* and branches. The lowest CBH activity values were observed in biofilms of fresh leaves, sand and gravel substrata. Biofilm on sand and gravel showed the highest PO activity compared with that in organic substrata communities (Scheffé test; Figure 4.1). Among leaf species, the highest PO activity was measured in the biofilm on fresh and decaying leaves of *Alnus glutinosa* while the lowest was on fresh *Populus nigra* leaves. The highest P activity was registered in sand and gravel biofilm, although Scheffé test did not show statistically significant differences between substrata (Figure 4.1). Among leaves, the highest P activity was detected in biofilm on fresh and decaying *Populus nigra* leaves while the lowest was on fresh *Platanus acerifolia* leaves.

Sources	CBH	PO	P	Ergo
(1)				
Time	$F_{6,161}$=6.43	$F_{6,162}$=2.97	$F_{6,162}$=1.08	$F_{6,71}$=90.01
	p<0.0001	p<0.01	ns	p<0.0001
Substrata	$F_{8,161}$=37.65	$F_{8,162}$=23.18	$F_{8,162}$=2.61	$F_{8,71}$=5.64
	p<0.0001	p<0.0001	p<0.05	p<0.0001
Time × Substrata	$F_{39,161}$=2.55	$F_{39,162}$=2.78	$F_{39,162}$=0.93	$F_{36,71}$=2.23
	p<0.0001	p<0.0001	ns	P<0.005
(2)				
Time	$F_{6,201}$=1.67	$F_{6,202}$=6.16	$F_{6,202}$=1.74	$F_{6,108}$=53.71
	ns	p<0.0001	ns	p<0.0001
Substrata	$F_{1,201}$=12.37	$F_{1,202}$=124.65	$F_{1,202}$=10.36	$F_{1,108}$=18.43
	p<0.005	p<0.0001	p<0.005	p<0.0001
Time × Substrata	$F_{6,201}$=0.49	$F_{6,202}$=6.52	$F_{6,202}$=1.94	$F_{6,108}$=4.27
	ns	p<0.0001	ns	p<0.005

Table 4.3. Two-way analysis of variance performed between enzymatic activities of streambed substrata and over time (1), and grouping organic (leaves and branches) and inorganic (sand and gravel) substrata (2). F-statistics and probabilities for the different sources of variation and interactions are shown.

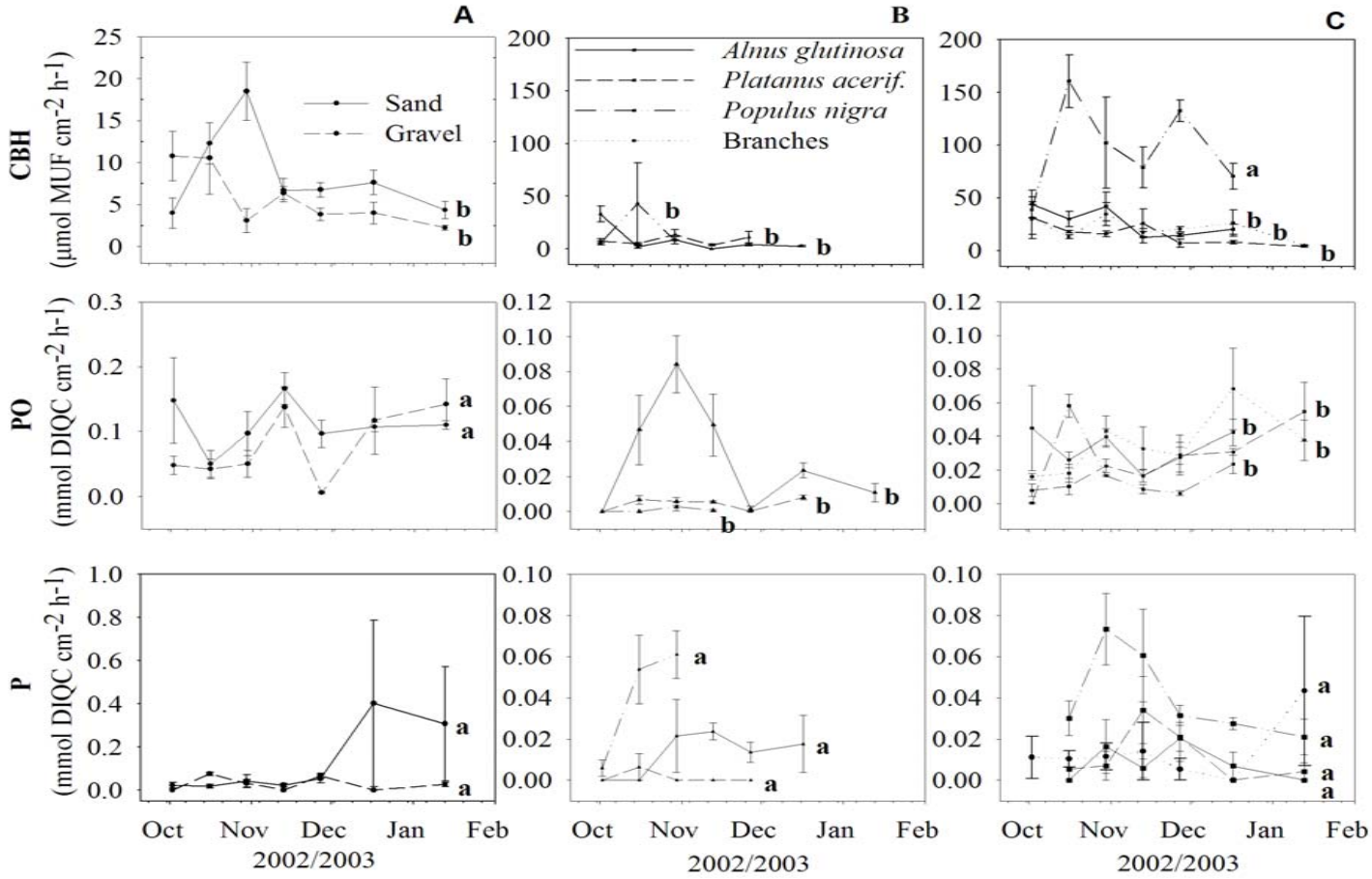


Figure 4.1. Enzymatic activities (CBH, PO and P) in (A) sand and gravel, (B) fresh leaves, (C) decaying leaves and branches during 2002/2003 in Fuirosos stream. Values are means and SE of activity in each substratum (4 replicates) during the 7 sampling dates. Marked in small letter the groups of substrata with signifficant differences (a>b) obtained with the Scheffé test (α=0.05) are shown. Legend in graph B was also used in graph C where branches were represnted. (MUF= methylumbellyferone; DIQC= 2,3-dihydroindole-5,6-quinone-2-carboxilate).

Ergosterol content

A general increase in fungal biomass occurred at the end of autumn in all the substrata (Figure 4.2). Ergosterol content showed differences over time and among substrata (Table 4.3). Fungal biomass was higher on inorganic substrata, particularly on sand, than on leaves and branches. Ergosterol on sand was *c*. 10-fold higher that on gravel. Among leaf species, *Alnus glutinosa* accounted for the highest fungal biomass.

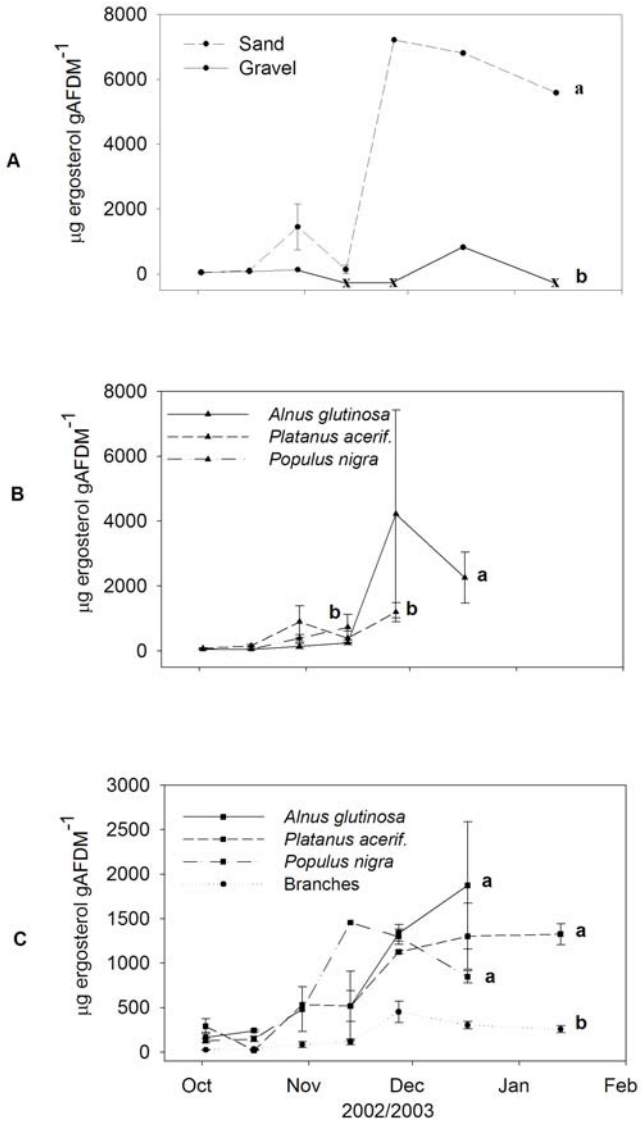


Figure 4.2. Ergosterol content on the streambed substrata during autumn-winter 2002/03 in the Fuirosos stream. (a) Sand and gravel, (B) fresh leaves, (C) decaying leaves and branches. Values are means and SE of ergosterol in each substratum (3 replicates) during the 7 sampling dates. Marked in small letters the groups with significant differences (a>b) obtained with the Scheffé test (α= 0.05) are shown. (x) indicate samples under detection limit of ergosterol content method (approx. 0.252 μg ergosterol gAFDM^{-1}). Some samples have no SE since the lack of replicates.

Stream reach capacity on OM decomposition

The stream reach capacity for the different enzyme activities was calculated after considering the activities by the corresponding percent of substrata occupying the streambed. High values of CBH were characteristic of the whole study period. Both PO and P increased after several weeks (Figure 4.3). Biofilm in sand and gravel were responsible for the high values of PO and P activities. CBH showed higher values in biofilms on organic substrata, being initially higher on fresh leaves and later increasing on decaying leaves. Water temperature was negatively correlated with the PO activity in biofilms on decaying leaves and branches, but positively correlated with the CBH activity in sand-gravel biofilms (Table 4.4). Dissolved inorganic nitrogen (DIN=nitrate+nitrite+ammonia) was positively correlated with PO in biofilm on decaying leaves and P in biofilm on sand-gravel (Table 4.4).

Activ	Substr	Temp	Disch	DIN	DIC	CBH	PO	P
CBH	DL	ns	0.823*	ns	ns	1	ns	ns
	S-G	0.89**	ns	ns	ns	1	ns	ns
PO	FL	0.76*	ns	ns	ns	ns	1	ns
	DL	-0.76*	ns	0.76*	ns	ns	1	ns
	B	-0.86*	ns	ns	-0.93*	ns	1	ns
P	S-G	ns	0.82*	0.96**	-0.96**	ns	ns	1
Ergost.	DL	ns	ns	ns	ns	0.88*	0.87*	ns
	B	ns	ns	ns	-0.80*	ns	0.88*	ns
	S-G	-0.82*	ns	0.76*	-0.91*	ns	ns	0.82*

*Table 4.4. Correlations (Pearson coefficients) calculated for CBH, PO and P activities in terms of surface occupied by each substratum on the streambed and physico-chemical variables (discharge, temperature, dissolved inorganic nitrogen (DIN) and dissolved inorganic carbon (DIC)). Streambed substrata are indicated as FL (fresh leaves), DL (decaying leaves), B (branches), and S-G (sand and gravel). Substrata and physico-chemical variables with any significant correlation are not included in the table. Level of significance is indicated by asterisks: * p<0.05 and ** p<0.001.*

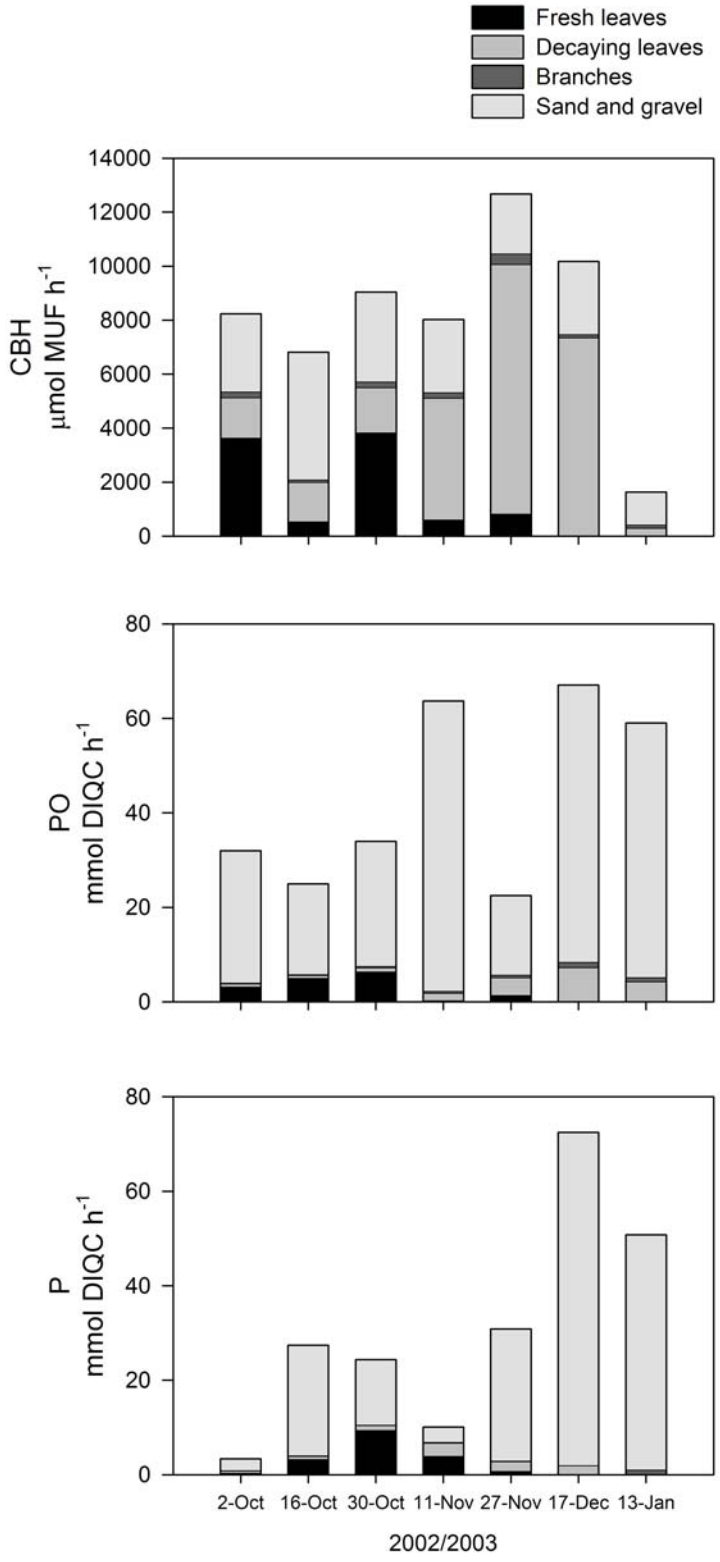


Figure 4.3. Enzymatic activity values corrected by real surface area occupied by each substratum in the studied reach. Units are µmol MUF (CBH) and mmol DIQC (PO and P) h^{-1} in the whole reach.

Maximum fungal biomass was reached in November and December. Ergosterol was first accumulated on fresh and decaying leaves (November) but was later more abundant on sand-gravel (Figure 4.4). Lignocellulosic activities were correlated with the ergosterol content of decaying leaves and branches (PO activity) and sand-gravel substrata (P activity, Table 4.4). The peroxidase activity in the reach (obtained as the integration of the enzymatic activity values in each substrata) was significantly correlated with DIN (r = 0.964, $p < 0.0001$). Integrated fungal biomass per stream reach was also correlated with DIN (r = 0.770, $p < 0.0001$).

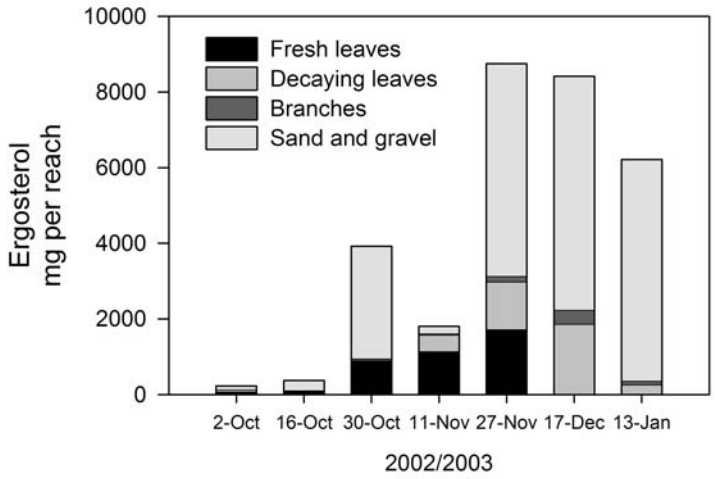


Figure 4.4. Ergosterol content corrected by real surface area accupied by each substratum in the studied reach. Units are mg ergosterol in the whole reach.

Discussion

Organic matter decomposition showed a clear temporal pattern and remarkable differences in the enzymatic activities and ergosterol content between substrata. According to the model of Berg (1986), the decomposition of fresh leaves begins with the easily mineralised fractions of non-lignified carbohydrates, whereas later stages are characterized by mineralization of more recalcitrant fractions of lignified carbohydrates. In the Fuirosos stream, after leaf fall peak (October), CBH activity was high for the whole period, while P and PO activities increased only after several weeks. This observation indicates that OM decomposition began with cellulose decomposition followed by degradation of lignin compounds. However, physico-chemical parameters, such as discharge and DIN, may also determine the time-pattern of the enzymatic activity. The abrupt flows in autumn or early winter in Mediterranean streams (Gasith & Resh, 1999) may mobilise most of the nitrate in the catchment of the Fuirosos stream (Bernal et al., 2002). After the dry period (summer), the first storms caused the weathering of dissolved and particulate OM accumulated on soil, and N concentration

in streamwater was positively correlated with lignocellulosic activities (P and PO). Therefore, fungal activities were enhanced by nitrate availability in a system where N may be a limiting resource (Romaní et al., 2004). An enhancement of lignocellulosic activities by the water N content has been observed in other systems (Alvarez & Guerrero, 2000). However, Carreiro et al. (2000) described the inhibition of these activities by high N concentrations.

Differences between biofilms colonising organic or inorganic substrata were evident in Fuirosos stream. Lignocellulosic activities were higher on inorganic substrata biofilm (developed on sand and gravel) while CBH was higher in biofilm developed on organic substrata (leaves and branches). High ligninolytic activities in sand and gravel biofilm were caused by the large accumulation of fine detritic material derived from decomposition of coarse particulate organic matter (CPOM). The largest accumulation of this material occurred in the slow-moving habitats, coinciding with stream pools or littoral zones where sand and gravel were the main substrata. The fine detritus accumulated in these substrata might be composed by a higher proportion of lignin (Yeager & Sinsabaugh, 1998; Sinsabaugh & Findlay, 1995). The lower CBH activity of biofilm growing on sand and gravel indicates the low availability of cellulose compounds in fine detritic particles. In contrast, high CBH activity in biofilms developed on organic substrata could reflect the availability of cellulolytic compounds on leaves and branches.

The enzymatic activities of biofilms differed on the leaves of different species, which might be attributable to differences in leaf composition (C: N ratio, lignin content, polyphenol content, leaf durability; Griffin, 1994). The lower enzymatic activities for the biofilm on *Platanus acerifolia* was already observed in previous studies developed on leaf decomposition in soil and in other aquatic habitats. The high C:N ratio measured in this substratum (Bernal et al., 2003; Ostrofsky, 1997) may be pointed out as the cause of low mineralization observed for this material. Slower breakdown of *Platanus* leaves than other indigenous Mediterranean leaf species (e. g. *Populus nigra*; Casas & Gesner, 1999) is probably caused by the higher lignin and other recalcitrant compounds content in the former (Ostrofsky, 1997). In contrast, high CBH and P activities were recorded in biofilms on *Populus nigra* leaves, while the highest PO was measured on *Alnus glutinosa* biofilm. Differences in PO activities between biofilms of leaf material of these plant species are probably related to the inhibition effect of phenolic compounds (Pind et al., 1994). The lower polyphenol content of *Alnus glutinosa* than *Populus nigra* leaves (Pereira et al., 1998) may imply a higher PO activity. This distinct enzymatic behaviour determines a faster decomposition of *Alnus*

glutinosa leaves in the stream reach, followed by those of *Populus nigra* and finally by *Platanus acerifolia*.

Fungal biomass was generally related with the enzymatic activities measured in the different substrata, indicating that fungi were responsible for most of decomposition processes that occurred in the stream in autumn (Griffin, 1994). The similar PO activity values measured in biofilm in sand and gravel substrata, but the lower fungal biomass in gravel, may be related to a higher proportion of fungi with PO ability (white rot fungi, Dix & Webster, 1995) on gravel than on sand. However, this activity in gravel could also be produced by some microorganisms (e. g. bacteria) using at least part of the degradation intermediates of lignin generated by fungi (Rüttimann et al., 1991). This was supported by Baldy et al. (1995) where demonstrated the importance of bacteria in the late stages of the breakdown process of leaves.

The estimates of ergosterol concentration per stream reach showed a progression of fungal biomass throughout the study period, which decreased only after most of the material had been processed. The fungi could be considered as facultative microorganisms in the sense of selection and colonization of riverbed substrata during the fall. They use the new allochthonous CPOM that entered the system during autumn. When leaf material was carried downstream, the fungi remained active on the fine detritic materials derived from the breakdown of leaves and branch materials, therefore achieving a complete decomposition of all the material that entered the reach during this season.

Chapter 3

Relating nutrient molar ratios of microbial attached communities to organic matter utilization in a forested stream

Abstract

In Mediterranean forested streams, the large input of particulate organic matter (POM) in autumn structures the benthic microbial community into two interconnected habitats: that associated with the POM itself (leaves and branches) and that colonizing gravel and sandy substrata. Relationships were examined between microbial decomposition activities (β-glucosidase, β-xylosidase, cellobiohydrolase, phenoloxidase, peroxidase, peptidase and phosphatase) at different stream substrata (leaves, branches, sand and gravel) and total carbon (C), nitrogen (N) and phosphorus (P) content of attached microbial communities and stream water matter fractions (particulate and dissolved nutrient concentrations). Microbial communities associated to leaves and branches showed a higher C: N and lower N: P molar ratios (averaging 21.7 ± 1.4 and 5.4 ± 2.1, respectively) and higher polysaccharide degrading activity (sum of β-glucosidase, β-xylosidase and cellobiohydrolase activities). Instead, biofilms on sand and gravel, where algae accumulates and finer particulate materials were more available, showed lower C: N and higher N: P molar ratios (averaging 10.3 ± 0.6 and 19.9 ± 3.7, respectively) and greater ligninolytic (sum of phenol oxidase and peroxidase activities) and peptidase activities. These results suggest that enzyme activities of microbial attached communities are linked to their nutrient molar ratios and, at the same time, these might be modulated by the different nature of available organic matter (OM) in each substratum and the microbial groups accumulated (algae, bacteria, fungi, micro- and meiofauna). However, similarities (C:N) and divergences (N:P) between stream water and nutrient molar ratios in microbial communities may also affect nutrient demands and in consequence, the expression of extracellular enzymes. The obtained results show a relationship between function (extracellular enzyme activities) and nutrient molar ratios of attached microbial communities.

Introduction

Benthic microbial communities develop on solid substrata of living or dead, organic or inorganic materials (Charaklis et al., 1990; Wetzel, 2001), and are responsible for most organic matter decomposition in fluvial ecosystems (Lock & Hynes, 1976). The substrata and their associated microbial communities are located heterogeneously in the streambed, and their respective relevance may also shift with time, according to hydrology and organic matter inputs (Acuña et al., 2005). As soon as a new substratum is submerged in the stream, an organic film accrues on its surface within hours (Peterson, 1987). Fungi and bacteria rapidly colonize POM (Sampaio et al., 2001; Romaní et al., 2006), while algae (mainly diatoms) and bacteria prevail in the

hard and relatively inert substrata, such as cobbles and gravel (Stevenson, 1996; Battin et al., 2003). Within days, the surface of these substrata is covered by microbes, and the community continues to develop through the growth of populations within the biofilm and by additional recruitment from the water column (Jackson et al., 2001). Micro- and meiofauna populations also appear in the early colonization stages (i. e. detritus; Franco et al., 1998; Gaudes et al., 2008), while macroinvertebrate feeders appeared later (Abelho, 2001).

Stream benthic microbial communities are characterized not only by their microbial composition but also by their metabolism and role in the OM cycling within the stream ecosystem (Marxsen & Witzel, 1990; Romaní & Sabater, 2001; Fellows et al., 2006). The different microbial groups forming the benthic community show a wide range of trophic strategies that may change depending on the availability of nutrients in their surrounding media. But also, microbial activity may be influenced by the grazing pressure that higher trophic levels exert on the microbial community (i. e. between meiofauna and bacteria; Perlmutter & Meyer, 1991). In general, algae and bacteria take the nutrients from the water column by diffusion mechanisms, even passive or active, through the cell membrane (Brochardt, 1996). Conversely, microbial heterotrophs (bacteria and fungi) have the capacity to breakdown high molecular weight molecules into low molecular weight compounds using extracellular enzymes, and thereafter assimilate those small molecules through the cell membrane. Some extracellular enzymes are expressed constitutively by the microbes, but many more are induced only under specific circumstances (Arnosti, 2003). Enzymatic activities related to organic matter decomposition processes are in general stimulated by increasing the available organic substratum. Thus, peptidase activity will be stimulated by increasing available proteins or peptides while polysaccharide degrading enzyme activities (β-glucosidase, β-xylosidase, cellobiohydrolase) will be stimulated by increasing available hemicellulose, cellulose and other polysaccharides. However, enzyme activities and the use of specific materials might be also modulated by nutrient imbalances of the microbial community (C: N: P molar ratios; Sterner & Elser, 2002). Determining the nutrient molar ratios (C:N, C:P and N:P) of the attached microbial communities might help us to link element imbalances to ecosystem processes such as organic matter cycling (Cross et al., 2005; Frost et al., 2002). At the same time, the organic matter use and thus the expression or inhibition of a specific enzyme is affected by the availability and nutrient ratios of dissolved inorganic nutrients in stream water (Alvarez & Guerrero, 2000; Romaní et al., 2004; Sala et al., 2001).

In this study, to determine whether patterns of organic matter utilization (extracellular enzyme activities) of stream benthic microbial communities are related to

their nutrient molar ratios (biofilm C:N and N:P) was aimed. This possible linkage might be different for the microbial communities developing at the different streambed substrata (leaves and branches, sand and gravel) where the community will be affected by the substratum characteristics and by the stream water nutrient molar ratios as well. For this purpose, the total nutrient content (C, N and P content) and parameters related to biomass of benthic microbial communities (bacteria, fungi and algae) were analyzed together with the extracellular enzyme activities involved in the decomposition of cellulose and hemicellulose, lignin, organic phosphorus compounds and peptides in the different stream substrata. The study was performed in the autumn-winter period (2002/2003) of a forested Mediterranean stream, coinciding with the major total input of OM due to the litter fall process (1117 ± 581 mgC m^{-2} day^{-1}; Acuña et al., 2004) and major leaf litter accumulation in the stream channel (34.4 ± 6.3 % of coverage of the stream bed; Chapter 2).

Materials and methods

Sampling

Coinciding with the sampling performed in the second experiment (autumn-winter 2002/2003; Chapter 2), additional samples of benthic substrata (*Platanus* and *Populus* fresh and decaying leaves, branches, sand and gravel) were taken to analyze total carbon (C), nitrogen (N) and phosphorus (P) content of microbial attached communities (section 2.4.1 of the methods), as well as total C, N, P concentrations in the dissolved and particulate water fractions (section 2.7.1). Moreover, bacterial density and chlorophyll-*a* concentrations (sections 2.2.2 and 2.2.3, respectively), total polysaccharide content (section 2.4.2) and four additional enzymatic activities involved in C (β-glucosidase, β-xylosidase), N (leucine-aminopeptidase) and P (phosphatase activity) compounds decomposition (section 2.6.1) were also measured for each substratum at each sampling time. All these measurements were combined with the results obtained in the Chapter 2 (ergosterol concentration, cellulolytic and ligninolytic activites and water physical and chemical parameters) in order to determine whether relationships between organic matter utilization (extracellular enzyme activities) and nutrient molar ratios of microbial benthic communities exist.

Total C, N and P content of microbial communities and stream water fractions (total dissolved and particulate nutrient concentrations) were expressed as C:N and N:P molar ratios. The polysaccharide degrading activity was the sum of β-glucosidase, β-xylosidase and cellobiohydrolase activities, while the lignin degrading activity was the sum of phenol oxidase and peroxidase activities. Leucine-aminopeptidase (peptidase)

and phosphatase activities were expressed as single activities. Enzyme activity ratio calculations (polysaccharide: lignin degradation and leucine-aminopeptidase: phosphatase) were performed to determine the prevailing demand of nutrient by the microbial communities. Thus, the polysaccharide: lignin degradation ratio was calculated as the quotient between (∑ β-glucosidase, β-xylosidase and cellobiohydrolase) and (∑ phenoloxidase and peroxidase), while the peptidase: phosphatase ratio was calculated directly from the quotient of these two enzymatic activities.

Statistical analyses

Differences in enzymatic activities, microbial biomass indicators, polysaccharide concentration and C: N and N: P molar ratios of benthic microbial communities among various stream substrata were examined by a multivariate analysis of variance (MANOVA) on the log (X = log (X+1)) transformed data. A post hoc multiple comparison test (*Tukey* HSD) was used to examine the differences between substrata. To evaluate whether nutrient molar ratios of microbial communities were related to the nutrient molar ratios of the stream water materials a correlation analysis (*Pearson*) was performed, whereas, a *T*-test was used to compare the differences between the C: N and N: P molar ratios of microbial communities and stream water dissolved and particulate fractions. Further correlations between the extracellular enzyme activities and nutrient molar ratios of microbial community were also performed. The statistical analyses were carried out using SPSS for Windows (Version 12.0, SPSS Inc. 2003).

Results

Physicochemical characteristics

During the study period (n=7) water temperature was moderate (11.1 ± 1.8 ºC) and decreased steadily from 16ºC in October to 3ºC in January. Underwater light was very low (19.9 ± 5.1 μmol photons m^{-2} h^{-1}). Water conductivity (268.6 ± 28.1 μS cm^{-2}), pH (7.6 ± 0.01) and dissolved oxygen (8.9 ± 0.6 mgO_2 L^{-1}) did not highly differ from average values encountered in the Fuirosos (Acuña et al., 2004).

C, N and P composition of stream water

C: N molar ratios of the dissolved and particulate fractions in the stream water were similar during the study (18 ± 1.7 and 18.3 ± 5.6, respectively; Figure 5.1 a). However, the N: P molar ratio of the dissolved fraction in the water was 15 times higher than that measured for the particulate fraction (334.5 ± 38.7 and 22.9 ± 1.2,

respectively; Figure 5.1 b). Those large differences were caused by the high N concentration, especially dissolved organic nitrogen (DON) forms, in the stream water.

C, N and P composition of microbial communities

The nutrient molar ratios (C: N and N: P) of microbial communities varied considerably between streambed substrata (MANOVA, $p < 0.0001$, Figure 5.1). C: N molar ratios in leaves and branches communities were significantly higher (21.7 ± 1.4 in average) than those measured in sand and gravel communities (10.3 ± 0.6 in average, *Tukey's* test, $p < 0.05$, Figure 5.1 a). These differences were because of the higher C and lower N content in leaves and branches communities (i. e. 79.3 ± 3.5 μgC cm^{-2} and 3.3 ± 0.4 μgN cm^{-2} in *Platanus* fresh leaves, average values n=7) than those developed on sand and gravel (i. e. 59.4 ± 9.9 μgC cm^{-2} and 7.9 ± 1.6 μgN cm^{-2} in gravel; average values n=7). The C: N molar ratio of leaves and branches microbial communities were not significantly different than those measured for the particulate and dissolved fractions of the stream water (*T*-test, $p = 0.24$ for both fractions).

High N: P molar ratios characterized the microbial communities on inorganic substrata, as well as those colonising branches (*Tukey's* test, $p < 0.05$), and were close to the values measured for the particulate fraction of the stream water (Figure 5.1 b). In contrast, *Platanus* and *Populus* microbial colonizing communities showed in average N: P molar ratios of 1 ± 0.3. A higher P content was detected in leaf communities (i. e. 13.8 ± 1.2 and 22.9 ± 4 μgP cm^{-2} in average in *Platanus* and *Populus* decaying leaves, respectively) than those colonizing sand and gravel (1.2 ± 0.1 and 2.5 ± 0.5 μgP cm^{-2} in average in gravel and sand, respectively). N: P molar ratios of attached microbial communities (from 0.5 to 35) were much lower than those measured in the dissolved fraction of the stream water (334.5 ± 38.7, Figure 5.1 b).

Microbial communities and polysaccharide concentration

The bacterial density, chlorophyll-*a*, and ergosterol concentrations varied between stream substrata (MANOVA, $p < 0.005$). Bacterial density was higher on sand biofilms while densities on decaying leaves (DL) and branches exceeded those measured on the recently deposited leaves (FL) (*Tukey's* test, $p < 0.05$; Figure 5.2 a). Ergosterol was higher in branches while chlorophyll-*a* was higher on sand and gravel (*Tukey's* test, $p < 0.05$; Figure 5.2 b & c). Leaf microbial communities, and especially those formed on recently deposited *Populus* leaves, showed the highest polysaccharide concentration (*Tukey's* test, $p < 0.05$; Figure 5.2 d).

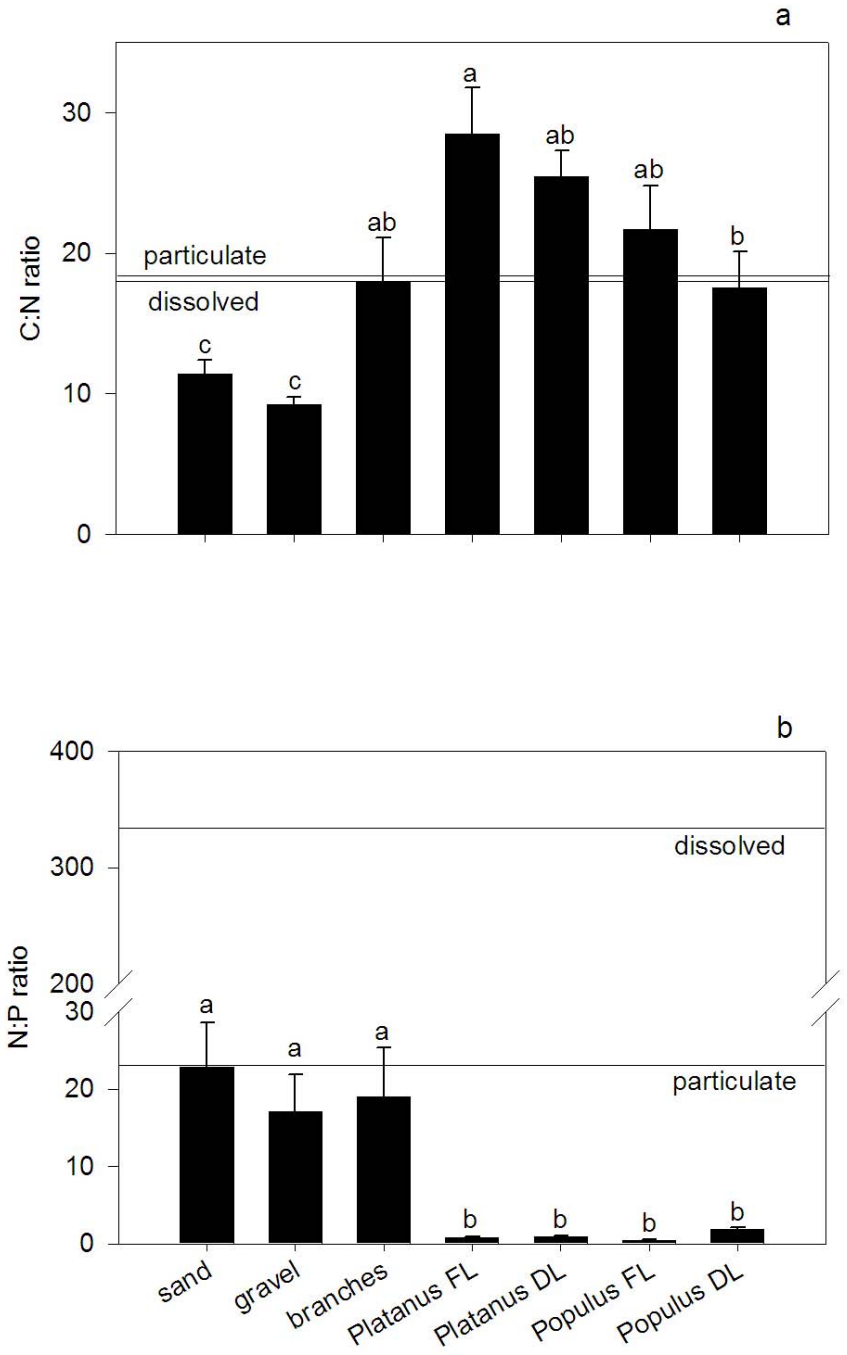


Figure 5.1. C:N (a) and N:P (b) molar ratios of microbial communities colonizing different substrata and those from the particulate and dissolved fractions of stream water. Values are means and standard errors of seven sampling dates. Significantly different groups (a>b>c) obtained with the Tukey's test (α=0.05) are also shown.

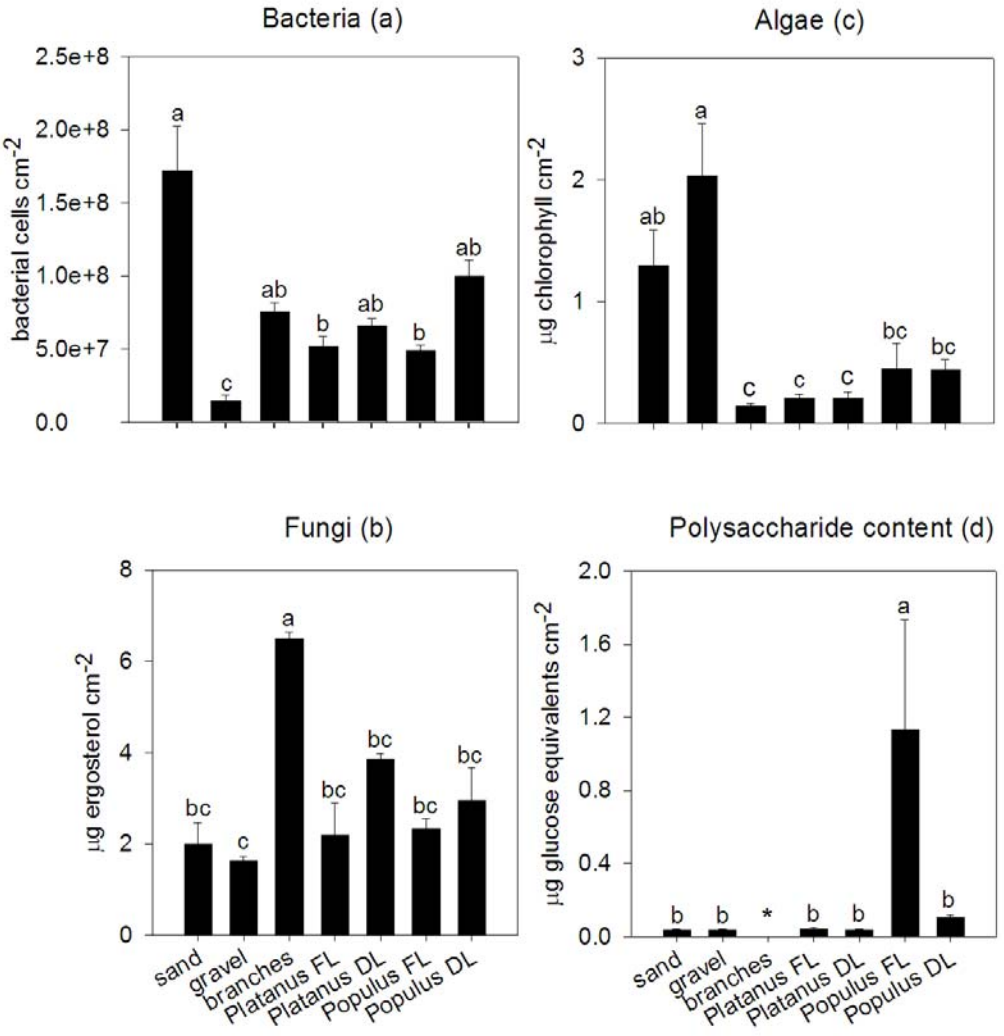


Figure 5.2. Biomass measurements of bacteria (a), fungi (b) and algae (c) and polysaccharide content (d) in microbial communities colonizing the different substrata analyzed (means and standard errors). Significantly different groups obtained with Tukey's test (a>b>c, α=0.05) are also shown. Missing values are represented by (*).

Extracellular enzyme activities

Activities involved in the degradation of polysaccharides (β-glucosidase, β-xylosidase and cellobiohydrolase) were higher in microbial communities developing on decaying leaves (DL), especially in those of *Populus* (*Tukey's* test, $p < 0.05$, Figure 5.3 a). In contrast, those on sand and gravel showed higher capacity to degrade lignin (*Tukey's* test, $p < 0.05$, Figure 5.3 b). Peptidase activity was significantly higher in sand and gravel biofilms, but also in *Populus* decaying leaves (*Tukey's* test, $p < 0.05$, Figure 5.3 c), while phosphatase activity was higher in decaying leaves, branches and sand (Figure 5.3 d).

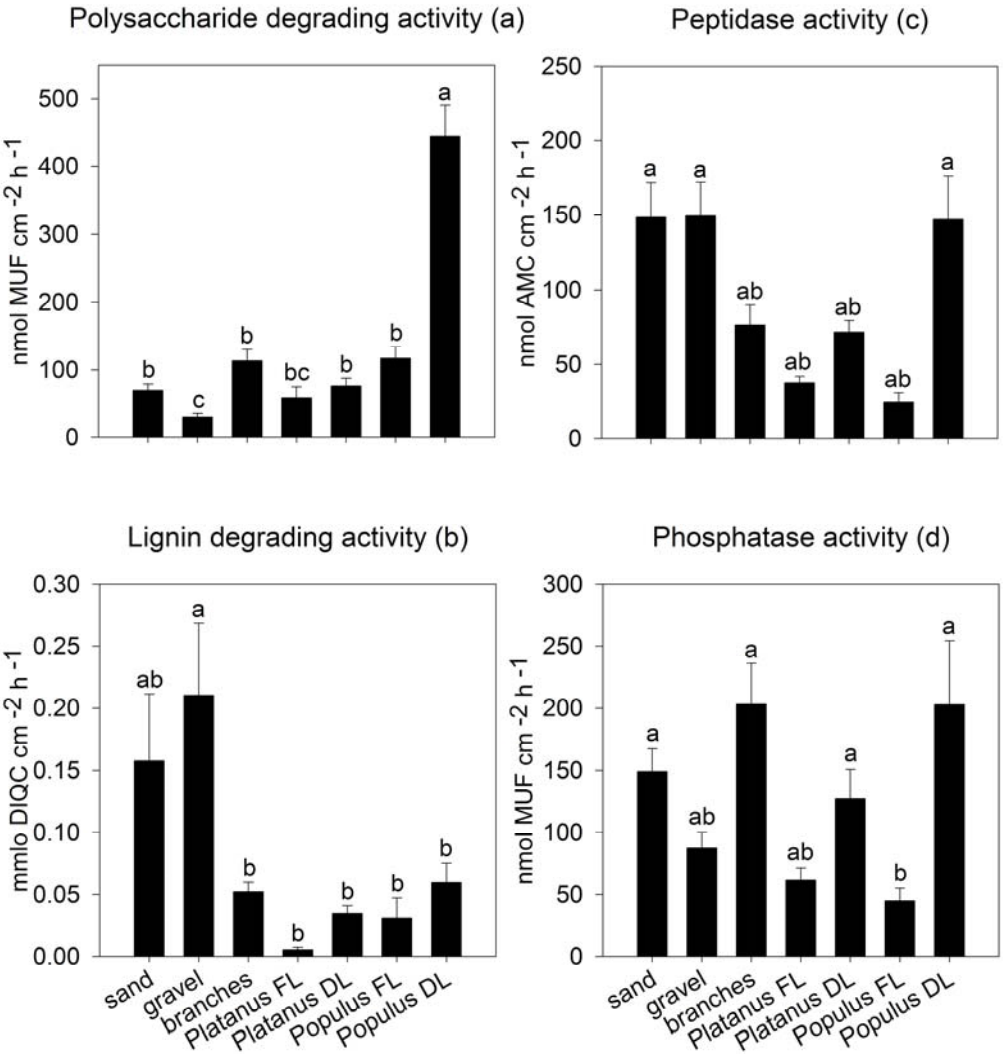


Figure 5.3. Extracellular enzyme activities measured in each substratum during the study. The polysaccharide degrading activity (a) is the sum of β-glucosidase, β-xylosidase, cellobiohydrolase activities while the lignin degrading activity (b) is the sum of phenoloxidase and peroxidase activities. Peptidase (c) and phosphatase (d) activities are also shown. Values are means and standard errors of the seven sampling dates. Significantly different groups (a>b>c) obtained by means of Tukey's test (α=0.05) are shown.

The polysaccharide: lignin degradation ratio indicated that prevailing activities on microbial communities differed according to the substrata where they developed (MANOVA, $p < 0.05$; Table 5.1). The ratio reached higher values in leaves and branches, especially in the microbial communities on *Populus* leaves, and was lower in those on sand and gravel (*Tukey's* test, $p < 0.05$). The peptidase: phosphatase ratio indicated that in most substrata there was a preferential transformation of organic phosphorus compounds (higher phosphatase activity). However, microbial communities growing on gravel had higher transformation of peptidic compounds (higher peptidase activity).

The C: N molar ratio of microbial communities (n = 49) tended to decrease when the ligninolytic and peptidase activities increased (r = -0.656, $p < 0.001$; r = -0.323, $p < 0.05$; respectively). Also, ligninolytic activity and N: P molar ratios of microbial communities increased accordingly (r = 0.412, $p < 0.001$). However, peptidase and ligninolytic activities were also sensitive to the C: N and N: P molar ratios of the dissolved water fraction. Specifically, the peptidase activity of biofilms and the C:N molar ratio of the dissolved fraction of the water tended to increase at the same time (r = 0.456, $p < 0.001$).

	Polysaccharide : lignin degradation		peptidase : phosphatase	
	Mean	*SE*	Mean	*SE*
Sand	0.5	*(0.2)*	0.7	*(0.1)*
Gravel	0.3	*(0.1)*	2.3	*(1.1)*
Branches	2.5	*(0.5)*	0.6	*(0.2)*
Platanus FL	5.8	*(1.6)*	0.8	*(0.2)*
Platanus DL	3.1	*(1.0)*	0.6	*(0.2)*
Populus FL	8.1	*(2.4)*	0.7	*(0.2)*
Populus DL	9.3	*(1.8)*	1.1	*(0.7)*

Table 5.1. Polysaccharide: lignin degradation ratio and peptidase: phosphatase ratio measured along with the values of enzymatic activities in the different stream substrata. Values are means and standard errors of the seven sampling dates

Discussion

The combined analysis of extracellular enzyme activities and nutrient molar ratios of benthic microbial communities developed on different stream substrata suggested a link between the differential capacity for organic matter decomposition and the C: N and N: P molar ratios of each microbial community. Biofilms on inorganic substrata (sand and gravel) showed a major lignin and peptide decomposition capacity and a higher proportion of N (lower C: N and higher N: P molar ratios). In contrast, higher C: N and lower N: P molar ratios were measured in biofilms developed on organic substrata (leaves and branches), which also showed major capacities for polysaccharide decomposition (cellulose and hemicellulose). This linkage could be related to the differential organic matter available at different streambed substrata but also to the quality and quantity of materials in the flowing water. Nevertheless, the accrual of microbial biomass (algae, bacteria, fungi) as well as other biological groups

(i. e. micro- and meiobenthic fauna) colonizing the benthic substrata, may also be relevant in determining biofilm nutrient molar ratios.

The stream habitat (sand, gravel, accumulated leaves and branches) may affect the functioning and nutrient molar ratios of the attached microbial communities due to several reasons: 1) the quantity and quality of available organic matter sources (Findlay et al., 2002), 2) the physical characteristics of the different substrata (Pusch et al., 1998), 3) the changes of nutrient availability in the water (Masseret et al., 1998; Stelzer et al., 2003), and 4) the interactions between different biological groups colonizing the substratum [i. e. algae-bacteria in the epilithon (Francoeur & Wetzel, 2003) and bacteria-meiofauna in leaves (Perlmutter & Meyer, 1991)]. In sand and gravel habitats, finer particulate organic matter with high lignin content (Yeager & Sinsabaugh, 1998) and low C:N molar ratio (C:N = 17.7 ± 0.5, unpublished data) may accumulate, enhancing ligninolytic enzyme activities and a higher N content of the microbial communities. Cycling of nitrogenous compounds in microbial communities on sand and gravel is further showed by the high peptidase activity. High peptidase activity in aquatic biofilms has been usually related to the use of peptides released by algae (Romaní & Sabater, 2000; Francoeur & Wetzel, 2003; Rier et al., 2007), which might occur in benthic microbial communities developing on sand and gravel. The higher N concentration of sand and gravel microbial communities (lower C: N and higher N: P) might be in concordance to the major use of nitrogenous compounds (lignin and peptides).

In contrast to sand and gravel habitats, debris dams and leaf accumulation habitats are stream sites of high heterotrophic activity (Pusch et al., 1998) due to the decomposition of plant material. Degradation of celluloses and hemicelluloses is an early process in the decomposition of leaf litter (Berg & McClaugherty, 2003), and this was shown by the high polysaccharide degrading activities (β-glucosidase, β-xylosidase and cellobiohydrolase) that microbial communities in leaves and branches exhibit (higher polysaccharide: lignin ratio). The major use of C compounds by bacteria and fungi on these substrata may determine the higher C: N, in contrast to that on sand and gravel. Although contamination of leaf material when detaching the leaf microbial community could occur, the methodological verification by observation of leaf material before and after sonication and by comparing the much higher C:N molar ratio in leaves to those measured in the detached material, evidenced that sonication did not cause any significant inclusion of leaf particles (section 2.4.1). However, major differences were detected for the lower N:P molar ratios measured in *Platanus* and *Populus* leaves in contrast to sand and gravel. The higher P content of these communities was not related to a higher phosphatase activity rate, but probably to the

use of labile organic P compounds released during leaching. Finally, consistent differences between C:N and N:P molar ratios in leaves and branches versus those measured for sand and gravel could be further modulated by the specific composition of the microbial community developed in each substratum (algae, bacteria, fungi, micro and meiofauna) which might show differences in C, N and P content (Sterner & Elser, 2002).

Even the effects and differences between benthic habitats, microbial functioning might be also influenced by stream water and its nutrients molar ratios in dissolved and particulate fractions. While C:N molar ratios of attached communities were similar to those measured in the stream water (both dissolved and particulate), the N:P ratios were much lower than those measured in the dissolved water fraction (Figure 5.1). Previous studies have shown that dissolved N availability (mainly nitrate) in the Fuirosos water is very high during punctual rainfall episodes, because of N mobilisation from the watershed soils (Bernal et al., 2002). The larger N bulk in stream water (mainly due to the dissolved organic nitrogen, DON) may exceed the demands by the microbial community and/or an important proportion of this DON might be less biodegradable. The availability of dissolved nitrogen in the water seem to influence some enzymatic activities, as suggested by the positive correlations between the C:N molar ratio of the water and the peptidase activity of the biofilm.

During the large autumnal input of particulate organic matter in forested streams, the microbial benthic community is distributed in two major habitats, which become dynamic and interconnected. The high availability of allochthonous OM (materials with a high C: N molar ratio) during the study period (autumn-winter) affects both the nutrient ratios and the enzyme activities of microbial communities. The POM, mainly leaves, may support a microbial community with a higher polysaccharide matrix and high C and P content, which feeds on the substrata itself. As decomposition process takes place, plant material is transformed into smaller organic particles, which get included to the stream bed sediments. Inorganic substrata in streambed support communities with high N content that rely upon fine detritic materials accumulated and on peptide molecules produced by algae within the biofilm. Even though microorganisms (bacteria, fungi and algae) are essential in shaping the nutrients imbalance and metabolism of stream biofilms, recent studies have demonstrated that meiofauna participate to a variety of trophic processes within the benthic community that can contribute to these differences (Robertson et al., 2000; Schmid-Araya & Schmid, 2000). Probably the lower allochthonous OM input and higher autotrophic production (high authochthonous OM input) characteristic of open streams would determine different patterns between the community nutrient molar ratios and

enzymatic activities. Further studies addressed at different stream ecosystems considering both microbial and other biological groups (i. e. micro- and meiobenthic fauna as well as macrofauna) will be essential to validate the relationships between function and nutrient molar ratios of attached communities.

Chapter 4

Relationships between microbial biomass and extracellular enzyme activities during leaf litter breakdown in a forested stream

Abstract

Microbial decomposition of leaf litter in streams is mostly carried out by fungal and bacterial extracellular enzyme activities that enable the breakdown of large organic matter molecules into small molecules. In this study, microbial biomass (fungi and bacteria) and extracellular enzyme activities involved in polysaccharide (β-glucosidase, β-xylosidase and celloiohydrolase) and lignin (phenol oxidase) decomposition were analysed during leaf breakdown process in the stream. A three-month litter bag experiment was carried out using two different leaf species (*Platanus acerifolia* and *Populus nigra*). The effect of microbial activity on leaves was analyzed through the changes in their physical and chemical properties (carbon, nitrogen, phosphorus and lignin content and leaf toughness). Different patterns of fungal and bacterial biomass accumulation were found: while bacterial biomass increased sharply during the first week, fungal biomass slightly decreased, and the steepest fungal biomass increase occurred after day 17. At day 44, fungal biomass stabilized and bacterial biomass increased until the end of the experiment (day 112). It was between days 7 and 17, when the widest and fastest leaf structural changes (steepest decrease in toughness and C:N and C:P ratios) and the maximum enzyme activities per unit of microbial carbon took place. This indicates a high efficient organic matter use by the fungi and bacteria during this short period. There was an exponential relationship between bacterial biomass and most of the enzyme activities measured in the two leaf species. Moderate increases in bacterial biomass accrual coincide with rapid increases of enzymatic activity. This was more pronounced in *Populus* (higher slope of the curves) than in *Platanus* leaves. Fungal biomass and activity showed exponential relationships only in *Platanus* leaves, though not in *Populus*. The use of mixed species in the same litter bag made difficult to separate the biomass establishment from the enzyme activity expression, though our results suggested that fungi probably required of a higher stability to develop and degrade substrates than bacteria. Consequently, the higher durability of *Platanus* species might have been favouring the establishment of fungi and enhancing the relationship between fungal biomass and activity.

Introduction

The breakdown of leaf litter is a key process in the stream ecosystem metabolism (Cummins, 1988). From early studies, breakdown process of leaf litter has been mainly referred to leaf mass loss and defined in three different phases that are separated in a temporal scale: i) lixiviation of soluble leaf constituents, ii) conditioning by microbial heterotrophs, and iii) fragmentation by shredders (Webster & Benfield, 1986; Maltby,

1992). Nevertheless, more recent perspectives on litter breakdown process suggest that these phases usually overlap, and proposed that the process should be also viewed in terms of the products of litter breakdown (Gessner et al., 1999). In fact, microbial hetertrophs (fungi and bacteria) are present during the whole breakdown process, although they are considered more relevant (in terms of leaf carbon degradation) at the beginning (fungi) and late phases (bacteria; Gessner et al., 1991). In a study based on the differences in immigration, colonization and fungal-bacterial interactions during leaves colonization, Bengtsson (1992) reported that fungal mycelia doubled in biomass, roughly ten times faster than bacterial biomass and suggested that this might explain the dominance of fungi in leaves.

Leaf carbon loss is controlled mostly by fungi (Hieber & Gessner, 2002) and, therefore, net carbon transformation in leaves is a reflection mainly of fungus degradative ability. However, bacteria may account also for a sizeable fraction of total microbial production in leaves (Baldy et al., 2002). Fungi and bacteria are able to decompose leaves through the production of extracellular hydrolytic and oxidative enzymes (Mansfield, 2005). An early study from Sinsabaugh et al. (1994), confirmed a consistent correlation between enzyme activities and leaf mass loss in freshwater environments. Therefore, these activities must be considered as an important factor in the overall leaf decay process in streams. It is generally assumed that the decomposition of lignin and complex polysaccharide molecules (i. e. cellulose) is mainly carried out by fungi in leaves, and that bacteria degrade smaller carbon molecules or uptake simple compounds released by fungi during enzymatic activities (Velicer, 2003; Romaní et al., 2006). Despite this assumption of defined and separate roles, fungi and bacteria have been shown to coexist in synergistic relationships (Bengtsson, 1992), as well as to compete with each other for the uptake of leaf nutrients (Mille-Lindblom & Tranvik, 2003).

Several studies have attempted to evaluate the relationship between microbial biomass and enzyme activity in soils (Frankenberger & Dick, 1983; Nannipieri et al., 1983), and some of them found strong positive correlations (i. e. Xiangzhen & Pariente, 2003). These results suggested that organic carbon and enzyme activity should be related to each other via microbial biomass (Bergstrom et al., 1998). To define whether fungal and bacterial biomass standing stock in leaves is responsible of variations on the enzyme activity may give some light on the role of these groups during leaf breakdown process. Nevertheless, few studies have been focused on these relationships in freshwater environments and most of them were performed in lentic environments (Vrba et al., 1992; Hoppe et al., 1998).

In this study, the evolution of microbial biomass (fungi and bacteria) and extracellular enzyme activities (β-glucosidase, β-xylosidase, cellobiohydrolase and phenol oxidase) was followed in leaves (*Platanus acerifolia* and *Populus nigra*) submerged in a Mediterranean forested stream during 112 days. Variations in the microbial community structure and metabolism were analyzed together with the the changes observed in leaf properties (C, N, P and lignin content and leaf toughness). Specifically, the following three questions were asked: i) How microbial biomass and enzyme activities evolved during the breakdown process of *Platanus acerifolia* and *Populus nigra* leaves? ii) What was the relationship between fungal and bacterial biomass and extracellular enzyme activities during the leaf breakdown process? iii) What was the relationship between microbial enzyme activities and changes in leaf physical and chemical properties during breakdown of materials?

Materials and Methods

Sampling strategy

Recently fallen leaves of *Platanus acerifolia* (from now referred as *Platanus* in the text) and *Populus nigra* (referred as *Populus*) were collected from the riparian forest and transported to the laboratory. Once there, leaves were first dried at room temperature for 48h, and then placed in sterile bags for autoclave. A simple sterilization cycle at 121 ºC for 15 min. on leaf materials was performed to ensure a microbial blank before the breakdown experiment begins. In the field, sterilized leaves were carefully transferred from the sterile bags into nylon net bags (1 mm of mesh size) and rapidly immersed. In total, 33 bags were submerged along the reach. Each bag contained a mixture of *Platanus* and *Populus* leaves, similar to natural accumulation of mixed leaf species in the stream channel (4 leaves of *Platanus* and 6 of *Populus*). Bags were immersed on October 6, 2003 and retrieved at days 1, 2, 4, 7, 17, 28, 44, 58, 73, 93 and 112. At each sampling time, three litter bags were collected at random in the reach. Leaves were removed from the bags and rinsed with water in order to eliminate fine detritic materials and/or silt particles accumulated. Then, leaf disks were obtained from clean leaves and used for subsequent analysis of the attached microbial community. Physical and chemical characteristics of the stream water (temperature, conductivity, pH, discharge, nitrate and phosphate concentrations) were measured in the field with portable meters, while additional water samples were taken to analyze nitrate and phosphate concentrations (section 2.7.1).

Fungal and bacterial biomass (sections 2.2.1 and 2.2.2, respectively) and a range of extracellular enzyme activities involved in polysaccharide (β-glucosidase, β-xylosidase and cellobiohydrolse) and lignin (phenol oxidase) decomposition (section

2.6.1) were analyzed during the breakdown experiment of *Platanus* and *Populus* species. Simultaneously, changes in leaf physical (toughness; section 2.5.2) and chemical (total C, N, P and lignin content; sections 2.5.1 and 2.5.2, respectively) properties and streamwater characteristics (temperature, discharge, conductivity, pH and nitrate and phosphate concentrations) were analyzed and related to microbial biomass and activity parameters during the breakdown process.

In this study, all measurements corresponding to the attached microbial community in leaves (enzyme activity, biomass, CNP content) were referred to the ash free dry mass (AFDM) of leaf disks.

Statistical analyses

Repeated measures analysis of variance (ANOVA) on log-transformed data was used to analyze the temporal evolution of enzyme activity, microbial biomass and leaf characteristics during the breakdown experiment, but also, to determine differences between species (*Platanus* and *Populus*). The Dunn-Sidak correction was applied to resultant ANOVA probability values (*P values*) of all sources analyzed. Furthermore, relationship between microbial biomass and extracellular enzyme activity during breakdown process was analyzed by means of regression analysis. Linear regression and a sort of non linear regression models (exponential, logistic, logarithmic, quadratic, inverse) were assayed on fungal and bacterial biomass and enzyme activity data obtained for the two species. Moreover, relationships between extracellular enzymes and leaf physico-chemical properties and stream water characteristics were analyzed through product moment Pearson correlation analysis. The statistical analyses were performed using the SPSS for Windows (Ver. 12.0, SPSS Inc. 2003).

Results

Physical and chemical properties of stream water

Results of water physical and chemical characteristics are summarized in Table 6.1. Water nutrient concentration and temperature did not show drastic changes during the experiment, but a punctual increase in the water flow (from 20 Ls^{-1} up to 110 Ls^{-1} at day 10) made also decrease values of conductivity in the water. This episode may have been influencing on the leaf mass loss, especially in *Populus* species.

	Mean	SE
temperature (ºC)	10.8	0.9
conductivity ($\mu S\ cm^{-1}$)	229.8	18.6
pH	7.02	0.5
nitrate ($\mu g\ NO_3^-\ L^{-1}$)	857.1	59.9
phosphate ($\mu g\ PO_4^{3-}\ L^{-1}$)	24.5	5
discharge ($L\ s^{-1}$)	31.4	31.6

Table 6.1. Results of physical and chemical parameters measured in the stream water during the litter breakdown experiment. Values are means and standard errors (SE) of the 12 sampling dates.

Physical and chemical properties of leaves

Observations in the field suggested that *Populus* leaves decomposed faster than *Platanus*; after day 58 only the latter remained in the litter bags. This faster breakdown of *Populus* was confirmed by the faster decrease on leaf toughness of this species (day 7) than in *Platanus* (day 17; Time × Species effect, Table 6.2, Figure 6.1 a). Lignin content was similar between leaf species (Table 6.2), but percent of lignin remaining showed a slight increase during breakdown process (Figure 6.1 b).

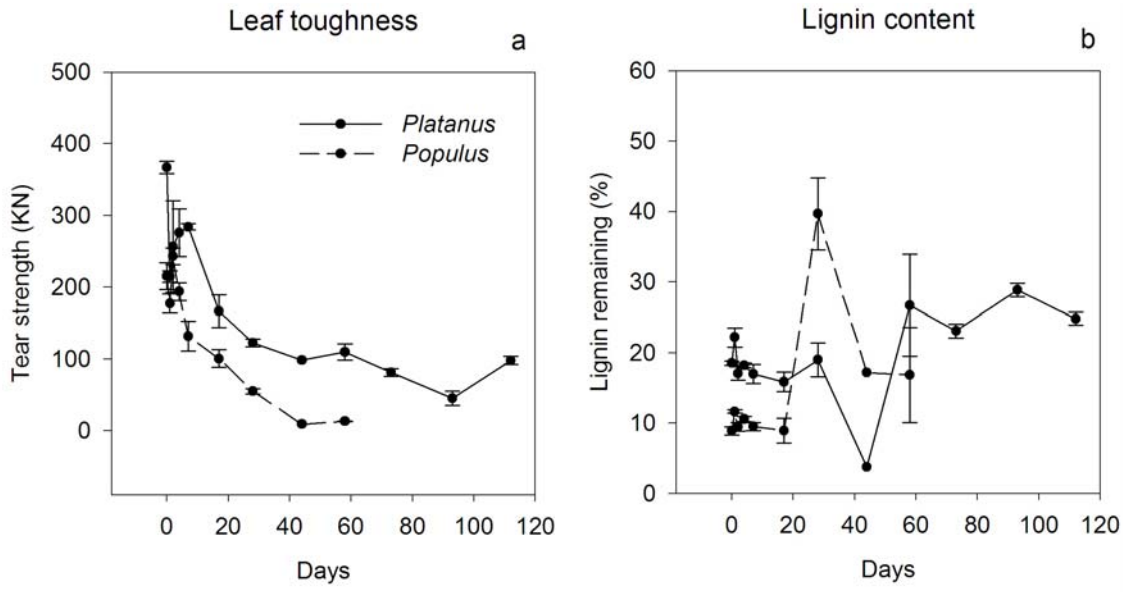


Figure 6.1. Leaf tear strength (a) and percent of lignin remaining (b) of the two leaf species (Platanus and Populus) during the litter bag experiment. Values are means (n = 3) and standard errors of each sampling date.

Parameters	Time		Species		Time × Species	
	F-ratio	*P value*	*F-ratio*	*P value*	*F-ratio*	*P value*
fungi	2.6	0.990	0.7	1	0.6	1
bacteria	134.1	**< 0.005**	7.6	0.329	1.5	1
β-glucosidase	149.6	**< 0.005**	139.9	**< 0.005**	7.7	0.232
β-xylosidase	169.5	**< 0.005**	2.8	0.978	9.4	0.712
cellobiohydrolase	129.3	**< 0.005**	159.9	**< 0.005**	23.1	0.258
phenol oxidase	35.9	**< 0.005**	3.6	0.945	0.2	1
C:N	17.7	**< 0.005**	1.6	1	0.02	1
N:P	2.3	1	1.4	1	1.7	1
C:P	8.6	0.233	1.9	1	3	0.996
lignin	6.5	0.840	9.3	0.646	11.1	0.621
toughness	125.7	**< 0.005**	31	**< 0.005**	251.9	**< 0.005**

Table 6.2. Results of repeated-measures ANOVA on microbial biomass, enzyme activities and leaf properties during litter bag experiments. F-ratios and probability are indicated for all sources of variation: Time effect, Species effect and Time × Species effect. Probability within groups was corrected for sphericity by the Greenhouse-Geisser correction. All the probability values were adjusted by the Dunn-Sidak correction. Significant P values are marked in boldface type letter.

C: nutrient molar ratios (C:N and C:P) in leaves decreased mainly between days 7 and 17 of the breakdown experiment (Table 6.3), although only the C:N ratio decrease was statistically significant (Time effect, Table 6.2). These ratios decreased coinciding with the increase of total N and P content in leaves. Conversely, N:P ratio values in leaves did not show drastic changes during the experiment. Differences in nutrient molar ratios between *Platanus* and *Populus* species were not consistent (Table 6.2).

Days	C:N		C:P		N:P	
	Platanus	*Populus*	*Platanus*	*Populus*	*Platanus*	*Populus*
0	77.7 (0.8)	91.5 (3.3)	841.9 (133.8)	712.6 (70.1)	71.7 (12.3)	51.1 (2.7)
1	65.7 (9.3)	96.6 (15.4)	868.3 (136.2)	2247.7 (494.8)	95.4 (26.4)	151.8 (14)
2	94.2 (13.1)	97.7 (22.8)	840.7 (287.7)	1879.5 (794.4)	67.2 (28.2)	131.2 (44)
4	82.7 (2.3)	90.7 (6.5)	1358.5 (93.4)	1309 (201.2)	109.9 (8.7)	97.6 (17.1)
7	93.1 (5.2)	70.3 (6.8)	992.3 (104.1)	1079.2 (97)	72.2 (10.4)	102.8 (5.9)
17	58.8 (5.5)	47.9 (1.9)	566.3 (60.8)	393 (31.4)	64.2 (2.4)	54.5 (2.3)
28	39.5 (1.7)	38.2 (1.2)	453.6 (30.7)	390.4 (36.1)	76.7 (4.6)	68.3 (6.5)
44	53.8 (0.3)	46.9 (1.1)	510.2 (65.1)	409.4 (64.5)	63.4 (8.4)	58.5 (9.8)
58	45.7 (7.9)	43.6 (3.8)	494.3 (137.8)	622.2 (139)	68.8 (10.1)	93.3 (13.6)
73	50.6 (3.3)		659.1 (31.4)		87.2 (2.5)	
93	36.2 (3.4)		324.7 (38.5)		62.1 (12)	
112	56.3 (1.2)		480.4 (32.2)		57.2 (4.9)	

Table 6.3. C:N, C:P and N:P molar ratios of the two leaf species during the litter bag experiment. Values are means (n = 3) and standard errors (in parenthesis) at each sampling time.

Fungal and bacterial biomass in leaves

Fungal and bacterial biomass increased in the two leaf species during the breakdown experiment, although only the increase of bacteria was statistically significant (Time effect, Table 6.2). Fungi were responsible for most microbial biomass standing stock in leaves during the whole breakdown process (98% fungal C versus 2% bacterial C, average of the two species; Figure 6.2). Biomass accrual of fungi and bacteria was similar between species.

Fungal and bacterial biomass evolved in different manner during the breakdown process. Bacterial biomass in *Platanus* leaves sharply increased during the first week, reaching a plateau between days 7 to 17 which was maintained until day 44. After day 44, bacterial density increased again following a less pronounced but linear slope until the end of the experiment (day 112, Figure 6.2 a). Bacterial biomass dynamics in *Populus* was similar to that described for *Platanus* for the first week (sharp increase) but then continued to increase until the complete disappearance of the leaves (day 58).

In contrast to bacteria, fungal biomass showed a slight decrease during the first week in the two species and only after day 17 showed a sharp increase, reaching a maximum at day 44. Afterwards, fungal biomass values kept in the same range until the end of the experiment (Figure 6.2 b).

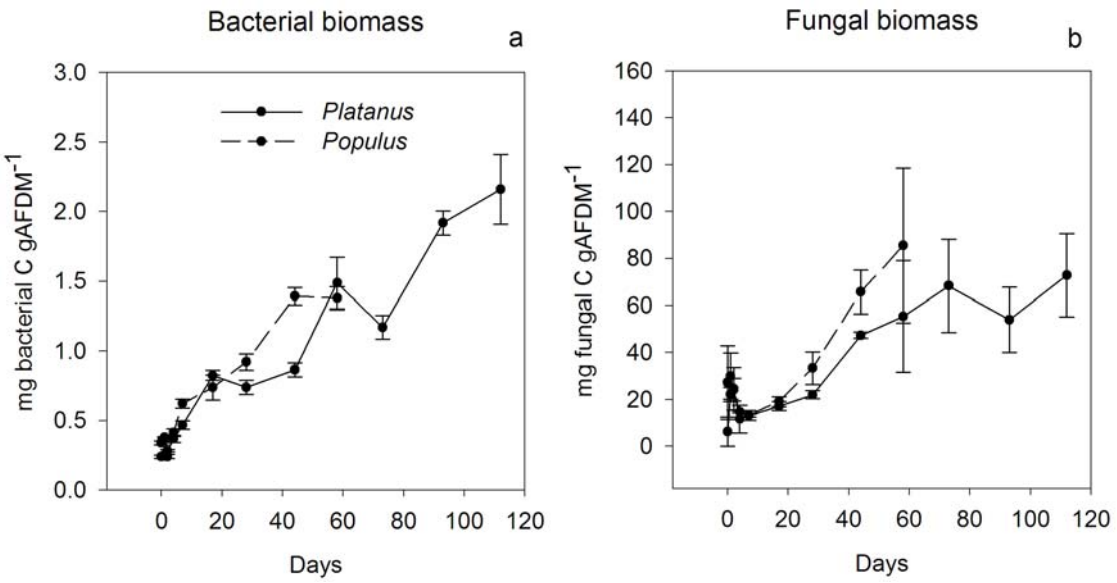


Figure 6.2. Bacterial (a) and fungal (b) biomass in terms of carbon (C) per g AFDM measured in the two leaf species (Platanus and Populus) during the litter bag experiment. Values are means (n=3) and standard errors of each sampling date.

Extracellular enzyme activity in leaves

Polysaccharide (β-glucosidase, β-xylosidase and cellobiohydrolase activities) and lignin (phenol oxidase activity) degrading activities increased during the breakdown experiment (Time effect, Table 6.2), but evolved in different manner between leaf species (Figure 6.3). Polysaccharide degrading activities in *Populus* increased sharply during the second week (days 7 to 17), peaked at day 44, and then decreased at day 56 (Figure 6.3 a, b & c). A similar increase of activities was observed in *Platanus* leaves, but those did not peak as abruptly as in *Populus*. The temporal evolution of phenol oxidase activity was similar between leaf species, increasing after day 7 and reaching a plateau at day 17, which extended until the end of the experiment (Figure 6.3 d).

β-glucosidase and cellobiohydrolase activity per unit of AFDM was higher in *Populus* than in *Platanus* leaves, while the rest of activities were similar between species (Species effect, Table 6.2). Instead, biomass-specific enzyme activity calculations of β-xylosidase, cellobiohydrolase and phenol oxidase enzymes were slightly higher for *Platanus* than for *Populus* leaves (Figure 6.4 b, c & d). This suggested that the microbial biomass on *Platanus* was more efficient in decomposing organic matter than that on *Populus*. Moreover, the enzyme activity per unit of microbial carbon tends also to peak early in the experiment and decrease afterwards (Figure 6.4).

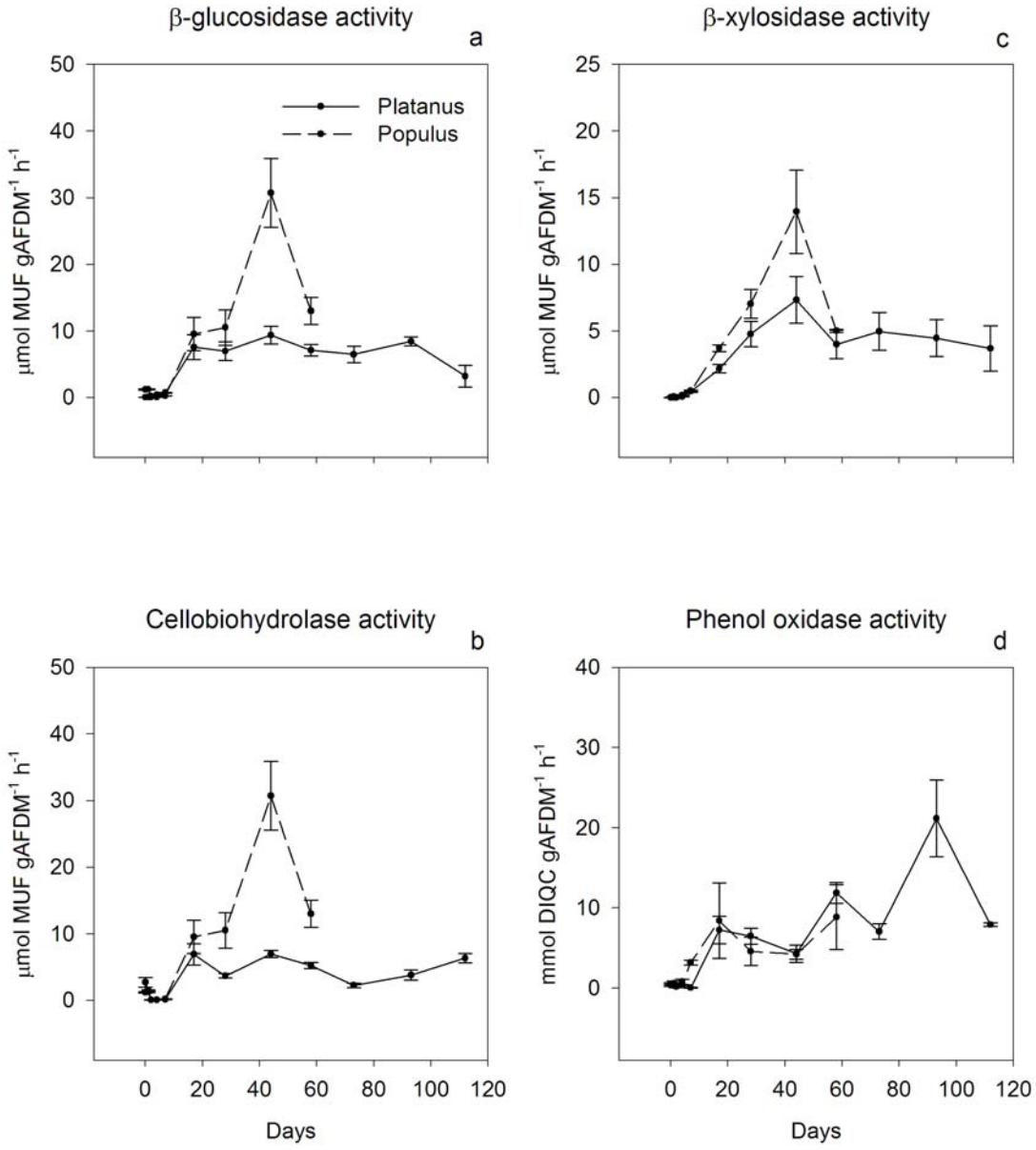


Figure 6.3. Values of β-glucosidase (a), β-xylosidase (b), cellobiohydrolase (c) and phenol oxidase (d) activities during the breakdown of the two leaf species. Units of enzymatic activities are expressed as the amount of methylumbelliferone (MUF for β-glucosidase, β-xylosidase and cellobiohydrolase) and 2,3-dihydroindole-5,6-quinone-2-carboxilate (DIQC for phenol oxidase) released per unit of leaf AFDM and time. Values are means (n=3) and standard errors of each sampling date.

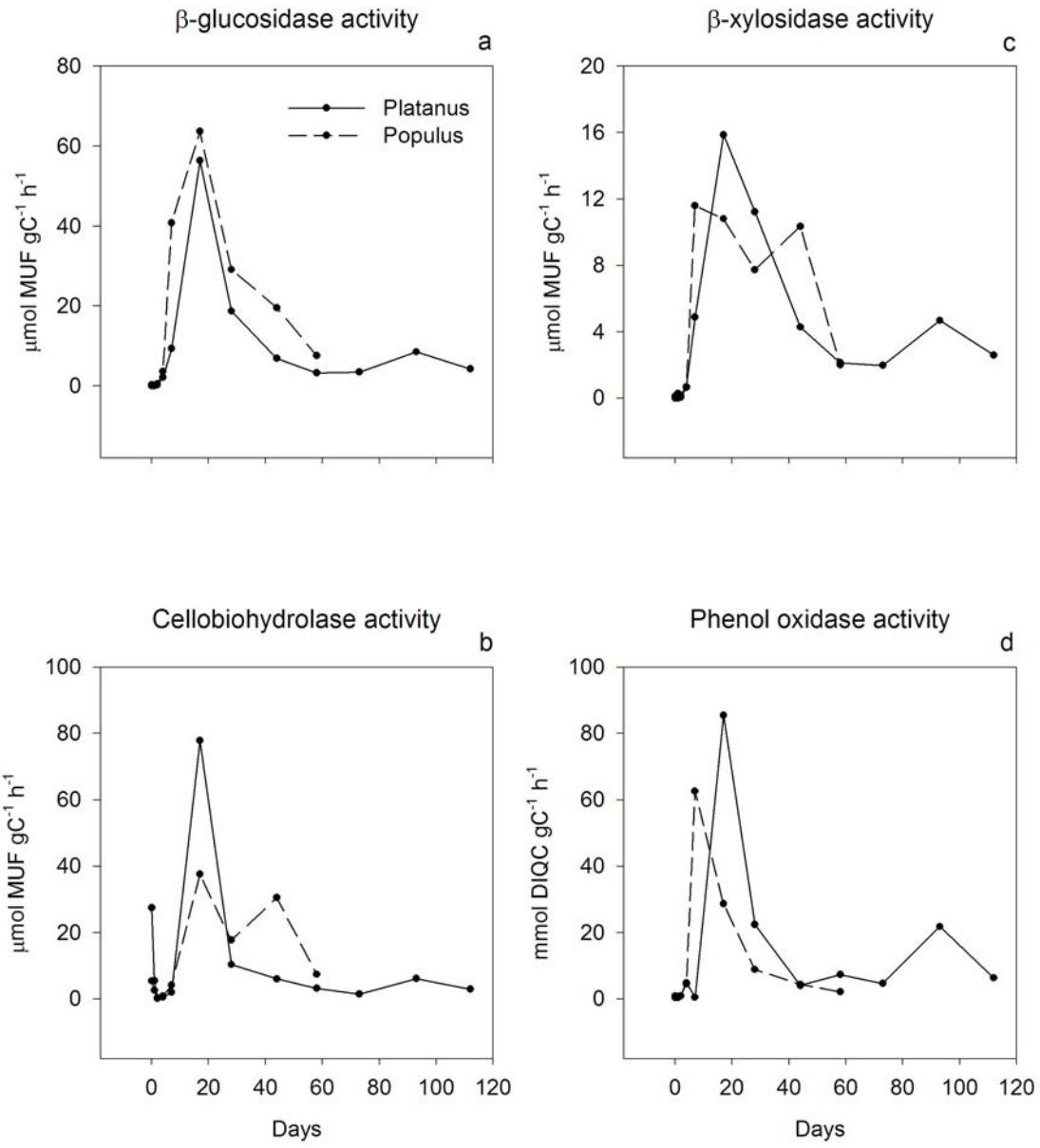


Figure 6.4. Biomass-specific enzyme activities [β-glucosidase (a), β-xylosidase (b), cellobiohydrolase (c) and phenol oxidase (d)] in the two leaf species during the breakdown experiment. Values are the quotient between average values of enzymatic activity and total microbial carbon (fungal plus bacterial C) at each sampling time.

Relationships between microbial biomass, enzyme activity and leaf properties

Linear and non linear regression analysis between microbial biomass and enzyme activity data were observed during breakdown process of leaves. Bacterial biomass and the four enzyme activities showed an exponential relationship in the two leaf species (Figures 6.5 & 6.6), though β-xylosidase and cellobiohydrolase activity in *Populus* showed a linear relationship (Figure 6.6 b & c). Moreover, it was in *Populus*

species where the highest slopes of the curves were obtained (Figure 6.6 a, b, c & d). In contrast to bacteria, regression between fungal biomass and enzyme activities was consistent in *Platanus*, but not in *Populus* (Figures 6.5 & 6.6).

Significant correlations (Pearson) between the four extracellular enzyme activities and leaf physical and chemical properties were observed (Table 6.4). The decrease in leaf toughness and the increase in C:N and C:P ratios of leaves was correlated to the increase of enzyme activities and microbial biomass in leaves. However, fungal biomass did not explain variations in leaf C:N, C:P and toughness in the *Populus* species. Non significant relationships were found between water related parameters (temperature, nutrients and discharge) and enzyme activities.

	C:N	C:P	toughness
Platanus (n = 12)			
β-glucosidase	- 0.837**	- 0.792**	- 0.841**
β-xylosidase	- 0.80**	- 0.753**	- 0.860 **
cellobiohydrolase	- 0.670*	- 0.758**	- 0.611 *
phenol oxidase	- 0.798**	- 0.749**	- 0.782 **
bacterial biomass	- 0.694*	- 0.714**	- 0.790**
fungal biomass	- 0.616*	- 0.627*	- 0.828**
Populus (n = 9)			
β-glucosidase	- 0.920**	- 0.751*	- 0.889**
β-xylosidase	- 0.766*	ns	- 0.841**
cellobiohydrolase	- 0.726*	ns	- 0.832**
phenol oxidase	- 0.801**	- 0.677*	- 0.801*
bacterial biomass	-0.895**	- 0.693*	- 0.968**
fungal biomass	ns	ns	ns

Table 6.4. Correlations (Pearson coefficients) between extracellular enzyme activities and leaf physical and chemical properties (C:N and C:P molar ratios and leaf toughness) in Platanus and Populus leaves during the breakdown experiment. Significant correlations are indicated by asterisks [(*) for P < 0.05 and (**) for P < 0.005] while the not significant are indicated by (ns).

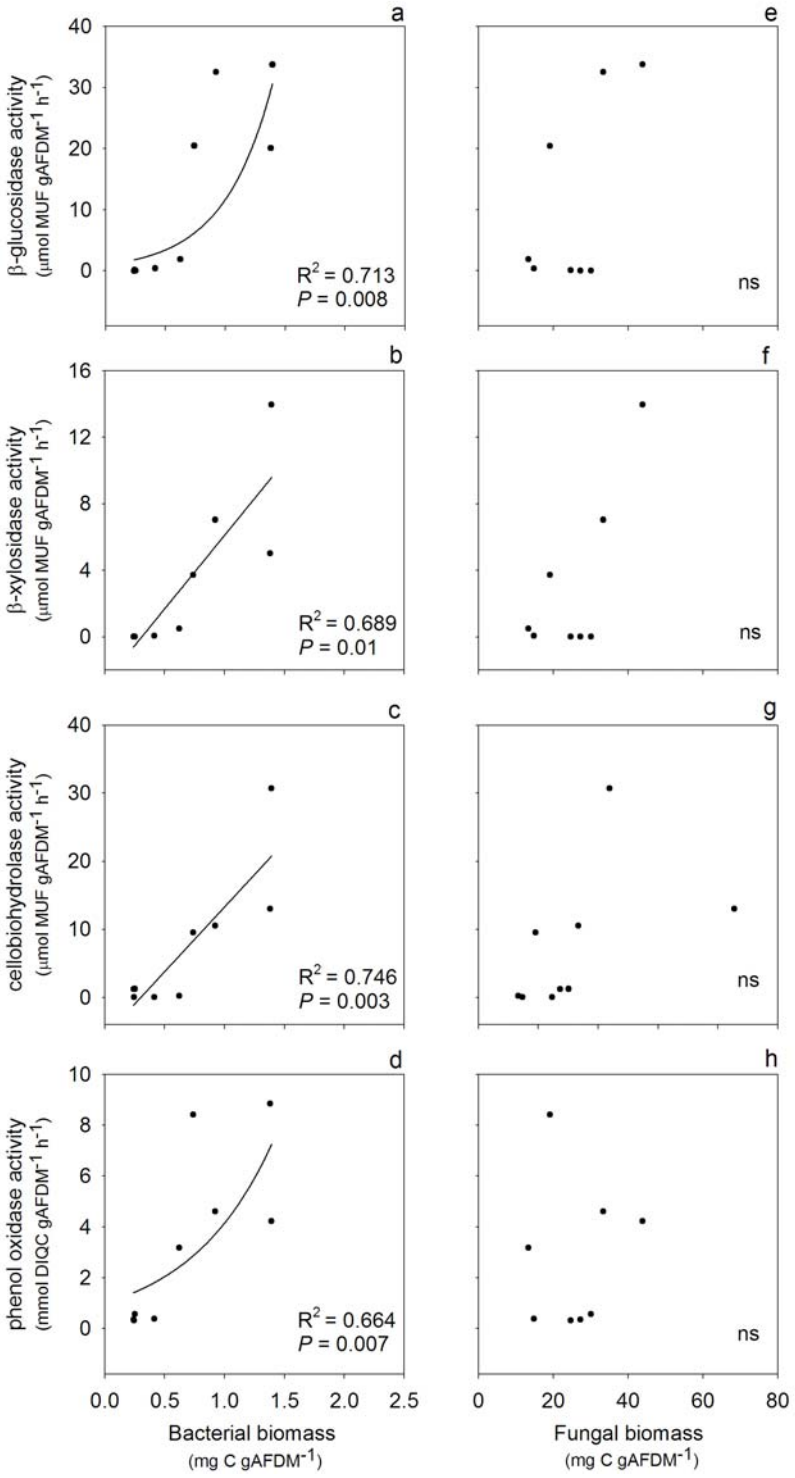


Figure 6.5. Regression analysis between enzyme activity (β-glucosidase, β-xylosidase, cellobiohydrolase and phenol oxidase) and microbial biomass [bacterial (plot a to d) and fungal (plot e to h)] data obtained from Poplus species analysis. R-square values (R^2) and probability (P) values are also shown.

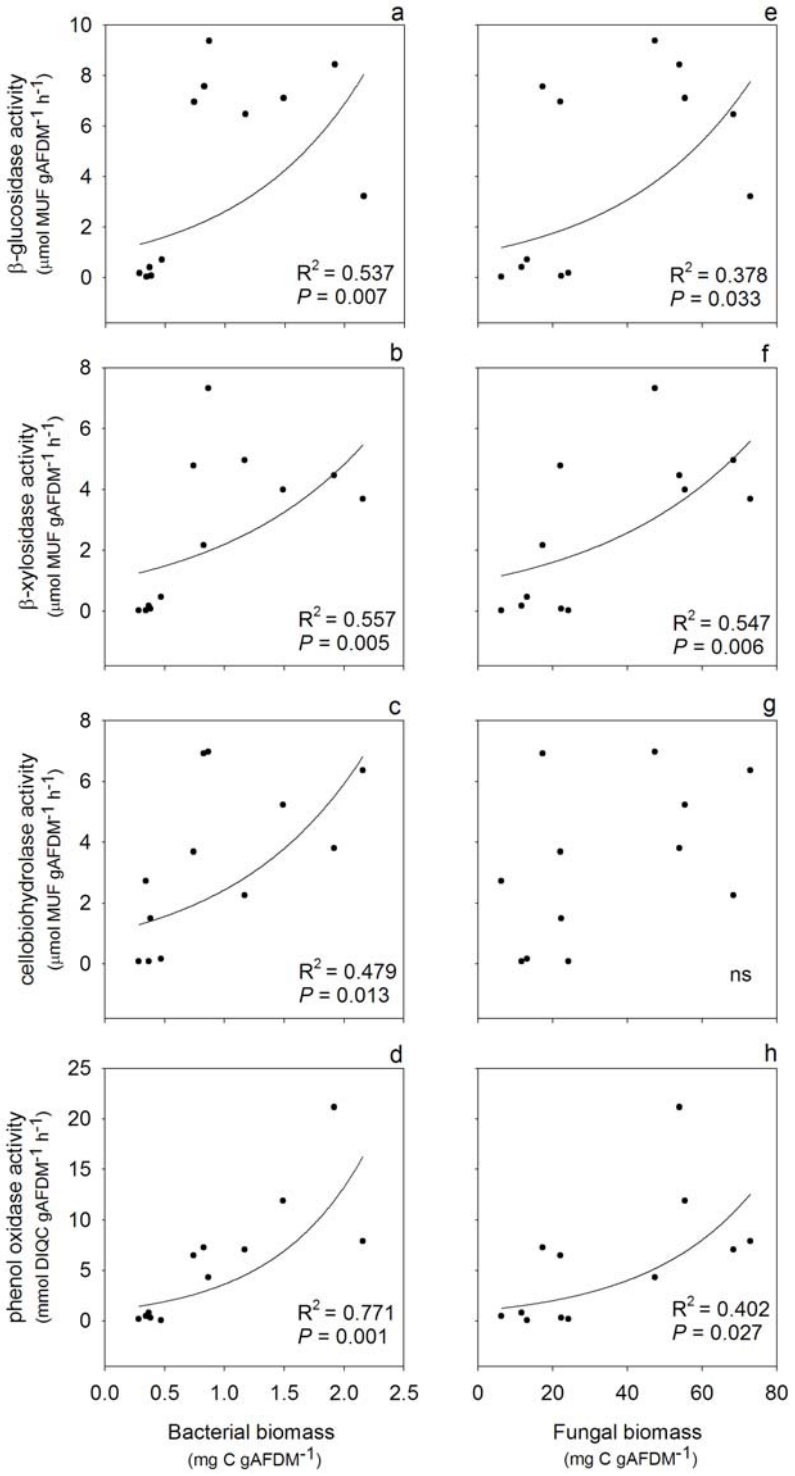


Figure 6.6. Regression analysis between enzyme activity (β-glucosidase, β-xylosidase, cellobiohydrolase and phenol oxidase activities) and microbial biomass [bacterial (plot a to d) and fungal (plot e to h)] data obtained from Platanus species analysis. R-square values (R^2) and probability (P) values are also shown.

Discussion

Fungal biomass and activity generally dominated microbial breakdown process of leaves in streams, while bacteria become relevant in advanced stages of decomposition process (Baldy et al., 1995; Weyers & Suberkropp, 1996; Gulis & Suberkropp, 2003a; Hieber & Gessner, 2002). This trend has been observed in the present study of *Platanus* and *Populus* leaves breakdown, demonstrated by the continued increase in bacterial biomass after the stabilization of fungal biomass at day 44. However, this study has also shown the existence of contrasting colonization patterns for bacteria and fungi during the first colonization stages of leaves. While bacterial biomass increased sharply during the first week, fungal biomass slightly decreased, and the steepest fungal biomass increase occurred after day 17 when bacterial biomass was slowly increasing (Figure 6.2). The fast increase of bacterial biomass during the first week might be related to their ability to metabolize leaf leachates (simple sugars and proteins) released at the beginning of the decay process (Sala & Güde, 2004). Nevertheless, the delayed fungal biomass increase may be related to the high amount of biomass lost (in form of conidia) through sporulation process during the first weeks of colonization (Gessner et al., 1993). Sridhar & Bärlocher (2000) found that more than 50% of the total fungal production is invested in the sporulation process.

Although sterilization of leaves (autoclaving) may have produced a previous leaching and altered leaf properties (i. e. toughness), the evolution of microbial biomass and enzyme activities was similar to studies described elsewhere (Baldy et al., 1995; Weyers & Suberkropp, 1996; Sampaio et al., 2001). The temporal scale in which all these processes took place in the present study might have been brought forward because of the sterilization step, but also by the sudden increase of water discharge at day 17 of the experiment. The physical abrasion generated on leaves during the flooding episode might be responsible of the fast breakdown of *Populus* leaves (only 58 days; Gaudes et al., 2008).

Assuming that separate measures of microbial biomass and enzyme activity in *Platanus* and *Populus* species may have been influenced by the species mixture inside the litter bag (Blair et al., 1990), this study revealed that enzyme activity pattern between species was different. *Populus* leaves decomposed faster than *Platanus* and differences in extracellular enzyme activity (a higher magnitude and different timing of β-glucosidase and cellobiohydrolase activity) are also expressed in the faster decomposition of *Populus* leaves.

The occurrence of microbial biomass accumulation in leaves was parallel with the extracellular enzyme expression during the breakdown process. Most of the studies

focused on statistical relationships between extracellular enzyme activity and microbial parameters often show equivocal results, and suggest that biomass parameters are insufficient to explain enzyme activity results in complex microbial communities (Vrba et al., 1992; Hoppe et al., 1998). In this study, bacterial biomass and enzyme activity showed a clear exponential relationship in the two species analyzed. However, the relationship between fungal biomass and enzyme activity was only consistent in the *Platanus* species, but not in the *Populus*. These results could be resulting from the fact that the two leaf species were incubated together in the leaf bags and therefore the microbial community responsible for enzymatic decomposition might be not only those attached to each specific leaf but the whole community included in the leaf material of the leaf bag. Therefore, fungal biomass accumulated at the *Platanus* leaves could be responsible for decomposition of *Populus* leaf materials which in turn, shows a more palatable composition than the main *Platanus* leaves. This possible reason would be in accordance to the known non-additive and accelerating effects of leaf mass loss when leaf species are mixed, although this might be depending on the specific species which are mixed (Kominoski et al., 2007; Taylor et al., 2007). The species mixed litter bag was planned in this study in order to mimic the multispecies leaf packs usually found in forested streams, and specifically in the studied Fuirosos stream, dominated by *Platanus* and *Populus* leaf accumulations. The results suggest that fungi probably required of a higher stability to develop and degrade substrates than bacteria, because of their differences in colonization strategies (Bengtsson, 1992). Consequently, the higher durability of *Platanus* species might have been favouring fungal biomass establishment, and enhancing the decomposition of the most labile polysaccharides in *Populus* leaves (showing higher β-glucosidase and cellobiohydrolase enzyme activities). Although it is important to remark that other invertebrate animals and eukariotes (i. e. protozoa, diatoms) present in the microbial community should be considered as potential enzyme producers (Vrba et al., 2004), the changes in microbial biomass explained an important part of the variation of extracellular enzyme activities during leaf breakdown process.

Microbial colonization and the expression of its extracellular enzyme activity was highly related to changes in leaf properties which showed a maximum during a short but metabolically intensive period of time (week 2 after immersion). The activity of most enzymes was tightly related to the decrease in leaf toughness underlinig the tight relationship between extracellular enzymes and mass loss described by Sinsabaugh et al. (1994). Specifically, the widest and fastest changes in leaf physical and chemical properties (steep reduction in toughness and C:N and C:P ratios) occurred between days 7 and 17, coinciding with the sharp increase of biomass-specific enzyme activities

(enzyme activities per unit of microbial carbon). The decrease in C:N and C:P ratios (N and P enrichment) might be further attributed to the microbial colonization (i. e. Webster & Benfield, 1986). This high efficient use of organic matter period suggests a synergistic relationship between fungi and bacteria; bacteria benefiting from the organic compounds pre-treated by the fungi (Velicer, 2003; Romaní et al., 2006).

The present study shows that the use of mixed species in the same litter bag made difficult to separate the biomass establishment from the enzyme activity expressed by the attached microbial community. However, fungi seemed to keep the relationship between biomass and activity in the hardest species (*Platanus*) while bacteria were able to colonize and produce enzymes irrespectively of the leaf species within the litter bag. In general, fungal biomass accumulation in leaves is slow and dominant over time while bacterial biomass increased in those periods of greater simple organic compounds availability (i. e. during the first week after immersion and after the fungal action). The most important changes in leaf properties coincided with the episode of highest enzyme activity per unit of microbial carbon. This coincidence indicates that fungi and bacteria are efficient in using available organic matter, especially after the first to second week of immersion.

Chapter 5

Patterns of epilithic biofilm formation in two streams of different bioclimatic regions: analysis on microbial community structure and metabolism

Abstract

Patterns of biofilm formation were investigated in two streams from different bioclimatic regions: a Central European (the Walzbach) and a Mediterranean (the Fuirosos). The succession of bacterial and algal populations in the biofilm was analyzed through bacterial rDNA sequences analysis (16S rDNA and 16S-23S intergenic sequence) and diatom taxa identification over a 60-days colonization experiment. Moreover, structural (microbial biomass and polysaccharide content in EPS) and metabolic (β-glucosidase, leu-aminopeptidase and β-glucosaminidase activity) parameters of the biofilms were also analyzed. The analysis of the whole biofilm colonization sequence showed a greater bacterial biomass and diversity at the Fuirosos biofilms as well as higher EPS-polysaccharide content. In contrast, greater diatom diversity was measured at biofilms developing at the Walzbach. The successional patterns of bacterial and diatom populations in the Fuirosos showed clear discontinutities coinciding with flood episodes while at the Walzbach the time sequence was more gradual. The results suggest that microbial community structure development is highly related to the physical characteristics of the different sites (specially the stream hydrology) where the high bacterial diversity and EPS development as well as the presence of rapid colonizing diatom species at the Mediterranean stream might be an adaptation to hydrological changes. In contrast, major algal diversity can be developed in the more constant hydrology of the central-european stream, also showing high cell-specific enzyme activity being more efficient in the use of organic matter. However, the hystorical evolutionary pressure from the different bioclimatic regions could be also affecting the microbial community composition.

Introduction

As soon as a new substratum (i. e. a cobble) is submerged in the stream, an organic film mainly composed of bacteria and algae will accrue on the surface within hours (Peterson, 1987; Costerton et al., 1987). At this step of the colonization process, both bacteria and algae stimulate the biosynthesis and deposition of extracellular polymeric compounds, which facilitate the adhesion of cells to substratum (Cooksey & Wigglesworth-Cooksey, 1995). Within days, the surface is covered by microbes, and the community continues to develop through the growth of populations within the biofilm and by additional recruitment from the water column (Jackson et al., 2001). However, it is difficult to define a “mature” biofilm since its high dynamism. Dead and release as well as recruitment of new microorganisms are continuously occurring in biofilms (Jenkinson & Lappin-Scott, 2001).

A variety of physical and chemical determinants, including nutrient availability, light, hydrology and temperature, as well as biological processes (i. e. grazing), are dynamically shaping the biofilm (Lawrence & Neu, 2003; Besemer et al., 2007). Physical and chemical characteristics may differentially affect biofilms of different ages. Previous studies have shown that thick and mature biofilms differ from thin and young biofilms in their ability to escape from the conditions in the overlaying water (Paul & Duthie, 1989; Pringle, 1990). Therefore, changes in the stream water characteristics (i. e. nutrient availability) might have a major reflection in young than in mature biofilms. Nutrients often enhance microbial biomass in the epilithon (Mallin et al., 2004; Rier & Stevenson, 2006), thereby increasing biofilm structural complexity (i. e. thickness) and prompting changes in microbial assemblages. Although some studies did not find clear effects of nutrients on algal and bacterial diversity in biofilms (Stelzer & Lamberti, 2001; Lyautey et al., 2003), many others showed clear relationships between nutrients and changes in biofilm species composition (Winter & Duthie, 2000; Féray & Montuelle, 2003; Lawrence et al., 2004; Olapade & Leff, 2004). Nevertheless, the effect of light often prevails over the effect of nutrients in biofilms developed in forested streams (Ylla et al., 2007). Stream hydrology is also a relevant factor in controlling biofilm structure and metabolism. Moderate increases in the overlying water velocity may increase the total microbial biomass accrual, the potential heterotrophic metabolism (i. e. denitrification) and the relative abundance of algal species in biofilms (Arnon et al., 2007). Greater increases in water current velocity may reduce biofilm thickness, as well as the internal cycling of carbon (Battin et al., 2003). Drastic flood events may produce complete resets in stream benthic biological communities (Sabater et al., 2006). Diatom assemblages have been used as indicators of flooding influence in rivers (Weilhoefer & Pan 2003), while studies relating epilithic bacterial assemblages to changes in stream hydrology are inexistent.

The objective of this research was to look insight the process of colonization of a bare substratum by algae and bacteria at two similar stream sites from two bioclimatic regions, focusing on the changes in microbial community. The intensive analysis of colonization sequences looking at the same time to the microbial community composition (by molecular analysis, DGGE and sequencing and microscopical analysis), biofilm structure (microbial biomass and polysaccharide content in EPS) and metabolism (extracellular enzyme activities) lead us to relate structural and metabolic parameters during the biofilm formation process. The study is planned in two intensive colonization experiments, performed in a Mediterranean and a central European stream showing different hydrological regime and water nutrient content. Our aim was to know whether there is a significant effect of the study site on

the sequence of biofilm formation in terms of microbial diversity, structure and metabolism. Specifically, three questions were formulated: i) Were differences in bacterial and algal succession remarkable in the two study sites?, ii) Were the differences expressed in the microbial diversity?, and iii) Were the structural differences between biofilms influencing the use of organic matter?

Materials and methods

Study sites

The biofilm experiments were performed in reaches of 50 m in length and 3 to 5 m in width of the Walzbach (SW of Germany, 49º00'N / 8º36'E) and the Fuirosos (NE of Spain 41º41'N / 2º34'E) streams (see General introduction). Both streams were of low order, with similar slopes and with a rather well developed riparian forest. Hazelnut and ash trees (*Corylus avellana* and *Fraxinus* sp.) and few shrubs (*Rubus ulmifolia*, *Asparagus acutifolia*) composed the riparian forest of the Walzbach, while plane trees, black poplar and alder (*Platanus acerifolia*, *Populus nigra*, *Alnus glutinosa*) and dense populations of shrubs were dominant in the Fuirosos riparian forest.

Images of the Walzbach (on the left) and Fuirosos (on the right) stream reaches where biofilm experiments were performed.

Epilithic biofilm sampling

Two colonization expriments were performed during the autumn 2005 in the Walzbach and the Furiosos. Sampling of epilithic biofilms was performed using sandblasted glass tiles of 4.2 cm^2 of surface area as rocky substrata surrogates. Arrays of tiles were glued onto 3 different flagstones (approx. 150 tiles per stone), which were randomly submerged along the reaches to depths of 10 to 20 cm. Biofilm samples were recovered at days 1, 2, 3, 4, 7, 14, 21, 28, 44, and 60. On each date, bacterial and algal biomass standing stock (sections 2.2.2 and 2.2.3, respectively) and polysaccharide content in EPS (section 2.4.2) were measured in biofilms. Three

extracellular enzyme activities (β-glucosidase, leucine-aminopeptidase and β-glucosaminidase; section 2.6.1) were assayed on each date and samples for bacterial community analysis (section 2.3.2) and diatom taxa identification (section 2.3.3) were also taken. Triplicate samples (one from each flagstone) were considered for each parameter and sampling date.

Measurements of water physical and chemical parameters were performed in the field at each sampling date with portable meters, while additional stream water samples were taken for further dissolved nutrient analysis (nitrate and phosphate concentrations, section 2.7.1) in the laboratory.

Statistical analyses

Repeated measures analysis of variance (ANOVA) was used to detect temporal changes on biofilm structural (microbial biomass and EPS content) and metabolic (enzyme activities) parameters during biofilm experiments and to evaluate whether differences between sampling sites exist. All data were log transformed (X = log (X+1)) before statistical analyses and the Dunn-Sidak correction was applied on probability values (P-values) of the whole sources analyzed. Temporal correlations between structural and functional variables from biofilms were analyzed by means of the product-moment Pearson coefficient. The statistical analyses were performed using the SPSS for Windows (Ver. 12.0, SPSS Inc. 2003).

Differences in the bacterial and diatom community composition throughout colonization sequences were analyzed by means of similarity indexes. Homology between bacterial band pattern at each sampling date was calculated by means of the Jaccard index (Jaccard, 1901), while data abundance on diatom taxa at days 3, 7, 30 and 60 were used to calculate the Bray-Curtis index (Bray & Curtis, 1957). Both the Jaccard and Bray-Curtis scores were used to classify the samples by a joining tree method based on the Unweighted Pair-Group Average (UPGA, Euclidean distances between measures) using STATISTICA for Windows (StatSoft® Ver. 6, 2001). Differences in bacterial band richness between the Walzbach and the Fuirosos were determined by means of a paired T-test (SPSS, 2003).

Results

Physical and chemical characteristics of the stream water

Main differences between reaches were the hydrological regime and the water nutrient concentration, as summarized in Table 7.1. Although average values of water temperature and discharge were similar between study sites, two drastic flooding episodes took place at days 10 (682 L s^{-1}) and 40 (195 L s^{-1}) in the Fuirosos (Table

7.1). Ammonia, nitrate and phosphate concentrations in the water were higher in the Walzbach than in the Fuirosos. Moreover, differences in stream water phosphate concentrations between sites produced consistent differences in the dissolved inorganic nitrogen: soluble reactive phosphorus concentration ratio (DIN:SRP; Table 7.1).

Light incidence was slightly lower in the Walzbach than in the Fuirosos, because of its greater forestry canopy. The stream bed at the two reaches was made up of similar coarse (rocks and cobles) and fine (sand and gravel) inorganic substratum coverage. However, a higher amount of particulate organic matter standing stock was found in the Fuirosos stream channel (Table 7.1).

Parameters	Walzbach	Fuirosos
Temperature (ºC)	9.4 (0.2)	10.8 (0.7)
Discharge (L s^{-1})	65 (4.1)	57.6 (20.3)
Ammonia (mg L^{-1})	0.2 (0.005)	0.1 (0.002)
Nitrate (mg L^{-1})	1.9 (0.1)	0.4 (0.01)
Phosphate (μg L^{-1})	63 (1.1)	7.5 (2.1)
DIN: SRP ratio	33.3 (0.09)	66.6 (0.01)
Canopy cover (%)	70 (5)	60 (10)
Rocks abundance (%)	50 (2.5)	60 (10)
Leaf litter standing stock (%)	10.5 (4.1)	38.5 (5.8)

Table 7.1. Average values of physical and chemical characteristics of the Walzbach and Fuirosos stream water during the colonization experiment. The dissolved inorganic nitrogen and soluble reactive phosphorus ratio (DIN: SRP) was calculated as the quotient between nitrogen forms (ammonia plus nitrate) and phosphate concentrations. The relative cover of rocks, the forestry canopy cover and the accumulated leaf litter in the stream channel were determined visually. Moreover, light incidence was determined as % of forest canopy cover impeding light entrance into the channel. Values are means and standard errors (in parenthesis) of measures performed at each sampling date (n = 10).

Bacterial population analysis in biofilms

PCR amplification of DNA extracted from epilithic biofilms was successful in most of the cases, except for the Walzbach biofilm samples at day 3 (ISR analysis) and 21 (16S rDNA analysis; Table 7.2, Figure 7.1). The size of the amplicons in the 16S ribosomal gene sequences analysis coincided with the expected amplicon sizes of 566 base pairs (bp), while due to the high sequence variations of intergenic ribosomal regions (ISR) the amplicon lengths ranged from 200 to 1500 bp. Overall, the highest

PCR efficiencies were observed for the ISR analysis than for the 16S rDNA (Table 7.2, Figure 7.1).

Biofilm age (days)	Walzbach		Fuirosos	
	16S	16S-23S	16S	16S-23S
1	2	16	9	27
2	6	14	11	28
3	8	Inhib.	15	30
4	12	15	12	17
7	10	13	8	32
14	12	23	8	29
21	Inhib.	15	11	25
28	11	22	11	28
44	10	24	10	22
60	6	17	11	28
Mean	8.5 (1.1)	17.7 (1.4)	10.6 (0.6)	26.6 (1.4)

Table 7.2. Number of total bacterial DNA bands scored in 16S rDNA-DGGE analysis and 16S-23S-PAGE analysis in the Walzbach and the Fuirosos biofilms during colonization. Inhibitions in PCR reaction (Inhib.) are marked in the table. Also, average values and standard errors (in parenthesis) of the bands scored by each gene sequence during colonization experiments are shown.

Successional changes in bacterial band pattern were obvious during biofilm colonization at the two study sites (Figure 7.1). Cluster analysis obtained from 16S rDNA-DGGE scores grouped apart samples of days 1-7 from samples of days 14-60 in the Fuirosos, while day 1 was separated from the rest of the dates in the cluster of the Walzbach (Figure 7.2 a & c). Despite this strong difference in the Walzbach communities, cluster analysis tended to group apart older (day 28-60) from younger (day 2-14) populations. ISR-PAGE scores covered changes in the succession of bacterial populations during biofilm development, but did not evolve according to the age of the biofilm (Figure 7.2 b & d).

The average band number of 16S and ISR analysis during biofilm experiments confirmed a higher bacterial biodiversity in the Fuirosos than in the Walzbach (*T*-test, $P < 0.07$ and $P < 0.005$, in the 16S and ISR analysis, respectively). Bacterial band richness of the 16S analysis evolved in different manner during biofilm experiments in the two study sites (Table 7.2). In the Fuirosos, band richness increased between days 1 and 3 (15 bands), decreased on days 7 to 14 (8 bands), and then recovered on day 21 (11 bands) until the end of the experiment. Conversely, band richness in the

Walzbach increased between days 1 to 4 (12 bands), kept stabilized between days 7 to 44 (10 to 11 bands), and finally decreased at day 60 (6 bands).

Strong and dominant DGGE bands from bacterial populations were excised from the gels, re-amplified, and subjected to electrophoresis and sequencing (Figure 7.1). Sequence alignments using 16S rDNA sequences available in the GenBank databases and ARB software package resulted in a taxonomical affiliation to beta (β) and gamma (γ) *Proteobacteria*, two groups of gram positive bacteria (*Firmicutes* and *Actinobacteria*), as well as to *Cyanobacteria*, which was especially relevant during the first week of colonization in the Fuirosos biofilms (strong and dominant band; Figure 7.1, Table 7.3). β- *Proteobacteria* appeared later, after flooding episodes in the Fuirosos biofilms, but also during the first week of colonization. Biofilm of day 1 in the Walzbach was characterized by two bands corresponding to groups of actinobacteria and firmicutes (Table 7.3).

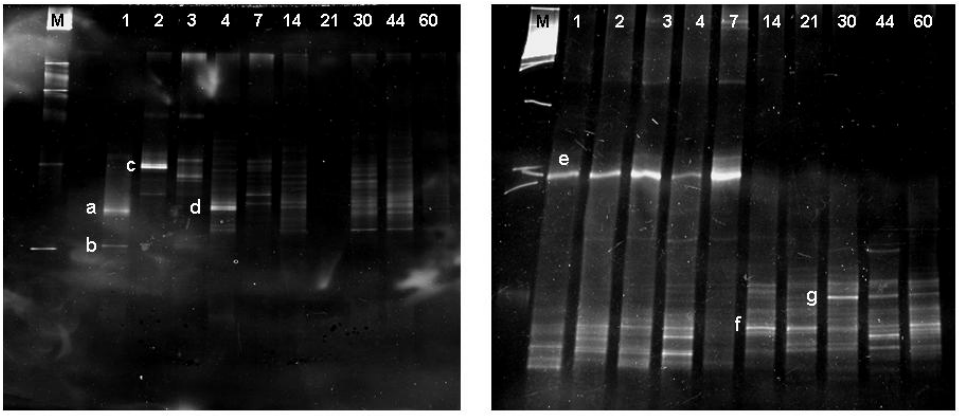


Figure 7.1. Changes in bacterial populations revealed by 16S rDNA-DGGE (40-70% urea gradient) analysis during biofilms colonization in the Walzbach (left) and the Fuirosos (right). Days of colonization (day 1 to 60) and the molecular weight marker (M) are marked in the gel. Lower cap letters indicate the bands excised for subsequent sequencing analysis.

Site	Band	Sequence affiliation	Day
Walzbach	a	Firmicutes	1
	b	Actinobacteria	1
	c	β-proteobacteria	2
	d	β-proteobacteria	4
Fuirosos	e	Cyanobacteria	1
	f	β-proteobacteria	14
	g	γ-proteobacteria	28

Table 7.3. Affiliation of the sequences excised from the 16SrDNA-DGGE obtained from the Fuirosos and Walzbach experiments. Lower cap letters correspond to the bands marked in the gels (Figure 7.1).

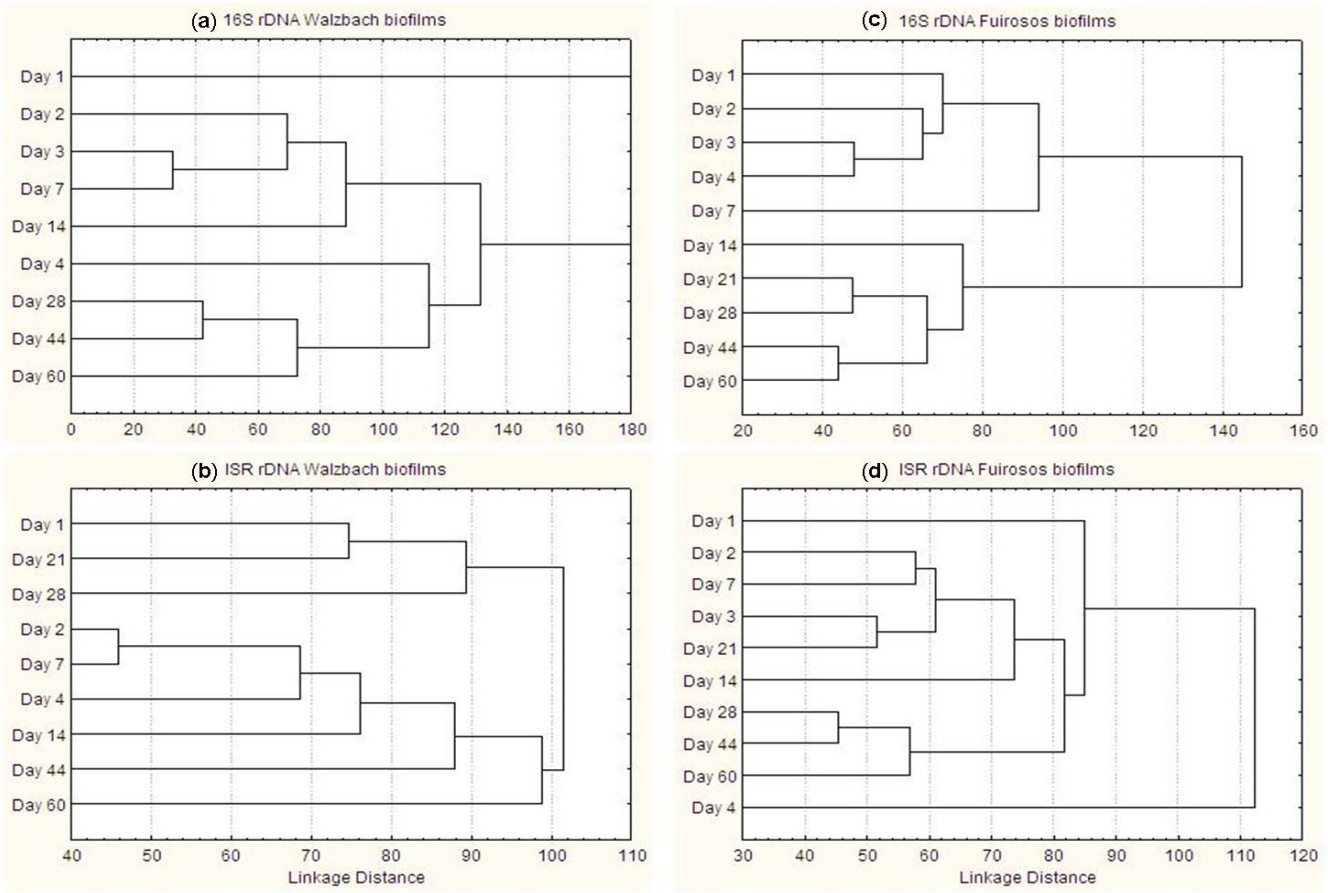


Figure 7.2. Unweighted Pair-Group Average tree of linkage of distances based on Jaccard's similarity index inferred from 16S and 16S-23S rDNA sequences analysis in the Walzbach (a and b) and the Fuirosos (c and d) biofilms during the colonization sequences. Day 3 (16S-23S analysis) and day 21 (16S analysis) are missing in the Walzbach clusters (plots a and b, respectively) because of inhibitions in the PCR.

Diatom population analysis in biofilms

A total of 49 diatom taxa were identified in the Walzbach biofilms during biofilm experiments, while 38 taxa were identified in the Fuirosos. Cluster analysis on diatom populations of the Walzbach revealed that diatom species composition varied according to the time and maturation of the assemblage, as well as we observed for the bacterial populations (Figure 7.3 a). However, diatom populations in the Fuirosos grouped days 3, 7 and 60 apart from day 28 (Figure 7.3 b). This different cluster ordination coincided with the flooding episodes pattern observed in the Fuirosos. Few diatom taxa dominated biofilms in the Fuirosos, while higher diversity was detected in the Walzbach (Figure 7.4). In the Fuirosos, the species *Achnanthes minutissima* appeared during the first week (day 3), but also after flood episodes (days 28 and 60; Figure 7.4). In the Walzbach, the species *Denticula tenuis*, *Amfora pediculus*, *Gomfonema angustatum* and *Navicula viridula* dominated the day 3 community. The species of the genus *Achnanthes* (*Achnanthes biasolettiana*, *Achnanthes lanceolata* ssp. *frequentissima* and *Achnanthes minutissima*) appeared later in the Walzbach biofilms (Figure 7.4).

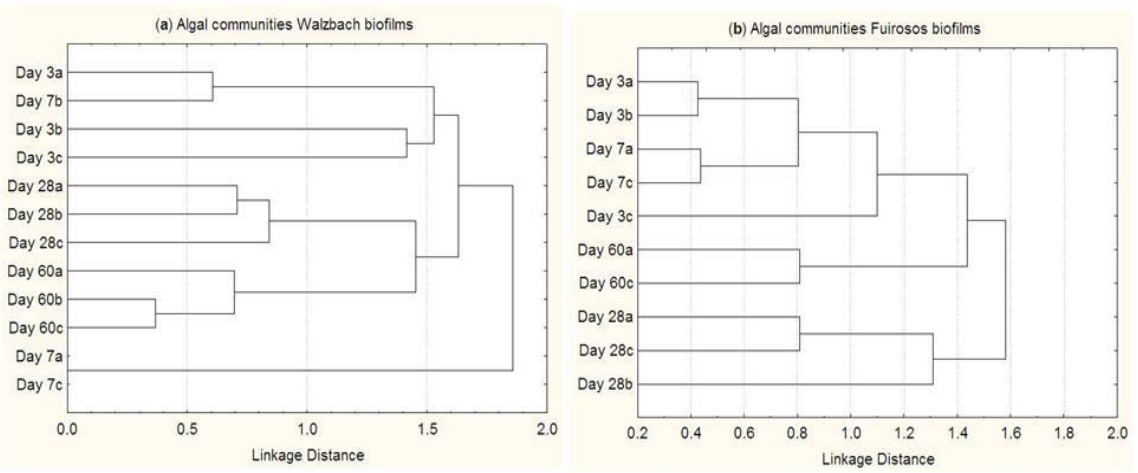


Figure 7.3. Unweighted Pair-Group Average tree of linkage of distances based on Bray-Curtis similarity index inferred from diatom taxa identification in the Walzbach (a) and the Fuirosos (b) biofilm samples of days 3, 7, 28 and 60. Each sampling date has three replicates, except for the days 7 and 60 in the Fuirosos which only had two.

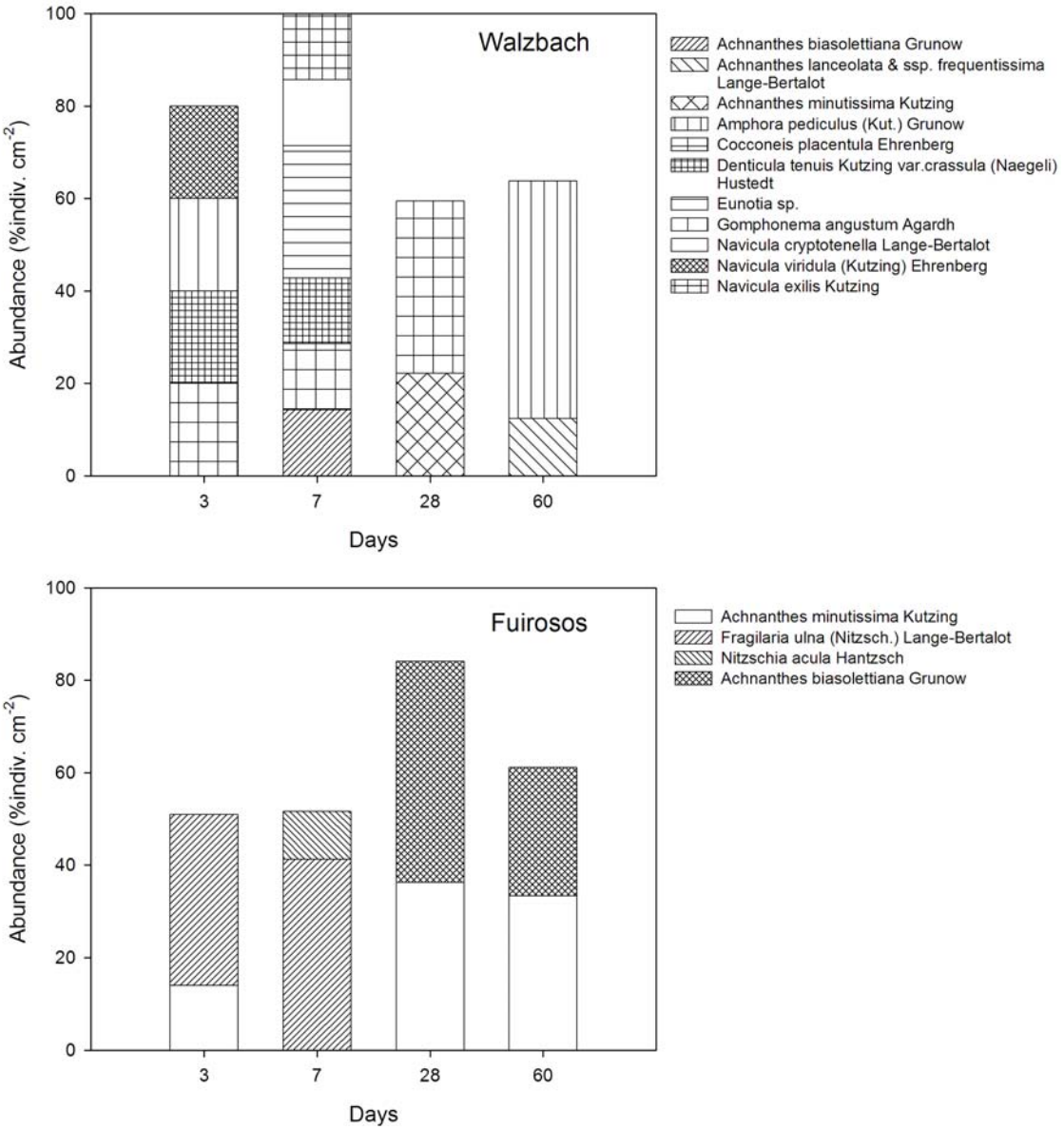


Figure 7.4. Main diatom taxa colonizing the Fuirosos and the Walzbach biofilms. Only the diatom taxa with abundances > 10% of individuals per unit of biofilm surface area are represented in the plot. Values are means of the three replicates at each sampling time.

Microbial biomass and EPS-polysaccharide content in biofilms

Biomass of algae and bacteria increased during colonization experiments in the two study sites (ANOVA *T* effect, Table 7.4), although the increase was faster in the Fuirosos communities (day 7 for bacteria and algae), and more gradual in the Walzbach (day 28 for bacteria and day 44 for algae, Figure 7.5). In the Fuirosos biofilms, the algal biomass showed highest variations (ANOVA *T* × *S* effect, Table 7.4). The two drastic decreases (at days 14 and 44) coincided with the flooding episodes of days 10 and 40 (Figure 7.5 a). Total biomass of biofilms was higher in the Fuirosos than in the Walzbach, especially for the bacteria (*S* effect, Table 7.4).

The polysaccharide content in the EPS tended to increase during the first week of colonization in the two study sites. The highest EPS-polysaccharide accumulation was detected in the Fuirosos biofilms (*T* effect and *S* effect, Table 7.4, Figure 7.6).

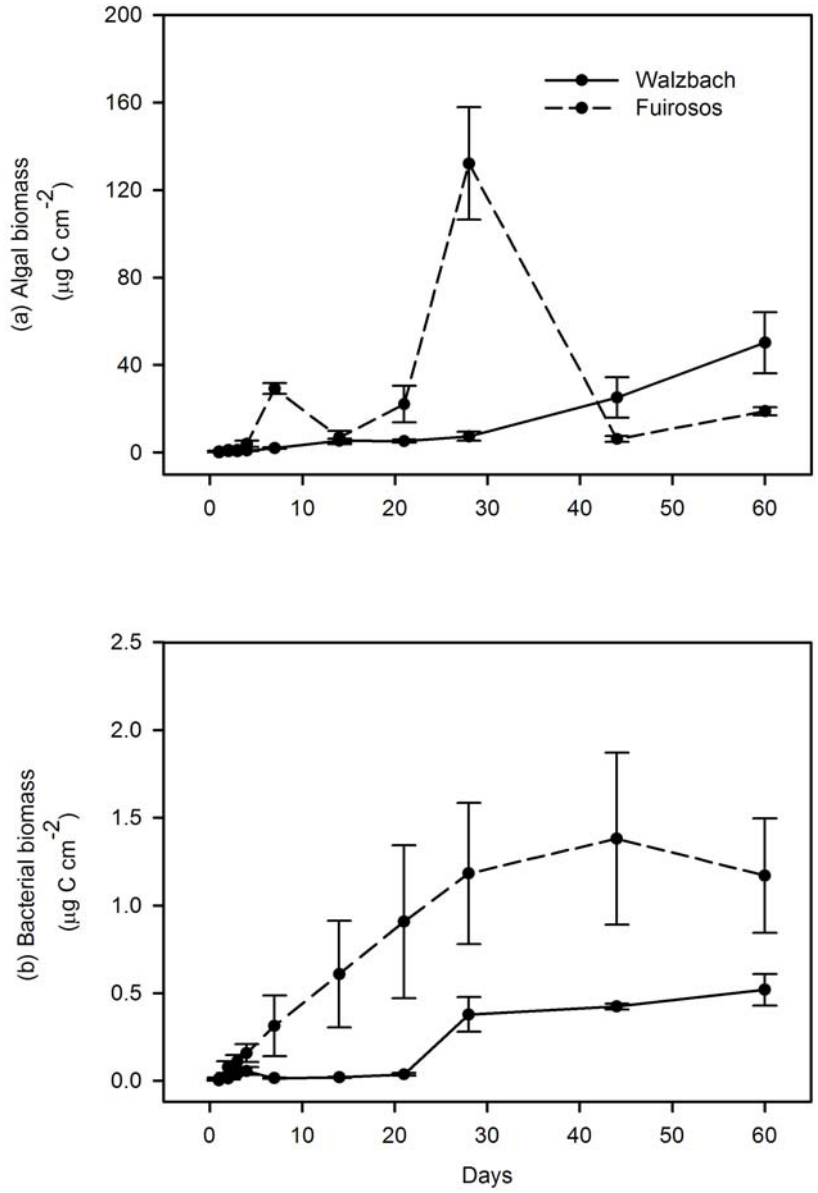


Figure 7.5. Biomass of algae (a), bacteria (b) in terms of Carbon and per unit of biofilm surface area measured in the Walzbach and the Fuirosos biofilms during the colonization experiments. Values are means (n = 3) and SE of each sampling date.

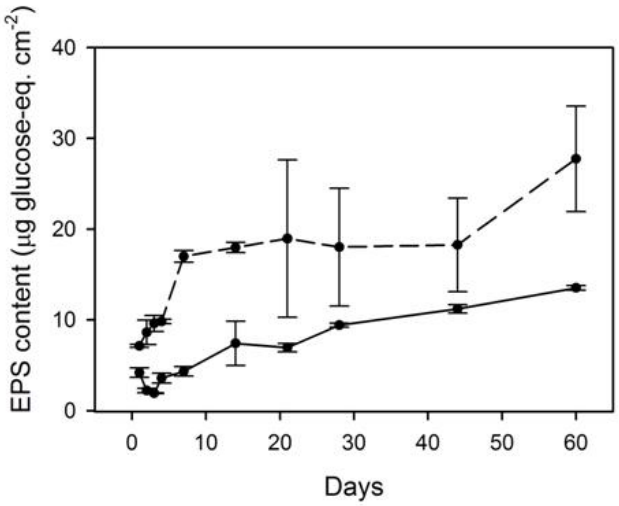


Figure 7.6. Extracellular polysaccharide content (EPS) of biofilms is expressed as the amount of glucose equivalents and per unit of biofilm surface area. Values are means and SE of each sampling date.

Extracellular enzyme activity

Extracelular enzyme activities increased over time in biofilms of the two study sites, except for the β-glucosaminidase (T *effect*, Table 7.4). Most of the activities kept stabilized after day 14 of the experiment (Figure 7.7). Biofilms of the Fuirosos decomposed simple polysaccharide and peptide molecules (higher β-glucosidase and leu-aminopeptidase activity) in a higher rate than those of the Walzbach (S effect, Table 7.4, Figure 7.7 a & b). Conversely, the highest rates to decompose chitin and peptidoglycan molecules were detected in the Walzbach biofilms (higher β-glucosaminidase activity, Figure 7.7 c).

Enzyme activity expressed per unit of bacterial cell was higher in the Walzbach than in the Fuirosos biofilms (Figure 7.8). Activity per bacterial cell increased drastically at the beginning of the sequence and decreased afterwards, except for the β-glucosaminidase (Figure 7.8 c).

Enzyme activity (β-glucosidase and leucine-aminopeptidase) were correlated to bacterial biomass ($r = 0.65$ and $r = 0.82$, $P < 0.01$) and EPS content ($r = 0.65$ and $r = 0.78$, $P < 0.01$) in the Fuirosos biofilms. However, these relationships were not consistent in the Walzbach communities.

	Time (*T*)		Site (*S*)		*T* × *S*	
	F	*P*	*F*	*P*	*F*	*P*
Algae	31.1	**< 0.005**	8.6	0.765	26	**< 0.005**
Bacteria	115.2	**< 0.005**	563.8	**< 0.005**	11.9	0.329
Fungi	3.26	0.979	3	0.996	2.38	0.998
EPS	15	0.064	101.5	**< 0.05**	1.91	0.999
β-glucosidase	25.3	**< 0.005**	58.1	**< 0.005**	17	**< 0.005**
Leu-aminopeptidase	85	**< 0.005**	148.2	**< 0.05**	70	**< 0.005**
β-glucosaminidase	7	0.306	27.1	**< 0.05**	5.2	0.566

Table 7.4. Results of the repeated measures ANOVA performed on biofilm structural (microbial biomass and EPS content) and metabolic (β-glucosidase, β-glucosaminidase, Leu-aminopeptidase activities) parameters measured during colonization experiments (Time effect) in the two study sites (Site effect). F-statistics are indicated and probability within groups was corrected for sphericity by the Greenhouse-Geisser correction. Moreover, all the probability (P) values were adjusted by the Dunn-Sidak correction. Significant P values are marked in boldface type letter.

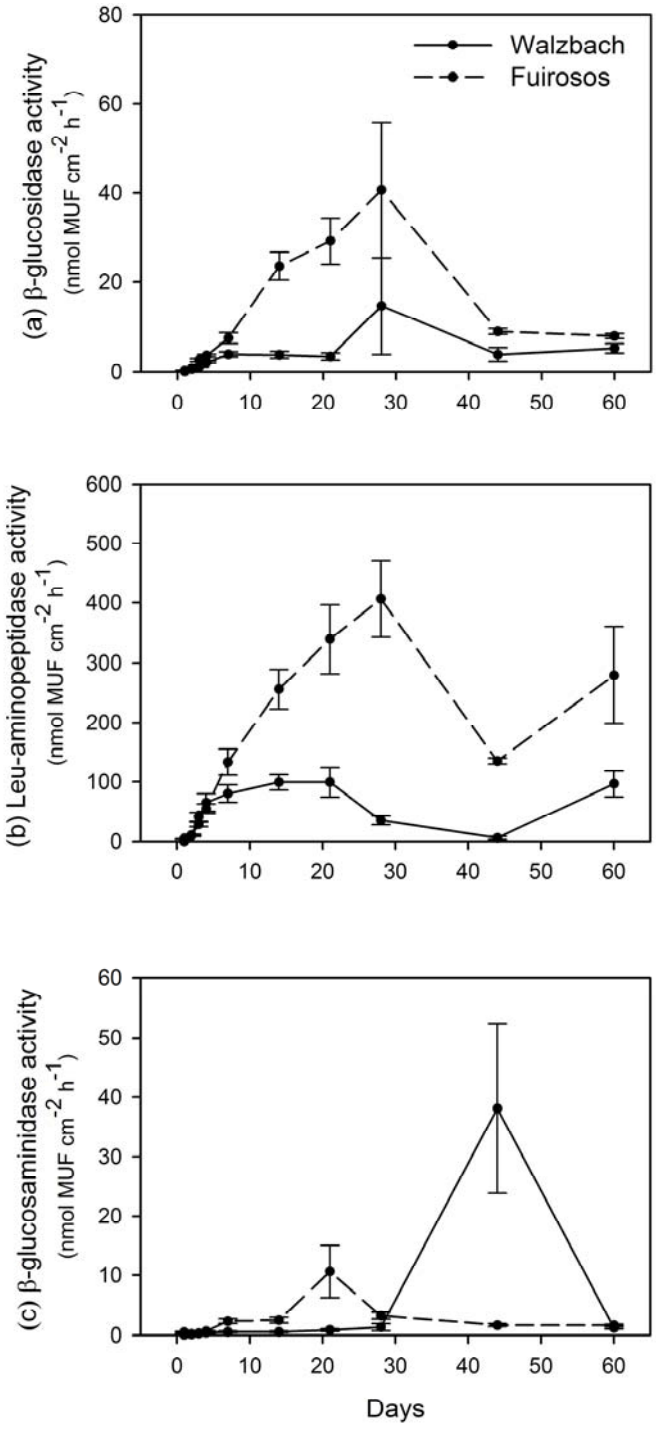


Figure 7.7. Activity of the β-glucosidase (a), leu-aminopeptidase (b) and β-glucosaminidase (c) extracellular enzymes measured during biofilm formation in the Walzbach and the Fuirosos streams. Units of enzyme activities were expressed as the amount of methylumbelliferone (MUF for β-glucosidase, β-glucosaminidase) and/or aminomethyl-coumarin (AMC for leucine-aminopeptidase) released per unit of biofilm surface area (cm^2) and time (hour).

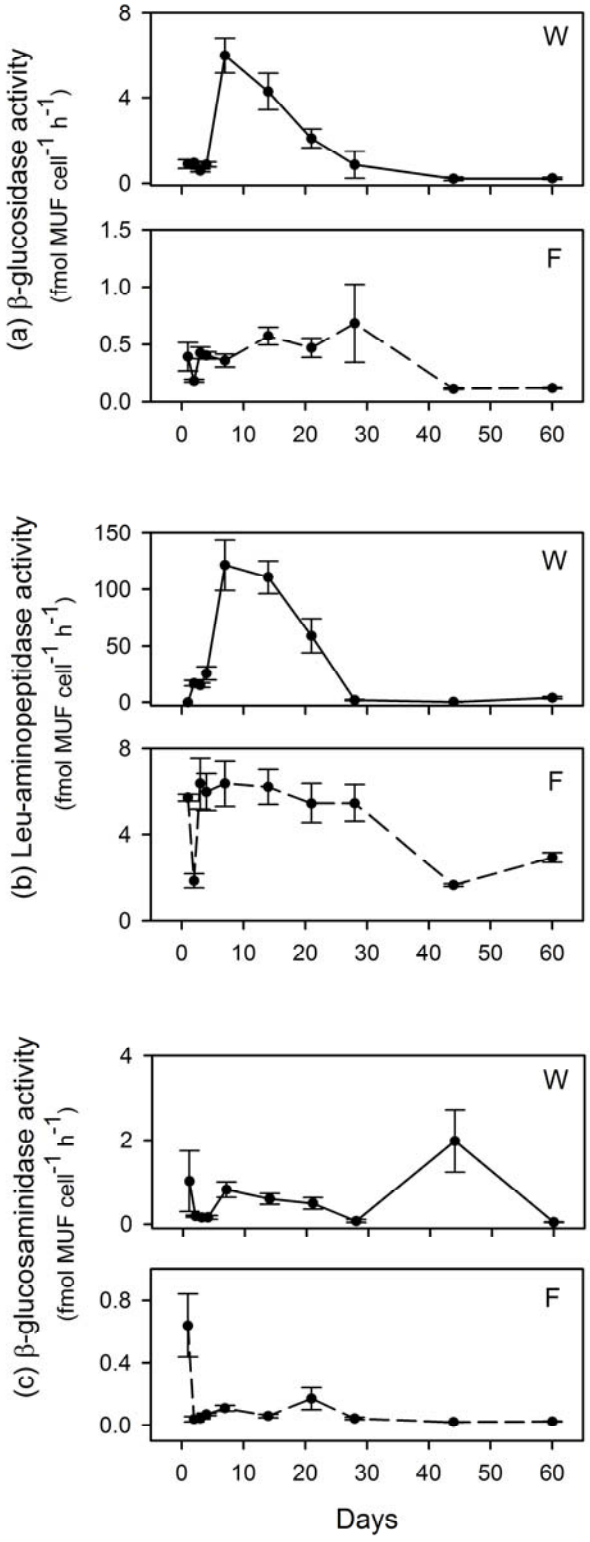


Figure 7.8. Biomass-specific enzyme activity of the β-glucosidase (a), leu-aminopeptidase (b) and β-glucosaminidase (c) enzymes measured during biofilm formation in the Walzbach (W) and the Fuirosos (F) streams. Units of enzyme activity are expressed as the amount of MUF and AMC released per bacterial cell and time.

Discussion

The colonization of algae and bacteria to form a biofilm from a bare substratum showed differences between the two studied streams, mainly due to greater bacterial biomass and diversity and polysaccharide content in EPS at the Mediterranean stream biofilms but a higher algal diversity at the Central-European stream. The 60 days time sequence of substratum colonization at the two sites was similar for all analyzed parameters except for a major fluctuation of algal biomass and β-glucosidase and leucine-aminopeptidase enzyme activities at the Fuirosos. Although both study sites being typical forested streams of similar size, the specific physical and chemical characteristics during the study period (nutrient content, incident light, discharge) might be affecting the colonization sequence of the different groups. Specifically, hydrological changes at the Fuirosos (two flood events occur) in contrast to stable hydrology at the Walzbach might be one of the major effect. At the same time, long-time effects due to be in a different bioclimatic region could be also affecting the microbial biodiversity differences. Differences in species diversity between biological populations may be related to geographical and climatic factors, as well as to productivity of the ecosystem (Begon et al., 1987).

Both rDNA approaches confirmed higher bacterial species richness in the Fuirosos biofilms. However, a major bacterial diversity could be expected at the Walzbach due to the higher hydrological stability and higher nutrient concentration in the flowing water. Two possible explanations may justify these differences between sites: i) the higher microbial biomass accrual during biofilm formation at the Fuirosos might favour bacterial species richness, since epilithon biomass can control species diversity in bacterial assemblages (Jackson et al., 2001; Lyautey et al., 2003), and ii) the potential effect of flooding episodes (days 10 and 40 at the Fuirosos) tends to reduce biotic interactions (i. e. competitive exclusion) in stream biological communities and prompt biological biodiversity (Gasith & Resh, 1999). The effect of flood events at the Fuirosos is stressed by the observed fluctuations of bacterial diversity and drastic reduction of algal biomass after floods. Bacterial species richness (inferred from the number of DGGE bands) in the Fuirosos evolved in three stages: i) an initial increase of species richness (15 bands, day 3), ii) an intermediate stage characterized by a limited number of populations (8 bands, days 7 to 14), and iii) a late stage characterized by higher diversity (11 bands, day 21). These results were similar to the pattern of bacterial assemblage development described by Jackson et al. (2001). In contrast, the species richness in the Walzbach increased between days 1 to 4 (12 bands), kept steady between days 7 to 44 (10 to 11 bands), and finally decreased at day 60 (6 bands). In this case, the reduction of species richness in the intermediate

stage was not as pronounced as in the Fuirosos biofilms. The grouping of the bacterial sequences also shows a drastic change in bacterial community structure before and after the main flood event in Fuirosos. Sequences affiliated to β-proteobacteria group were found in biofilms of the two study sites. Nevertheless, cyanobacteria were especially relevant between days 1-7 in the Fuirosos communities suggesting that this group might be responsible for the fast increase of bacterial biomass (Roeselers et al., 2007). After flooding episodes (days 14 and 60), repeatable autogenic succession of bacterial species did not occur, because in these dates bacterial assemblages were not dominated by cyanobacteria, but the group of β-proteobacteria prevailed. Previous studies have shown that β-proteobacteria species are very important colonizers (Kalmbach et al., 1997; Emtiazi et al., 2004), but also may persist during biofilm development stabilizing the overall community structure.

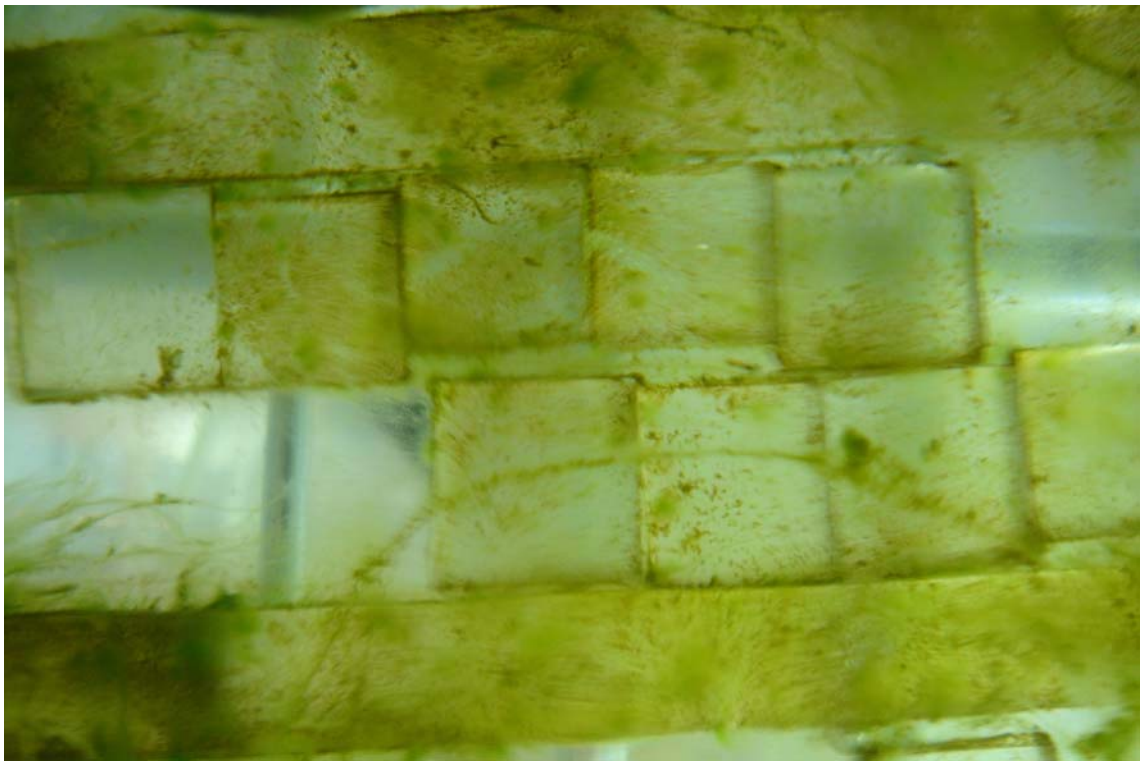
Succession of bacterial populations during biofilm formation in the two streams (the Walzbach and the Fuirosos) was detected by means of the 16S rDNA gene-based PCR-DGGE technique (Muyzer et al., 1993). Nevertheless, the ribosomal intergenic target amplification (ISR) resulted in a higher PCR efficiency with an increased number of DNA bands after PAGE visualisation. Both rDNA approaches covered successional changes in bacterial populations (Jackson et al., 2001; Araya et al., 2003; Lyautey et al., 2005; Lear et al. 2008). However, scores of intergenic experiments (band richness) did not evolve in any clear temporal pattern, while bacterial 16S-DGGE analysis revealed clear changes in a temporal scale. This was probably related to the potential bias of ISR community profiling in identifying different bacterial species of a given community that possess multiple amplicons of identical length (Jensen et al., 1993; Nagpal et al., 1998). For this reason, the use of the 16S rDNA-DGGE technique seemed to be more appropriate to analyze temporal changes in bacterial populations during biofilm formation.

In contrast to bacteria, the highest diatom species richness was found in the more stable environment, the Walzbach. The lower diatom taxa richness and the particular succession of diatom populations during biofilm formation in the Fuirosos might be again tightly related to the flooding episodes (Horner et al., 1990; Battin et al., 2003). While cluster analysis arranged diatom community patterns according to the time and maturation of Walzbach biofilms, a repeatable autogenic succession took place in the Fuirosos after flooding episodes (i. e. linkage distance between days 3, 7 and 60 was shorter than distance between days 30 and 60). In the Fuirosos, the species *Achnanthes minutissima* appeared during the first days, but also after the flooding episodes. Species of this genus can be very rapid colonizers of the epilithon (Soininen & Eloranta, 2004).

Characteristic microbial community composition in the studied biofilms are accompanied by characteristic structural and metabolic parameters. This is shown by the major EPS accumulation at Fuirosos biofilms. Elevated exopolysaccharide / cell ratios in biofilms would improve adhesion under elevated shear stress and thus reflect a functional response of biofilms to avoid detachment through erosion and sloughing during high flow episodes (Battin et al., 2003). Metabolic differences were mainly due to a greater decomposition capacity for polysaccharides and peptides at Fuirosos biofilms while chitin and peptidoglycan compounds degradation (β-glucosmainindase activity) prevailed in the Walzbach. At the Fuirosos, β-glucosidase and peptidase enzyme activities were significantly correlated to the EPS content suggesting that not only cell linked enzymes but also EPS matrix enzymes might be relevant in this biofilm (Romaní et al. 2008). In contrast, calculations of the biomass-specific enyme activity (activity per bacterial cell) suggested that bacterial cells of the Walzbach were much more efficient in degrading polysaccharide, peptide and chitin compounds than those at the Fuirosos biofilms.

The studied 60 days colonization sequences at the central European and Mediterranean streams showed a greater complexity and greater fluctuations of structural and metabolic parameters of the biofilm developed at Fuirosos probably adapted to hydrological changes. In contrast, the hydrological stability and the high water nutrient concentration in the Walzbach generated biofilms with greater algal diversity and a more efficient use of the OM resources. In conclusion, the biofilm development in streams of different bioclimtic regions may have a different structural organization and functioning.

Chapter 6



Relevance of polymeric matrix enzymes during biofilm formation

Abstract

Extracellular polymeric substances (EPS) contribute to biofilm stability and adhesion properties. The EPS matrix might also be a site for free extracellular enzyme activity; however, little is known about participation of enzyme activity in EPS during biofilm formation. Here it has been analyzed the activities of β-glucosidase, leu-aminopeptidase and β-glucosaminidase during the colonization of artificial substrata (glass tiles) in a stream distinguishing enzyme activity in EPS matrix (matrix-enzymes) and total biofilm extracellular enzyme activity. The use of cation exchange resin as extraction procedure seems appropriate to measure matrix-enzymes in freshwater biofilms, although the methodological limitation for using a biofilm suspension instead of an undisrupted biofilm. Total biofilm activities and matrix-enzyme activities showed similar capabilities to decompose organic matter compounds, with a greater capacity for peptide decomposition (leu-aminopeptidase) than for polysaccharides (β-glucosidase), and a low decomposition of chitin and peptidoglycan (β-glucosaminidase). Matrix-enzyme activity increased with colonization time, but showed a lower slope than that of total enzyme activity. At the beginning of the colonization experiment (days 1-4) matrix-enzymes accounted for 65-81% of total biofilm enzyme activity. Higher EPS relative content and matrix-enzyme activities per μg of polysaccharides in the EPS were measured during the first 1-3 days of biofilm formation, indicating a high rate of enzyme release into the matrix during this period. Relative contribution of matrix-enzyme activities decreased as biofilm matures but was maintained at 13-37% of total enzyme activity at the 42-49 days-old biofilm. These enzymes retained and conserved in the EPS may contribute to community metabolism. When analyzing extracellular enzymes in biofilms, the contribution of matrix-enzymes must be considered, especially for young biofilms.

Introduction

Biofilms are structured communities of bacteria, algae, cyanobacteria, fungi and protozoa embedded in a polymeric matrix (Lock et al., 1984; Marshall, 1992). They are present in various compartments of flowing waters (e.g. benthic, hyporheic, aquifer sediments) and play a key role in the uptake and retention of inorganic and organic nutrients (Lock, 1993; Romaní et al., 2004a). Most microorganisms found in biofilms produce extracellular polymers, which lead to adhesion to the substrate (Cooksey & Wigglesworth-Cooksey, 1995; Low & White, 1989; Marshall, 1971) and comprise the polymeric matrix responsible for biofilm integrity (Hamilton, 1987; Lock, 1993).

Extracellular polymeric substances (EPS) are rich in high molecular weight polysaccharides as well as other non-sugar compounds such as proteins (Bhaskar & Bhosle, 2005). The EPS matrix is a crucial structural parameter for biofilm stability and architecture and provides a refuge for the microbial community against shear stress and protection against desiccation (Ramasamy & Zhang, 2005). EPS may adsorb or entrap soluble and particulate matter, as well as metals and toxic compounds, thereby removing them from the flowing water (Freeman & Lock, 1995; Bhaskar & Bhosle, 2005). Within the biofilm, extracellular enzymes and products from hydrolysis, involved in the recycling of organic molecules, are protected by the EPS matrix (Freeman & Lock, 1995). Relevant aminopeptidase and phosphatase activity in the polymeric matrix of mature biofilms (Thompson & Sinsabaugh, 2000) and also peptidase activity in the EPS of activated sludge (Frølund et al., 1996) have been measured, underlining the enhanced metabolism within biofilms (Costerton et al., 1995). Even decomposition of the main EPS, as sources of proteins and polysaccharides for the microbial community, can occur in the biofilm (Zhang & Bishop, 2003).

The structure and metabolism of the biofilm change throughout its formation. During the early stages, EPS are prevalent, while colonization by algae and bacteria occurs later on (Barranguet et al., 2004). Changes in C, N, and P content and in the density (and consequently diffusion properties) of the biofilm have also been reported (Romaní et al., 2004b; Yallop et al., 2000). Autotrophic and heterotrophic metabolic changes also take place throughout biofilm formation associated with structural changes (Romaní et al., 2004b). Specifically, proteolytic and polysaccharidic enzymes are positively associated with the accrual of algal biomass as well as with photosynthetic activity in the biofilm (Francoeur & Wetzel, 2003; Romaní & Sabater, 2000). However, these extracellular enzymatic measurements include the activity of ectoenzymes (enzymes bound to the cell; Chróst, 1991), and matrix-enzymes (enzymes in the polymeric matrix, including free and linked to EPS). The function of extracellular enzymes for the biofilm microbial community is mainly the acquisition of N and C compounds for their growth and reproduction (Chróst, 1991). Extracellular enzymes may be also involved in other functions such as lysis of microbial cell walls for microbial growth or protozoa grazing (i.e. bacterivory). In this regard, the most relevant enzymes, as well as those most studied in freshwater biofilms, include proteolytic and polysaccharidic enzymes such as leucine-aminopeptidase (leu-aminopeptidase) (involved in peptide decomposition) and β-glucosidase (involved in the final step of cellulose and polysaccharide decomposition). β-glucosaminidase activity, which is involved in the decomposition of peptidoglycan and chitin, might also be found in

biofilms, especially when protozoa and fungi are present (Chamier, 1985; Vrba et al., 1993).

Here the activity of matrix-enzymes and their relationship with total biofilm enzyme activity was examined during biofilm formation on a solid surface in an undisturbed stream. The role and activity of ectoenzymes and matrix-enzymes might change throughout the formation of the biofilm. There is probably a relevance of matrix-enzymes at the first steps of the colonization, while in the growing and mature biofilm, ectoenzymes might be enhanced. Matrix-enzymes originate from direct release and excretion by algae and bacteria or from release by cell lysis and protozoa grazing (Lock et al., 1984). The activity of matrix-enzymes might be further affected by the microenvironmental conditions within the biofilm, such as pH and ionic strength and/or presence of humic substances (Freeman & Lock, 1992; Hoppe, 1983). Here it has been reported that the activity patterns of the matrix-enzymes leu-aminopeptidase, β-glucosidase and β-glucosaminidase differ to those of total extracellular enzymes during biofilm formation. The method for the EPS extraction with cation exchange resin for the measurement of matrix-enzymes in natural biofilms is also checked.

Materials and methods

Biofilm sampling

During spring 2006 (May-June), freshwater biofilm was cultivated on sandblasted glass tiles measuring 1 cm^2 that were fixed on 5 heavy concrete slabs with silicone. The slabs were lowered onto the riverbed (Fuirosos stream, Romaní et al. 2004b) with a minimum water level of 10 cm over the tiles and were distributed along the reach (*c*. 50 m in length) to cover a range of light and current conditions. The tiles were collected on days 1, 2, 3, 4, 7, 14, 21, 31, 42 and 49 after immersion, immersed in river water and taken to the lab in a cooling bag. During the study period, the average water nutrient content of the Fuirosos stream was 10.6 ± 2.4 μg $P\text{-}PO_4$ L^{-1} and 46.9 ± 5.8 μg $N\text{-}NO_3$ L^{-1} and with a mean pH of 7.1 ± 0.2 (values are means and SD of weekly values). Average current velocity on the tiles was 0.07 ± 0.04 m/s, light ranged between 40-150 μmol photons m^{-2} s^{-1}, and water temperature between 12-14 ºC.

On each sampling date, eight tiles were removed from each of the five immersed slabs. Three of these tiles were used for assays related to the EPS matrix (polysaccharide content and matrix-enzymes measurements). EPS extraction and polysaccharide content determination was performed according to the protocol described in section 2.4.2 of the methods, while measures of matrix-enzyme activity (β-

glucosidase, leu-aminopeptidase and β-N-acetylglucosaminidase) was performed according to section 2.6.1. The other five tiles were used for whole biofilm assays (total β-glucosidase, leu-aminopeptidase, β-N-acetylglucosaminidase enzyme activity, total polysaccharide content and C/N content of biofilms; sections 2.6.1, 2.4.2 and 2.4.1, respectively).

In most cases, results were referred to the biofilm surface area (1 cm^{-2}). But also, matrix-enzyme activities were expressed as nmol MUF (or AMC) per μg of polysaccharides in EPS. The relevance of matrix-enzymes throughout colonization was calculated as the % of matrix-enzyme activity versus total biofilm enzyme activity.

Checking EPS extraction and matrix- enzyme measurements

In spring 2007, about 200 glass tiles 1 cm^2 were colonized at Fuirosos stream and 14 days-old, 30 days-old and 42 days-old biofilms were used to further check the extraction procedure of EPS and the measurement of matrix-enzymes in natural aquatic biofilms.

To check the efficiency of EPS extraction of freshwater biofilms by using cation exchange resin, extractions at 0, 0.5, 1, 2, and 6 hours (3 replicates at each extraction time) were performed with 14 days-old and 30 days-old biofilms following the same procedure described in section 2.4.2. The possible appearance of living cells at the extracted EPS fraction as well as the possible damage to biofilm cells due to the extraction procedure was checked for the 14 days-old biofilm by using the live/dead *Bac*Light Bacterial Viability kit (Molecular Probes). This double staining consists in a mixture of 3.34 mM SYTO® 9 (which labels all cells) and 20 mM propidium iodide (which stains only cells with damaged membranes). Subsamples (200 μL) from the supernatant and the centrifuged pellet fractions were diluted (10 times, with miliQ autoclaved water) and the mixture of SYTO® 9 and propidium iodide (1:1, 3 μL) was added. After 15 minutes, samples were filtered (0.2 black polycarbonate filter, Nuclepore Track-Etch Membrane, Whatman), mounted with oil (Mounting oil, Molecular Probes), and observed under the epifluorescence microscope (Nikon Eclipse 600W). Green and red (live and dead) bacterial cells were counted at 20 fields per filter.

To check the relationship between matrix-enzymes and total biofilm enzyme activities, 30 days-old biofilms (5 replicates) were analyzed for three fractions: 1-total enzyme activity (activity at the suspension of the whole biofilm), 2- enzyme activity at the EPS matrix fraction (activity at the supernatant after 1h extraction with the cation exchange resin), 3- enzyme activity at the cell-linked fraction (activity at the centrifuged pellet after 1h extraction with the cation exchange resin). To obtain the pellet fraction, all the supernatant was carefully eliminated with a pipette and phosphate buffer was

added (1.5 ml) to obtain a suspension which was gently homogenized by shaking and subsamples taken for all measurements. At the three biofilm fractions, β-glucosidase and leu-aminopeptidase activities, and polysaccharide content were measured following the procedures described in sections 2.6.1 and 2.4.2.

The effect of using a biofilm suspension instead of an undisrupted biofilm was also checked for β-glucosidase and leu-aminopeptidase activities by using 14 days-old, and further 42 days-old biofilm for leu-aminopeptidase. For the biofilm suspension glass tiles were detached from the substrata (sterile silicone cell scraper) while colonized glass tiles were incubated directly for the undisrupted biofilm measurements. The possible effect of the phosphate buffer for enzyme activity measurements was checked by using 14 days-old colonized biofilms. The phosphate buffer effect was both checked for the suspended and undisrupted biofilm (3 replicates each).

Statistical analyses

Time differences in C, N, total polysaccharides, EPS-polysaccharides, total enzyme activities, and EPS-enzyme activities during colonization were analysed by means of a MANOVA. All data were logarithmically transformed to stabilize the variance. Post-hoc comparisons (Tukey's HSD test) were used to analyze time differences and define significantly different groups along the colonization experiment. Correlation analyses (Pearson coefficient) were performed to examine covariation between EPS-enzyme and total enzyme activities as well as the EPS and C and N content during colonization.

Further ANOVAs were used to test for the effect of the extraction time to the polysaccharide content and bacterial density at the centrifuged pellet and the supernatant fractions. T-test of paired values was used to test for possible differences due to the use of phosphate buffer and the use of a suspended or undisrupted biofilm.

Results

C, N and polysaccharides

The colonizing biofilms showed a significant increase in biomass (C and N) after one week (day 7), followed by a much smaller increase from days 14-30. At 42-49 days biofilm biomass seems to be stabilized (Figure 8.1). On the basis of the MANOVA-Tukey analyses (Figure 8.1), we defined three stages during the biofilm colonization: 1-4 days-old biofilm, 14-31 days-old biofilm and 42-49 days-old biofilm.

Total and extracellular polysaccharide content followed a similar trend to that of C and N (Figure 8.2), as shown by the highly significant correlation between EPS and the content of C and N (Table 8.1). The proportion of EPS-polysaccharides versus total biofilm polysaccharides was very high for the young biofilm (79%) and decreased along colonization to 36% in the 42-49 days-old biofilm (Figure 8.2, Table 8.2).

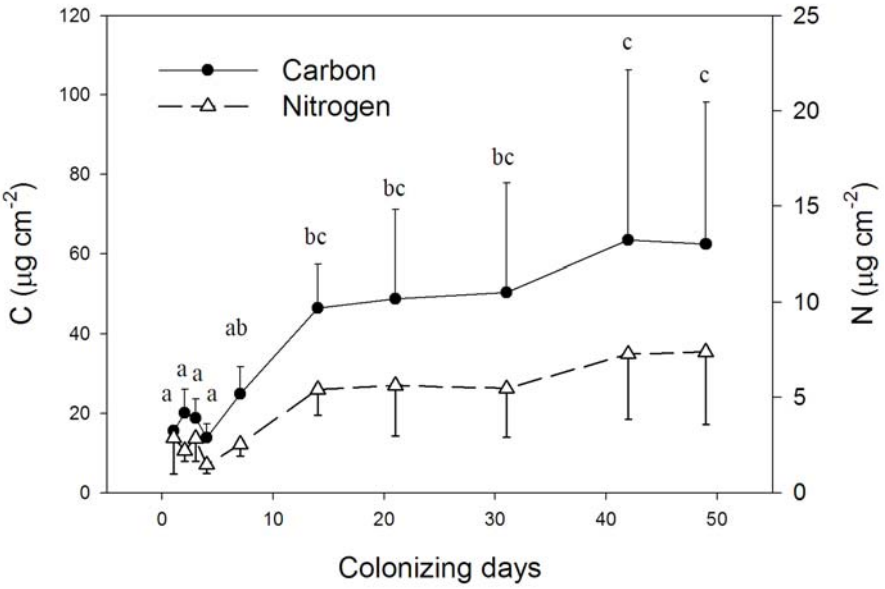


Figure 8.1. C and N biofilm content on the colonized biofilms. Values are means + SD for C and means – SD for N (n=5). Lower-case letters indicate significantly different groups found by Tukey's HSD test (p<0.05).

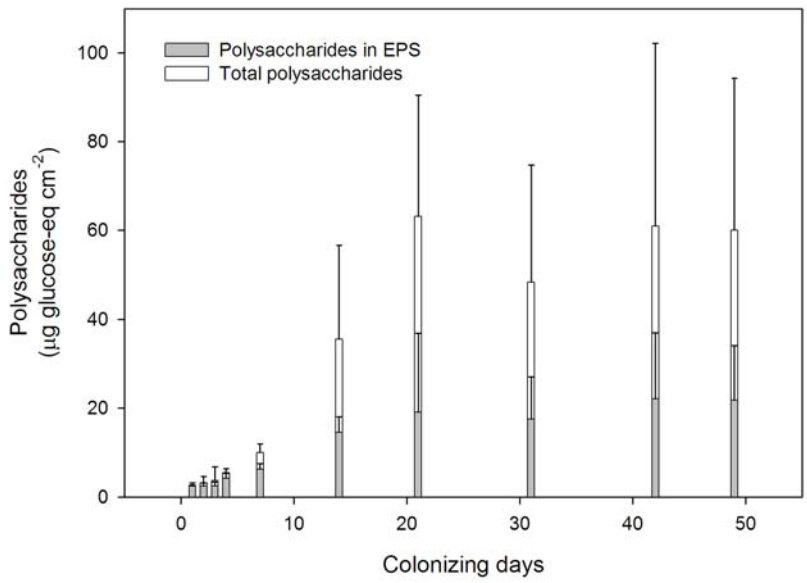


Figure 8.2. Total polysaccharide biofilm content and polysaccharide content of EPS on the colonized biofilms. Values are means + SD (n=5).

	PepE	GluE	GlucosE	PepT	GluT	GlucosT	C	N
PepE	1							
GluE	0.33	1						
GlucosE	0.15	-0.25	1					
PepT	0.76*	0.50	0.48	1				
GluT	0.40	0.96**	-0.05	0.68*	1			
GlucosT	0.78**	0.38	0.46	0.86**	0.48	1		
C	0.93**	0.71*	0.27	0.86**	0.82**	0.74*	1	
N	0.90**	0.72*	0.22	0.85*	0.82**	0.71*	0.98**	1
EPS	0.90**	0.68*	0.31	0.90**	0.81**	0.73*	0.98**	0.96**

*Table 8.1. Pearson correlation coefficients between total enzyme activity (T) and EPS matrix-enzyme activit (E) of leu-aminopeptidase (Pep), β-glucosidase (Glu) and β-glucosaminidase (Glucos) and the C, N and EPS polysaccharide content in the biofilm during colonization (days 1-49, n=10). Significance is indicated by the asterisk: *p<0.05, **p<0.01.*

Matrix-enzyme and total enzyme activities

The biofilm showed a higher leu-aminopeptidase than β-glucosidase activity, while β-glucosaminidase was the lowest throughout colonization (Figure 8.3). This trend was found both for the total enzymes and for matrix-enzymes. The ratio β-glucosidase: leu-aminopeptidase was lower than 0.3 for most of the biofilms showing no significant differences between total enzyme and matrix-enzyme activities (*t*-test, p=0.55, Table 8.2).

The increase in total extracellular enzyme activity during biofilm formation was slightly delayed compared with the increase recorded for C, N and polysaccharide content. In particular, leu-aminopeptidase and β-glucosidase did not show any significant increase till day 21, while β-glucosaminidase showed a slight peak at day 7, but increased at the end of the experiment (Figure 8.3). However, total extracellular enzyme activities were significantly and positively correlated with C, N and EPS-polysaccharide content. Furthermore, the three activities measured were positively correlated (Table 8.1).

The activity of the matrix-enzymes β-glucosidase and leu-aminopeptidase increased with colonization time, but showed a lower slope than that of total enzyme activity (Figure 8.3). Both enzymes were positively correlated with C, N and EPS-polysaccharide content (Table 8.1). In contrast, β-glucosaminidase matrix-enzyme activity was low, varying with time and showing no significant correlation with biofilm

biomass (Table 8.1). Correlation between total enzyme activity and matrix-enzyme activity was found for leu-aminopeptidase and β-glucosidase (Table 8.1).

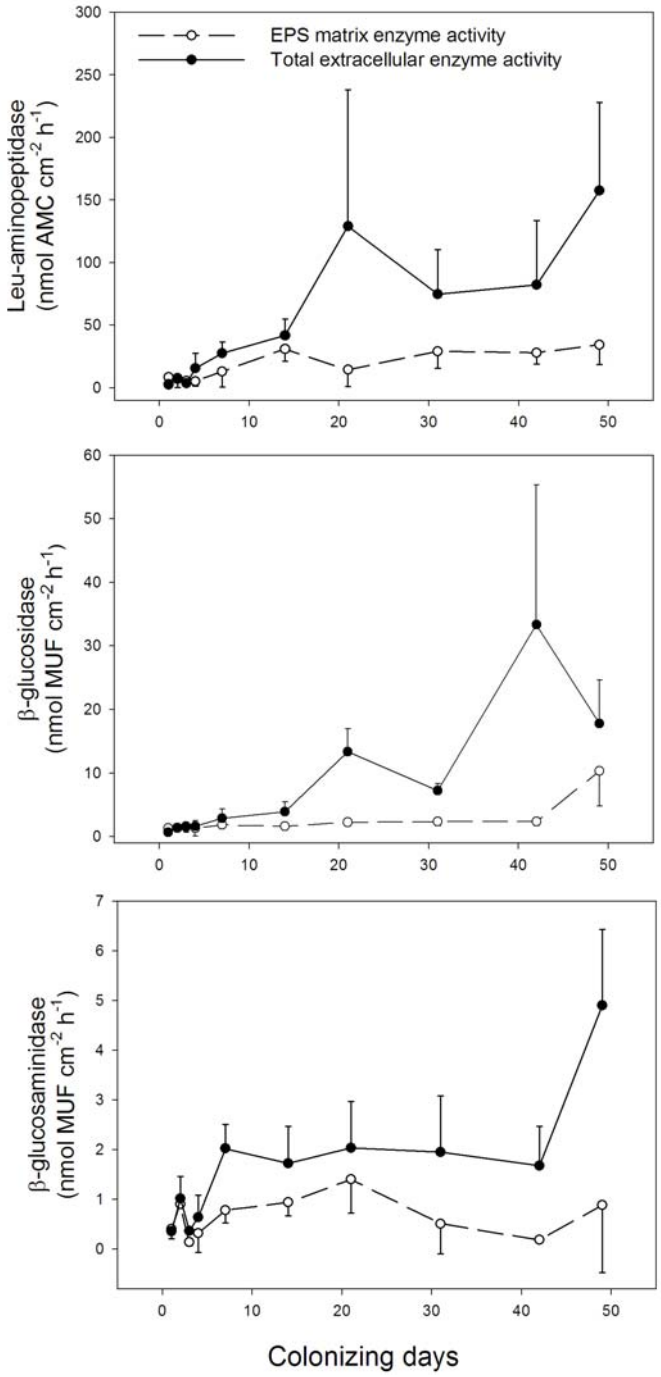


Fig. 8.3. Total biofilm extracellular enzyme activity and matrix-enzyme activity of leu-aminopeptidase, β-glucosidase and β-glucosaminidase on the colonized biofilms. Values are means + SD for total enzymes and means – SD for matrix-enzymes (n=5).

The calculation of the % of matrix enzymes with respect to total enzyme activity was subjected to a high variability especially for the 1-4 days-old biofilm and the leu-aminopeptidase activity, but clearly showed a decrease with colonizing time (Figure 8.3, Table 8.2). Matrix-enzyme activities accounted for 65-81% of total biofilm enzyme activity in the 1-4 days-old biofilm (Figure 8.3, Table 8.2). At the 42-49 days-old biofilm, leu-aminopeptidase and β-glucosidase matrix-enzymes activities reached 36% of total activity, while β-glucosaminidase matrix-enzyme activity was the lowest decreasing to 13% of total activity (Figure 8.3, Table 8.2).

When the matrix-enzyme activities were calculated with respect to μg of EPS-polysaccharides, significant higher activities were obtained for the 1-4 days-old biofilm than for the older biofilm (days 1-2 for leu-aminopeptidase; days 1-3, but also day 42, for β-glucosidase; day 2 for β-glucosaminidase, MANOVA-tukey test, $p<0.05$).

		Biofilm age		
		1-4-days-old	7-31-days-old	42-49-days-old
Extracellular enzyme activity (nmol cm^{-2} h^{-1})				
Leu-aminopeptidase	Total	7.4 *(1.2-36.7)*	68.3 *(5.7-204.2)*	119.9 *(18.5-283.9)*
	% in EPS	73.7 *(14.2-100.0)*	59.2 *(4.2-100.0)*	36.6 *(13.4-96.0)*
β-glucosidase	Total	1.3 *(0.4-2.7)*	6.8 *(2.1-18.4)*	25.6 *(9.9-59.7)*
	% in EPS	80.8 *(46.1-100.0)*	41.6 *(13.5-83.4)*	35.6 *(15.6-47.1)*
β-glucosaminidase	Total	0.6 *(0.3-1.7)*	1.9 *(0.7-3.2)*	3.3 *(0.9-6.4)*
	% in EPS	64.5 *(21.7-100.0)*	51.6 *(2.7-98.2)*	13.2 *(2.2-37.9)*
β-glucosidase : leu-aminopeptidase ratio				
	Total	0.25 *(0.07-0.52)*	0.10 *(0.06-0.44)*	0.26 *(0.11-0.75)*
	In EPS	0.21 *(0.11-1.96)*	0.09 *(0.03-1.76)*	0.26 *(0.13-0.46)*
Polysaccharide content (μg cm^{-2})				
	Total	3.7 *(0.2-7.4)*	39.3 *(7.5-89.3)*	60.5 *(28.7-134.5)*
	% in EPS	78.6 *(41.2-100.0)*	42.7 *(19.2-84.2)*	36.3 *(33.8-41.5)*

Table 8.2. Summary of the relevance of EPS-enzyme activities for 1-4 days old, 7-31 days old and 42-49 days old biofilm. The β-glucosidase: leu-aminopeptidase ratio and polysaccharide content of EPS for the three biofilm stages are also shown. Values are means and range (in parenthesis) for each stage.

Checking EPS extraction and matrix- enzyme measurements

The EPS-polysaccharide of the 14 days-old biofilm changed with extraction time (ANOVA, p=0.011). The measured EPS-polysaccharide content increased significantly after 1h and differences between 1h, 2h and 6h were not significant (Figure 8.4, Tukey's test). For the 30 days-old biofilm, at 0 hours significant amount of EPS-polysaccharide was measured and slightly increased with extraction time, although extraction time effect was not statistically significant (Figure 8.4, ANOVA, p=0.21). At the extracted EPS live bacteria were scarce (two orders of magnitude lower than those measured for the pellet) and dead bacteria were most abundant (Table 8.3). Significant differences were observed due to extraction time (ANOVA, p=0.025, p=0.005, for live bacteria and percentage of live bacteria, respectively), live bacteria increasing significantly after 6 h of extraction time (Tukey's test, p<0.05, Table 8.3). Bacterial density and percentage of live bacteria at the centrifuged pellet were not affected by extraction time (ANOVA, p=0.7, p=0.8, respectively), the values being about 7.7 x 10^6 cell cm^{-2} with 46% of live bacteria (Table 8.3).

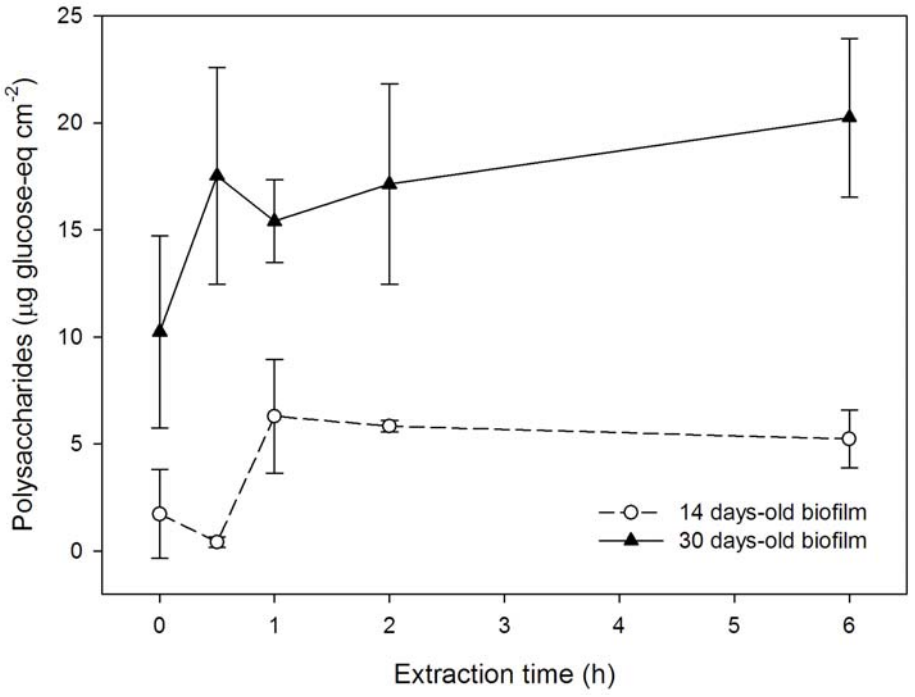


Figure 8.4. Polysaccharide content of the extracted EPS with cation exchange resin at different extraction times for 14 days-old and 30 days-old biofilms colonized at the Fuirosos stream. Values are means ± SD (n=3).

Biofilm fraction		Extraction time (hours) 0	0.5	1	6
Supernatant (matrix)	Live bacteria (cell 10^4 cm^{-2})	4.30 ± 1.88	8.04 ± 1.72	8.98 ± 2.55	15.35 ± 5.76
	% live bacteria	8.3 ± 4.1	11.5 ± 1.8	13.1 ± 4.1	27.0 ± 4.9
Pellet (cell-linked)	Live bacteria (cell 10^6 cm^{-2})	7.74 ± 1.73	6.26	9.81 ± 4.74	6.99 ± 2.52
	% live bacteria	44.2 ± 5.9	44.1	50.0 ± 6.6	47.8 ± 0.6

Table 8.3. Density of live bacteria and percentage of live bacteria at the supernatant (EPS matrix fraction) and pellet (cell-linked fraction) after cation exchange resin extraction at different times. The biofilms used were 14 days-old biofilm colonized in the stream. Values are means ± SD (n=3, but n=1 for 0.5 hours extraction time).

The analysis of matrix and cell-linked polysaccharides and enzyme activities showed together slightly higher values than those obtained for the total biofilm measurements (Table 8.4). A high variability was specially shown for the cell-linked values (centrifuged pellet).

Differences between suspended and undisrupted 14 day-old biofilm were not significant (p=0.2 and p=0.1 for leu-aminopeptidase, p=0.9, p=0.4 for β-glucosidase, without and with phosphate buffer). For the 42 day-old biofilm, significant lower leu-aminopeptidase activity was measured for the suspended biofilm (t-test, p= 0.021), the enzymatic activity accounting for 87% of that measured at the undisrupted biofilm (Table 8.5). There was no significant effect of the use of phosphate buffer for the β-glucosidase and leu-aminopeptidase measurements, irrespectively of the use of the suspended or undisrupted biofilm (t-test, p>0.2, Table 8.5).

	Matrix	Cell-linked	Total	Total/Total expected
β-glucosidase (nmol cm^{-2} h^{-1})	37.25 ± 16.74	14.49 ± 7.00	38.41 ± 15.41	0.78 ± 0.24
Leu-aminopeptidase (nmol cm^{-2} h^{-1})	33.83 ± 19.22	250.51 ± 127.6	188.25 ± 43.72	0.80 ± 0.44
Polysaccharide (μg cm^{-2})	19.54 ± 1.29	81.68 ± 26.50	58.76 ± 1.30	0.58 ± 0.04

Table 8.4. Enzyme activities and polysaccharide content at the matrix fraction, cell-linked fraction and total biofilm of 30 days-old biofilm colonized in the stream. The matrix and cell-linked fractions correspond to the supernatant and the pellet after one hour extraction with cation exchange resin. The relationship between total values and expected total biofilm values (sum of matrix and cell-linked) is also shown. Values are means ± SD (n=5).

Biofilm age	Incubation solution	Enzyme	Undisrupted biofilm	Biofilm suspension
14 days-old	River water (pH 7)	Peptidase	72.22 (11.77)	59.93 (5.95)
		Glucosidase	3.26 (0.72)	3.04 (1.87)
	Phosphate buffer (pH 7)	Peptidase	74.44 (3.69)	54.31 (11.47)
		Glucosidase	4.80 (1.22)	4.69 (1.59)
30 days-old	River water	Peptidase	999.7 (68.1)	873.8 (71.1)

Table 8.5. Effects of using suspended versus undisrupted biofilm, and of using phosphate buffer versus river water for enzyme activity measurements (leu-aminopeptidase and β-glucosidase). Values are means (in nmol cm^{-2} h^{-1}) and SD (n=3 for 14 days-old biofilm, n=5 for 30 days-old biofilm).

Discussion

The process of freshwater biofilm formation includes the adhesion of microbial cells (algae, bacteria) followed by an accrual and changes of the microbial community along with structural changes such as decrease in the polysaccharide relative content, decrease in the C/N ratio, changes in diffusion properties (Barranguet et al., 2004; Romaní et al., 2004b; Yallop et al., 2000). In general, biofilm colonization studies show that metabolic measurements, including total extracellular enzyme activities, increase together with structural parameters (Romaní & Sabater, 1999; Sabater & Romaní, 1996). Similarly, in this study, total extracellular enzyme and matrix-enzyme activities

positively correlate with C, N and EPS content, but, it is also shown that the relative proportion of matrix-enzyme and enzyme activity directly linked to the cell is varying along the colonization process. The matrix-enzymes leu-aminopeptidase, β-glucosidase and, in a lower amount, β-glucosaminidase, are especially high in 1-4 days-old biofilms (65-81% of total biofilm) and decrease as biofilm matures (13-37% after 49 days). The varying proportion of matrix-enzyme activity along colonization might modulate the interpretation of total biofilm enzyme activity measurements. Since the presence of matrix-enzymes results from previous release processes, they might not be linked to actual cell regulation mechanisms. Especially for young biofilm, an important fraction of the enzyme activity would not be directly linked to active cells although contributing to the whole metabolism. This must be taken into account when total enzymes are calculated in a cell basis (activity per cell; Romaní & Sabater, 1999). At the same time, the obtained results indicate that both total biofilm activities and matrix-enzyme activities show similar capabilities to decompose organic matter compounds, with a greater capacity for peptide decomposition (leu-aminopeptidase) than for polysaccharides (β-glucosidase), and a low decomposition of chitin and peptidoglycan (β-glucosaminidase). The higher leu-aminopeptidase activity could be related to the bacterial-algal coupling (stimulation by photosynthetic activity) in such light-grown biofilms (Francoeur et al., 2001).

Biofilms from the beginning of the colonization process (1-4 days-old) can be clearly distinguished from those at the end (42-49 days-old). During the initial biofilm colonization, high release rate of enzyme activity into the EPS-matrix by the first biofilm colonizers might occur as shown by the prevalence of EPS but also the major enzyme activity in the EPS matrix itself. This higher proportion of EPS and matrix-enzymes in young biofilm could be related to the process of early biofilm formation such as cell attachment (Cooksey & Wigglesworth-Cooksey, 1995; Costerton et al., 1995). Conditions like this first colonization steps might periodically occur along a natural biofilm “life”. Collapse (dramatic decrease of cells) and recolonization (start to increase the number of cells) can occur periodically in biofilms (Ács & Kiss, 1993). In rivers and streams biofilm attachment and detachment processes are frequent, especially after high flood events, when shear forces decrease the accumulated biomass to previous colonization steps, a new colonization process begins, and metabolism (respiration) is enhanced (Blenkinsopp & Lock, 1992). During these recolonization periods, increases in the relative proportion of EPS and matrix-enzyme activity might be expected.

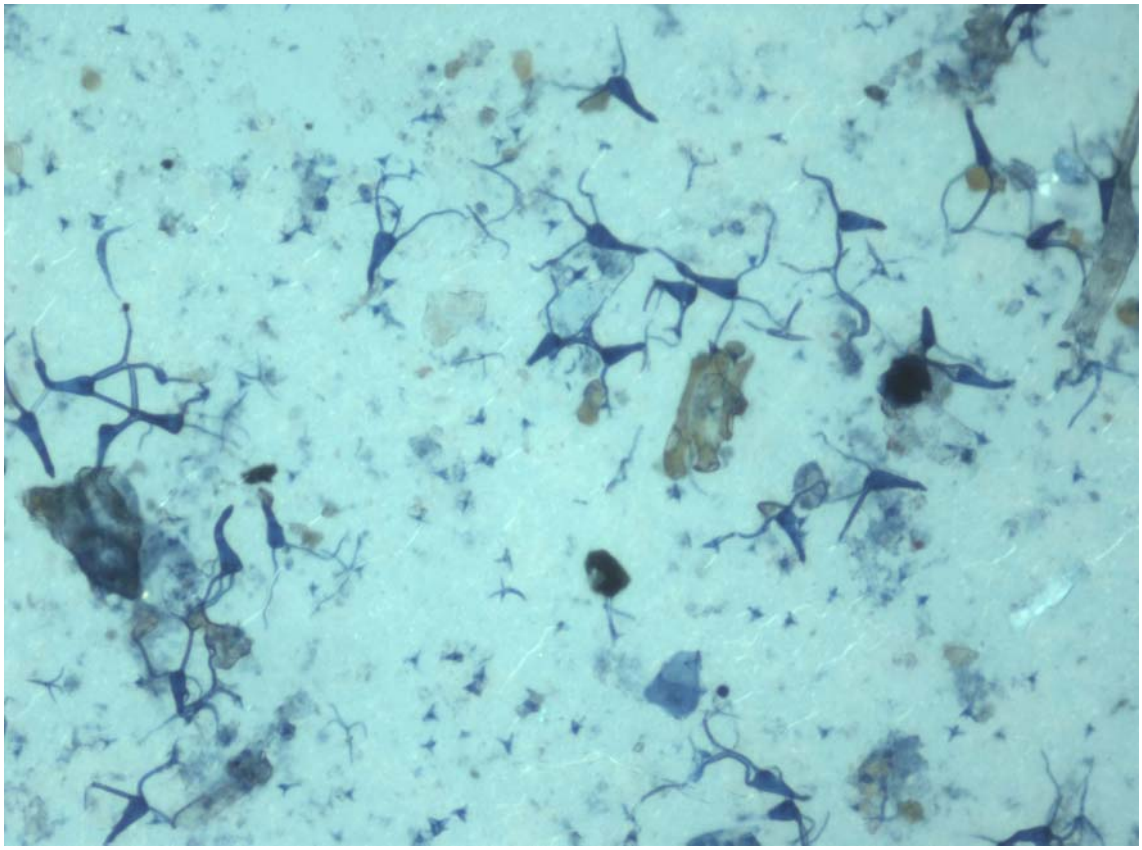
As the biofilm is gaining in structure (thickness and architecture, Battin et al., 2003), the potential activity of enzymes linked to the cells became more important than

the matrix-enzymes. After 42-49 days of colonization, matrix-enzymes leu-aminopeptidase and β-glucosidase accounted for about 37% of total biofilm enzyme activity, which is consistent with results in other mature biofilms (Thompson & Sinsabaugh, 2000). The uncoupling between total and matrix-enzyme activities for β-glucosaminidase as well as the decrease in matrix β-glucosidase and leu-aminopeptidase might be further affected by the unfavorable conditions within the biofilm media for matrix-enzymes, such as pH and osmotic strength (Hoppe, 1983) or inhibition by humic materials (Freeman & Lock, 1992). This hypothesis was also proposed by Thomson & Sinsabaugh (2000), who found a lower affinity for the substratum (higher Km) for the matrix aminopeptidase in mature biofilms. However, although the contribution of matrix-enzymes decrease as biofilm matures, it seems to be maintained to a certain level, evidencing the role of the extracellular matrix as a reservoir for extracellular enzymes. The release of enzymes into the matrix is in concordance with the theory of the "altruistic" behavior of biofilms (Costerton et al., 1994; Kreft, 2004), when due to the physical proximity between microbial cells within the biofilm, EPS and related enzymes and their products may be used not only by their producers but shared by the whole microbial community.

The technique for the analysis of matrix-enzymes by using cation exchange resin seems to be appropriate for natural biofilms. After one hour extraction most of the EPS material potentially extracted by the cation exchange resin should be extracted and few bacterial cells are included. Furthermore, the damage of the extraction procedure to the biofilm cells is negligible as shown by any effect on the live bacteria at the centrifuged pellet which were maintained at about 45% of total bacteria (within the range commonly found in natural river biofilms, Ylla unpublished). The use of the phosphate buffer to counteract the acidity of the cation exchange resin is not affecting the enzyme activities measured, although this should be probably taken into account when measuring phosphatase activities (Chróst, 1991). On the other hand, this technique imposes a limitation for biofilm research which is the requirement of a biofilm suspension instead of working with an undisrupted biofilm. Biofilm metabolism measurements should be preferably performed with an undisrupted biofilm in order to avoid alterations in the biofilm structure which could lead to over (or under) estimation of metabolism (Barranguet et al., 2004). Investigations of biofilm by considering its three-dimensional structure (by using Confocal Laser Scanning Microscopy) show the patchiness structure of aquatic biofilms (Neu & Lawrence, 1997) but the spatially metabolic biofilm measurement is still a technical challenge (Barranguet et al., 2004; Francoeur et al., 2001). Although in the studied natural biofilms not significant

differences in enzyme activities were observed between the undisrupted and the suspended biofilm, the use of the biofilm suspension might be a source of variability specially when fractionating and subsampling it for the different parameters. Thus, analysis of matrix-enzymes in natural aquatic biofilms by this technique must be performed with thin biofilms grown under similar and controlled physical and chemical conditions in order to minimize variability. This method can be further developed in, at least, two aspects. First, it should be possible to separate the free enzyme activity in the matrix (loose) to the more conserved EPS-linked enzyme activity. Secondly, different biofilms from different environments and under different physical-chemical conditions should be tested. Biofilms can change its metabolism, thickness, density and composition depending on its own evolution and/or physical-chemical conditions (Stoodley et al., 2001). Many studies show changes in total extracellular enzyme activities (Francoeur & Wetzel, 2003; Romaní et al., 2004b) and biofilm structure due to changes in light and nutrients (Neu et al., 2005; Zippel & Neu, 2005). Therefore, changes in the activity and contribution of matrix-enzymes to the whole biofilm metabolism due to those factors should be also expected, the present study being a specific example.

Chapter 7



Effect of nutrients on the sporulation and diversity of aquatic hyphomycetes on submerged substrata in a Mediterranean stream

Abstract

Eutrophication in streams often results in enhanced decomposition rates and chemical conditions that greatly affect the suitability of the habitat for many species. We performed an enrichment experiment in a 50-m reach of a Mediterranean stream by the continuous addition of N and P (enriched reach) while another consecutive reach upstream served as the reference (unenriched reach). After 1.5 years of enrichment, conidia production and species composition of aquatic hyphomycetes were measured in communities colonizing four streambed substrata (*Platanus acerifolia* and *Populus nigra* leaves, epipsammic and epilithic biofilms). Samples collected from the two reaches were subjected to two nutrient treatments in the laboratory [low nutrient (-NP) and high nutrient (+NP) concentrations] to test for short-term (48 h) nutrient effects. Long-term nutrient additions did not affect potential sporulation rates while short-term nutrient enrichment produced insignificant increase in these rates on most substrata collected in the unenriched reach. Major differences in potential sporulation rates were observed between substrata, the highest rate being for leaves (especially *Platanus acerifolia*). However, enrichment produced significant changes in fungal community composition. *Clavariopsis aquatica*, *Alatospora acuminata* and *Lemonniera* sp. were dominant in the enriched reach while *Heliscella stellata*, *Trisceloporus acuminatus* and *Clavatospora longibrachiata* were characteristic of the unenriched reach. Our results suggest that fungal N demands can be fulfilled at relatively low levels of dissolved nitrate and further increases in the nutrients availability may not result in enhanced fungal activity.

Introduction

The high availability of organic matter and light limitation in low-order forested streams enhance in-stream heterotrophic processes (Vannote et al., 1980), which are mostly performed by bacteria and fungi. Among the latter, aquatic hyphomycetes are the prime microbial decomposers of leaf litter (Suberkropp, 1992) and in turn, play a key role in structuring food webs in forested stream ecosystems (Bärlocher, 1992; Hieber & Gessner, 2002).

Aquatic hyphomycetes colonize mainly organic stream substrata such as leaves and wood. However, other sites accumulate organic matter, such as epilithic (on rocks) and epipsammic (on sand) habitats, and may also be relevant for the fungal activity (Artigas et al., 2004). Substrate preference by fungi is still unclear and only a few studies have demonstrated trends in substrate specificity (Charcosset & Gardes, 1999). Gulis (2001) observed that the size of the substrate unit and its chemical

composition (especially lignin content) caused selective colonization by some fungal species. However, other studies have proposed that environmental variables (i.e.: temperature, conductivity, water current velocity and dissolved oxygen concentration), as well as stream water nutrient concentrations are structuring factors for hyphomycete communities (Suberkropp, 1984; Gönczöl et al., 2003; Pascoal & Cassio, 2004). Analysis of fungal colonization of distinct stream substrata has revealed that the presence of species or phylotypes is governed mainly by seasonal conditions (Nikolcheva & Bärlocher, 2003).

Conidia production is one of the most energy-consuming processes undertaken by fungi, in which they invest more than 50% of total production (Suberkropp, 1991; Sridhar & Bärlocher, 2000). Suberkropp (1998) observed that at increased availability of dissolved inorganic nutrients, resources are mainly invested to the sporulation process rather than to mycelium growth in leaf litter. Moderate stream fertilization experiments have demonstrated the positive effect of inorganic nutrients on the sporulation rates of the hyphomycete community (Gulis & Suberkropp, 2003a; Gulis & Suberkropp, 2004; Abelho & Graça, 2006). These effects were also observed at the species level (Suberkropp, 1998). However, the tendency in heavily nutrient-rich rivers may be the opposite. Pascoal & Cassio (2004) observed a 100-fold decline in fungal sporulation rates in a nutrient-rich river (> 2500 μg L^{-1} $N\text{-}NO_3^-$ and > 300 $\mu g L^{-1}$ $P\text{-}PO_4^{3-}$) with low dissolved oxygen concentration. Lecref et al. (2006) proposed that certain levels of nutrients (i.e.: high ammonium concentrations) have a negative effect on the functional aspects of stream biota. In the case of aquatic hyphomycetes, the stream nutrient concentrations that might affect their reproductive output remain unclear.

This study analyses the effect of nutrients on conidia production by aquatic hyphomycetes and their community composition on several stream substrata (*Platanus acerifolia* and *Populus nigra* leaves, epipsammic and epilithic biofilms). The effect of short-term laboratory nutrient addition (48 hours) was tested on colonized substrata taken from enriched (N and P addition) and unenriched stream reaches. Specifically, the following questions were addressed: i) does long-term reach enrichment (unenriched *vs.* enriched) affect the potential sporulation rates and hyphomycete community composition on stream substrata? ii) are there differences between the substrata analyzed? and iii) does short-term nutrient addition affect conidia production on the substrata of unenriched and enriched reaches?

Materials and methods

Sampling

Two reaches (*c*. 50 m in length) were defined in the Fuirosos stream. The reference reach was upstream from the enriched reach. The enriched reach was artificially fertilized by increasing basal N concentrations up to 2 times and P up to 3 times (Table 9.1). Fertilization was achieved by the continuous addition of an ammonium nitrate and ammonium phosphate solution stored in a tank of 200 L beside the stream channel. Nutrient solution was replaced weekly and adjusted considering the upstream reach nutrient concentrations and discharge.

	Unenriched reach	Enriched reach
$P\text{-}PO_4^{3-}$ ($\mu g\ L^{-1}$)	10.3 *(9.4)*	30.1 *(13.1)*
$N\text{-}NH_4^{+}$ ($\mu g\ L^{-1}$)	24.6 *(23.5)*	185 *(192)*
$N\text{-}NO_3^{-}$ ($\mu g\ L^{-1}$)	364.1 *(471)*	580 *(544)*
N:P molar ratio	85	56
Discharge ($L\ s^{-1}$)	10.90 *(1.60)*	10.90 *(1.60)*

Table 9.1. Nutrient concentration and discharge measurements in the Fuirosos stream from June 2004 to December 2005. Values are means and SE of a weekly sampling during the enrichment experiment.

Preliminary studies revealed that the two reaches (unenriched and enriched) were quite identical in terms of physical-chemical conditions, streambed substrata cover and benthic microbial biomass before the enrichment experiment (autumn-winter 2003). Physical and chemical characteristics of stream water, such as water temperature (7.6 +/- 2.5), pH (7.6 +/- 0.1), and conductivity (179.6 +/- 7.8 $\mu S\ cm^{-1}$) were not significantly different between the two reaches. The two reaches showed 70% of the streambed surface area covered by rocks, 30% by sand, and leaves may cover up to 50% of the bed surface in autumn. Moreover, not significant differences in the fungal and bacterial biomass on rocks were observed between the unenriched (125.1 +/- 63.3 μg fungal C cm^{-2} and 3.3 +/- 0.6 μg bacterial C cm^{-2}) and enriched reaches (128.2 +/- 42.2 μg fungal C cm^{-2} and 2.9 +/- 0.6 μg bacterial C cm^{-2}) before the enrichment experiment.

Fungal sporulation rates and species composition were analysed in communities associated with four streambed substrata (*Platanus acerifolia* and

Populus nigra leaves, sand and rocks) during early winter 2005. On 17th November 2005, six litterbags (1mm-mesh size, each containing 6 leaves of *Platanus acerifolia* and 18 leaves of *Populus nigra*, previously dried 7 days at room temperature) and 6 rocks (each with 12 unglazed clay tiles of 1 cm^{-2} glued onto the flat rock surface) were immersed in the stream. Leaves were placed in litterbags in order to control immersion time and thereby ensure samples at similar decomposition stages for the analysis of potential sporulation rates and hyphomycete communities, while clay tiles were used as surrogates for the epilithic biofilm. Three litter bags and rocks were immersed in the unenriched reach and another three bags and rocks in the enriched reach. The substrates remained immersed for 40 days to achieve complete colonization. On 27th December 2005, leaf bags and colonized tiles were removed from the two reaches and three sand cores (Perspex cylinders 4.3 cm in diameter) were collected from each site. Materials were carefully cleaned with sterilized water (filtered trough 0.2 μm) in order to eliminate deposited particles from substrata surfaces.

Three replicates of each substratum (*Platanus* and *Populus* leaves, epipsammic and epilithic biofilms) collected from the unenriched and enriched stream reaches were incubated under two short-term nutrient treatments: i) low N and P (-NP) and ii) high N and P (+NP) concentrations. The -NP treatment took the filtered stream water directly from the unenriched site while the +NP treatment increased the phosphate and nitrate concentrations up to 7 times by the addition of ammonium phosphate and ammonium nitrate, respectively. Stimulation of sporulation in the different substrata and conidia identification was performed according to the protocols described in the methods section (sections 2.6.2 and 2.3.1, respectively). Moreover, analysis of inorganic nutrients (nitrate and phosphate) was periodically controlled during both stream and laboratory water enrichment (section 2.7.1). Sporulation rates were expressed as number of conidia per surface area and time.

Additional samples were taken for SEM observations of microbial communities attached on different stream bed substrata and subjected to the nutrient treatments (section 2.3.1).

Statistical analysis

Analyses of variance on log (x+1) transformed data were used to test the stream enrichment effects (unenriched *vs.* enriched) and the short-term nutrient addition effects (+NP *vs.* -NP) on the sporulation rates of aquatic hyphomycetes. Separate analysis were performed on organic (*Platanus acerifolia* and *Populus nigra* leaves) and inorganic (sand and rocks) substrata (Ver. 12.0, SPSS Inc. 2003). A principal component analysis (PCA) on square root-transformed aquatic hyphomycete

taxon data was used to reveal the ordination trends of the substrata using CANOCO Ver. 4.5 (ter Braak and Smilauer, 2002).

Results

Effect of nutrients on sporulation rates and SEM

Sporulation rates differed between the two leaf species but not between epipsammic and epilithic biofilms (Table 9.2). Rates measured on the organic substrata (leaves) were one order of magnitude higher than those measured on the inorganic substrata (epipsammic and epilithic biofilms). Overall, *Platanus acerifolia* leaves showed the highest sporulation rates (ANOVA Table 9.2, Figure 9.1). Long-term enrichment experiment only affected the sporulation on the inorganic substrata, while short-term nutrient addition did not generate any significant effect (Table 9.2).

Sources	Sporulation rates
Substrata (S)	$F_{3,32} = 168.75$
	$\mathbf{P < 0.0001}$
Long-term Enrichment (E)	$F_{1,32} = 8.26$
	$\mathbf{P < 0.01}$
Short-term Nutrient Addition (N)	$F_{1,32} = 0.20$
	$P = 0.656$
S x E	$F_{3,32} = 8.16$
	$\mathbf{P < 0.0001}$
S x N	$F_{3,32} = 1.23$
	P = 0.316
E x N	$F_{1,32} = 0.66$
	$P = 0.423$
S x E x N	$F_{3,32} = 6.10$
	$\mathbf{P < 0.005}$

Table 9.2. Results of an analysis of variance considering the factors substrata (sand, rocks and the Platanus acerifolia and Populus nigra leaves), long-term reach enrichment (unenriched vs. enriched) and short-term nutrient addition (-NP vs. +NP) on sporulation rates. F-ratios and degrees of freedom are shown and the significant probabilities (P) are marked in bold.

The lower sporulation rates registered in epipsammic and epilithic biofilms from the enriched reach were mainly due to significantly lower rates in the epipsammic samples (Table 9.2, S × E effects, Figure 9.1), while no clear differences in sporulation

rates were detected for the epilithic samples. Although the effect of short-term nutrient treatment was not significant, a slight increase in sporulation rates for the + NP treatment was detected for most of substrata (Table 9.2, Figure 9.1) in the unenriched reach, except in epilithic biofilms.

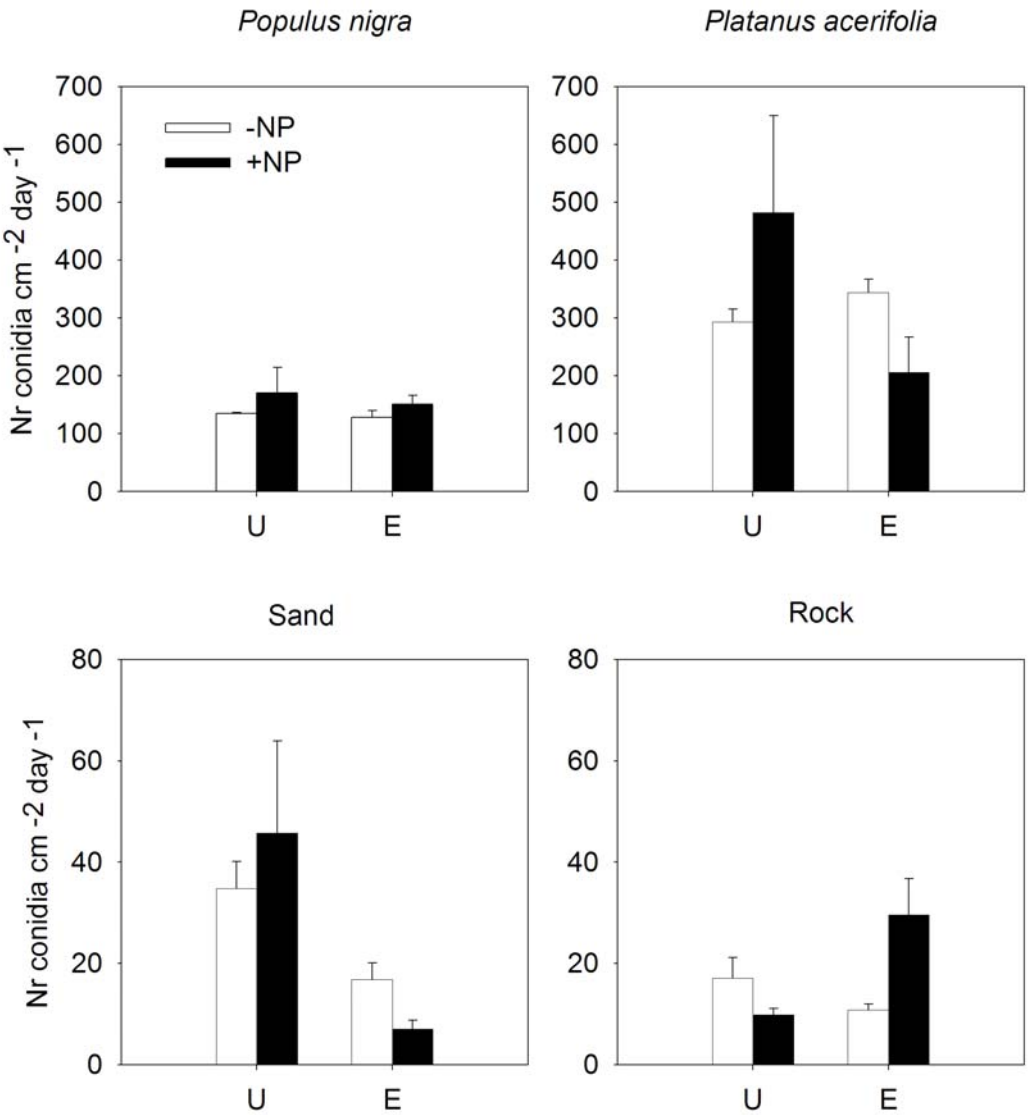


Figure 9.1. Sporulation rates of aquatic hyphomycetes associated with the four stream substrata in the two short-term nutrient treatments [low nutrient concentrations (-NP) and high nutrient concentrations (+NP)] in the unenriched (U) and enriched (E) stream reaches. Means and SE are shown (n=3).

SEM photographs confirmed that aquatic hyphomycetes were common on leaves. Fungal hyphae colonized inside and outside the leaf tissue (Figure 9.2 A & B). However, fungal hyphae were also seen in epipsammic and epilithic biofilms (Figure 9.2 C & D). Fragmented hyphae forming chains of arthroconidia were observed in epilithic biofilms (Figure 9.2 D).

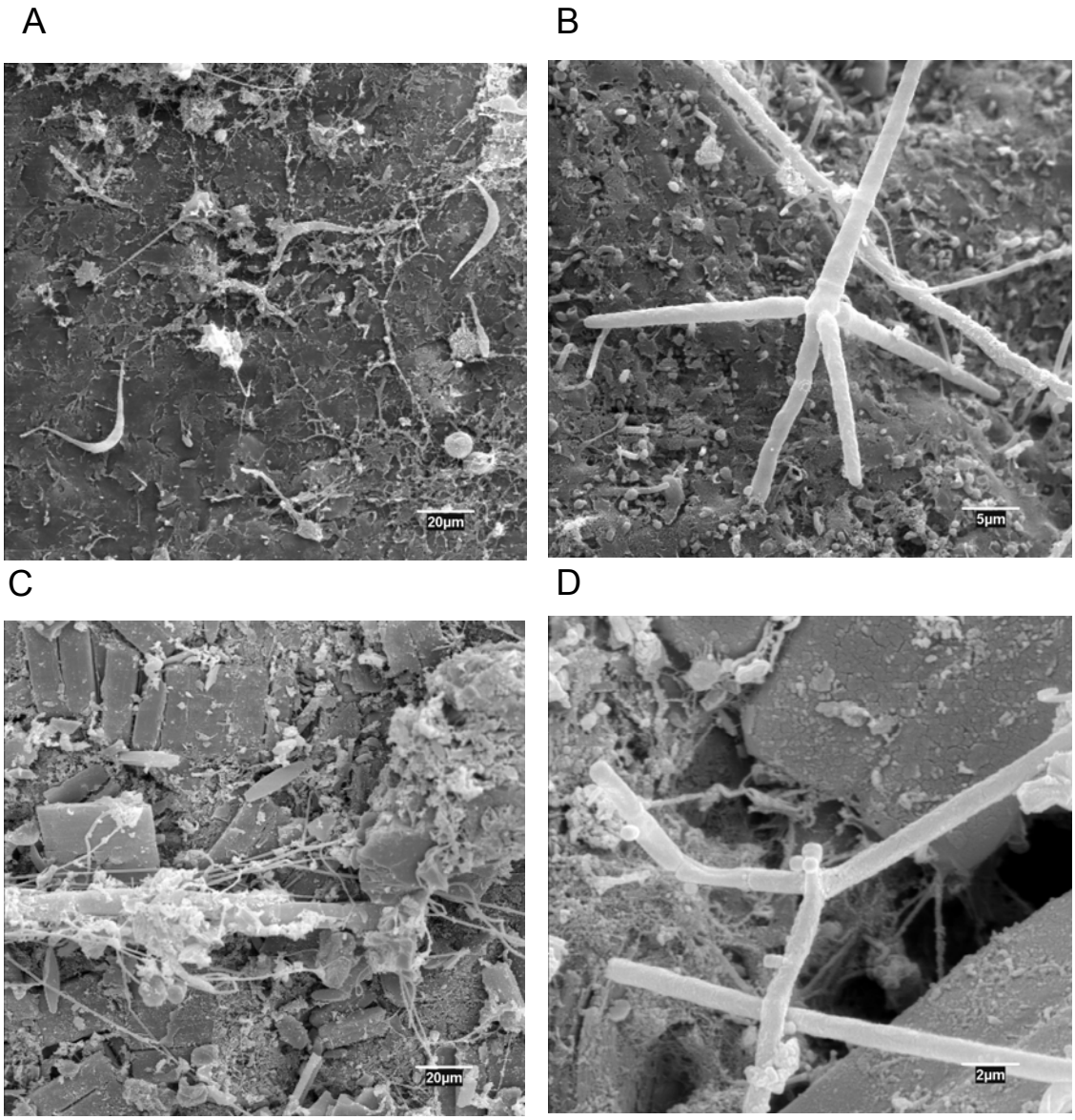


Figure 9.2. SEM photographs of the four incubated stream substrata from the unenriched reach. A) overview on Populus nigra leaves surface with attached conidia of Lunulospora curvula, (B) detail of Trisceloporus sp. conidia shortly before its detachment in Platanus acerifolia leaves, (C) group of hyphae surrounding a filament of algae and embedded into extrapolymeric substances on a sand grain, and (D) fragmented hyphae forming a chain of arthroconidia in the epilithic biofilm.

Effect of nutrients on species diversity

The average species richness was higher in leaves (11 species) than in epipsammic and epilithic communities (7 species). *Helsicella stellata* showed the highest sporulation rates in leaves (132.4 +/- 47.4 conidia cm^{-2} day^{-1} and 46 +/- 5.4 conidia cm^{-2} day^{-1}, in *Platanus* and *Populus*, respectively) while *Alatospora acuminata* prevailed in epipsammic and epilithic biofilms (1.9 +/- 0.4 and 1.5 +/- 0.3 cm^{-2} day^{-1}, respectively).

The principal component analysis (PCA) showed two biologically meaningful directions for the ordination of hyphomycete species (Figure 9.3).

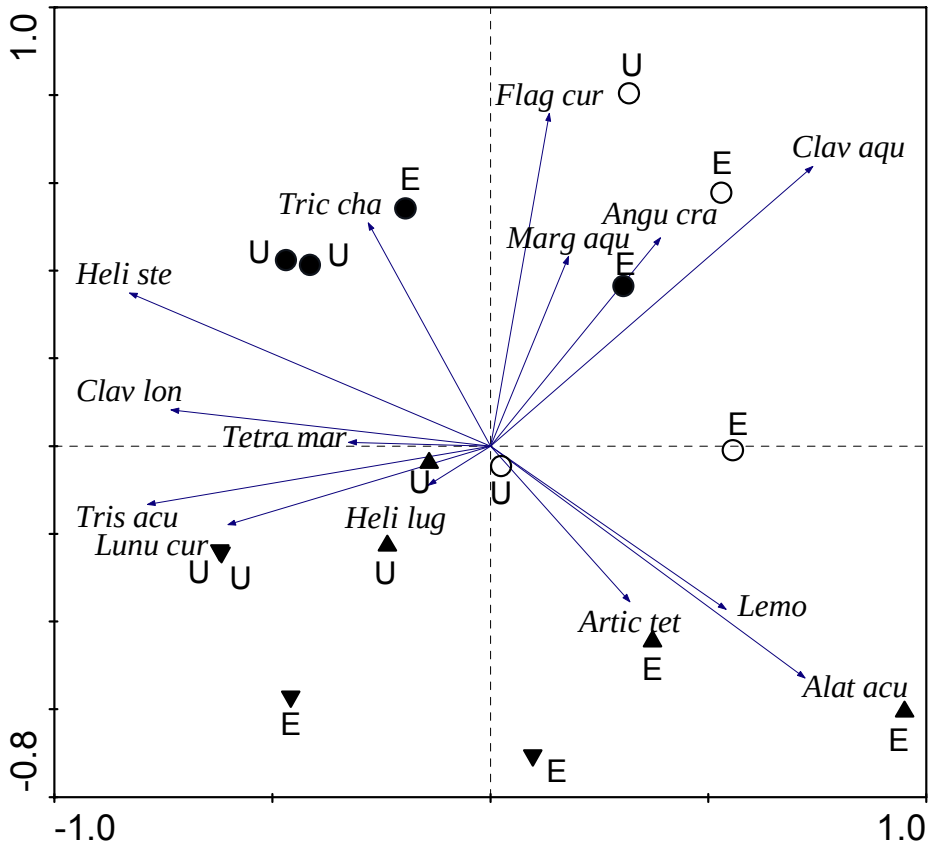


Figure 9.3. Plot of the first and second axes of the PCA carried out with the hyphomycete species data associated with the two short-term nutrient treatments of the four substrata colonized in the unenriched and the enriched stream reaches. Identification of acronyms [unenriched reach (U), enriched reach (E)] and symbols [(Platanus acerifolia (●), Populus nigra (○), episammic biofilms (▲) and epilithic biofilms (▼)] used for samples are shown. Species acronyms (Alat acu – Alatospora acuminata; Angu cra – Anguillospora crassa; Artic tet – Articulospora tetracladia; Clav aqu – Clavariopsis aquatica; Clav lon – Clavatospora longibrachiata; Flag cur – Flagellospora curvula; Heli ste – Heliscella stellata; Heli lug – Heliscus lugdunensis; Lemo – Lemonniera sp.; Lunu cur – Lunulospora cuvula; Marg aqu – Margaritispora aquatica; Tetra mar – Tetracladium marchalianum; Tric cha – Tricladium chaetocladium; Tris acu – Triscelophorus acuminatus) are shown in the same plot.

The first component explained the 43.12 % of the variance and arranged the species on the basis of origin (unenriched *vs.* enriched, Figure 9.3). Taxa from the unenriched reach (*Heliscella stellata*, *Trisceloporus acuminatus*, *Clavatospora longibrachiata* and *Lunulospora curvula*) were separated from those inhabiting the enriched reach (*Clavariopsis aquatica*, *Alatospora acuminata* and *Lemonniera sp.*; Table 9.3). Differences in species diversity during short-term nutrient treatments were not consistent.

The second component (18.17 % of the variance, Figure 9.3) separated species on the basis of preferential substrate for colonization (*Platanus* and *Populus* leaves and epipsammic and epilithic biofilms). *Flagellospora curvula*, *Clavariopsis aquatica* and *Trisceloporus acuminatus* occurred mostly on leaf substrata while *Alatospora acuminata* was more characteristic in sandy and rocky habitats (Table 9.3). *Heliscus lugdunensis* had a very low abundance and was detected only on sand.

	Factor 1	Factor 2
Alatospora acuminate (Ingold)	**0.721**	**-0.527**
Anguillospora crassa (Ingold)	0.390	0.474
Articulospora tetracladia (Ingold)	0.320	-0.354
Clavariopsis aquatica (De Willd.)	**0.739**	**0.638**
Clavatospora longibrachiata (Ingold, Marvanová & S. Nilsson)	**-0.732**	0.082
Flagellospora curvula (Ingold)	0.135	**0.758**
Heliscella stellata (Ingold & Cox, Marvanová)	**-0.828**	0.349
Heliscus lugdunensis (Sacc. & Thérry)	-0.143	-0.09
Lemonniera sp.	**0.54**	-0.372
Lunulospora cuvula (Ingold)	**-0.602**	-0.179
Margaritispora aquatica (Ingold)	0.179	0.432
Tetracladium marchalianum (De Wild.)	-0.325	0.009
Tricladium chaetocladium (Ingold)	-0.281	**0.509**
Trisceloporus acuminatus (Ingold)	**-0.787**	-0.133
Eigenvalues	0.431	0.182
Explained variance	43.1	18.2

Table 9.3. Factor loadings, eigenvalues and explained variance of the principal components analysis performed with aquatic hyphomycete taxa from the Fuirosos substrata samples. Highest loading factors (larger than 0.5) at axes extremes (positive and negative) are marked in bold.

Discussion

The long-term nutrient enrichment experiment in the Fuirosos stream produced changes in the fungal community composition but did not significantly affect the potential sporulation rates of aquatic hyphomycetes, except for a slight decrease in those from epipsammic biofilms. Short-term nutrient additions caused only a slight increase in sporulation rates on most substrata collected in the unenriched reach. Major differences in fungal community structure and reproductive capacity were due to the type of benthic substratum.

In general, the sporulation rates on leaf litter in this experiment are lower than those reported in other studies (Srihdar & Bärlocher, 2000; Pascoal & Cassio, 2004; Gulis et al., 2006). This difference is probably because we measured these rates after the expected sporulation peak (1-2 week of leaves immersion). Typically, fungal biomass and conidia production on decomposing leaves tend to decrease through time after they peak (Abelho & Graça, 2006; Grattan & Suberkropp, 2001). However, the simultaneous measurements of sporulation from all substrata required at least 6 weeks of colonization to develop a stable community on clay tiles (Sabater & Romaní, 1996). Consistent differences in the fungal community and its sporulation rates were observed between the substrata analyzed, especially between those on leaves (*Platanus acerifolia* and *Populus nigra* leaves) and communities inhabiting sand and rocks. The major relevance of the leaf substrata for the development of stream fungal communities is reflected by the greater species richness and sporulation rates. Other studies have reported distinct species composition in communities colonizing leaf species that greatly differ in their physical and chemical characteristics (Gulis, 2001; Ferreira et al., 2006). In contrast, differences between the fungal communities of *Platanus* and *Populus* leaves were detected only for sporulation rates and not for species composition. The higher sporulation in *Platanus acerifolia* leaves might be related to their physical-chemical traits, such as the higher initial N content (lower C:N molar ratio) and slower decrease in phosphorus than in *Populus nigra* leaves during decay (Gulis & Suberkropp, 2003a; Artigas unpublished data). Although fungal hyphae attachment and growth on inorganic substrata is not optimal considering their colonization strategy (mycelial growth), production of spores on these substrata has been detected. The possibility that the spores on these substrata could be deposited from the water column during the colonization, and then resuspended during the incubations in the laboratory, was reduced by carefully cleaning substrata with sterile water. Furthermore, inorganic substrata hold a distinct fungal community to that developed on leaves (the former characterized by *Alatospora acuminata*), which might be important in decomposition of fine detritic material accumulated on these substrata

(Chapter 2). Scanning electron microscopy observations revealed that fungal hyphae mainly colonize the leaf tissue, but sporadically present in epipsammic and epilithic communities. However, our results indicate that fungal mycelia accumulation and/or activity in epilithic and epipsammic biofilms is minor compared to those on leaves. Previous analysis comparing the fungal biomass accumulated on different stream substrata (autumn-winter 2003) revealed that accumulation in leaves (1876.4 +/- 242.4 μg fungal C cm^{-2}) was 5 to 14 times higher than that observed on sand and rocks (336.2 +/- 39.1 and 125.1 +/- 63.3 μg fungal C cm^{-2}, respectively).

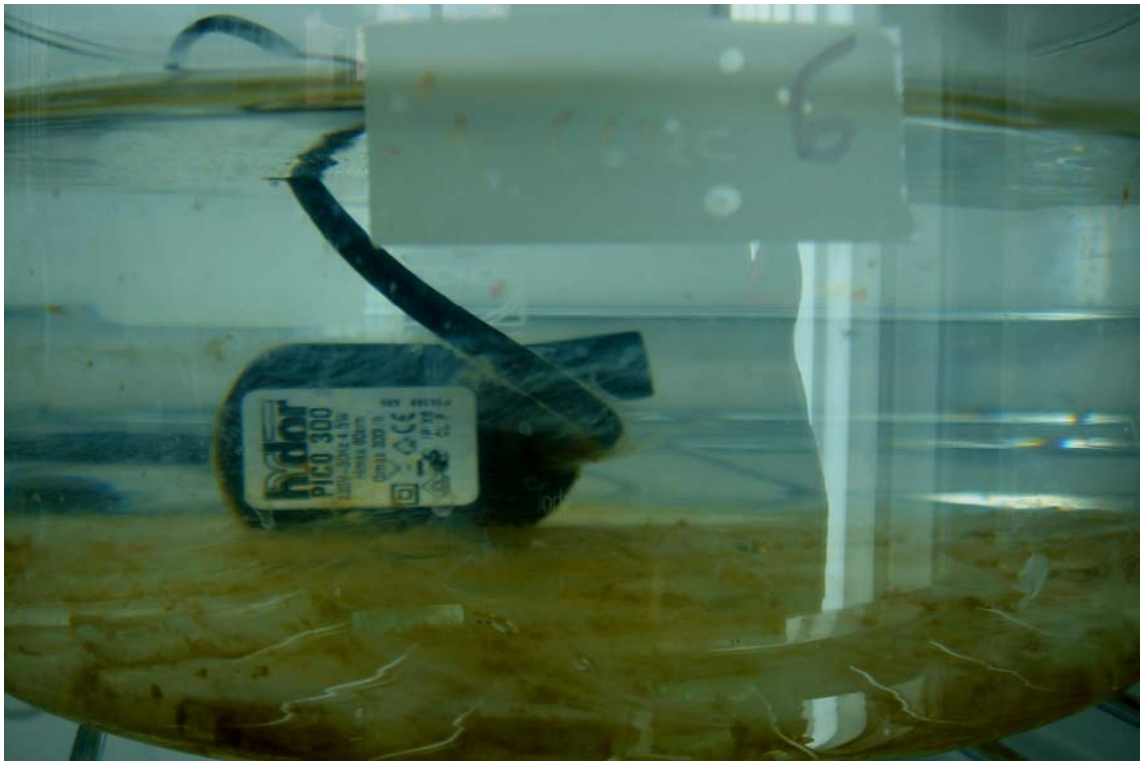
Most field and laboratory studies have emphasized that inorganic nutrients (mainly nitrate and phosphate concentrations) increase conidia production by aquatic hyphomycetes to a greater extent than biomass accumulation and rates of leaf litter (Suberkropp & Chauvet, 1995; Weyers & Suberkropp, 1996; Srihdar & Bärlocher, 2000; Grattan & Suberkropp, 2001). However, Robinson & Gessner (2000) observed a null effect of nutrients on the biomass accumulation and sporulation of hyphomycetes in alder (*Alnus viridis*) leaves. Neither did Royer & Minshall (2001) found an effect of nutrient enrichment on the decomposition rates in three leaf species. Nutrient availability may produce distinct effects on the biomass and activity response of microbes (bacteria and fungi) in streams (Stelzer et al., 2003; Tank & Dodds, 2003). In the Fuirosos stream, long-term enrichment did not significantly affect the sporulation rates in most of the substrata analyzed. There are two possible explanations to this observation: i) bacteria were stimulated by nutrients, competed with fungi and negatively affected aquatic hyphomycete sporulation, or ii) saturating nutrient concentrations that met fungal demands were already present in the unenriched reach, and further nutrient additions did not increased fungal sporulation. The non-significant increase or even decrease in the sporulation rates might be explained by the competition between fungi and bacteria during the decomposition of leaf litter (Romaní et al., 2006). Bacteria may also take advantage of simple compounds released during the fungal decomposition of recalcitrant plant polymers (Velicer, 2003). During the enrichment experiment, bacterial biomass has significantly increased in epilithic biofilms from the enriched reach (from 0.3 +/- 0.5 μg bacterial C cm^{-2} in the unenriched up to 1.4 +/- 0.2 μg bacterial C cm^{-2} in the enriched reach) while fungal biomass was not significantly different between reaches (196.2 +/- 26.6 and 232.2 +/- 50.8 μg fungal C cm^{-2} in the unenriched and enriched reaches, respectively) (Artigas unpublished). These indirect observations cannot rule out that relatively high nitrate levels at the unenriched reach may explain the null effect of nutrient additions on the fungal activity. Microbial N demands can be fulfilled at relatively low levels of dissolved nitrate

(Ferreira et al., 2006) and further increases in the nutrient availability (experimental nutrient additions) may not result in enhanced fungal activity.

The fungal community in the enriched reach, although having similar potential sporulation rates to that in the unenriched reach, showed differences in species composition, probably as an adaptation both to higher nutrient availability and to the changes in the benthic community structure (higher bacterial biomass). The hyphomycete species identified on substrata of the unenriched reach coincided with the taxa that Gessner et al. (1993) attributed to colonization in mature and late successional stages of leaf decomposition (i.e: *Clavatospora logibrachiata* and *Heliscella stellata*). In the enriched reach, *Clavariopsis aquatica*, *Lemmoniera* sp. and *Alatospora acuminata* were characteristic. The differential adaptation of species to the nutrient conditions of each stream reach is also supported by the differential responses to the short-term nutrient addition observed. This was especially clear for epipsammic biofilm in the unenriched reach. Those substrata showed a slight increase in sporulation rate with nutrient addition that was not observed in those of the enriched reach.

On the basis of our results, we conclude that the effect of long-term stream enrichment on the fungal community greatly depends on the characteristics of the stream ecosystem, "i.e." basal nutrient concentrations (e. g. nitrate), benthic organic matter availability, and especially the proportion of benthic substrata (sand, rocks, leaves), which would determine the fungal community structure. Our results show that long-term enrichment produces changes in the species composition of the fungal community. However, when fungal demand for dissolved inorganic nutrients (nitrate) is already met, further increases in available nutrients may not significantly enhance fungal activity on the different stream benthic substrata.

Chapter 8



Effect of water nutrient concentration and N:P molar ratio on biofilm structure and metabolism

Abstract

The response of epilithic biofilms to changes in water nutrient concentrations and N:P molar ratio were performed in a mesocosm experiment using artificial glass substrata. Mature biofilms grown in the field at similar nutrient conditions were exposed to four water treatments N:P= 16:1 and N:P= 56:1, each at high (HN) and low (LN) nutrient concentrations during 35 days. Biofilms were analyzed for algal and bacterial biomass, extracellular polysaccharides (EPS) and C, N and P content, as well as for three extracellular enzyme activities (cellobiohydrolase, leucine-aminopeptidase, phosphatase) during seven times. Water nutrient concentration had positive effects on algal and bacterial biomass accrual in biofilms, as well as on the stimulation of peptidase activity. The increase of bacterial peptidases suggested a preferential use of nitrogenous compounds produced mostly by algae within the biofilm. Instead, the increase of water N:P ratio (56:1 treatments) reduced bacterial biomass and stimulated phosphatase activity. The sensitivity of bacterial biomass to the water N:P imbalance (low P availability) was stronger than that experienced by algae. Nevertheless, the algal response to high water N:P consisted on the EPS production. Contrary to our predictions, the interaction between the two factors (nutrient concentration and N:P ratio) was not statistically significant for any of the biofilm analyzed parameters. This suggested that each factor had independent effects in modulating biofilm structural and metabolic parameters. Less than 35 days were enough to modify extracellular enzyme activities (day 7) and microbial biomass (day 3 for bacteria and day 21 for algae) in biofilms, while nutrient molar ratios (C:N and C:P) remained stable for longer (until day 28). Biofilm C:N ratio decreased according to the water nutrient concentration, suggesting a preferential uptake of N than P by microbes. However, biofilm C:P responded only to changes in the water N:P. The fast response of biofilm structural and metabolic parameters to changes in water nutrient availability suggested a high dependence between biofilms and nutrients in the overlying water.

Introduction

Over the last four decades, eutrophication has been mostly referred to human processes (i. e. agricultural runoff, domestic sewage and food processing industries) that lead to deterioration of freshwater environments (Tusseau-Vuillemin, 2001). Eutrophication in streams is mainly understood as a process of nitrogen (N) and phosphorus (P) enrichment (Smith, 2003). Most enrichment experiments have been focused on the effect of P limitation in the river periphyton (Mohamed et al., 1998; Stelzer & Lamberti, 2001; Dodds, 2003) since it is deficient for biological communities in comparison to other major biological elements (Hutchinson, 1957). However,

periphyton may be also limited by N depending on the type of stream (Tank & Dodds, 2003).

Several studies have been focused on the effect of nutrients in algal-bacterial interactions in biofilms (Rier & Stevenson, 2001; Niyogy et al., 2003; Carr et al., 2005). In general, the biomass of both groups increase when dissolved nutrient concentrations in the water are increased (Brochardt, 1996; Francoeur et al., 1999; Rier & Stevenson, 2006), though algal-bacterial production may be decoupled under high nutrient conditions (Scott et al. 2008). Bacteria are more efficient than algae for the dissolved phosphorus uptake, especially when dissolved phosphate concentrations in the water are low (Currie et al., 1986). However, bacterial utilization of phosphorus may be limited by the availability of dissolved organic carbon (DOC; Cotner & Wetzel, 1992). An early study of Haack & McFeters (1982), demonstrated that algae may supply bacteria with DOC resulting from excretion processes during photosynthesis (exudates) and after algal cell death in biofilms. Espeland et al. (2001) observed that several extracellular enzyme activities involved in the polysaccharide degradation (α-glucosidase, β-glucosidase, β-xylosidase) can be stimulated by algal photosynthesis (see also Romaní & Sabater, 2000). The response of biofilms to the changing nutrient conditions might be modulated also by changes in biofilm structural characteristics (such as extracellular polymeric substances) which might buffer nutrient changes at the flowing water (Freeman & Lock, 1995; Lawrence & Neu, 2003).

Few studies have evaluated the effect of water N:P in biofilms and most of them evaluate its effect on algae and bacteria, separately (Mohamed et al., 1998; Stelzer & Lamberti, 2001). It is hypothesized that low nutrient concentrations and high N:P ratio (low P availability) in the water would limit biofilm development, rather than the sole effect of decreasing nutrient concentrations. Moreover, the changes in water N:P ratio might alter biofilm stoichiometry and the metabolic processes associated (i. e. OM cycling; Frost et al., 2002; Cross et al., 2005). The importance to combine changes in water nutrient concentration and N:P ratios are relevant to understand biofilm functioning in systems where nutrient inputs are imbalanced (i. e. streams nearby food processing industries, wastewater treatment plants).

In this study, mature biofilms grown at similar nutrient conditions were subjected to changes in the water nutrient concentration (high and low) and N:P molar ratio (16:1 and 56:1). The study aims to evaluate the effect of changes in water nutrient concentration (*C*) and N:P ratio (*R*) on biofilm structural and metabolic parameters and to determine whether an interaction effect between these two factors exist ($C \times R$). Specifically, the following three questions were asked: i) Did exist interaction between the changes in water nutrient concentration and N:P ratio for any of the biofilm

analyzed parameters?, ii) How the relationships between nutrient molar ratios in the water and in the biofilm vary during the experiment?, and iii), Were changes in nutrient concentrations and N:P ratio affecting algae and bacteria in different manner?

Materials and methods

Mesocosms experiments

Sandblasted glass tiles of 1 cm^2 surface area (from now referred as biofilm samples) were collected after two months colonization at the Fuirosos stream and transported cold (4ºC) to the laboratory. Once there, biofilm samples were placed in mesocosms (Schott Duran, Germany, *c*. 2 L) and incubated under four different nutrient treatments: i) N:P= 16:1 and high nutrient concentration (16:1-HN; 750 $\mu g\ L^{-1}$ $N\text{-}NO_3^-$ and 101 $\mu g\ L^{-1}$ $P\text{-}PO_4^{3-}$), ii) N:P= 16:1 and low nutrient concentration (16:1-LN; 75 $\mu g\ L^{-1}$ $N\text{-}NO_3^-$ and 10.1 $\mu g\ L^{-1}$ $P\text{-}PO_4^{3-}$), N:P= 56:1 and high nutrient concentration (56:1-HN; 750 $\mu g\ L^{-1}$ $N\text{-}NO_3^-$ and 27 $\mu g\ L^{-1}$ $P\text{-}PO_4^{3-}$), and iv) N:P= 56:1 and low nutrient concentration (56:1-LN; 75 $\mu g\ L^{-1}$ $N\text{-}NO_3^-$ and 2.7 $\mu g\ L^{-1}$ $P\text{-}PO_4^{3-}$). Three mesocosm replicates were considered for each treatment (12 mesocosms in total). A recirculating pump (Hydor Pico 300, 300 L h^{-1}, Italy) was submerged in each mesocosms to achieve similar water current conditions than that observed in the field. In order to maintain nutrient levels in the mesocosm, water was replaced each 3 days. It was previously tested that after 3 days phosphorus concentration decreased at the mesocosms. The 12 mesocosms were placed inside an incubator (AGP-570 Radiber, Spain) in order to maintain daily temperature and light conditions at 14 hours of light (103.8 ± 0.4 μmol photons $m^{-2}\ s^{-1}$) and 18 ± 0.5 ºC and 10 hours of darkness (0 μmol photons $m^{-2}\ s^{-1}$) and 16 ± 0.5 ºC.

The experiment lasted 35 days and biofilm samples were recovered at days 1, 3, 7, 14, 21, 28 and 35. At each date, chlorophyll-*a* concentration and bacterial density (sections 2.2.2 and 2.2.3), carbon, nitrogen and phosphorus content (section 2.4.1) and extracellular polysaccharide content in EPS (section 2.4.2) were analyzed in the biofilms. Also, three enzyme activities involved in the C (cellobiohydrolase activity), N (leucine-aminopeptidase activity) and P (phosphatase activity) organic compounds decomposition were assayed in biofilm samples (section 2.6.1). Each biofilm parameter was analyzed in triplicate for each water treatment (one tile from each mesocosm, except for the extracellular enzyme activities where two tiles from each mesocosm were considered). Results were expressed in terms of biofilm surface area (cm^2).

Statistical analyses

Repeated measures analyses of variance (ANOVA) was used to analyze the effect of water nutrient concentration (*C*) and N:P ratio (*R*) on the structural (microbial biomass, C, N, P content and EPS) and metabolic (enzymatic activities) parameters of biofilms. All data were log transformed (X = log (X+1)) before statistical analysis and Dunn-Sidak correction was applied on the probability values (*P values*) of the whole sources analyzed. Correaltion (n=28) between structural and metabolic variables of biofilms during incubations were analyzed through the product-moment Pearson coefficient. All statistical analyses were performed using SPSS for Windows (Ver. 12.0, SPSS Inc. 2003).

Results

Algal and bacterial biomass in biofilms

Biomass of algae was affected by changes in the water nutrient concentration (*C* effect), while bacteria respond to changes in nutrient concentration and N:P ratio (*C* and *R* effect, Table 10.1, Figure 10.1). Highest algal and bacterial biomass was detected in biofilms subjected to high nutrient concentration treatments (HN). The biomass of both groups was positively correlated with N and P concentrations in the water during the experiments (Table 10. 2). However, bacterial biomass was also decreased in the 56:1 treatments (low P availability in the water). The fastest decrease in bacterial biomass was detected in the 56:1 and low nutrient concentration treatments (56:1-LN, Figure 10.1 b). The response of bacteria to changes in nutrient availability (day 3) was faster than that observed by algae (day 21).

Nor algal neither bacterial biomass in the biofilm was affected by the interaction between water nutrient concentration and N:P ratio during biofilm treatments.(*C* × *R* effect, Table 10.1). Therefore, each factor had independent effects in modulating microbial biomass in biofilms. Differences between field and laboratory conditions probably caused that algal and bacterial biomass tended to decrease during experiments (*T* effect, Table 10.1).

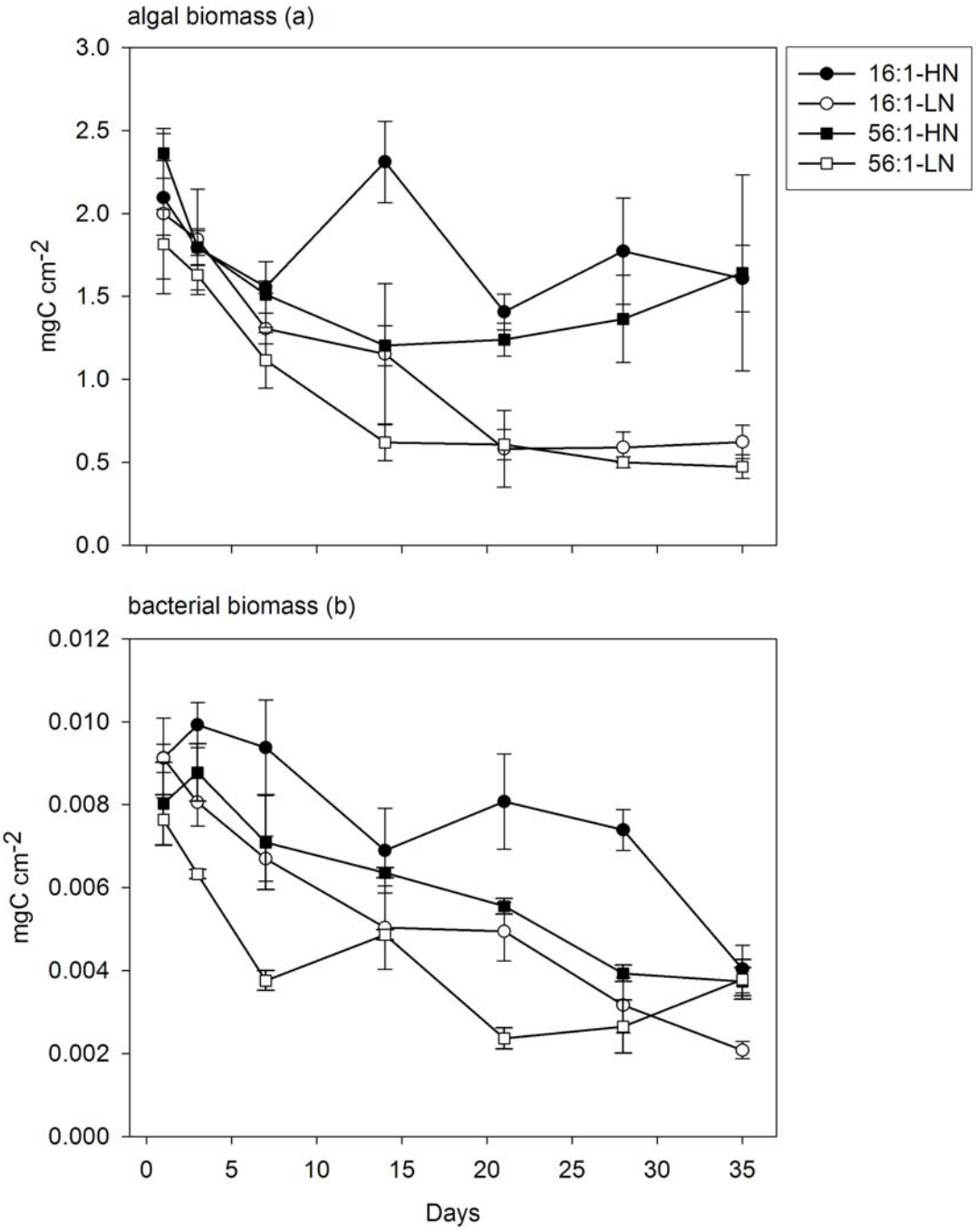


Figure 10.1. Biomass of algae (a) and bacteria (b) in terms of biofilm surface area. The four nutrient treatments 16:1 and 56:1, each at high (HN) and low (LN) nutrient concentrations are represented in the plot. Values are means (n=3) and SE of the seven sampling dates.

Parameter	Sources of variation	*P*	Sources of variation	*P*
bacterial C	Ratio (*R*)	**< 0.01**	Time	**< 0.01**
	Concentration (*C*)	**< 0.01**	Time × *R*	1
	R × *C*	0.993	Time × *C*	1
			Time × *R* × *C*	0.995
algal C	Ratio (*R*)	1	Time	**< 0.01**
	Concentration (*C*)	**<0.05**	Time × *R*	1
	R × *C*	1	Time × *C*	**< 0.01**
			Time × *R* × *C*	1
C:N biofilm	Ratio (*R*)	1	Time	**< 0.05**
	Concentration (*C*)	**< 0.05**	Time × *R*	1
	R × *C*	1	Time × *C*	1
			Time × *R* × *C*	1
N:P biofilm	Ratio (*R*)	0.155	Time	1
	Concentration (*C*)	1	Time × *R*	1
	R × *C*	1	Time × *C*	1
			Time × *R* × *C*	1
C:P biofilm	Ratio (*R*)	**< 0.01**	Time	0.320
	Concentration (*C*)	0.940	Time × *R*	0.590
	R × *C*	1	Time × *C*	1
			Time × *R* × *C*	1
EPS content	Ratio (*R*)	**< 0.01**	Time	**< 0.01**
	Concentration (*C*)	0.546	Time × *R*	0.397
	R × *C*	0.950	Time × *C*	0.397
			Time × *R* × *C*	0.286
phosphatase	Ratio (*R*)	**< 0.01**	Time	**< 0.01**
	Concentration (*C*)	1	Time × *R*	**< 0.05**
	R × *C*	1	Time × *C*	0.325
			Time × *R* × *C*	1
peptidase	Ratio (*R*)	1	Time	**< 0.01**
	Concentration (*C*)	**< 0.01**	Time × *R*	0.462
	R × *C*	1	Time × *C*	0.943
			Time × *R* × *C*	0.462
cellobiohydrolase	Ratio (*R*)	1	Time	**< 0.01**
	Concentration (*C*)	1	Time × *R*	0.362
	R × *C*	1	Time × *C*	0.519
			Time × *R* × *C*	1

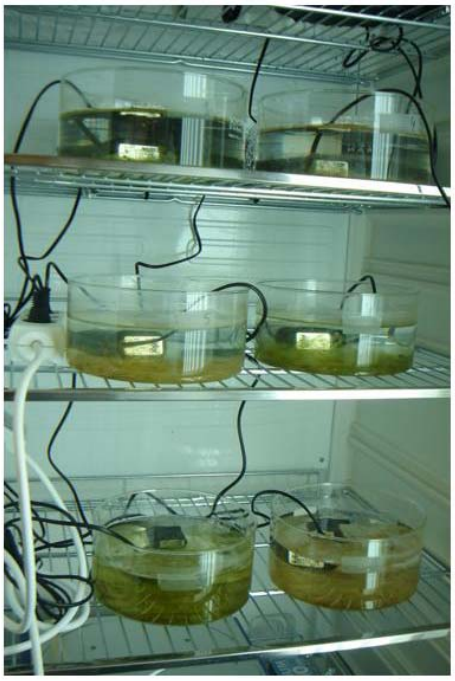
Table 10.1. Repeated measures ANOVA results considering the effect of water nutrient concentration (C) and nutrient ratios (R) on biofilm structural and metabolic parameters. Probability within groups of all the sources analyzed was corrected for sphericity by the Greenhouse-Geisser correction and P-values adjusted by the Dunn-Sidak correction. Values < 0.05 and < 0.01 are indicated in boldface type.

CNP and EPS content of biofilms

C:N molar ratio of biofilms was affected by the nutrient concentration (*C* effect), while C:P ratio changed according to the water N:P (*R* effect, Table 10.1). The increase of water nutrient concentrations (HN treatments) provoked an increase in biofilm N content and the consequent decrease in biofilm C:N (Figure 10.2 a). The high N:P treatments (56:1) reduced P content of biofilms and increased C:P ratio (Figure 10.2 c). Changes in C:N and C:P ratios of biofilms were detected after 28 days of exposure (Figure 10.2 a & c). The interaction between the two factors (*R* × *C*) was not affecting biofilm nutrient molar ratios (Table 10.2).

Biofilm N:P was not significantly altered by the nutrient treatments, though adapted rather well to water N:P at the end of the experiment (19.8 +/- 2.9 for the 16:1 treatment and 44.7 +/- 9.6 for the 56: treatment, Figure 10.2 b). Relationships between the water and the biofilm N:P ratio were observed during experiments (Table 10.2). P concentration in the water was tightly related to the biofilm P content.

EPS content of biofilms increased in all water treatments (Time effect, Table 9.1; Figure 9.3), though the highest increase was detected in the 56:1 treatments (*R* effect, Table 10.1). Low P concentration in the water was tightly related to the EPS increase in biofilms after 21 days of exposure ($r = -0.559$, $P < 0.005$).



Set of mesocosms inside the incubator

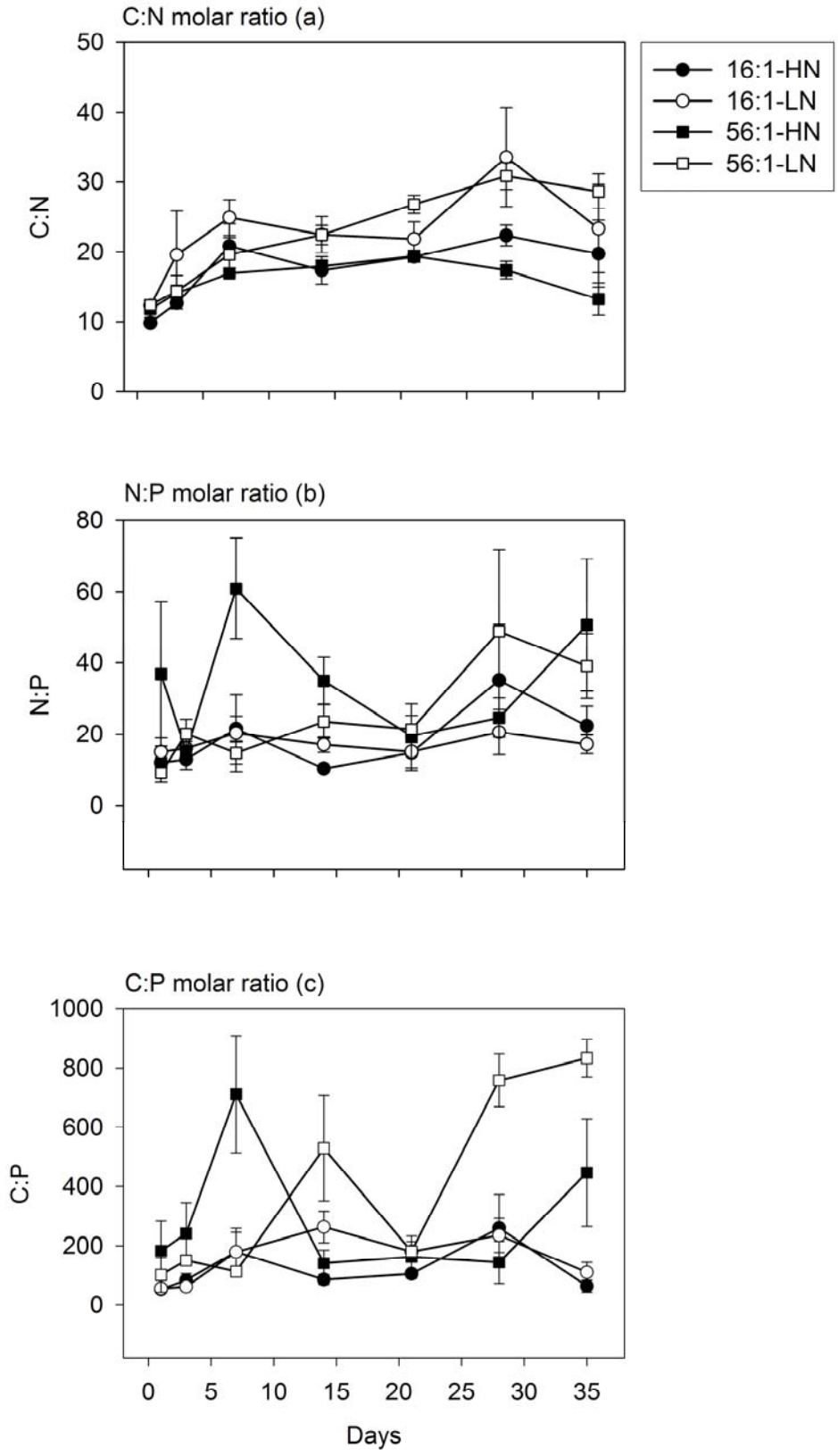


Figure 10.2. C:N (a), N:P (b) and C:P (c) molar ratios of biofilms subjected to the four water treatments during the 35 days of experiment. Values are means (n=3) and SE of the seven sampling dates.

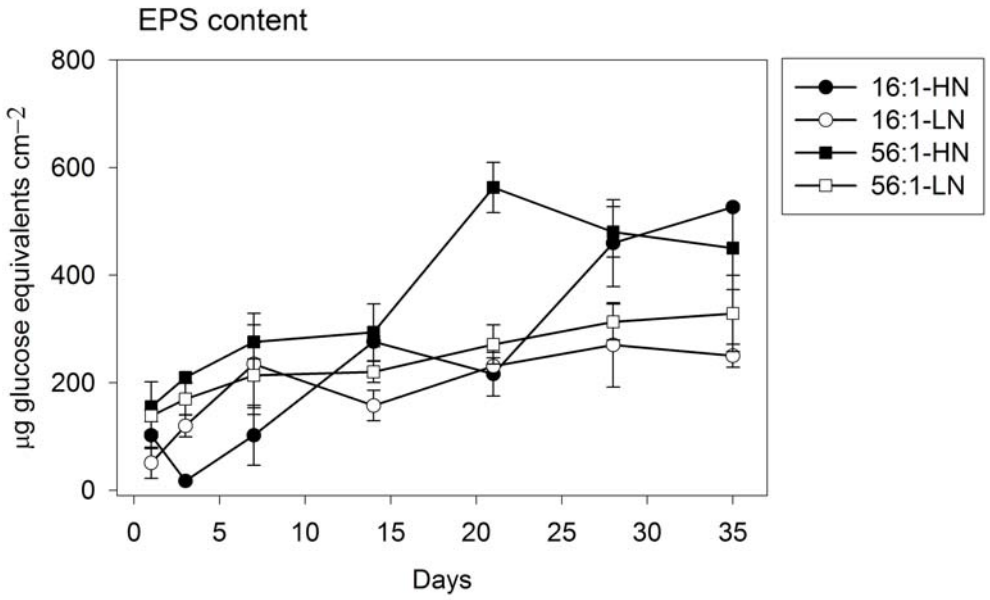


Figure 10.3. Extracellular polymeric substances (EPS) content of biofilms subjected to the four water treatments. EPS is expressed as the amount of glucose equivalents (extracted from standard glucose curves) per unit of biofilm surface area. Values are means (n=3) and SE of the seven sampling dates.

Extracellular enzyme activities of biofilms

The response of the enzyme activities to changes in water nutrient availability was faster than any other parameter analyzed in biofilms, changes were observed after 3 days of exposure. Phosphatase activity was affected by changes in water N:P ratio (*R* effect), while nutrient concentration had effects on leucine-aminopeptidase activity (*C* effect, Table 10.1, Figure 10.4). Conversely, cellobiohydrolase activity was not affected by the nutrient treatments. The increase of water N:P ratio (56:1 treatments) stimulated phosphatase activity in the biofilm because of the low P availability in the water (Figure 10.4 a). In contrast, leucine-aminoeptidase activity increased according to the nutrient concentration (Figure 10.4 b). The interaction between the two factors (*R* × *C*) was not affecting in the response of the enzyme activities analyzed (Table 10.1).

Nutrient composition of biofilms was tightly related to the expression of enzyme activities. Cellobiohydrolase activity and C:N ratio of biofilms increased accordingly, while biofilm P content explained variations in phosphatase activity (Table 10.2). Moreover, phosphatase activity was tightly related to the EPS accumulation and to the decrease of microbial biomass in biofilms.

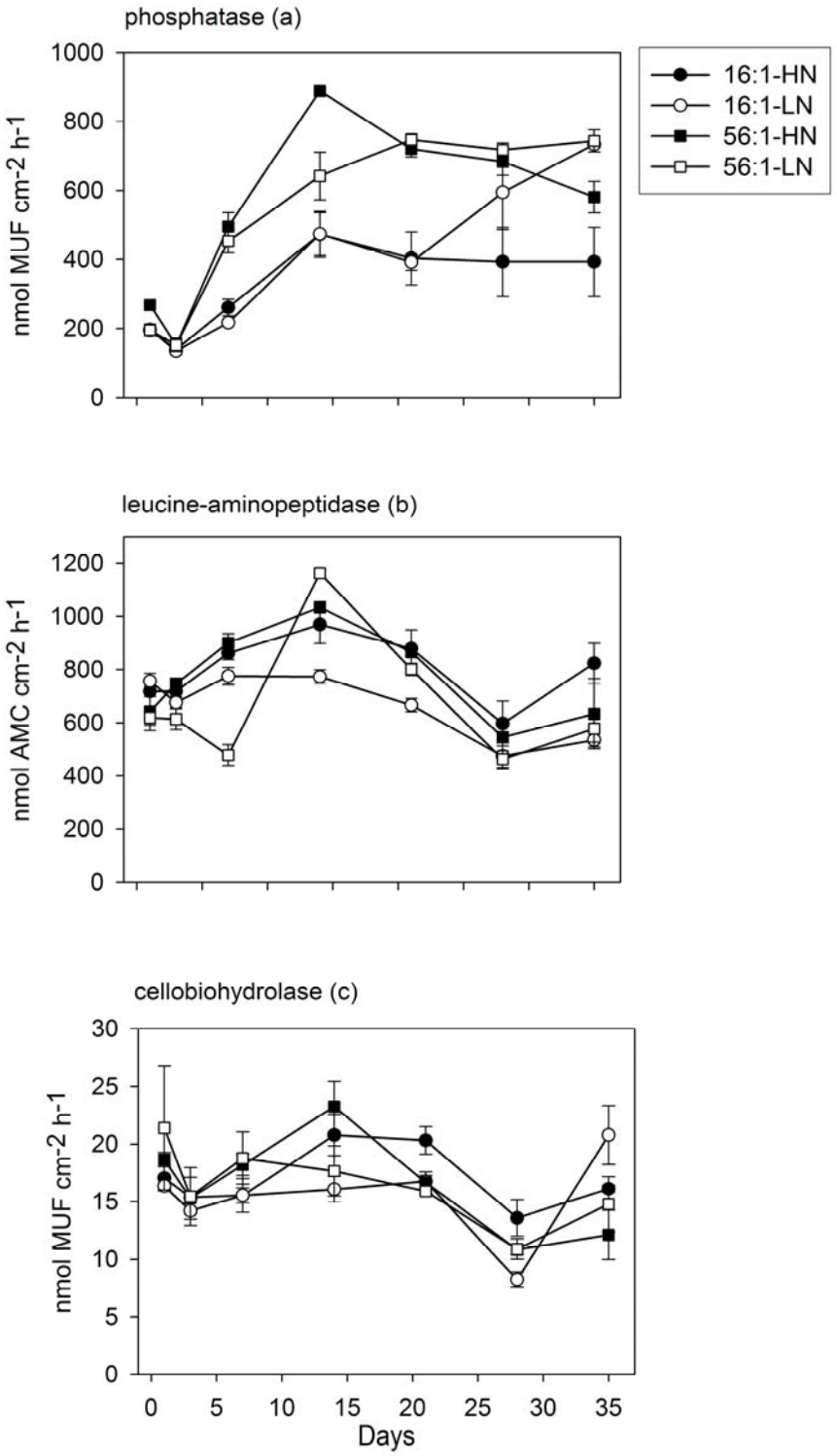


Figure 10.4. Phosphatase (a), leucine-aminopeptidase (b) and cellobiohydrolase (c) activity measured in biofilms subjected to the four water treatments. Phosphatase and cellobiohydrolase activities were expressed in nmol MUF (methylumbelliferone) while leucine-aminopeptidsae was expressed in nmol AMC (aminomethyl-coumarin). Mean values (n=6) and SE of the three enzyme activities at each sampling time are shown in the plots.

	N water	P water	N:P water	phosphatase	cellobiohydrolase
algal C	0.566 **	0.523 **	ns	-0.664 **	ns
bacterial C	0.442 *	0.497 **	ns	-0.739 **	ns
C:N biofilm	-0.491 **	ns	ns	ns	-0.401 *
N:P biofilm	ns	ns	0.440 *	0.392 *	ns
P biofilm	ns	0.412 *	-0.388 *	- 0.683 **	ns
EPS	ns	ns	ns	0.625 **	ns

Table 10.2. Perason's correlation coefficients between biofilm structural and metabolic parameters and between those parameters and water chemistry are shown. The analysis was performed considering four water treatments together (n= 28). Probability values are expressed as follows: P < 0.005 (**), P < 0.05 (*) and not significant correlations (ns).

Discussion

Changes in water nutrient concentration (nitrate plus phosphate) and N:P ratio modulate the structural and metabolic characteristics of stream biofilms. The present study confirmed that changes in water nutrient concentration affected algal and bacterial biomass and modulated peptidase activity in biofilms. Instead, changes in the water N:P ratio affected only bacterial biomass and stimulated phosphatase activity. Although early studies suggested about the irrelevance of water N:P affecting periphyton biomass and productivity (Bothwell, 1985; Stelzer & Lamberti, 2001), this study found that the N:P imbalance (N:P = 56) decreased drastically bacterial biomass. Recent studies support that the combined addition of C, N and P in the water increased algal biomass and reduced bacteria in biofilms, though the single addition of N (high water N:P) depress bacterial biomass in a higher rate (New et al., 2005). The response of bacteria to the nutrient treatments was faster than the observed for algae (first week for bacteria and third week for algae), though nutrient effects had a major persistence in controlling algal biomass. Contrary to our prediction, the interaction between the two factors (nutrient concentration and N:P ratio) was not statistically significant for any of the biofilm analyzed parameters. Therefore, the P limitation generated by the high water N:P and low nutrient concentration (56:1-LN treatments) did not show a stronger effect on the integrity of biofilm parameters. Results suggested that both factors had independent effects in modulating biofilm structural and metabolic parameters.

Nutrient molar ratios of biofilms may be affected by changes in the water nutrient concentrations and N:P ratios (Stelzer & Lamberti, 2001). Biofilm C:N was

lower in the high nutrient concentration treatments. Although it was expected that increases in total nutrient concentration would affect both ratios (C:N and C:P; Peterson et al., 1993; Hillebrand & Kahlert, 2001), results suggested that biofilms incorporated N in a higher rate than P. The preference of biofilms for the dissolved N uptake instead of P was probably related to the nutrient demands for algal growth (Liess & Hillebrand, 2006), since algal C dominated biofilm biomass. Nutrient limitation in benthic algae varies between streams (Brochardt, 1996 Tank & Dodds, 2003), being the communities used for this study clearly limited by N. In contrast to biofilm C:N, biofilm C:P respond to water N:P increases. The low P availability in the water probably influenced biofilm P content as shown by the positive relationship between these two parameters during the experiments. Though water N:P imbalance had a stronger effect on bacteria than on algae, biofilm structural responses (i. e. EPS accumulation) evidenced also stress on algae (Miklestad, 1995). Polysaccharide production has been reported to be highest when excess of carbohydrate in the media was available while other nutrients such as N, P or S were limited (Sutherland, 1977 & 1982).

The EPS matrix is effective at accumulating extracellular enzymes (Thompson & Sinsabaugh, 2000) and this accumulation may partially account for the metabolic resistance of biofilms to organic matter fluctuations (Sinsabaugh et al., 1991; Lawrence & Neu, 2003). The increase of EPS and phosphatase activity was a clear response of biofilms to the water N:P imbalance (Klotz, 1992; Neu et al., 2005). This response was sufficient to sustain algal biomass in biofilms, but not to sustain biomass of bacteria. One might expect that the low P availability in the 56:1 treatments would have larger effects on algae because bacteria are more efficient for the dissolved phosphorus uptake at low nutrient concentrations (Currie et al., 1986). However, bacteria show a higher P demand for growth than higher trophic levels in the stream food web (Sterner & Elser, 2002). Moreover, the nutrient storage capacity of bacterial cells is probably minor than the capacity of algal cells. Therefore, bacterial cells are more dependent than algae to nutrient fluctuations in the media. Although meiofauna was not analyzed during this study, bacterial biomass reduction might be also explained by the meiofauna grazing pressure on bacterial cells (Perlmutter & Meyer, 1991).

Peptidase activity increased in biofilms subjected to high nutrient concentrations. The stimulation of peptidase activity in biofilms might be provoked by dissolved N limitation in the water and/or by an increase in N substrates availability. N limitation was ruled out since ambient N concentrations in the HN treatments (750 μg N L^{-1}) were not limitant for periphyton growth (50-90 μg N L^{-1}; Grimm & Fischer, 1986; Lohman et al., 1991). However, the increase of algal biomass and peptidase activity in the high nutrient concentration treatments suggested that an important part of algal

exudates were degraded by bacterial peptidases (Francoeur & Wetzel, 2003; Romaní et al., 2004). This agrees with the more general observation that dissolved organic carbon (DOC) released by photosynthesizing algae can be utilized by bacteria in stream biofilms (Geesey et al., 1978; Haack & McFeters, 1982). However, cellobiohydrolase activity (cellulose compounds breakdown) did not increase in the high nutrient concentration treatments. Probably, the DOC released by algae was made up of lower size molecules than cellulose (i. e. disaccharides and xylooligosaccharides; Espeland et al., 2001) and did not stimulate cellobiohydrolase in biofilms. The decrease of biofilm C:N ratios in the high nutrient concentration treatments might be explained also by the internal N recycling in biofilms (Romaní et al., 2004a).

Less than 35 days were sufficient to modify the structural and metabolic parameters of stream biofilms subjected to changes in the water nutrient concentration and N:P ratio. This suggested a high sensitivity of mature biofilms to changes in nutrient availability in the overlying water (Paul & Duthie, 1989; Pringle, 1990). Extracellular enzyme activities and bacterial biomass were the first parameters that respond to the nutrient treatments (first week), followed by algal biomass and EPS accumulation (third week) and finally by changes in nutrient molar ratios (fourth to fifth week). N:P ratios of biofilms adapted rather well to the water N:P ratios at the end of the experiment (19.8 for the 16:1 treatment and 44.7 for the 56: treatment). This enforced the tight connection between water and biofilm nutrients in streams. Assuming that changes in water nutrient concentrations and N:P may affect the nutrient molar ratios of biofilms, such changes would therefore be reflected in the stoichiometry of higher trophic levels of the stream food web.

Aquatic microbial ecology: new trends and future perspectives

Many studies in microbial ecology are concerned with examining the adaptive features that permit particular microbial species to function in particular habitats. Nevertheless, to determine which microbes of a given complex microbial community are responsible of a certain function (i. e. organic matter use) is still a challenge for microbial ecologists. For instance, biofilms in streams are composed of various microbial groups (bacteria, fungi, cyanobacteria, protozoa) with the potential to produce extracellular enzymes involved in OM breakdown. However, conventional measures of the enzyme activity (MUF substrates) usually reflect the potential activity of the whole microbial community, making difficult to assing separate roles in degradation abilities between the different microbial groups forming the community. Investigations in this direction, have made possible the design of specific probes (i. e. ELF fluorescent substrates) able to detect and label alkaline phosphatase activity in phytoplancton. Combining this technique with confocal laser scanning microscopy (CLSM) has permitted to determine which elements of the plancton were responsible of phosphatase activity (Dhyrman & Palenik 1999). Although recent studies using specific fluorescent probes in biofilms have been published (Huang et al. 1998; Sirová et al., 2006), few of them combine this technique with CLSM (Francoeur et al. 2001). In my opinion, future research in functional measurements in stream benthic microbial communities should integrate both conventional and the more recent advances in enzyme activity detection. Knowing total enzyme activity of a given community and knowing the percentage of microbial goups stained by ELF substrates, one might approach to the functional role of each microbial group in organic matter processing in stream benthic communities. Genomics and proteomics may be also important tools for identification of microbial species and the corresponding synthesized proteins responsibles of the enzymatic activity (Thomas et al. 2006). Nevertheless, large databases are required, since organism's genome is constant, but the proteome differs from cell to cell. Analysis of genes and proteins offer an accurate detection of the whole species and proteins present in the community, although they are not representative of the intensity in which microbes express proteins during the normal community functioning.

Despite the accurate information of microbiological processes given by the new emerging molecular tools, aquatic microbial ecology should try to integrate these

findings to better explain ecological processes at the ecosystem level (i. e. nutrient recycling). The issue of scales has recently gained more scientific importance, also in river ecology (Naiman et al., 1992). The river scaling concept aims to be the basis for the assesment for ecological integrity and involves two steps, down- and upscaling (Habersback, 2000). Parallel analyses of the microbial benthic community in tributaries and different reaches of the Furisosos stream (data not presented in this thesis) revealed consistent differences between reaches within the same watershed. These differences were probably related to differences in geomorphology and environmental characteristics of the river section analyzed. Therefore, when one attempts to upscale river measures up to the watershed scale, reach scale characterization become essential. In my opinion, river upscaling for microbial ecology studies in streams should consist of the following steps: i) precise analysis of the type of water courses in the stream basin (main course, tributaries) ii) selection and precise analysis of the reaches-type present in the basin (microhabitat scale measures), and iii) use of geographic information systems (GIS) to characterize the surface area occupyed in the catchment by each reach-type. It is obvious that river upscaling often reduce the punctual information of processes occurring at the microhabitat scale. Nevertheless, the processes that still persist, even after scaling up of measures, must be considered as really relevant processes of the ecosystem functioning.

REFERENCES

Abelho, M. (2001) From litterfall to breakdown in streams: a review. The Scientific World Journal, 1, 656-680.

Abelho, M. & Graça, M.A.S. (2006) Effects of nutrient enrichment on decomposition and fungal colonization of sweet chestnut leaves in an Iberian stream (Central Portugal). Hydrobiologia, 560, 239-247.

Ács, E. & Kiss, K.T. (1993) Colonization processes of diatoms on artificial substrates in the River Danube near Budapest (Hungary). Hydrobiologia, 269/270, 307-315.

Acuña, V., Giorgi, A., Muñoz, I., Sabater, F. & Sabater, S. (2007) Meteorological and riparian influences on organic matter dynamics in a forested Mediterranean stream. Journal of the North American Benthological Society, 26, 54-69.

Acuña, V., Muñoz, I., Giorgi, A., Omella, M., Sabater, F. & Sabater, S. (2005) Drought and postdrought recovery cycles in an intermittent Mediterranean stream: structural and functional aspects. Journal of the North American Benthological Society, 24, 919-933.

Acuña, V., Giorgi, A., Muñoz, I., Uehlinger, U. & Sabater, S. (2004) Riparian forest and hydrology influences on the ecosystem structure and function of a Mediterranean stream. Freshwater Biology, 49, 960-971.

Allan, J.D. (1995) Stream Ecology. Structure and function of running waters. 388 pp., Chapman & Hall, London.

Alvarez, S. & Guerrero, M.C. (2000) Enzymatic activities with decomposition of particulate organic matter in two shallow ponds. Soil Biology and Biochemistry, 32, 1941-1951.

Amalfitano, S., Fazi, S., Zoppini, A., Caracciolo, A.B., Grenni, P. & Puddu, A. (2008) Responses of benthic bacteria to experimental drying in sediments from Mediterranean temporary rivers. Microbial Ecology, 55, 270-279.

Amann, R.I., Ludwig, W. & Schleifer, K.H. (1995) Phylogenetic identification and in situ detection of microbial cells without cultivation. Microbiological Reviews, 59, 143-159.

American Public Helath Association (APHA, 1989) Standard Methods for the examination of water and wastewater, 17 edn. American Public Health Association, Washington.

Arnon, S., Packman, A.I., Peterson, C.G. & Gray, K.A. (2007) Effect of overlying velocity on periphyton structure and denitrification. Journal of Geophysical Research, doi: 10.1029/2006JG000235.

Arnosti, C. (2003) Microbial extracellular enzymes and their role in dissolved organic matter cycling. In Aquatic Ecosystems: interactivity of dissolved organic matter (eds S.E.G. Findlay & R.L. Sinsabaugh), pp. 315-342. Academic Press, California.

Baldy, V., Gobert, V., Guerold, F., Chauvet, E., Lambrigot, D. & Charcosset, J.Y. (2007) Leaf litter breakdown budgets in streams of various trophic status: effects of dissolved inorganic nutrients on microorganisms and invertebrates. Freshwater Biology, 52, 1322-1335.

Baldy, V., Chauvet, E., Charcosset, J.Y. & Gessner, M.O. (2002) Microbial dynamics associated with leaves decomposing in the mainstem and floodplain pond of a large river. Aquatic Microbial Ecology, 28, 25-36.

Baldy, V. & Gessner, M.O. (1997) Towards a budget of leaf litter decomposition in a first-order woodland stream. Comptes Rendus de l'Academie des Sciences Series III, 320, 747-758.

Baldy, V., Gessner, M.O. & Chauvet, E. (1995) Bacteria, fungi and the breakdown of leaf litter in a large river. Oikos, 74, 93-102.

Banoub, M.W. & Williams, P.J.L. (1972) Measurements of microbial activity and organic material in the western Mediterranean Sea. Deep-Sea Research, 19, 433-443.

Barber, H.G. & Haworth, E.Y. (1981). A guide to the morphology of the diatom frustule. The Freshwater Biological Association, Ambleside.

Bärlocher, F. (1992) The ecology of aquatic hyphomycetes Springer-Verlag, Berlin.

Bärlocher, F., Canhoto, C. & Graça, M.A.S. (1995) Fungal colonization of alder and eucalypt leaves in two streams in central Portugal. Archiv für Hydrobiologie, 133, 57-68.

Barranguet, C., van Beusekom, S.A.M., Veuger, B., Neu, T.R., Manders, E.M.M., Sinke, J.J. & Admiraal, W. (2004) Studying undisturbed autotrophic biofilms: still a technical challenge. Aquatic Microbial Ecology, 34, 1-9.

Battin, T.J., Kaplan, L.A., Newbold, J.D., Cheng, X.H. & Hansen, C. (2003) Effects of current velocity on the nascent architecture of stream microbial biofilms. Applied and Environmental Microbiology, 69, 5443-5452.

Bavykin, S.G., Lysov, Y.P., Zakhariev, V., Kelly, J.J., Jackman, J., Stahl, D.A. & Cherni, A. (2004) Use of 16S rRNA, 23S rRNA, and gyrB gene sequence analysis to determine phylogenetic relationships of *Bacillus cereus* group microorganisms. Journal of Clinical Microbiology, 42, 3711-3730.

Beche, L.A. & Resh, V.H. (2007) Short-term climatic trends affect the temporal variability of macroinvertebrates in California 'Mediterranean' streams. Freshwater Biology, 52, 2317-2339.

Begon, M., Harper, J.L. & Townsend, C.R. (1996) Ecology: individuals, populations and communities, 3 edn. 886pp., Blackwell Science, Oxford.

Bengtsson, G. (1992) Interactions between fungi, bacteria and beech leaves in a stream mesocosm. Oecologia, 89, 542-549.

Berg, B. (1986) Nutrient release from litter and humus in coniferous forest soils: a mini review. Scandinavian Journal of Forest Research, 1, 359-369.

Berg, B. & McClaugherty, C. (2003). Decomposition as a process. In: Plant litter decomposition, humus formation, carbon sequestration (eds B. Berg & C. McClaugherty), pp. 11-29. Springer-Verlag, Berlin.

Bergstrom, D.W., Monreal, C.M., Millette, J.A. & King, D.J. (1998) Spatial dependence of soil enzyme activities along a slope. Soil Science Society of America Journal, 62, 1302-1308.

Bernal, S., Butturini, A., Nin, E., Sabater, F. & Sabater, S. (2003) Leaf litter dynamics and nitrous oxide emission in a Mediterrean riparian forest: implications for soil nitrogen dynamics. Journal of Environmental Quality, 32, 191-197.

Bernal, S., Butturini, A. & Sabater, F. (2002) Variability of DOC and nitrate responses to storms in a small Mediterranean forested catchment. Hydrology and Earth System Sciences, 6, 1031-1041.

Berner, E.K. & Berner, R.A. (1987) The global water cycle: geochemistry and environment. Prentice-Hall, Englewood Cliffs.

Bhaskar, P. & Bhosle, N.B. (2006) Bacterial extracellular polymeric substance (EPS): A carrier of heavy metals in the marine food-chain. Environment International, 32, 191-198.

Biggs, B.J.F. (1999) Eutrophication of streams and rivers: dissolved nutrient-chlorophyll relationships for benthic algae. Journal of the North American Benthological Society, 19, 17-31.

Blair, J.M., Parmelee, R.W., & Beare, M.H. (1990) Decay rates, nitrogen fluxes, and decomposer communities of single- and mixed-species foliar litter. Ecology, 71, 1976-1985.

Blenkinsopp, S.A. & Lock, M.A. (1992) Impact of storm-flow on electron transport system activity in river biofilms. Freshwater Biology, 27, 397-404.

Bothwell, M.L. (1985) Phosphorus limitation of lotic periphyton growth rates: an intersite comparison using continuous-flow troughs (Thompson River system, British Columbia). Limnology and Oceanography, 30, 527-542.

Bratbak, G. & Dundas, I. (1984) Bacterial dry matter content and biomass estimations. Applied and Environmental Microbiology, 48, 755-757.

Bray, J.R. & Curtis, J.T. (1957) An ordination of the upland forest communities of southern Wisconsin. Ecological Monographs, 27, 325-349.

Brochardt, M.A. (1996). Nutrients. In: Algal Ecology. Freshwater Benthic Ecosystems (eds R.J. Stevenson, M.L. Bothwell & R.L. Lowe), pp. 184-218. Academic Press, San Diego.

Buesing, N. & Gessner, M.O. (2002) Comparison of detachment procedures for direct counts of bacteria associated with sediment particles, plant litter and epiphytic biofilms. Aquatic Microbial Ecology, 27, 29-36.

Butturini, A., Bernal, S., Sabater, S. & Sabater, F. (2002) The influence of riparian-hyporheic zone on the hydrological responses in an intermittent stream. Hydrology and Earth System Sciences, 6, 515-525.

Butturini, A. & Sabater, F. (2000) Seasonal variability of dissolved organic carbon in a Mediterranean stream. Biogeochemistry, 51, 303-321.

Canhoto, C. & Graça, M.A.S. (2006) Decomposition of *Eucaliptus globulus* leaves and 3 native leaf species (Alnus glutionsa, Castanea sativa and Quercus faginea) in a Portuguese low order stream. Hydrobiologia, 333, 79-85.

Cardinale, B.J., Palmer, M.A., Swan, C.M., Brooks, S. & Poff, N.L. (2002) The influence of substrate heterogeneity on biofilm metabolism in a stream ecosystem. Ecology, 83, 412-422.

Carr, G.M., Morin, A. & Chambers, P.A. (2005) Bacteria and algae in stream periphyton along a nutrient gradient. Freshwater Biology, 50, 1337-1350.

Carreiro, M.M., Sinsabaugh, R.L., Repert, D.A. & Parkhurst, D.F. (2000) Microbial enzyme shifts explain litter decay responses to simulated nitrogen deposition. Ecology, 81, 2359-2365.

Caruso, B.S. (2002) Temporal and spatial patterns of extreme low flows and effects on stream ecosystems in Otago, New Zealand. Journal of Hydrology, 257, 115-133.

Casas, J. & Gessner, M.O. (1999) Leaf litter breakdown in a Mediterranean stream characterized by travertine precipitation. Freshwater Biology, 41, 781-793.

Chamier, A.C. (1985) Cell-wall-degrading enzymes of aquatic hyphomycetes: a review. Botanical Journal of the Linnean Society, 91, 67-81.

Characklis, W.G., McFeters, G.A. & Marshall, K.C. (1990). Physiological ecology in biofilm systems. In: Biofilms (eds W.G. Characklis & K.C. Marshall), pp. 341-394. John Wiley and Sons Inc., New York.

Charcosset, J.Y. & Chauvet, E. (2001) Effect of culture conditions on ergosterol concentration in mycelium of aquatic hyphomycetes. Applied and Environmental Microbiology, 67, 2051-2055.

Charcosset, J.Y. & Gardes, M. (1999) Intraspecific genetic diversity and substrate preference in the aquatic hyphomycete *Tetrachateum elegans*. Mycological Research, 103, 736-742.

Chróst, R.J. (1991) Microbial enzymes in aquatic environments. Springer-Verlag, New York.

Cilia, V., Lafay, B. & Christen, R. (1996) Sequence heterogeneities among 16S ribosomal RNA sequences and their effect on phylogenetic analyses at the species level. Molecular Biology and Evolution, 13, 451-461.

Cole, J.J. (1982) Interactions between bacteria and algae in aquatic ecosystems. Annual Review of Ecology and Systematics, 13, 291-314.

Comte, S., Guibaud, G. & Baudu, M. (2006) Relations between extraction protocols for activated sludge extracellular polymeric substances (EPS) and EPS complexation properties Part I. Comparison of the efficiency of eight EPS extraction methods. Enzyme and Microbial Technology, 38, 237-245.

Cooksey, K.E. & Wigglesworth-Cooksey, B. (1995) Adhesion of bacteria and diatoms to surfaces in the sea: a review. Aquatic Microbial Ecology, 9, 87-96.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R. & Lappin-Scott, H.M. (1995) Microbial biofilms. Annual Review of Microbiology, 49, 711-745.

Costerton, W.J., DeBeer, D. & Lewandowski, Z. (1994) Biofilms, the customized niche. The Journal of Bacteriology, 176, 2137-2142.

Costerton, J.W., Cheng, K.J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M. & Marrie, T.J. (1987) Bacterial biofilms in nature and disease. Annual Review of Microbiology, 41, 435-464.

Cotner, J.B. & Wetzel, R.G. (1992) Uptake of dissolved inorganic and organic phosphorus compounds by phytoplankton and bacterioplankton. Limnology and Oceanography, 37, 232-243.

Couteaux, M.M., Bottnera, P. & Berg, B. (1995) Litter decomposition, climate and liter quality. Trends in Ecology and Evolution, 10, 63-66.

Cox, E.J. (1990) Studies on the algae of a small softwater stream. I. Occurrence and distribution with particular reference to the diatoms. Archiv für Hydrobiologie, 83, 525-552.

Cross, W.F., Benstead, J.P., Frost, P.C. & Thomas, S.A. (2005) Ecological stoichiometry in freshwater benthic systems: recent progress and perspectives. Freshwater Biology, 50, 1895-1912.

Cummins, K.W. (1992). Invertebrates. In: The rivers handbook. I. Hydrological and ecological principles (eds R. Calow & G.E. Petts), pp. 234-250. Blakwell Scientific, Oxford.

Cummins, K.W. (1988). The study of stream ecosystems: a functional view. In: Concepts of ecosystem ecology (eds R.L. Pomeroy & J.J. Albert), pp. 247-262. Springer-Verlag, New York.

Cummins, K.W. (1962) An evaluation of some techniques for the collection and analysis of benthic samples with special emphasis on lotic waters. The American Midland Naturalist, 67, 477-504.

Currie, D.J., Bentzen, E. & Klaff, J. (1986) Does algal-bacterial phosphorus partitioning vary among lakes? A comparative study of orthophosphate uptake and alkaline phosphatase activity in freshwater. Canadian Journal of Fisheries and Aquatic Sciences, 52, 828-835.

Decho, A.W. (2000) Microbial biofilms in intertidal systems: an overview. Continental Shelf Research, 20, 1257-1273.

Diez, J., Elosegi, A., Chauvet, E. & Pozo, J. (2002) Breakdown of wood in the Aguera stream. Freshwater Biology, 47, 2205-2215.

Dix, N. & Webster, J. (1995) Fungal Ecology Chapmann and Hall, New York.

Dodds, W.K. (2003) The role of periphyton in phosphorus retention in a shallow freshwater aquatic ecosystem. Journal of Phycology, 39, 840-849.

Dodds, W.K., Hutson, R.E., Eichem, A.C., Evans, M.A., Gudder, D.A., Fritz, K.M. & Gray, L. (1996) The relationship of floods, drying, flow and light to primary production and producer biomass in a prairie stream. Hydrobiologia, 333, 151-159.

Dubois, M., Giles, K.A., Hamilton, J.K., Rebers, P.A. & Smith, F. (1956) Colorimetric method for the determination of sugars and related substances. Analytical Chemistry, 28, 350-356.

Dyhrman, S. T. & Palenik B. (1999) Phosphate stress in cultures and field populations of the dinoflagellate *Prorocentrum minimum* detected by a single-cell alkaline phosphatase assay. Applied and Environmental Microbiology, 65, 3205-3212.

Emtiazi, F., Schwartz, T., Marten, S.M., Krolla-Sidenstein, P. & Obst, U. (2004) Investigation of natural biofilms formed during the production of drinking water from surface water embankment filtration. Water Research, 38, 1197-1206.

Espeland, E.M., Francoeur, S.N. & Wetzel, R.G. (2001) Influence of algal photosynthesis on biofilm bacterial production and associated glucosidase and xylosidase activities. Microbial Ecology, 42, 524-530.

Fellows, C.S., Clapcott, J.E., Udy, J.W., Bunn, S.E., Harch, B.D., Smith, M.J. & Davies, P.M. (2006) Benthic metabolism as an indicator of stream ecosystem health. Hydrobiologia, 572, 71-87.

Féray, C. & Montuelle, B. (2003) Chemical and microbial hypothesis explaining the effect of wastewater treatment plant discharge on the nitrifying communities in freshwater sediment. Chemosphere, 50, 919-928.

Ferreira, V., Gulis, V. & Graça, M.A.S. (2006) Whole-stream nitrate addition affects litter decomposition and associated fungi but not invertebrates. Oecologia, 149, 718-729.

Findlay, S., Tank, J., Dye, S., Valett, H.M., Mulholland, P.J., McDowell, W.H., Johnson, S.L., Hamilton, S.K., Edmonds, J., Dodds, W.K. & Bowden, W.B. (2002) A cross-system comparison of bacterial and fungal biomass in detritus pools of headwater streams. Microbial Ecology, 43, 55-66.

Findlay, S., Strayer, D., Goumbala, C. & Gould, K. (1993) Metabolism of streamwater dissolved organic carbon in the shallow hyporheic zone. Limnology and Oceanography, 38, 1493-1499.

Findlay, S.G. & Arsuffi, T.L. (1989) Microbial growth and detritus transformations during decomposition of leaf litter in a stream. Freshwater Biology, 21, 261-269.

Fischer, S.G. & Likens, G.E. (1973) Energy flow in Bear Brook, New Hampshire: an integrative approach to stream ecosystem metabolism. Ecology, 43, 421-439.

Franco, C., Esteban, G.F. & Tellez, C. (1998) Colonization and succession of ciliated protozoa associated with submerged leaves in a river. Limnologica, 28, 275-283.

Francoeur, S.N. & Wetzel, R.G. (2003) Regulation of periphytic leucine-aminopeptidase activity. Aquatic Microbial Ecology, 31, 249-258.

Francoeur, S.N., Wetzel, R.G. & Neely, R.K. (2001) New spatially explicit method for detecting extracellular protease activity in biofilms. Applied and Environmental Microbiology, 67, 4329-4334.

Francoeur, S.N., Biggs, B.J.F., Smith, R.A. & Lowe, R.L. (1999) Nutrient limitation of algal biomass accrual in streams: seasonal patterns and a comparison of methods. Journal of the North American Benthological Society, 18, 242-260.

Frankenberger, W.T. & Dick, W.A. (1983) Relationships between enzyme activities and microbial growth and activity indices in soil. Soil Science Society of America Journal, 47, 945-951.

Freeman, C. & Lock, M.A. (1995) The biofilm polysaccharide matrix: A buffer against changing organic substrate supply? Limnology and Oceanography, 40, 273-278.

Freeman, C. & Lock, M.A. (1992) Recalcitrant high-molecular-weight material, an inhibitor of microbial metabolism in river biofilms. Applied and Environmental Microbiology, 58, 2030-2033.

Frølund, B., Palmgren, R., Keiding, K. & Nielsen, P.H. (1996) Extraction of extracellular polymers from activated sludge using a cation exchange resin. Water Research, 30, 1749-1758.

Frølund, B., Griebe, T. & Nielsen, P.H. (1995) Enzymatic activity in the activated sludge floc matrix. Applied and Environmental Microbiology, 43, 755-761.

Frost, P.C., Stelzer, R.S., Lamberti, G.A. & Elser, J.J. (2002) Ecological stoichiometry of trophic interactions in the benthos: understanding the role of C : N : P ratios in lentic and lotic habitats. Journal of the North American Benthological Society, 21, 515-528.

Gallardo, A. & Merino, J. (1993) Leaf decomposition in two Mediterranean ecosystems of southwest Spain: influence of substrate quality. Ecology, 74, 152-161.

Garcia-Martinez, J., Acinas, S.G., Antón, A.I. & Rodríguez-Valera, F. (1999) Use of the 16S-23S ribosomal genes spacer region in studies of procaryotic diversity. Journal of Microbiological Methods, 36, 55-64.

Garcia-Martinez, J., Martinez-Murcia, A.J., Anton, A.I., Rodríguez-Valera, F. & Zorraquino, I. (1996) Molecular evidence supporting the existence of two major groups in uropathogenic *Escherichia coli*. FEMS Immunology and Medical Microbiology, 14, 231-244.

Gasith, A. & Resh, V.H. (1999) Streams in Mediterranean climate regions: abiotic influences and biotic responses to predictable seasonal events. Annual Review of Ecology and Systematics, 30, 51-81.

Gaudes. A., Artigas, J., Romaní, A. M., Sabater, S. & Muñoz, I. (2008) Contribution of microbial and invertebrate community to leaf litter colonization in a Mediterranean stream. Journal of the North American Benthological Society, *in press*.

Geider, R.J. & MacIntyre, H.L. (1996) A dynamic model of photoadaptation in phytoplankton. Limnology and Oceanography, 41, 1-15.

Gessey, G.G., Mutch, R. & Costerton, J.W. (1978) Sessile bacteria: An important component of the microbial populations in small mountain streams. Limnology and Oceanography, 23, 1214-1223.

Gessner, M.O. (1997) Fungal biomass, production and sporulation associated with particulate organic matter in streams. Limnetica, 13, 33-44.

Gessner, M.O. & Newell, S.Y. (2002). Biomass, growth rate and production of filamentous fungi in plant litter. In: Manual of environmental microbiology (eds C.J. Hurst, G. Knudsen, M. McInerey, L.D. Stetzenbach & M. Walter), pp. 295-308. American Society for Microbiology, Washington.

Gessner, M.O., Chauvet, E. & Dobson, M. (1999) A perspective on leaf litter breakdown in streams. Oikos, 85, 377-384.

Gessner, M.O., Suberkropp, K., & Chauvet, E. (1997). Decomposition of plant litter by fungi in marine and freshwater ecosystems. In: The Mycota: Environmental and Microbial Relationships (eds D.T. Wicklow & B. Söderström), Vol. 4, pp. 303-322. Springer-Verlag, Berlin.

Gessner, M.O. & Schmitt, A.L. (1996) Use of solid-phase extraction to determine ergosterol concentrations in plant tissue colonized by fungi. Applied and Environmental Microbiology, 62, 415-419.

Gessner, M.O. & Chauvet, E. (1994) Importance of stream microfungi in controlling breakdown rates of leaf litter. Ecology, 75, 1807-1817.

Gessner, M.O. & Chauvet, E. (1993) Ergosterol-to-biomass conversion factors for aquatic hyphomycetes. Applied and Environmental Microbiology, 59, 502-507.

Gessner, M.O., Thomas, M.I., Jean-Louis, A.M. & Chauvet, E. (1993) Stable successional patterns of aquatic hyphomycetes on leaves decaying in a summer cool stream. Mycological Research, 97, 163-172.

Gönczöl, J., Csontos, P. & Révay, A. (2003) Catchment scale patterns of aquatic hyphomycetes. The role of physicochemical variables and substrate composition in structuring conidial communities. Archiv für Hydrobiologie, 157, 249-266.

Gordon, N.D., MacMahon, T.A. & Finlayson, B.L. (1992) Stream Hydrology. An Introduction for Ecologists. John Wiley & Sons, Cichester.

Graça, M.A.S. & Canhoto, C. (2006) Leaf litter processing in low order streams. Limnetica, 25, 1-10.

Graça, M.A.S. & Zimmer, M. (2005). Leaf toughness. In: Methods to study litter decomposition: a practical guide (eds M.A.S. Graça, F. Bärlocher & M.O. Gessner), pp. 121-125. Springer, Dordrecht.

Grasshoff, K., Ehrhardt, M. & Kremling, K. (1983) Methods of seawater analysis. Second, Revised and Extended Edition. Verlag Chemie, Weinheim.

Grattan, R.M. & Suberkropp, K. (2001) Effects of nutrient enrichment on yellow poplar leaf decomposition and fungal activity in streams. Journal of the North American Benthological Society, 20, 33-43.

Griffin, D.H. (1994) Fungal physiology, 2 edn. Wiley-Liss, New York.

Grimm, N.B. & Fischer, S.G. (1986) Nitrogen limitation in an Sonoran Desert stream. Journal of the North American Benthological Society, 5, 2-15.

Gulis, V. (2001) Are there any substrate preferences in aquatic hyphomycetes? Mycological Research, 105, 1088-1093.

Gulis, V., Ferreira, V. & Graça, M.A.S. (2006) Stimulation of leaf litter decomposition and associated fungi and invertebrate by moderate eutrophication: implications for stream assessment. Freshwater Biology, 51, 1655-1669.

Gulis, V., Rosemond, A.D., Suberkropp, K., Weyers, H.S. & Benstead, J.P. (2004) Effects of nutrient enrichment on the decomposition of wood and associated microbial activity in streams. Freshwater Biology, 49, 1437-1447.

Gulis, V. & Suberkropp, K. (2004) Effects of whole-stream nutrient enrichment on the concentration and abundance of aquatic hyphomycete conidia in transport. Mycologia, 96, 57-65.

Gulis, V. & Suberkropp, K. (2003a) Effect of inorganic nutrients on relative contributions of fungi and bacteria to carbon flow from submerged decomposing leaf litter. Microbial Ecology, 45, 11-19.

Gulis, V. & Suberkropp, K. (2003b) Leaf litter decomposition and microbial activity in nutrient-enriched and unaltered reaches of a headwater stream. Freshwater Biology, 48, 123-134.

Haack, T.K. & McFeters, G.A. (1982) Microbial dynamics of an epilithic mat community in a high alpine stream. Applied and Environmental Microbiology, 42, 702-707.

Habersack, H. M. (2000) The river scaling concept (RSC): a basis for ecological assessments. Hydrobiologia, 422/423, 49-60.

Hamilton, W.A. (1987). Biofilms: Microbial interactions and metabolic activities. In: Ecology of Microbial Communities (eds M. Fletcher, J.R.G. Gray & J.G. Jones), pp. 361-385. Cambridge Univ. Press, Cambridge.

Hammel, K.E. (1997). Fungal degradation of lignin. In: Driven by nature: Plant litter quality and decomposition (eds G. Cadish & K.E. Giller), pp. 33-45. CAB International, Wallingford.

Hauer, F. & Lamberti, G.A. (1996) Methods in stream ecology, New York.

Hendel, B., Sinsabaugh, R.L. & Marxsen, J. (2005). Lignin-degrading enzymes: phenoloxidase and peroxidase. In: Methods to study litter decomposition. A practical guide (eds M.A.S. Graça, F. Bärlocher & M.O. Gessner), pp. 273-278. Springer, Dordrecht.

Hieber, M. & Gessner, M.O. (2002) Contribution of stream detrivores, fungi, and bacteria to leaf breakdown based on biomass estimates. Ecology, 83, 1026-1038.

Hill, W.R. (1996). Effects of light. In: Algal Ecology: Freshwater Benthic Ecosystems (eds R.J. Stevenson, M.L. Bothwell & R.L. Lowe), pp. 121-144. Academic Press, California.

Hill, W.R., Ryon, M.G. & Schilling, E.M. (1995) Light limitation in a stream ecosystem: responses by primary producers and consumers. Ecology, 76, 1297-1309.

Hillebrand, H. & Kahlert, M. (2001) Effect of grazing and nutrient supply on periphyton biomass and nutrient stoichiometry in habitats of different productivity. Limnology and Oceanography, 46, 1881-1898.

Hoppe, H.G., Giesenhagen, H.C. & Gocke, K. (1998) Changing patterns of bacterial substrate decomposition in a eutrophication gradient. Aquatic Microbial Ecology, 15, 1-13.

Hoppe, H.G. (1983) Significance of exoenzymatic activities in the ecology of brackish water: measurement by means of methylumbelliferyl-substrates. Marine Ecology Progress Series, 11, 299-308.

Horner, R.R., Welch, E.B., Seeley, M.R. & Jacoby, J.M. (1990) Responses of periphyton to changes in current velocity, suspended sediment and phosphorus concentratiion. Freshwater biology, 24, 215-232.

Huang, C. T., Xu, K. D., McFeters, G. A. & Stewart, P. S. (1998) Spatial patterns of alkaline phosphatase expression within bacterial colonies and biofilms in response to phosphate starvation. Applied and Environmental Microbiology, 64, 1526-1531.

Hutchinson, G.E. (1957) A treatise on limnology. John Wiley & Sons, New York.

Hynes, H.B.N. (1970) The ecology of running waters. University of Toronto Press, Toronto.

Iiyama, K. & Wallis, A.F.A. (1990) Determination of lignin in herbaceous plants by an improved acetyl bromide procedure. Journal of the Science of Food and Agriculture, 51, 145-151.

Ingold, C.T. (1957) An illustrated guide to aquatic and water-borne hyphomycetes (Fungi imperfecti) with notes on their biology. Freshwater Biological Association, Scientific publication No. 30, Ambleside.

International Pannel on Climate Change (IPCC, 2007). Climate change 2007: the physical science basis, Report No. 4, Switzerland.

Jaccard, P. (1901) Distribution de la flore alpine dans le Bassin des Dranses et dans quelques regions voisines. Bulletin de la Société Vaudoise des Sciences Naturelles, 37, 241-271.

Jackson, C.R., Churchill, P.F. & Roden, E.E. (2001) Successional changes in bacterial assemblage structure during epilithic biofilm development. Ecology, 82, 555-566.

Jackson, C.R., Foreman, C.M. & Sinsabaugh, R.L. (1995) Microbial enzyme activities as indicators of organic matter processing rates in a Lake Erie coastal wetland. Freshwater Biology, 34, 329-342.

Jeffrey, S.W. & Humphrey, G.F. (1975) New spectrophotometric equations for determining chlorophylls a, b, and c in higher plants, algae and natural phytoplankton. Biochemie und Physiologie der Pflanzen, 167, 191-194.

Jensen, M.A., Webster, J.A. & Straus, N. (1993) Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphysms. Applied and Environmental Microbiology, 59, 945-952.

Jones, S.E. & Lock, M.A. (1993) Seasonal determinations of extracellular hydrolytic activities in heterotrophic and mixed heterotrophic / autotrophic biofilms from two contrasting rivers. Hydrobiologia, 257, 1-16.

Kalmbach, S., Manz, W. & Szewzyk, U. (1997) Dynamics of biofilm formation in drinking water: phylogenetic affiliation and metabolic potential of single cells assessed by formazan reduction and in situ hybridization. FEMS Microbiology Ecology, 22, 265-279.

Kaplan, L.A. & Newbold, J.D. (2003). The role of monomers in stream ecosystem metabolism. In: Aquatic Ecosystems. Interactivity of dissolved organic matter (eds S.E.G. Findlay & R.L. Sinsabaugh), pp. 99-119. Academic Press, San Diego.

Kirchman, D.L. (2003). The contribution of monomers and other low-molecular weight compounds to the flux of dissolved organic material in aquatic ecosystems. In: Aquatic ecosystems: interactivity of dissolved organic matter (eds S.E. Findlay & R.L. Sinsabaugh), pp. 217-241, San Diego.

Klotz, R.L. (1992) Factors influencing alkaline phosphatase activity of stream epilithon. Journal of Freshwater Ecology, 7, 233-242.

Kominkova, D., Kuehn, K.A., Busing, N., Steiner, D. & Gessner, M.O. (2000) Microbial biomass, growth, and respiration associated with submerged litter of Phragmites australis decomposing in a littoral reed stand of a large lake. Aquatic Microbial Ecology, 22, 271-282.

Kominoski, J.S., Ball, B. A., Bradford, M. A, Coleman, D. C., Hall, D. B. & Hunter, M. D. (2007) Nonadditive effects of leaf litter species diversity on breakdown dynamics in a detritus-based stream. Ecology, 88, 1167-1176.

Krammer, K. & Lange-Bertalot, K. (1986 -1991) Bacillariophyceae. Süsswasserflora von Mitteleuropa G. Fischer, Jena.

Kreft, J.U. (2004) Biofilms promote altruism. Microbiology, 150, 2751-2760.

Lake, P.S. (2003) Ecological effects of perturbation by drought in flowing waters. Freshwater Biology, 48, 1161-1172.

Lamberti, G.A. (1996). The role of periphyton in benthic food webs. In: Algal Ecology. Freshwater Benthic Ecosystems (eds R.J. Stevenson, M.L. Bothwell & R.L. Lowe), pp. 533-572. Academic Press, San Diego.

Lange-Bertalot, H. (2001). *Navicula* sensu stricto, 10 genera separated from *Navicula* sensu lato, Frustulia, 2. In: Diatoms of Europe (ed H. Lange-Bertalot), pp. 526. Gantner Verlag, Ruggell.

Lawrence, J.R., Chenier, M.R., Roy, R., Beaumier, D., Fortin, N., Swerhone, G.D.W., Neu, T.R. & Greer, C.W. (2004) Microscale and molecular assessment of impacts of nickel, nutrients and oxygen level on structure and function of river biofilm communities. Applied and Environmental Microbiology, 70, 4326-4339.

Lawrence, J.R. & New, T.R. (2003) Microscale analyses of the formation and nature of microbial communities in river systems. Review in Environmental Sciences and Biotechnology, 2, 85-97.

Leblond-Bourget, N., Philippe, H., Mangin, I. & Decaris, B. (1996) 16S rRNA and 16S to 23S internal transcribed spacer sequence analyses reveal inter- and intraspecific *Bifidobacterium* phylogeny. International Journal of Systematic Bacteriology, 46, 102-111.

Lecref, A., Usseglio-Polatera, P., Charcosset, J.Y., Lambrigot, D., Bracht, B. & Chauvet, E. (2006) Assessment of functional integrity of eutrophic streams using litter breakdown and benthic invertebrates. Archiv für Hydrobiologie, 165, 105-126.

Lock, M.A. (1993). Attached microbial communities in rivers. In: Aquatic microbiology: an ecological approach (ed T.E. Ford), pp. 113-138. Blackwell, Oxford.

Lock, M.A., Wallace, R.R., Costerton, J.W., Ventullo, R.M. & Charlton, S.E. (1984) River epilithon: toward a structural-functional model. Oikos, 42, 10-22.

Lock, M.A. & Hynes, H.B. (1976) The fate of dissolved organic carbon derived from autumn-shed maple leaves (*Acer saccharum*) in a temperate hard-water stream. Limnology and Oceanography, 21, 436-443.

Lohman, K., Jones, J.R. & Baysinger-Daniel, C. (1991) Experimental evidence for nitrogen limitation in a northern Ozark stream. Journal of the North American Benthological Society, 19, 14-23.

Low, C.S.F. & White, D.C. (1989). Regulation of external polymer production in benthic microbial communities. In: Microbial Mats: physiological ecology of benthic microbial communities (eds Y. Cohen & E. Rosenberg), pp. 228-238. American Society for Microbiology.

Lyautey, E., Jackson, C.R., Cayrou, J., Rols, J.L. & Garabetian, F. (2005) Bacterial community succession in natural river biofilm assemblages. Microbial Ecology, 50, 589-601.

Lyautey, E., Teissier, S., Charcosset, J.Y., Rols, J.L. & Garabetian, F. (2003) Bacterial diversity of epilithic biofilm assemblages of an anthropised river section, assessed by DGGE analysis of a 16S rDNA fragment. Aquatic Microbial Ecology, 33, 217-224.

Mallin, M.A., McIver, M.R., Ensign, S.H. & Cahoon, L.B. (2004) Photosyntetic and heterotrophic impacts of nutrient loading to blackwater streams. Ecological applications, 14, 823-838.

Mallory, M.A. & Richardson, J.S. (2005) Complex interactions of light, nutrients and consumer density in a stream periphyton-grazer (tailed frog tadpoles) system. Journal of Animal Ecology, 74, 1020-1028.

Maltby, L. (1992). Detritus processing. In: The river handbook. Hydrological and ecological principles (eds P. Calow & G.E. Petts), Vol. 1, pp. 331-335. Blackwell, Oxford.

Mansfield, S.D. (2005). Extracellular fungal hydrolytic enzyme activity. In: Methods to study litter decomposition: A practical guide (eds M.A.S. Graça, F. Bärlocher & M.O. Gessner), pp. 239-248. Springer, Dordrecht.

Margalef, R. (1983) Limnología. 1010 pp., Omega, Barcelona.

Marshall, K.C. (1971) Mechanism of the initial events in the sorption of marine bacteria to surfaces. Journal of General Microbiology, 68, 337-348.

Marshall, K.C. (1992) Biofilms: an overview of bacterial adhesion, activity, and control at surfaces. American Society for Microbiology News, 58, 202-207.

Marxsen, J. & Witzel, K.P. (1990) Measurement of exoenzymatic activity in streambed sediments using methylumbelliferyl-substrates. Archiv für Hydrobiologie, 34, 21-28.

Mason, H.S. (1984) The chemistry of melanin. III. Mechanism of the oxidation dihydroxypheny-alanine by tyrosinase. Journal of Biological Chemistry, 172, 83-99.

Masseret, E., Amblard, C. & Bourdier, G. (1998) Changes in the structure and metabolic activities of periphytic communities in a stream receiving treated sewage from a waste stabilization pond. Water Research. 32, 2299-2314.

Mathuriau, C. & Chauvet, E. (2002) Breakdown of leaf litter in a neotropical stream. Journal of the North American Benthological Society, 21, 384-396.

Mehta, A. & Rosato, Y.B. (2001) Phylogenetic relationships of *Xylella fastidiosa* strains from different hosts, based on 16S rDNA and 16S-23S intergenic spacer sequences. Journal of Systematic and Evolutionary Microbiology, 51, 311-318.

Melillo, J.M., Naiman, R.J., Aber, J.D. & Linkins, A.E. (1983) The influence of substrate quality and stream size on wood decomposition dynamics. Oecologia, 58, 281-285.

Methvin, B.R. & Suberkropp, K. (2003) Annual production of leaf-decaying fungi in two streams. Journal of the North American Benthological Society, 22, 554-564.

Meybeck, M. & Helmer, R. (1989) The quality of rivers: fom pristine stage to global pollution. Paleogeography, Paleoclimatology, Paleoecology, 75, 283-309.

Mille-Lindblom, C. & Tranvik, L.J. (2003) Antagonism between bacteria and fungi on decomposing aquatic plant litter. Microbial Ecology, 45, 173-182.

Minshall, G.W. (1984). Aquatic insect-substratum relationships. In: The ecology of freshwater insects (eds V.H. Resh & D.M. Rosenberg), pp. 358 - 400. Praeger, New York.

Minshall, G.W., Petersen, R.C., Cummins, K.W., Bott, T.L., Sedell, J.R., Cushing, C.C. & Vanotte, R.L. (1983) Interbiome comparison of stream ecosystem dynamics. Ecological Monographs, 53, 1-25.

Mohamed, M.N., Lawrence, J.R. & Robarts, R.D. (1998) Phosphorus limitation of heterotrophic biofilms from the Fraser river, British Columbia, and the effects of pulp mill effluent. Microbial Ecology, 36, 121-130.

Moller, J., Miller, M. & Kjøller, A. (1999) Fungal-bacterial interaction on beech leaves: influence on decomposition and dissolved organic carbon quality. Soil Biology and Biochemistry, 31, 367-374.

Morrison, I.M. (1972) A semimicro method for the determination of lignin and its use in predicting the digestiblity of forage crops. Journal of the Science of Food and Agriculture, 23, 455-463.

Muyzer, G., Brinkhoff, T., Nuber, U., Santegoeds, C., Schafer, H. & Wawer, C. (1997). Denaturing gradient gel electrophoresis (DGGE) in microbial ecology. In: Molecular microbial ecology manual (eds A.D.L. Akkermans, J.D. van Elsas & F.J. de Bruijn), Vol. 1-27. Kluwer Academic, Dordrecht.

Muyzer, G., Waal, E.C. & Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology, 59, 695-700.

Myklestad, S.M. (1995) Release of extracellular products by phytoplankton with special emphasis on polysaccharides. Science of the Total Environment, 165, 155-164.

Nagpal, M.I., Fox, K.F. & Fox, A. (1998) Utility of 16S-23S rRNA spacer region methodology: how similar are interspace regions within a genome and between strains for closely related organisms? Journal of Microbiological Methods, 33, 211-219.

Naiman, A. J., Lonzarich, D. G., Beechie, T. J. & Ralph, S. C. (1992) General principles of classification and the assesment of conservation potential in rivers. In: River Conservation and Management (eds P. J. Boon, P. Calow & G. E. Petts). pp. 93-123. John Wiley & Sons, Cichester.

Nannipieri, P., Muccini, L. & Ciardi, C. (1983) Microbial biomass and enzyme activities: production and persistence. Soil Biology and Biochemistry, 15, 679-685.

Neu, T.R., Swerhone, G.D.W., Bockelmann, U. & Lawrence, J.R. (2005) Effect of CNP on composition and structure of lotic biofilms as detected with lectin-specific glycoconjugates. Aquatic Microbial Ecology, 38, 283-294.

Neu, T.R. & Lawrence, J.R. (1997) Development and structure of microbial biofilms in river water studied by confocal laser scanning microscopy. FEMS Microbiology Ecology, 24, 11-25.

Newell, S.Y. (1992). Estimating fungal biomass and productivity in decomposing litter. In: The fungal community: its organization and role in the ecosystem (eds G.C. Carroll & D.T. Wicklow), pp. 521-526. Marcel Dekker, New York.

Nikolcheva, L.G. & Bärlocher, F. (2003) Seasonal and substrate preference of fungi colonizing leaves in streams: traditional versus molecular evidence. Environmental Microbiology, 7, 270-280.

Niyogi, D.K., Lewis, W.M. & McKnight, D.M. (2003) Direct and indirect effects of mine drainage on bacterial process in mountain streams. Journal of the North American Benthological Society, 22, 276-291.

Olapade, O.A. & Leff, L.G. (2004) Seasonal dynamics of bacterial assemblages in epilithic biofilms in a northeastern Ohio stream. Journal of the North American Benthological Society, 23, 686-700.

Ostrofsky, M.L. (1997) Relationship beween chemical characteristics of autumn-shed leaves and aquatic processing rates. Journal of the North American Benthological Society, 16, 750-759.

Pan, Y., Stevenson, R.J., Hill, B.H., Herlihy, A.T. & Collins, G.B. (1996) Using diatoms as indicators of ecological conditions in lotic systems: a regional assessment. Journal of the North American Benthological Society, 15, 481-495.

Pascoal, C. & Cassio, F. (2004) Contribution of fungi and bacteria to leaf litter decomposition in a polluted river. Applied and Environmental Microbiology, 70, 5266-5273.

Paul, B.J. & Duthie, H.C. (1989) Nutrient cycling in the epilithon of running waters. Canadian Journal of Botany, 67, 2302-2309.

Pereira, A.P., Graça, M.A.S. & Molles, M. (1998) Leaf litter decomposition in relation to litter physico-chemical properties, fungal biomass, arthropod colonization, and geographical origin of plant species. Pedobiologia, 42, 316-327.

Perlmutter, D.G. & Meyer, J.L. (1991) The impact of a stream dwelling harpacticoid copepod upon detritally associated bacteria. Ecology, 72, 2170-2180.

Peterson, C.G. (1987) Influences of flow regime on development and dessication response of lotic diatom communities. Ecology, 68, 946-954.

Pind, A., Freeman, C. & Lock, M.A. (1994) Enzymic degradation of phenolic materials in peatlands- measurement of phenol oxidase activity. Plant and Soil, 159, 227-231.

Porter, K.G. & Feig, Y.S. (1980) The use of DAPI for identifying and counting aquatic microflora. Limnology and Oceanography, 25, 943-948.

Pozo, J., Basaguren, A., Elósegui, A., Molinero, J., Fabre, E. & Chauvet, E. (1998) Afforestation with *Eucaliptus globulus* and leaf litter decomposition in streams of northern Spain. Hydrobiologia, 373-374, 101-109.

Pringle, C.M. (1990) Nutrient spatial heterogeneity: Effects on community structure, physiognomy, and diversity of stream algae. Ecology, 71, 905-920.

Pusch, M., Fiebig, D., Brettar, I., Eisenmann, H., Ellis, B.K., Kaplan, L.A., Lock, M.A., Naegeli, M.W. & Traunspurger, W. (1998) The role of micro-organisms in the ecological connectivity of running waters. Freshwater Biology, 40, 453-495.

Quinn, P. & Keough, M. J. (2002) Experimental design and data analysis for biologists. (eds P. Quinn & M.J. Keough), 537pp., Cambridge University Press, Cambridge.

Ramasamy, P. & Zhang, X. (2005) Effects of shear stress on the secretion of extracellular polymeric substances in biofilms. Water Science and Technology, 52, 217-223.

Rier, S.T., Kuhen, K.A. & Francoeur, S.N. (2007) Algal regulation of extracellular enzyme activity in stream microbial communities associated with inert substrata and detritus. Journal of the North American Benthological Society, 26, 439-449.

Rier, S.T. & Stevenson, R.J. (2006) Response of periphytic algae to gradients in nitrogen and phosphorus in streamside mesocosms. Hydrobiologia, 561, 131-147.

Rier, S.T. & Stevenson, R.J. (2002) Effects of light, dissolved organic carbon, and inorganic nutrients on the relationship between algae and heterotrophic bacteria in stream periphyton. Hydrobiologia, 489, 179-184.

Rier, S.T. & Stevenson, R.J. (2001) Relation of environmental factors to density of epilithic lotic bacteria in 2 ecoregions. Journal of the North American Benthological Society, 20, 520-532.

Robertson, A.L., Rundle, S.D. & Schmid-Araya, J.M. (2000) Putting the meio- into stream ecology: current findings and future directions for lotic meiofaunal research. Freshwater Biology, 44, 177-183.

Robinson, C.T. & Gessner, M.O. (2000) Nutrient addition accelerates leaf breakdown in an alpine springbrook. Oecologia, 122, 258-263.

Rogall, T., Wolters, J., Flohr, T. & Böttger, E.C. (1990) Towards a phylogeny and definition of species at the molecular level within the genus *Mycobacterium*. International Journal of Systematic Bacteriology, 40, 323-330.

Roldán, A. (1988) Deuteromicetos acuáticos: taxonomía y ecología en cursos mediterraneos, PhD thesis, Universidad de Murcia, Murcia.

Romani, A., Fischer, H., Mille-Lindblom, C. & Tranvik, L.J. (2006) Interactions of bacteria and fungi on decomposing litter: differential extracellular enzyme activities. Ecology, 87, 2559-2569.

Romani, A.M., Giorgi, A., Acuña, V. & Sabater, S. (2004a) The influence of substratum type and nutrient supply on biofilm organic matter utilization in streams. Limnology and Oceanography, 49, 1713-1721.

Romani, A.M., Guasch, H., Munoz, I., Ruana, J., Vilalta, E., Schwartz, T., Emtiazi, F. & Sabater, S. (2004b) Biofilm structure and function and possible implications for riverine DOC dynamics. Microbial Ecology, 47, 316-328.

Romani, A.M. & Sabater, S. (2001) Structure and activity of rock and sand biofilms in a Mediterranean stream. Ecology, 82, 3232-3245.

Romani, A.M. & Sabater, S. (2000) Influence of algal biomass on extracellular enzyme activity in river biofilms. Microbial Ecology, 40, 16-24.

Romani, A.M. & Sabater, S. (1999) Effect of primary producers on the heterotrophic metabolism of a stream biofilm. Freshwater Biology, 41, 729-736.

Royer, T.V. & Minshall, W. (2001) Effects of nutrient enrichment and leaf quality on the breakdown of leaves in a hardwater stream. Freshwater Biology, 40, 603-610.

Rubin, M.A. & Leff, L.G. (2007) Nutrients and other abiotic factors affecting bacterial communities in an Ohio River (USA). Microbial Ecology, 54, 374-383.

Rüttimann, C., Vicuña, R., Mozuch, M.D. & Kirk, T.K. (1991) Limited bacterial mineralization of fungal degradation intermediates from synthetic lignin. Applied and Environmental Microbiology, 57, 3652-3655.

Sabater, S., Guasch, H., Muñoz, I. & Romaní, A.M. (2006) Hydrology, light and use of organic and inorganic materials as structuring factors of biological communities in Mediterranean streams. Limnetica, 25, 335-348.

Sabater, S., Acuña, V., Giorgi, A., Guerra, E., Muñoz, I. & Romani, A. (2005) Effects of nutrient inputs in a forested Mediterranean stream under moderate light availability. Archiv für Hydrobiologie, 163, 479-496.

Sabater, S., Bernal, S., Butturini, A., Nin, E. & Sabater, F. (2001) Wood and leaf debris input in a Mediterranean stream: the influence of riparian vegetation. Archiv für Hydrobiologie , 153, 91-102.

Sabater, S. & Romani, A.M. (1996) Metabolic changes associated with biofilm formation in an undisturbed Mediterranean stream. Hydrobiologia, 335, 107-113.

Sala, M. (1982) Datos quantitativos de los procesos geomorficos fluviales actuales en la cuenca e la riera de Fuirosos (Montnegre, macizo litoral catalan). In: Cuadrenos de Investigación Geográfica, pp. 53-70, Barcelona.

Sala, M. & Franch, F.S. (1980) Mediciones de reptación y lavado superficial en la cuenca de la riera de Furiosos (Montnegre, macizo litoral catalán). Métodos y resultados. Revista de Geografia, 14, 2-25.

Sala, M.M. & Güde, H. (2004) Ectoenzymatic activities and and heterotrophic bacteria decomposing detritus. Archiv für Hydrobiologie, 160, 289-303.

Sala, M.M., Karner, M., Arin, L. & Marrase, C. (2001) Measurement of ectoenzyme activities as an indication of inorganic nutrient imbalance in microbial communities. Aquatic Microbial Ecology, 23, 301-311.

Sampaio, A., Cortes, R. & Leâo, C. (2001) Invertebrate and microbial colonization in native and exotic leaf litter species in a mountain stream. International Review of Hydrobiology, 4/5, 527-540.

Schmid-Araya, J.M. & Schmid, P.E. (2000) Trophic relationships: integrating meiofauna into a realistic benthic food web. Freshwater Biology, 44, 149-163.

Sinsabaugh, R.L. & Findlay, S. (1995) Microbial production, enzyme activity, and carbon turnover in surface sediments of the Hudson River estuary. Microbial Ecology, 30, 127-141.

Sinsabaugh, R.L., Osgood, M.P. & Findlay, S. (1994) Enzymatic models for estimating decomposition rates of particulate detritus. Journal of the North American Benthological Society, 13, 160-169.

Sinsabaugh, R.L., Antibus, R.K., Linkins, A.E., McClaugherty, C.A., Rayburn, L., Repert, D. & Weiland, T. (1992) Wood decomposition over a first-order watershed: mass loss as a function of lignocellulose activity. Soil Biology and Biochemistry, 24, 743-749.

Sinsabaugh, R.L. & Linkins, A.E. (1990) Enzymic and chemical analysis of particulate matter from a boreal river. Freshwater Biology, 23, 301-309.

Sinsabaugh, R.L., Repert, D., Weiland, T., Golladay, S.W. & Linkins, A.E. (1991) Exoenzyme accumulation in epilithic biofilm. Hydrobiologia, 222, 29-37.

Sirová, D., Vrba, J. & Rejmánková, E. (2006) Extracellular enzyme activities in benthic cyanobacterial mats: comparison-between nutrient enriched and control sites in marshes of norther Belize. Aquatic Microbial Ecology, 44, 11-20.

Smith, V.H. (2003) Eutrophication of freshwater and coastal marine ecosystems. A global problem. Environmental Science and Pollution Research, 10, 126-139.

Sobczak, W.V. (1996) Epilithic bacterial responses to variations in algal biomass and labile dissolved organic carbon during biofilm colonization. Journal of the North American Benthological Society, 15, 143-154.

Sobczak, W.V. & Burton, T.M. (1996) Epilithic bacterial and algal colonization in a stream run, riffle, and pool: a test of biomass covariation. Hydrobiologia, 332, 159-166.

Soininen, J. & Eloranta, P. (2004) Seasonal persistence and stability of diatom communities in rivers: are there habitat specific differences? European Journal of Phycology, 39, 153-160.

Sokal, R. R. & Rholf F. J. (1995) Biometry- The principles and practice of statistics in biological research. 3rd Edition W. H. Freeman, New York.

Sridhar, K.R. & Bärlocher, F. (2000) Initial colonization, nutrient supply and fungal activity on leaves decaying in streams. Applied and Environmental Microbiology, 66, 1114-1119.

Staats, N., Stal, L.J., de Winder, B. & Mur, L.R. (2000) Oxygen photosynthesis as driving process in exopolysaccharide production of benthic diatoms. Marine Ecology Progress Series, 193, 261-269.

Stelzer, R.S., Heffernan, J., & Likens, G.E. (2003) The influence of dissolved nutrients and particulate organic matter quality on microbial respiration and biomass in a forest stream. Freshwater Biology, 48, 1925-1937.

Stelzer, R.S. & Lamberti, G.A. (2001) Effects of N : P ratio and total nutrient concentration on stream periphyton community structure, biomass, and elemental composition. Limnology and Oceanography, 46, 356-367.

Sterner, R.W. & Elser, J.J. (2002). The stoichiometry of autotroph growth: variation at the base of food web. In: Ecological stoichometry: the biology of elements from molecules to the biosphere (eds R.W. Sterner & J.J. Elser), pp. 80-133. Princeton University Press, New Jersey.

Stevenson, R.J. (1996). An introduction to algal ecology in freshwater benthic habitats. In: Algal Ecology (eds R.J. Stevenson, M.L. Bothwell & R.L. Lowe), pp. 3-30. Academic Press Inc, San Diego.

Stoodley, P., Wilson, S., Hall-Stoodley, L., Boyle, J.D., Lappin-Scott, H.M. & Costerton, J.W. (2001) Growth and detachment of cell clusters from mature mixed-species biofilms. Applied and Environmental Microbiology, 67, 5608-5613.

Suberkropp, K. (1998) Effect of dissolved nutrient on two aquatic hyphomycetes growing on leaf litter. Mycological Research, 8, 998-1002.

Suberkropp, K. (1995) The influence of nutrients on fungal growth, productivity, and sporulation during leaf breakdown in streams. Canadian Journal of Botany, 73, 1361-S1369.

Suberkropp, K. (1992). Interactions with invertebrates. In: The Ecology of Aquatic Hyphomycetes. Ecological studies series (ed F. Bärlocher), Vol. 94, pp. 118-134. Springer-Verlag, Berlin.

Suberkropp, K. (1991) Relationships between growth and sporulation of aquatic hyphomycetes on decomposing leaf litter. Mycological Research, 95, 843-850.

Suberkropp, K. (1984) Effect of temperature on seasonal occurrence of aquatic hyphomycetes. Transactions and Journals of the British Mycological Society, 82, 53-62.

Suberkropp, K. & Chauvet, E. (1995) Regulation of leaf breakdown by fungi in streams: Influences of water chemistry. Ecology, 76, 1433-1445.

Suberkropp, K. & Klug, M.J. (1976) Fungi and bacteria associated with leaves during processing in a woodland stream. Ecology, 57, 707-719.

Sundh, I. (1992) Biochemical composition of dissolved organic carbon derived from phytoplankton and used by heterotrophic bacteria. Applied and Environmental Microbiology, 58, 2938-2947.

Tank, J.L. & Dodds, W.K. (2003) Nutrient limitation of epilithic and epixylic biofilms in ten North American streams. Freshwater Biology, 48, 1031-1049.

Tank, J.L., Webster, J.R., Benfield, E.F. & Sinsabaugh, R.L. (1998) Effect of leaf litter exclusion on microbial enzyme activity associated with wood biofilms in streams. Journal of the North American Benthological Society, 17, 95-103.

Taulbee, W.K., Cooper, S.D. & Melack, J.M. (2005) Effects of nutrient enrichment on algal biomass across a natural light gradient. Archiv für Hydrobiologie, 164, 449-464.

Taylor, B.R., Mallaley, C. & Cairns, J. F. (2007) Limited evidence that mixing leaf litter accelerates decomposition or increases diversity of decomposers in streams of eastern Canada. Hydrobiologia, 592, 405-422.

Taylor, B.R., Parkinson, D. & Parsons, W.F.J. (1989) Nitrogen and lignin content as predictors of litter decay rates: a microcosm test. Ecology, 70, 97-104.

Theil-Nielsen, J. & Sondergaard, M. (1998) Bacterial carbon biomass calculated from biovolumes. Archiv für Hydrobiologie, 141, 195-207.

Thomas, D.P., Bachmann, S.P. & Lopez-Ribot, J.L. (2006) Proteomics for the analysis of the Candida albicans biofilm lifestyle. Proteomics, 6, 5795-5804.

Thompson, A.J. & Sinsabaugh, R.L. (2000) Matric and particulate phosphatase and aminopeptidase activity in limnetic biofilms. Aquatic Microbial Ecology. 21, 151-159.

Townsend, C.R. (1989) The patch dynamics concept of stream community ecology. Journal of the North American Benthological Society, 8, 36-50.

Triska, F.J., Kennedy, V.C., Avanzino, R.J., Zwellweger, G.W. & Bencala, K.E. (1989) Retention and transport of nutrients in a third-order stream: channel processes. Ecology, 70, 1877-1892.

Tusseau-Vuillemin, M.H. (2001) Do food processing industries contribute to the eutrophication of aquatic systems? Ecotoxicology and Environmental Safety, 50, 143-152.

Vannote, R.L., Minshall, G.W., Cummins, K.W., Sedell, J.R. & Cushing, C.E. (1980) The river continuum concept. Canadan Journal of Fischeries and Aquatic Sciences, 37, 130-137.

Vázquez, E., Romani, A.M., Sabater, F. & Butturini, A. (2007) Effects of the dry-wet hydrological shift on dissolved organic carbon dynamics and fate across stream-riparian interface in a mediterranean catchment. Ecosystems, 10, 239-251.

Velicer, G.J. (2003) Social strife in the microbial world. Trends in Microbiology, 11, 330-337.

Vrba, J., Callieri, C., Bittl, T., Šimek, K., Bertoni, R., Filandr, P., Hartman, P., Hejzlar, J., Macek, M. & Nedoma, J. (2004) Are bacteria the major enzyme producers of extracellular glycolytic enzymes in aquatic environments? International Review of Hydrobiology, 89, 102-117.

Vrba, J., Sĭmek, K., Nedoma, J. & Hartman, P. (1993) 4-Methylumbelliferyl-β-N-Acetylglucosaminide hydrolysis by a high-affinity enzyme, a putative marker of protozoan bacterivory. Applied and Environmental Microbiology, 59, 3091-3101.

Vrba, J., Nedoma, J., Šimek, K., & Seda, J. (1992) Microbial decomposition of polymer organic matter related to plankton development in a reservoir: activity of, β-glucosidase and β-N-acetylglucosaminidase and uptake of N-acetylglucosamine. Archiv für Hydrobiologie, 126, 193-211.

Webster, J.R. & Benfield, E.F. (1986) Vascular plant breakdown in freshwater ecosystems. Annual Review of Ecology and Systematics, 17, 567-594.

Weilhoefer, C.L. & Pan, Y. (2003) 170 wetland diatom assemblages as indicators of flooding influence and surrounding land use in the Willamette valley, OR. Journal of Phycology, 39, 59-59.

Weiss, M. & Simon, M. (1999) Consumption of labile dissolved organic matter by limnetic bacterioplankton: the relative significance of amino acids and carbohydrates. Aquatic Microbial Ecology, 17, 1-12.

Wentworth, C.K. (1922) A scale of grade and class terms for clastic sediments. The Journal of Geology, 30, 377-392.

Wetzel, R.G. (2001) Limnology, 3 edn. 1006 pp., Academic Press, California.

Wetzel, R.G. (1992) Gradient-dominated ecosystems: sources and regulatory functions of dissolved organic matter in freshwater ecosystem. Hydrobiologia, 229, 181-198.

Weyers, H.S. & Suberkropp, K. (1996) Fungal and bacterial production during the breakdown of yellow poplar leaves in 2 streams. Journal of the North American Benthological Society, 15, 408-420.

Wilczek, S., Fischer, H. & Pusch, M.T. (2005) Regulation and seasonal dynamics of extracellular enzyme activities in the sediments of a large lowland river. Microbial Ecology, 50, 253-267.

Winter, J.G. & Duthie, H.C. (2000) Stream epilithic, epipelic and epiphytic diatoms: habitat fidelity and use in biomonitoring. Aquatic Ecology, 34, 345-353.

Wright, R.T. & Hobbie, J.E. (1965) The uptake of organic solutes in lake water. Limnology and Oceanography, 10, 22-28.

Xiangzhen, L. & Pariente, S. (2003) Arylsulfatase activity of soil microbial biomass along a Mediterranean-arid transect. 35, 925-934.

Yallop, M.L., Paterson, D.M. & Wellsbury, P. (2000) Interrelationships between rates of microbial production, exopolymer production, microbial biomass, and sediment stability in biofilms of intertidal sediments. Microbial Ecology, 39, 116-127.

Yeager, P.E. & Sinsabaugh, R.L. (1998) Microbial diversity along a sediment detrital particle size gradient. Aquatic Ecology, 32, 281-289.

Ylla, I., Romani, A.M. & Sabater, S. (2007) Differential effects of nutrients and light on the primary production of stream algae and mosses. Fundamental and Applied Limnology, 170, 1-10.

Zhang, X. & Bishop, P.L. (2003) Biodegradability of biofilm extracellular polymeric substances. Chemosphere, 50, 63-69.

Zippel, B. & Neu, T.R. (2005) Growth and structure off phototrophic biofilms under controlled light conditions. Water Science and Technology, 52, 203-209.

CONCLUSIONS

Organic matter availability structures microbial biomass and activity in a Mediterranean stream

1. Microbial heterotrophic biomass (bacteria plus fungi) was generally higher than autotrophic biomass (algae), except during short periods of high light availability in the spring and winter. During these periods, sources of organic matter (OM) shifted towards autochthonous sources derived mainly from algae, which was demonstrated by high algal biomass and peptidase activity in benthic communities.

2. Heterotrophic activity peaked in the autumn. Bacterial and fungal biomass increased with the decomposition of cellulose and hemicellulose compounds from leaf material. Later, lignin decomposition was stimulated in fine (sand, gravel) and coarse (rocks, boulders and cobbles) substrata by the accumulation of fine detritus.

3. The Mediterranean summer drought provoked an earlier leaf fall. The resumption of the water flow caused the weathering of riparian soils and subsequently a large increase in dissolved organic carbon and nitrate, which led to growth of bacteria and fungi.

Organic matter decomposition by fungi in a Mediterranean forested stream: contribution of streambed substrata.

4. Cellulolytic activity was higher in microbial communities associated to organic substrata (leaves and branches), while highest ligninolytic activity was measured in inorganic substrata communities (sand and gravel).

5. Ligninolytic activity in biofilms increased together with dissolved inorganic nitrogen concentrations in the stream water. Lixiviation of the stream watershed during autumn rainfalls led to mobilise nutrients from soils to the stream water, and therefore, those nutrients may influence on fungal degrading activities in-stream.

6. Estimates of enzyme activity and ergosterol concentration in terms of stream reach showed that OM breakdown in the stream began with cellulose decomposition followed by degradation of lignin compounds. Fungal biomass and enzyme activities evolved

accordingly indicating that fungi were responsible for most of decomposition processes that occurred in the stream in autumn.

Relating nutrient molar ratios to organic matter utilization of microbial attached communities in a Mediterranean forested stream

7. Sand and gravel habitats had biofilms with higher N content (lower C: N and higher N:P molar ratios) and showed a major lignin and peptide decomposition capacity. Cycling of nitrogenous compounds in these biofilms was favoured by the presence of algae and fine detritic materials accumulating in sediments (both supplying N-compounds to microbes).

8. Debris dams and leaf accumulation habitats had communities with higher C and P content (C: N and lower N: P molar ratios) and major capacities for polysaccharides decomposition. Here, the major use of C compounds was tightly related to the availability of plant material polysaccharides.

9. Similarities (C:N) and divergences (N:P) between stream water and nutrient molar ratios in microbial communities may also affect nutrient demands and in consequence, extracellular enzymes expression. Dissolved nitrogen concentrations in the water correlated with peptidase activity.

Relationships between microbial biomass and extracellular enzyme activities during breakdown process of leaf litter in a forested stream

10. Fungi and bacteria showed contrasting colonization patterns in leaves. While bacteria increased sharply during the first week, fungi slightly decreased, and the steepest fungal increase occurred after day 17 when bacterial biomass was slowly increasing. It might be hipothesized that delayed fungal growth was because of biomass lost (in form of conidia) during sporulation process, while the early increase of bacteria was related to the use of leaf leachates.

11. Bacterial biomass and polysaccharide and lignin degrading activities showed a strong exponential relationship during breakdown process of leaves. This pattern was more pronounced in *Populus* than in *Platanus* leaves. Conversely, fungal biomass only kept this relationship in the *Platanus* species suggesting that the higher durability of

these materials might have been favouring the establishment of fungi and making possible the relationship between fungal biomass and activity.

12. Changes in leaf properties are related to the increase of microbial enzyme activities during breakdown process, but also by the microbial colonization. It was between days 7 and 17 of the breakdown experiment, when the widest and fastest leaf structural changes (steepest decrease in toughness and C:N and C:P ratios) coincided with the episode of maximum enzyme activities per unit of microbial carbon.

Patterns of epilithic biofilm formation in two streams of different bioclimatic regions: analysis on microbial community structure and metabolism

13. The analysis of the whole biofilm colonization sequence showed a greater bacterial biomass and diversity at the Fuirosos biofilms as well as higher EPS-polysaccharide content. In contrast, greater diatom diversity was measured at biofilms developing at the Walzbach, also showing high cell-specific enzyme activity being more efficient in the use of organic matter

14. Successional patterns of bacterial and diatom populations in the Fuirosos showed clear discontinutities coinciding with flood episodes while at the Walzbach the time sequence was more gradual.

15. The results suggest that microbial community structure development is highly related to the physical characteristics of the different sites (specially the stream hydrology). However, the hystorical evolutionary pressure from the different bioclimatic regions could be also affecting the microbial community composition.

Relevance of polymeric matrix enzymes during biofilm formation

16. The use of cation exchange resin as extraction procedure seems appropriate to measure matrix-enzymes in freshwater biofilms, although the methodological limitation for using a biofilm suspension instead of an undisrupted biofilm.

17. Higher EPS relative content and matrix-enzyme activities per μg of polysaccharides in the EPS were measured during the first 1-3 days of biofilm formation, indicating a high rate of enzyme release into the matrix during this period. Relative contribution of matrix-enzyme activities decreased as biofilm matures but was maintained at 13-37% of total enzyme activity at the 42-49 days-old biofilm.

18. The contribution of matrix-enzymes must be considered, especially for young biofilms.

Effect of nutrients on the sporulation and diversity of aquatic hyphomycetes on submerged substrata in a Mediterranean stream

19. Long-term stream enrichments experiments did not affect potential sporulation rates of aquatic hyphomycetes, but produced significant changes in fungal community composition. *Clavariopsis aquatica*, *Alatospora acuminata* and *Lemonniera* sp. species were dominant in the enriched reach while *Heliscella stellata*, *Trisceloporus acuminatus* and *Clavatospora longibrachiata* species were characteristic of the unenriched reach.

20. Short-term nutrient enrichments produced insignificant increases in fungal sporulation rates on distinct stream benthic substrata from the unenriched reach and did not affect fungal community composition. Major differences in sporulation rates were detected between substrata, the highest rate being for leaves (especially *Platanus acerifolia*).

21. Long- and short-term enrichment experiments suggested that fungal N demands can be fulfilled at relatively low levels of dissolved nitrate, and further increases in the nutrients availability may not result in enhanced fungal activity.

Effect of water nutrient concentration and N:P molar ratio on biofilm structure and metabolism

22. Water nutrient concentration had positive effects on algal and bacterial biomass accrual in biofilms, as well as on the stimulation of peptidase activity. Instead, the increase of water N:P ratio (56:1 treatments) reduced bacterial biomass and stimulated phosphatase activity.

23. Biofilm C:N ratio decreased according to the water nutrient concentration and by the increase of bacterial peptidases, suggesting a preferential uptake of N than P by microbes. The sensitivity of bacterial biomass to the water N:P imbalance (low P availability) was stronger than that experienced by algae. However, algae respond by increasing EPS in biofilms. Changes in water N:P modified also biofilm C:P.

24. Contrary to our predictions, the interaction between the two factors (nutrient concentration and N:P ratio) was not statistically significant for any of the biofilm analyzed parameters. This suggested that each factor had independent effects in modulating biofilm structural and metabolic parameters.

25. Less than 35 days were enough to modify extracellular enzyme activities (day 7) and microbial biomass (day 3 for bacteria and day 21 for algae) in biofilms, while nutrient molar ratios (C:N and C:P) remained stable for longer (until day 28). The fast response of biofilm parameters to changes in water nutrient availability suggested a high dependence between biofilms and nutrients in the water column.