Heterotrophic and autotrophic metabolism in Mediterranean streams

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HETEROTROPHIC AND AUTOTROPHIC METABOLISM IN MEDITERRANEAN STREAMS

Ph. D. Thesis

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METABOLISME HETEROTRÒFIC I AUTOTRÒFIC EN RIUS MEDITERRANIS

Tesi Doctoral presentada per Anna M. Romaní i Cornet
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Vist i plau del director de tesi

Sergi Sabater i Cortés
Professor titular d'Ecologia
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al Francesc, a l'Eloi i a tota la meva família

Riera dolça i lenta:
on vas avui matí?
Jo vaig avall sens pressa,
seguint el meu camí.

(cançó popular alemanya)

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AGRAÏMENTS

Agraïments

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INTRODUCCIÓ

Introducció

La distribució de l'activitat humana en una area geogràfica queda clarament definida pels rius que s'hi troben. Les aigües fluvials han estat, i encara són, utilitzades per la població com a font de riquesa (agricultura, energia elèctrica, indústria) i com a ambient per a activitats de temps lliure. Actualment, en zones altament poblades, com a Catalunya i a tot Europa en general, hi ha una gran pressió sobre els ecosistemes fluvials, sobrepassant sovint la seva capacitat d'autodepuració (e.g. Décamps i Naiman 1989, Margalef 1990). Margalef (1991) va descriure analògicament els rius com els ronyons que "netegen" els resultats de les activitats que tenen lloc a l'ecosistema terrestre.

L'ecosistema fluvial ha estat considerat com un sistema de flux d'energia que maximitza l'eficiència en la utilització d'energia i minimitza les pèrdues d'energia aigües avall (Margalef 1960, Vannote et al. 1980). En els rius de capçalera principalment té lloc el processat i transport de materials de l'ecosistema terrestre (McDowell i Fisher 1976, Newbold et al. 1982) mentre aigües avall (3er-6è ordre del riu) predomina l'autotròfia (Bott et al. 1985), i hi ha un retorn a l'heterotròfia en els trams finals del riu (Vannote et al. 1980). En la realitat però, molts rius (com els rius Mediterranis) s'allunyen d'aquesta teoria clàssica. Els rius de règim Mediterrani estan sotmesos sovint a una influència humana (construcció d'embassaments, alteracions del canal, pol.lució) i a una hidrodinàmica variable (períodes de sequera i avingudes) (Sabater i Sabater 1992, Sabater et al. 1995, Guasch 1995).

La matèria orgànica dissolta és la font més important de carboni reduit (Hobbie i Likens 1973, Kaplan i Bott 1983, Kuserk et al. 1984, Lock 1993, Volk et al. 1997) i és eliminada de l'aigua a través de processos biòtics (incorporació pels organismes) i abiòtics (adsorció física i/o química) (Dahm 1981, Ladd et al. 1982, Kuserk et al. 1984, Petersen et al. 1989). La font de matèria orgànica pot ser autòctona (procedent dels productors primaris del riu) o al.lòctona (procedent de la vegetació de ribera o altres materials provinents de fora del sistema fluvial). La quantitat i la font principal del material que entra al riu depèn del seu tamany (Naiman et al. 1987) així com del desenvolupament de la vegetació de ribera (González i Pozo 1997, Johnson i Covich 1997), de canvis de cabal, i de factors estacionals com la caiguda de fulles a la tardor (Lock i Ford 1986, Cummins et al. 1983, Fisher i Likens 1973, Hill et al. 1992).

En rius d'ordre baix la comunitat bentònica és la principal responsable de processar la matèria orgànica (Lock i Hynes 1976, Geesey et al. 1978, Naiman et al. 1987, Meyer 1988, Marxsen 1988). La comunitat microbiana bentònica (algues, bacteris, fongs i protozous) es desenvolupa en els diferents substrats del llit del riu (pedres, roques, grans de sorra, fulles, macròfits) formant el que s'anomena biofilm (p.e. Haack i McFeters 1982a). Aquesta estructura té un component de material mucilaginós (matriu de polisacàrids) que és en part responsable de la integritat del biofilm així com de la capactitat de retenció de materials orgànics, nutrients i enzims extracel.lulars (Lock et al. 1984, Hamilton 1987, Christensen i Characklis 1990, Blenkinsopp i Costerton 1991, Lock 1993) (veure Fig. 1 i Fig. 2 del capítol 1).

Els microorganismes no poden incorporar molècules orgàniques de més de 10,000 Da de pes molecular i la majoria dels compostos orgànics que formen part de la matèria orgànica dels ecosistemes aquàtics tenen un pes molecular més elevat (Lock 1990). Els enzims hidrolítics extracel.lulars degraden la matèria orgànica polimèrica i macromolecular en molècules de baix pes molecular que poden travessar la membrana cel.lular bacteriana (Rogers 1961) (veure Fig. 3 del capítol 1). La taxa de descomposició de la matèria orgànica depèn directament d'aquesta activitat enzimàtica i per tant és una activitat clau en el reciclatge del carboni en els ecosistemes aquàtics (Servais i Billen 1993, Chróst 1994, Turley 1994, Gajewski i Chróst 1995) (veure Fig. 4 del capítol 1). Els ectoenzims són enzims extracel.lulars lligats a la superfície dels microorganismes (principalment bacteris i fongs) que actuen fora de la cèl.lula (Chróst 1990). Els ectoenzims són induibles (la seva síntesi augmenta amb la presència de substrat) i regulats per repressió per catabolit (disminueix la seva síntesi quan el producte final s'acumula al medi) (Somville 1984, Chróst 1989, Chróst 1990, Chróst i Rai 1993) (veure Fig. 5 del capítol 1). El terme ectoenzims també es refereix, aquí, als possibles enzims lliures alliberats al medi o lligats a partícules detrítiques.

En aquest estudi s'han triat tres ectoenzims: la β-glucosidasa, β-xilosidasa i fosfatasa. La β-glucosidasa degrada cel.lobiosa (el principal component de la cel.lulosa) i altres polisacàrids amb enllaços β, alliberant β-D-glucosa (Atlas i Bartha 1987, Desphande i Eriksson 1988). La β-xilosidasa degrada xilobiosa (component de l'hemicel.lulosa) i altres xilooligosacàrids alliberant β-D-xilosa (Atlas i Bartha 1987, Lacke 1988). Aquests dos enzims polisacarídics indiquen, en part, quina és la font més important del material utilitzat per la comunitat microbiana: β-glucosidasa per l'autòcton i β-xilosidasa per l'al.lòcton. L'activitat fosfatasa degrada ésters de fosfat alliberant fósfor inorgànic (Jansson et al. 1988). Aquest enzim és un indicatiu de la degradació de compostos de fósfor però sobretot pot ser també un indicatiu d'una limitació de nutrients. Aquest enzim també es troba a les cèl.lules algals (Francko 1991, Cotner i Wetzel 1992).

Objectius

L'objectiu principal d'aquest estudi és determinar el paper dels heteròtrofs en la utilització de la matèria orgànica (autòctona i al.lòctona) en rius mediterranis. Els objectius més concrets queden resumits en els següents temes d'estudi:

1) Relació entre les algues i els bacteris que composen els diferents biofilms del rius. Paper dels productors primaris com a font de matèria orgànica autòctona.

2) Estudi de les característiques de la comunitat bacteriana bentònica (densitat, biomassa, capacitat hidrolítica, eficiència en la utilització de matèria orgànica) en rius mediterranis.

3) Factors físics i químics que controlen la variabilitat del metabolisme heterotròfic i autotròfic en biofilms de rius mediterranis. Efecte de l'estacionalitat (canvis de cabal, llum, temperatura, nutrients) en el funcionalisme dels sistemes mediterranis i comparació amb altres sistemes d'altres àrees geogràfiques.

4) Diferències en la capacitat hidrolítica entre els biofilms que es formen en cada substrat del llit del riu (pedres, sorra superficial, sorra subsuperficial, crosta cianobacteriana).

5) Recuperació del metabolisme autotròfic i heterotròfic del biofilm després d'un període de sequera.

6) Efectes de la tala de la vegetació de ribera sobre les activitats autotròfiques i heterotròfiques del biofilm del riu.

Desenvolupament d'aquesta memòria

Per tal d'aconseguir els objectius abans esmentats s'han realitzat les següents mesures: capacitat hidrolítica (activitats enzimàtiques potencials) de degradar polisacàrids i ésters de fosfat (enzims β-glucosidasa, β-xilosidasa i fosfatasa), densitat i biomassa bacteriana, biomassa (clorofil.la) i activitat (incorporació de $H^{14}CO_3$) algal, i activitat respiratòria (activitat de la cadena de transport d'electrons, ETS). L'estudi s'ha portat a terme al riu Ter i a dos dels seus afluents (rius no perturbats de segon ordre), la Riera Major i La Solana (veure Fig. 6 del capítol 1), que difereixen en la seva litologia (silícica versus calcària) i vegetació de ribera (riu forestat versus obert).

Durant el període gener 1994-febrer 1995 es va realitzar un estudi anual (amb mostrejos mensual) al riu Ter (capítol 6), La Solana (capítol 4.1) i Riera Major (capítol 3.1). A cada riu es van estudiar els diferents tipus de biofilms en funció del substrat: a Riera Major el biofilm epipsammic (diferenciant la sorra del mig del riu de la sorra del litoral) i epilític (utilitzant petits totxets de ceràmica com a substrats artificials), a La Solana la crosta cianobacteriana distingint les diferents taques que formen el biofilm estromatolític, i al riu Ter el biofilm epilític (utilitzant també petits totxets de ceràmica com a substrats artificials).

L'octubre de 1994 hi va haver una gran avinguda a Riera Major que va rentar el sediment. Aquest fet va ser aprofitat per a estudiar les diferències de metabolisme heterotròfic entre el sediment superficial i subsuperficial i el diferent patró temporal amb la presa de mostres (cores de sediment) quinzenals des de l'octubre fins al desembre del 1994 i mensuals des del desembre de 1994 fins a l'agost de 1995 (capítol 3.2).

A mitjans de febrer de 1995 es van talar els arbres d'una bona part de la vegetació de ribera de Riera Major justament en el mateix punt on s'havia realitzat el cicle anual. L'efecte d'aquesta perturbació sobre les comunitats bentòniques (algues i bacteris) va ser estudiada mitjançant un mostreig mensual durant el període març-juny 1995 i quinzenal durant el període juliol-agost 1995 (capítol 3.3).

Durant els mesos de juny i juliol de 1994 la riera de La Solana va quedar totalment seca, un fet que s'esdevé amb periodicitat intraanual. Es van prendre llavors mostres de la crosta cianobacteriana i es va estudiar (en un experiment de laboratori) la recuperació del metabolisme després d'immergir novament les mostres en aigua recirculant (capítol 4.2).

Durant l'any 1995 es va realitzar un estudi més profund de les activitats ectoenzimàtiques en els biofilms dels rius mediterranis de segon ordre. La cinètica enzimàtica (utilitzant l'aproximació de Michaelis-Menten) dels ectoenzims β-glucosidasa, β-xilosidasa i fosfatasa, va ser estudiada a La Riera Major (a la sorra i als petits totxets) i a La Solana (a la crosta cianobacteriana) amb un mostreig per cada estació de l'any (capítol 5). Es van obtenir les velocitats màximes de reacció (Vmax), les constants aparents de Michaelis (Km) el temps de renovació de la hidròlisis del substrat (Tt) per cada enzim, paràmetres que ens indiquen en part l'afinitat de l'enzim pel substrat i l'eficiència en la utilització del substrat.

Durant la tardor de 1995 es va realitzar un estudi al Breitenbach, un rierol de primer ordre situat al Nord Oest d'Alemanya (veure Fig. 7 del capítol 1). Les activitats ectoenzimàtiques epilítiques (utilitzant també els totxets de ceràmica com a substrats artificials) i la cinètica enzimàtica dels enzims β-glucosidasa, β-xilosidasa, fosfatasa i leucina-aminopeptidasa va ser estudiada a diferents punts del riu.

Els estudis anuals dels rius mediterranis feren sospitar de la importància de les algues com a font de matèria orgànica pels bacteris. Per investigar les possibles relacions entre les algues i els bacteris es va estudiar el procés de colonització (variació en la densitat i activitats autotròfiques i heterotròfiques) amb la utilització dels substrats artificials (totxets de ceràmica) al llarg de dos experiments a Riera Major. El primer es va realitzar durant l'estiu de 1994 (gran cobertura, poca disponibilitat de llum) analitzant el procés de colonització dels totxets per algues i bacteris (i les seves activitats) en comparació amb totxos ja colonitzats (posats prèviament al riu dos mesos abans) (capítol 8.1). El segon experiment es va realitzar durant la primavera de 1995 (poca cobertura, disponibilitat de llum, condicions màxim favorables pels productors primaris) comparant el procés de colonització del totxets col.locats al riu en condicions normals de llum (obtenció d'un biofilm autotròfic i heterotròfic) i totxets col.locats dins un tub semi-enterrat al riu (submergit), en condicions de foscor (obtenció d'un biofilm heterotròfic) (capítol 8.2).

Finalment s'ha realitzat una anàlisi conjunta de les dades per tal de descriure les possibles tendències generals en les activitats ectoenzimàtiques en biofilms de rius mediterranis. També s'ha analitzat la relació entre el quocient biomassa bacteriana/ biomassa algal per cada biofilm estudiat i les respectives activitats ectoenzimàtiques (capítol 9).

1. INTRODUCTION

1. Introduction

Rivers and streams define the distribution of human activity in a given geographic area. Flowing water has historically been used by the population as a source of wealth (agriculture, electric power, industry) as well as a leisure resort. Nowadays, in some highly populated areas, such as Catalonia and all of Europe, there is a great pressure on the river environment, exceeding its self-cleansing capacity (e.g. Décamps and Naiman 1989, Margalef 1990). The flow systems have been described as the kidneys which clean the results of the activities on the terrestrial environment (Margalef 1991).

The river ecosystem has been considered an energy flow transducer system which maximizes the efficiency of energy utilization and minimizes downstream energy loss (Vannote et al. 1980). Producer and consumer communities are distributed along the river in a continuum and in equilibrium with the dynamic physical conditions of each reach (discharge, riparian vegetation, streambed morphology, light) (Margalef 1960). Headwater streams are predominantly processors and transporters of materials from the terrestrial system and are mainly responsible for the efficient retention and oxidation of carbon (McDowell and Fisher 1976, Newbold et al. 1982). Downstream reaches (3rd-6th stream-order) show a predominance of autotrophy (Bott et al. 1985), and a return to heterotrophy in the lower stream reaches (Vannote et al. 1980). Although the river continuum concept was a sound theory, particular stream ecosystems such as desert streams, high humic streams and disturbed streams, could deviate from it (e.g. Meyer and Edwards 1990). More recently the effects of the especific watershed features as well as the hydraulics and seasonal effects has been included in the stream ecosystem theory (Naiman and Sedell 1981, Minshall et al. 1983, Statzner and Higler 1985, Minshall et al. 1985) and due to its complexity it has been suggested to incorporate chaos theory into models explaining stream ecosystems (Bretschko 1995). Mediterranean rivers could be an example of such a river system because of the important human influence (reservoirs, channel alterations, pollution) and the highly variable hydrodynamics (droughts and flood events) (Sabater and Sabater 1992, Sabater et al. 1995, Guasch 1995), which confer to them special characteristics.

Dissolved organic matter comprises most of the reduced carbon in streams and rivers (Hobbie and Likens 1973, Kaplan and Bott 1983, Kuserk et al. 1984, Lock 1993, Volk et al. 1997) and is removed from water through biotic (uptake) and abiotic (physical-chemical absorption) pathways (Dahm 1981, Ladd et al. 1982, Petersen et al. 1989). The organisms living in the stream utilize the organic matter compounds transported by the stream as a source of carbon (Kuserk et al. 1984). Dissolved organic carbon (DOC) sources may be autochthonous (synthesized within the stream environment, i.e. from primary producers) and/or allochthonus (synthesized out of the stream environment, i.e. leaves and plant material from the riparian vegetation and the watershed).

In low-order streams the benthic community is mostly responsible for organic matter processing (Lock and Hynes 1976, Geesey et al. 1978, Naiman et al. 1987). The highest

bacterial densities in low-order streams occur in the stream bed (Geesey et al. 1978, Meyer 1988, Marxsen 1988), and are important for the bioenergetic processes in the stream. The microbial compartment of the benthic community (bacteria, algae, fungi and protozoa) is usually structured on the solid surfaces of the streambed (rocks, sand grains, leaves, macrophytes) composing what is called the biofilm, an important place for energy transducing (Lock et al. 1984). The study of the autotrophic and heterotrophic processes in stream biofilms is a current research topic in stream ecology, to understand better the nutrient and carbon dynamics (Ward and Stanford 1991).

Stream biofilm structure

Biofilms are structured communities consisting of bacteria, algae, fungi and protozoa embedded in a polysaccharide matrix (Lock et al. 1984) (Fig. 1) and living on solid surfaces within an aqueous phase (Hamilton 1987, Characklis et al. 1990). Most bacteria produce extracellular polymers which lead to adhesion to the substrate (Marshall 1988, Low and White 1989) and compose the polysaccharide matrix responsible for the integrity of the biofilm (Hamilton 1987, Christensen and Characklis 1990, Lock 1993). Diatom adhesion is a more complicated process than that for bacteria; it requires metabolic energy, protein and glycoprotein synthesis (Cooksey and Wigglesworth-Cooksey 1995). It is suggested that the polysaccharide matrix retains and protects extracellular enzymes and products from their hydrolysis as well as being a site for entrapment of soluble and particulate matter (Lock 1981, Lock et al. 1984). The polysaccharide matrix could also serve as a place to concentrate nutrients (Hamilton 1987) through its nutrient entrapment mechanism mediated by ion exchange processes (Freeman et al.1995). The biofilm polysaccharide matrix may also act as a refuge for the microbial community from shear forces (Lock 1993).

The properties of biofilm vary with environmental factors such as population distribution in the film, nutrient loading rate, and hydrodynamic shear stress (Christensen and Characklis 1990). The current regime affects the benthic community in its resistance to biomass removal (Peterson and Stevenson 1992), also determining biomass accrual (Tett et al. 1978, Sabater and Sabater 1992) and thus the biofilm thickness. Trophic interactions such as herbivorousness of macroinvertebrates could also regulate the benthic biomass (Hart 1992, Wellnitz et al. 1996). Biofilm thickness, sometimes a function of biofilm age, determines diffusional length (Christensen and Characklis 1990). The decrease in nutrient diffusion in thick biofilms (e.g. Stevenson and Glover 1993) results in nutrient gradients (Hamilton 1987) where oxygen is of particular importance, which can lead to anoxic and reducing conditions close to the substratum surface (e.g. Kühl and Jorgensen 1992, Ramsing et al. 1993) (Fig. 1). Thick stream biofilms can increase internal nutrient cycling as a response to limited diffusion and thus to nutrient limitation (Mulholland et al. 1994). In sedimentary biofilms there is also a spatial heterogeneity of microbial activities related to the supply of organic material (Meyer-Reil 1994). Biofilm thickness

ranges from 50 µm for a laboratory pure culture to ca. 30 mm or higher for algal/bacterial mats (Christensen and Characklis 1990).

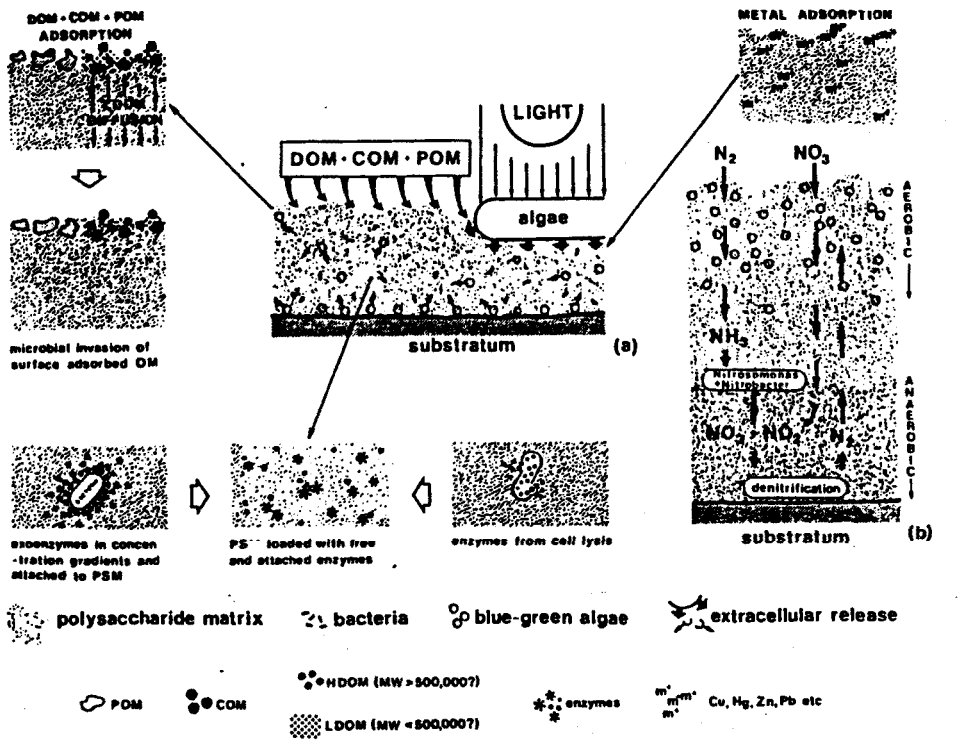


Fig.1. Structural-functional model of river epilithon. DOM= dissolved organic matter, COM= colloidal organic matter, POM= particulate organic matter, HDOM= high molecular weight DOM, and LDOM= low molecular weight DOM. From Lock et al. 1984.

Stream biofilms usually consist of bacteria, diatoms and encrusting or filamentous algae (e.g. Haack and McFeters 1982a). Close to the substrate there is a strongly attached zone while there is a more loose fraction in contact with the water flow (Oppenheim and Paterson 1990) (Fig. 2). The upper surfaces of stones tend to have algae and bacteria present (autoheterotrophic) while biofilms from within the streambed (hyporheic) will be predominantly heterotrophic (Fig. 2). Stream biofilms grow on the different benthic substrates such as rocks and stones (epilithic), sand (epipsammic), hyporheic zone sand (hyporheic), wood (epixylic), macrophytes (epiphytic) (Fig. 2).

Analogous biofilms also occur in industrial facilities (flow systems), water management equipment (water treatment plants, water distribution systems) (e.g. MacLeod et al. 1990) and within the human body (dental plaque, animal tissues). In spite of such heterogeneity, biofilms demonstrate a significant homogeneity of function (Hamilton 1987). The problems caused by biofilms (corrosion, slow down flowing) as well as the beneficial aspects (organic-nutrient trapping capabilities) (Blenkinsopp and Costerton 1991, Wolfaardt et al. 1995) are important to man.

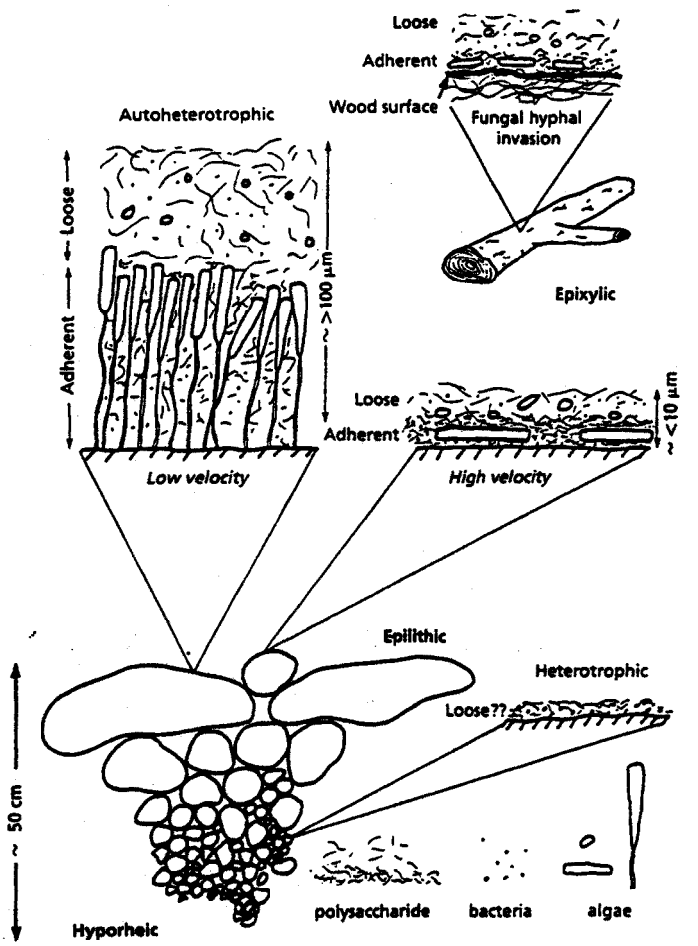


Fig. 2. Some biofilms from streambed surfaces. From Lock 1993.

Biofilm-organic matter interactions

The microbial community within stream biofilms utilize both autochthonous and allochthonous organic matter. The proportion of autochthonous or allochthonous sources of organic matter in a river and stream depends on its size (Vannote 1980, Conners and Naiman 1984, Naiman et al. 1987) but also on the local characteristics of each reach (González and Pozo 1997) such as riparian canopy (Johnson and Covich 1997). The input of dissolved organic matter quantity and composition changes between seasons (Lock and Ford 1986), but is generally associated with autumn leaf fall (Cummins et al. 1983) and discharge variations (Fisher and Likens 1973, Hill et al. 1992). Daily shifts in the organic matter pool have also been observed (Kuserk et al. 1984) and related to algal activity (Kaplan and Bott 1982). The quantity and quality of the materials transported by the stream are influenced by human disturbances such as removal of the riparian vegetation (Gregory et al. 1991).

The autochthonous organic matter provided the biofilm with high-quality compounds excreted from the algae (Kaplan and Bott 1989) although decaying algae and/or decaying heterotrophic organisms as well as possible heterotrophic exudates may also be a source of

autochthonous material. Healthy algae release a proportion of the carbon they fix in photosynthesis (Bjoernsen 1988, Sell 1994), which is related to availability of inorganic nutrients (Margalef 1997). These algal exudates contribute positively to the development of a nutrient-rich zone around themselves which could enhance attached bacterial growth (Murray et al. 1986). In contrast, bacteria may release organic compounds (such as vitamins) to algae (Lock 1993). Algal extracellular products include low molecular weight compounds (Chróst 1981, Bjoernsen 1988) including carbohydrates, lipids, peptides, organic phosphates, volatile substances, vitamins, toxins and antibiotics (Fogg 1966). Glycolic acid is the major component in phytoplankton excretion (Fogg 1977); it is also excreted by cyanobacteria (Fründ and Cohen 1992) but carbohydrates were 92-97% of the extracellular material from a cyanobacterial benthic community (Hall and Fisher 1983). In oligotrophic streams there seems to be a direct flux of soluble algal products to the bacteria (Haack and Mc Feters 1982b).

Allochthonous dissolved organic matter originates from terrestrial ecosystems via groundwater and surface or subsurface flows (Lock 1990). Dissolved organic compounds could also originate from leachates of particulate material such as leaves (Lock and Hynes 1976). A portion of the detritic material transported and/or accumulated in streams is absorbed on mineral-soil particle surfaces (Sollins et al. 1985). The major compounds identified in river waters are carbohydrates (100-2000 $\mu g\ L^{-1}$), amino acids (50-1000 $\mu g\ L^{-1}$) and fatty acids (50-500 $\mu g\ L^{-1}$), the rest (ca. 75%) being humic, fulvic acids and hydrophylic acids (Thurmann 1985). In a recent study (Volk et al. 1997) it has been reported that in a nutrient-rich Pennsylvania stream (in the headwaters) DOC consisted of 75% humic substances, 13% carbohydrates (predominantly polysaccharides), 2% aminoacids (nearly all combined), and 18% >100kDa, the biodegradable organic matter being 25% of the DOC.

Streambed bacterial uptake of dissolved organic molecules depends on their molecular size and composition (Kaplan and Bott 1983) with a preferential use for the low-molecular-weight molecules (Meyer et al. 1987). The heterotrophic metabolism is enhanced when increasing the low-molecular-weight component of DOC (Kaplan and Bott 1983), but is inhibited by recalcitrant high-molecular-weight material (Freeman and Lock 1992) and humic compounds (e.g. Wetzel 1993). However, a recent study reveals that a large proportion of high-molecular-weight compounds are more fresh and bioreactive than the low-molecular-weight compounds of the DOC in aquatic environments (Amon and Benner 1996) and therefore composition could be more important than molecular-weight.

In this study the heterotrophic microbial population has been referred to bacteria since they are the main organisms responsible for organic matter use in stream biofilms. However, other microorganisms play an important role in the stream's ecosystem function, such as fungi (Boulton and Boon 1991) and protozoa (Schönborn 1992). The microbial communities in streams are the main actor responsible for leaf decomposition in forest streams (Hill et al. 1992), especially bacteria and fungi (Gessner and Schwoerbel 1991, Boulton and Boon 1991, Gessner and Chauvet 1994).

Extracellular enzymes

Microorganisms are unable to uptake organic molecules much greater than 10,000 Da apparent molecular weight and the great proportion of organic matter in the environment is larger than this (Lock 1990). Extracellular hydrolytic enzymes are the primary mechanism for the degradation of polymeric and macromolecular organic matter into low-molecular-weight molecules which can cross the bacterial cell membrane (Rogers 1961) (Fig. 3).

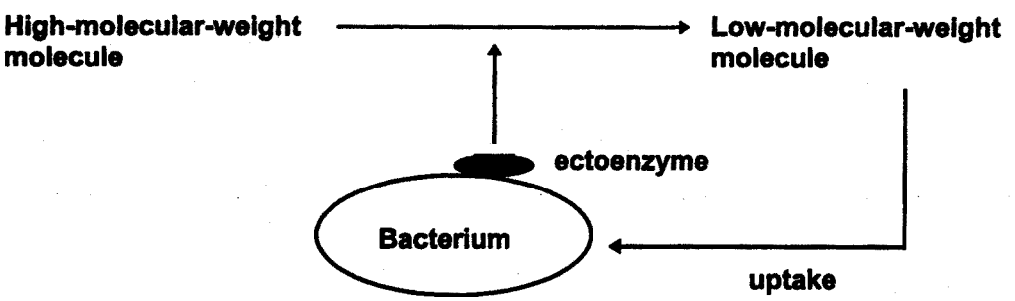


Fig. 3. Diagram of the action of bacterial ectoenzymes.

The rate of organic matter decomposition is dependent on this enzymatic activity (Lock 1990) which thus plays a key role in carbon cycling in aquatic environments (Servais and Billen 1993, Chróst 1994, Turley 1994). This microbial activity protects the environment against excessive accumulation of detrital organic matter, supplies photosynthetic organisms with nutrients, and is a clue to the growth and development of bacterial communitites (Gajewski and Chróst 1995) (Fig. 4). Through their heterotrophic activities, microorganisms are involved in the "microbial loop", the trophic system which was found to be separate from the typical photosynthetic organisms-grazers food chain, and which processes a considerable amount of organic matter (Azam et al. 1983, Mann 1988, Ducklow 1994).

Extracellular enzymes are found mainly in bacteria and fungi but also in algae and protozoa. Ectoenzymes are extracellular enzymes bound to the cell surface of microorganisms or in the periplasmic space in Gram-negative bacteria (usually predominant in aquatic environments) (Chróst 1991b) that act outside the cell (Chróst 1990). In this study the term ectoenzymes refers to the cell-bound enzymes defined by Chróst (1990) but also includes the possible free enzymes released in the environment (within the biofilm polysaccharide matrix and/or bound to detritic particles) which can be relevant when studying epipsammic biofilms (e.g Marxsen and Schmidt 1993). Extracellular enzymes may remain active (80% of their initial activity) for at least 3 days after being detached from the cell (Decho and Herndl 1995).

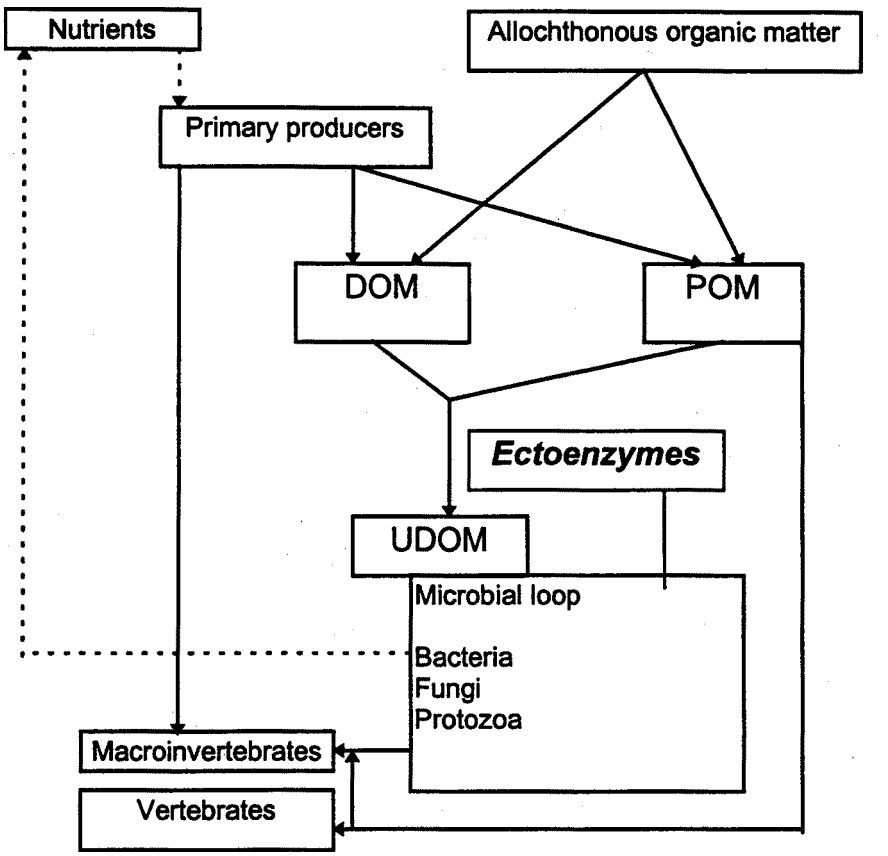


Fig. 4. The place and role of ectoenzymes in the transformation of organic matter and in the microbial loop formation in aquatic environments. Broken lines show nutrient regeneration pathways; solid lines show organic matter production and transformation. DOM= dissolved organic matter, POM= particulate organic matter, UDOM= readily utilizable dissolved organic matter. Adapted from Chróst 1990 and 1994.

The first studies of extracellular enzymes were performed in the soil habitat, stressing the importance of the bacterial community for the degradation of recalcitrant compounds (Burns 1983). The measurement of extracellular enzymes in aquatic environments began with the studies of Somville and Billen (1983) and Hoppe (1983) in the sea, although earlier studies were performed in lake waters (Reichardt et al. 1967, Jones 1971). From there, a rather wide study of these activities has been perfomed in the planktonic environment: in freshwater lakes (Chróst 1989, Münster et al. 1989, Vrba et al. 1992, Münster et al. 1992, Middelboe and Sondergaard 1993), in reservoirs (Vrba 1992), in marine environments (Vives-Rego et al. 1985, Hoppe et al. 1988, Hoppe et al. 1993, Karner and Rassoulzadegan 1995), in sediments (King 1986, Meyer-Reil 1986 and 1987, Boschker et al. 1995, Poremba 1995, Poremba and Hoppe 1995, Sala 1995), in wetlands (Jackson et al. 1995, Freeman et al. 1995), and within mucilagenous material such as diatom blooms (Smith et al. 1992 and 1995, Decho and Herndl 1995). In the past ten

years, enzymatic activities have also been measured in different habitats of river ecosystems: sediment (Marxsen and Schmidt 1993, Marxsen and Fiebig 1993, Sinsabaugh and Findlay 1995, Scholtz and Marxsen 1996), epilithic biofilms (Jones and Lock 1989, 1991 and 1993, Sinsabaugh et al. 1991a and 1991b, Chapell and Goulder 1992), wood and leaf detritus (Sinsabaugh et al. 1991a, 1992, 1993, 1994a and 1994b, Golladay and Sinsabaugh 1991), the periphyton (Scholtz and Boon 1993), and the water column (Admiraal and Tubbing 1991, Boon 1993, Chapell and Goulder 1995). In the majority of these studies it was concluded that the extracellular enzymes are important for the flux of organic matter and nutrients in streams (e.g. Marxsen and Witzel 1991) and in aquatic environments in general.

Ectoenzymes are inducible catabolic enzymes, and therefore their rates of synthesis and activity are strongly dependent on the presence of suitable polymeric substrates not continuously available in the environment (Somville 1984, Chróst 1989, Chróst 1990, Chróst and Rai 1993) (Fig. 5). Inducible enzymes are synthesized at a low basal rate in the absence of substrate, whereas when the substrate is available in the environment, there is a huge increase in the production rate of the particular enzyme (Chróst 1990). Since a particular ectoenzyme will be synthesized only when the available substrate is present, the ectoenzymatic activities could in part indicate the organic matter input in the habitat studied.

Ectoenzyme synthesis is also regulated by catabolic repression. The production of ectoenzymes is repressed by the end product that is derived from the substrate and accumulates in the cell or in the surrounding environment (Chróst 1990) (Fig. 5). A well known example is the repression of alkaline phosphatase synthesis by inorganic phosphate (e.g. Whitton 1991). Furthermore, the synthesis of ectoenzymes is in general repressed when there are sources of readily utilizable organic matter available (Chróst 1991a).

These two strategies which regulate ectoenzyme synthesis (inducible by substrate and repression by end product) avoid unnecessary waste of energy in enzyme synthesis when utilizable dissolved organic compounds are available and/or when there are no substrates available for enzyme reaction (Chróst 1991b).

Enzymatic activity and bacterial uptake are closely coupled processes (Azam and Cho 1987, Hoppe et al. 1988, Chróst 1989, Chróst 1990, Chapell and Goulder 1995), thus the microorganisms with the capacity to synthetize ectoenzymes have a competitive advantage over other organisms (Wetzel 1991, Gajewski and Chróst 1995) due to their ability to use molecules which are not directly available. Ectoenzymes are important for the survival of bacteria in oligotrophic conditions (Chróst 1990).

Hydrolases form the majority of extracellular enzymes in aquatic environments; among them the main enzymes are those which degrade polysaccharides, proteins and organophosphoric esters (López 1993).

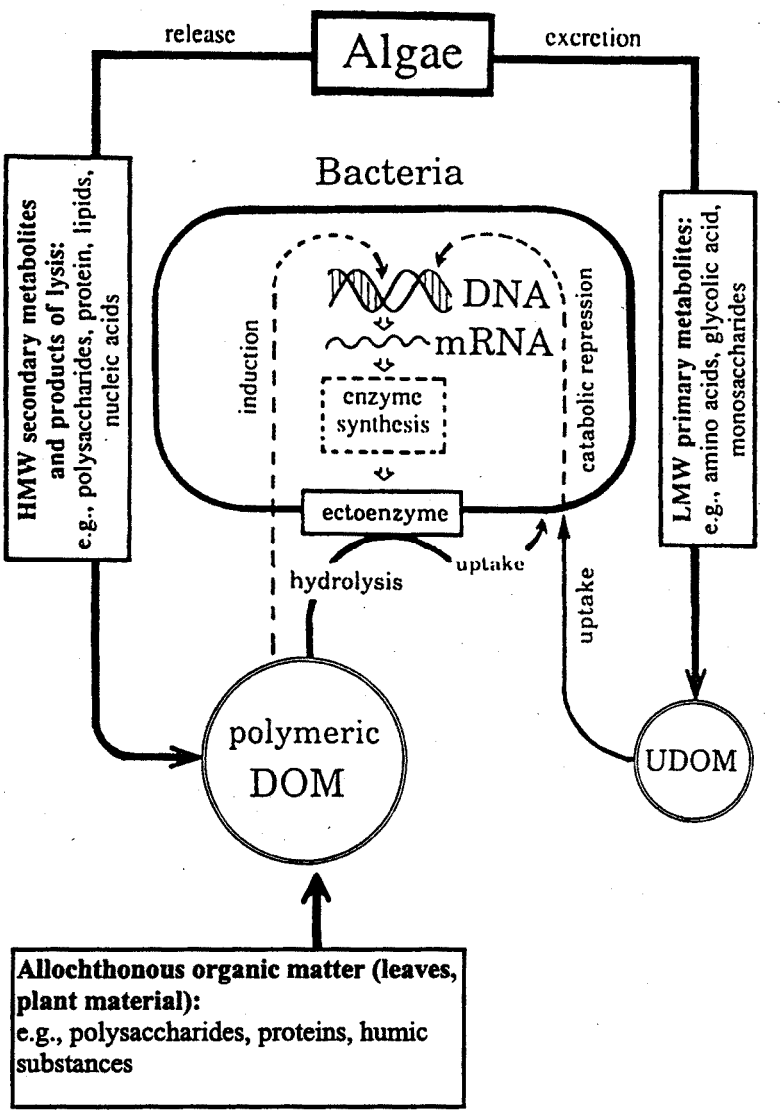


Fig. 5. Mechanisms of regulation of ectoenzyme synthesis. DOM= dissolved organic matter, UDOM= readily utilizable dissolved organic matter, HMW= high-molecular-weight, LMW= low-molecular-weight. Modified from Chróst 1991.

Three ectoenzymes were chosen for this study: the β-glucosidase, β-xylosidase and phosphatase. β-glucosidase is involved in the degradation of cellulose, which is one of the most abundant biopolymers in the world (Atlas and Bartha 1987). Degradation of cellulose (a linear chain of β 1-4 linked glucose units) involves cellulase enzymes (acting on native cellulose), endo- and exo- β 1-4 glucanases (converting the partially degraded molecules to cellobiose) and β-glucosidase (Atlas and Bartha 1987). This last enzyme degrades cellobiose and other relatively small oligomers containing β-D-glucose linkages (such as those from algal excretion) by splitting off the terminal β-D-glucose residue (Desphande and Eriksson 1988). This activity might be especially relevant since its end product, glucose, is energetically important for the microbial community (Chróst 1990).

β-xylosidase is involved in the degradation of hemicellulose, the second major class of plant constituents that enter soil habitats (Atlas and Bartha 1987). Hemicelluloses are more diverse than celluloses, often heteropolymeric (composed of various pentoses, hexoses and/or uronic acids) and branched (e.g. xylans, mannans, galactans) (Atlas and Bartha, 1987). The complexity of the hemicellulose molecules leads to the formation of a complexity of microbial degradation products. The β-xylosidase hydrolyses xylobiose or xylooligosaccharides to form D-xylose as the end product (Lachke 1988).

The two polysaccharidic enzymes were chosen as indicative, in part, of the source of the organic matter which is being used: β-glucosidase for autochthonous and β-xylosidase for allochthonous. This difference will not always be clear since both enzymes coincide in their activity when plant material and/or decaying algae are the main organic matter source since both cellobiosic and xylobiosic molecules are found. However, when there is an important input of polymers from algal excretion, β-glucosidase will be the mainly responsible for their degradation. In several chapters the β-xylosidase:β-glucosidase ratio has been calculated as a better indication of the organic matter source to be used.

Phosphatase activity indicates the degradation of phosphomonoesters to obtain inorganic phosphorus (Jansson et al. 1988). Apart from being an indication of the degradation of phosphorus organic materials, this activity was chosen for its relation to the nutrient content in stream water. An enhancement of this activity was postulated when phosphorus is a limiting nutrient as has been described for Mediterranean streams, especially in calcareous environments (Guasch et al. 1995).

While the polysaccharidic enzymes (β-glucosidase and β-xylosidase) are mainly restricted to bacterial cells (and fungi or protozoa), phosphatase activity is a more general enzyme, also present in algae (e.g. Francko 1991) where activity can be more important than bacterial phosphatase (Cotner and Wetzel 1992).

A short insight into proteolytic hydrolysis capacity was also performed by measuring the leucine aminopeptidase activity. This enzyme, an exopeptidase, is mainly produced by bacteria (Boon 1991) and breaks down peptid molecules from its endings releasing aminoacids (Jones and Lock 1991). This activity is related to the nitrogen cycling in aquatic environments and provides the microorganisms with nitrogenous compounds (Jacobsen and Rai 1991).

Besides microbial activity, the role of photochemical processes in degradation of organic polymers has recently been postulated, but there is still discussion as to the quantitative significance of this process in aquatic environments (Lindell et al. 1995), which might be more relevant in lakes and sea than in the flowing waters.

Objectives of this study

In early studies, the geochemical features of the river Ter and their relation to the watershed as well as the distribution, biomass and composition of the benthic communitiy have been investigated (Sabater F 1987, Sabater S 1987). The seasonal variation of physico-

chemical parameters, especially discharge and nutrients as well as local pollution effects from human activity, were the main factors affecting both the river geochemistry and the benthic community (Sabater F 1988, Sabater S 1988, Sabater and Sabater 1988). Following these studies, the epilithic metabolism (in terms of community primary production and respiration) and nutrient dynamics were investigated in Mediterranean streams (Guasch 1995, Martí 1995), defining the effects of the changes in the environmental conditions and the different geochemical features in two undisturbed second order streams tributaries of the river Ter. In these studies bacteria have been suggested as being responsible for several phenomena in both nutrient uptake and respiratory activity. As a following step, this study aims to focus on the role of the heterotrophs in organic matter utilization. The ecology of microorganisms have been studied in a wide variety of spanish aquatic ecosystems (e.g. Guerrero and Pedrós-Alió 1992), however, few data about the benthic stream microbial ecology of Mediterranean environments have been reported up to now. In addition there is a lack of functional studies of Mediterranean streams (Prat 1991).

The main objective of this study was to investigate the use of organic matter (autochthonous and/or allochthonous) by the microbial community of stream biofilms. The more specific objectives are summarized as follows:

1) The relationship between algae and bacteria was a main objective of this study. Algae may be a relevant organic matter source for bacteria living in the biofilm structure. This possible linkage in stream biofilms is suspected but no clear conclusions are yet available. This relationship, and therefore the role of autochthonous organic matter for the heterotrophs, was analysed in the different streams and biofilms studied.

2) A second general objective of this study was the characterization of the bacterial community in terms of density, biomass, hydrolytic capacity and efficiency in organic matter utilization in Mediterranean streams. Nowadays the knowledge of stream ecology has been widely developed in nutrient and carbon cycling, and the biotic and abiotic processes which are involved. The role of heterotrophs within the microbial loop is an important subject in the study of aquatic environments.

3) Following objective (2) more specifically, the physical and chemical parameters (DOC, light, nutrients, temperature, discharge) which control the variability of the heterotrophic and autotrophic metabolism in Mediterranean stream biofilms were investigated. Mediterranean rivers and streams are subject to drastic seasonality, which is clearly expressed in discharge variability: they can be dry in summer and support high flood events in autumn. The heterotrophic activities of the Mediterranean streams studied were also compared to those from other regions with different climatic regimes.

4) The hydrolytic capacity of a given stream biofilm would also depend on the structure which it has built and thus on the streambed substrate were it has grown. Therefore, the differences between the stream's benthic substrates were also analysed. A special examination of the biofilm metabolism on sandy substrates was made, distinguishing the surface from the subsurface zone. Furthermore, biofilm metabolism in sandy biofilms was compared to that in epilithic biofilms in order to find out which substrate will has a higher capacity to hydrolyse organic matter with respect to streambed surface area.

Another stream biofilm analysed was the thick stromatolitic cyanobacterial crust grown on a calcareous stream. The algal and bacterial metabolism in the different algal patches which constitute this cyanobacterial crust was studied to investigate the role of such a stromatolitic structure in the control of the hydrolytic capacity of the heterotrophs and whether there are differences between the different algal patches.

As minor objectives and using the "natural" disturbances which took place during the study period on the Mediterranean streams, the following purposes were adopted:

5) The recovery of heterotrophic and autotrophic metabolism of the cyanobacterial crust after a total drought of the streambed was investigated. The objective was to find out the recovery time and whether "normal" metabolic activities would recover when the biofilm structure was rewetted after a dry period.

6) The particular effect of a human disturbance, the removing of riparian vegetation, was investigated. The objective was to find out whether such removal would cause an increase or decrease in the autotrophic and heterotrophic activity in the stream biofilm.

Development of this report

For these purposes, the hydrolytic capacity (potential ectoenzymatic activity) of degrading cellulosic and hemicellulosic polysaccharides and organophosphoric esters was measured by the enzyme activity of β-glucosidase, β-xylosidase and phosphatase. Bacterial density was measured as being the main responsible for ectoenzymatic activities. Algal biomass (chlorophyll-a) and activity ($H^{14}CO_3$ incorporation) were analysed to investigate the possible algal-bacterial relationships and the importance of the autochthonous organic matter source. Heterotrophic activity of the biofilm community was measured by respiratory activity (electron transport system activity, ETS).

The study was performed in the river Ter and in two of its tributaries (second order undisturbed streams), Riera Major and La Solana (Fig. 6), which differ in watershed lithology (siliceous versus calcareous) and riparian vegetation (forest stream versus open stream) (Martí and Sabater 1996). The differences in the quantity and quality of the organic matter input between the two undisturbed streams would affect their enzymatic degradation by the microbial

community. The measurements in the river Ter biofilm will add information about microbial degradation capacity in a nutrient-rich river.

An annual study of the heterotrophic and autotrophic biofilm metabolism was performed in these Mediterranean streams and river. During the period January 1994-February 1995, ectoenzymatic activities (β-glucosidase, β-xylosidase, phosphatase), respiratory activity (ETS), photosynthetic activity ($H^{14}CO_3$ incorporation), bacterial density (DAPI stain) and chlorophyll-a, as well as physical and chemical parameters were analysed monthly. In each stream the different streambed substrates were considered: epilithic and epipsammic in Riera Major, cyanobacterial crust in La Solana, and epilithic in the river Ter. Artificial substrates (clay tiles) were used for the study of epilithic biofilms.

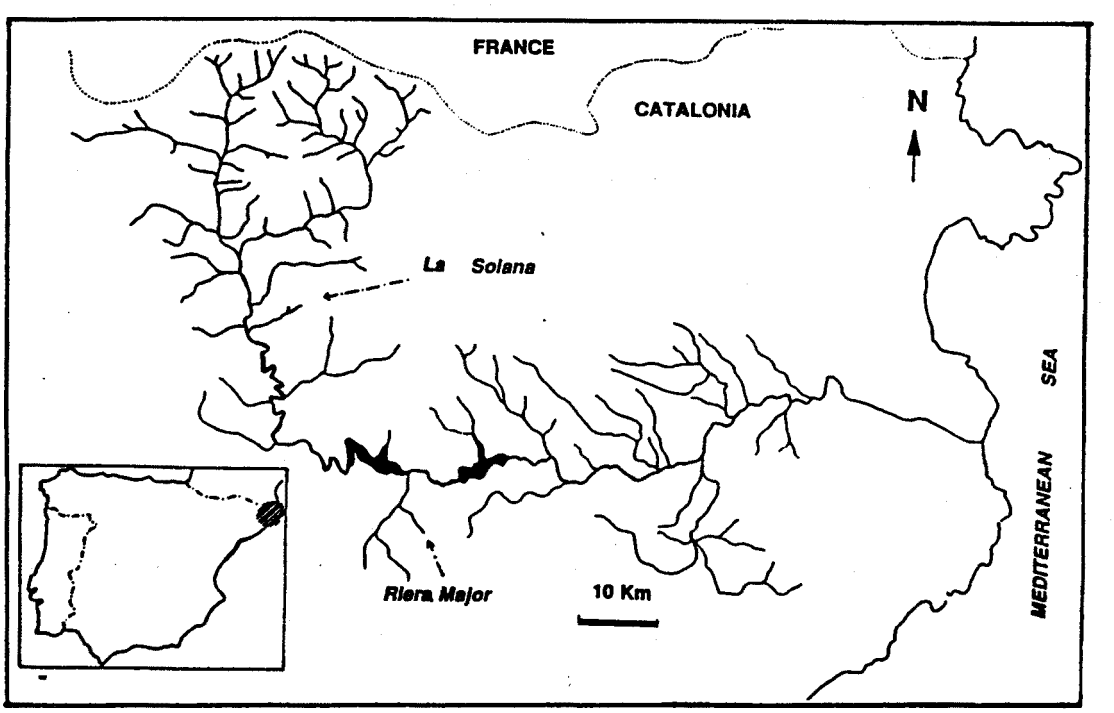


Fig. 6. Map of the river Ter watershed and the studied tributaries, Riera Major and La Solana.

Apart from these annual studies, the different disturbances which occurred in the studied streams were studied and/or "utilized" for a better knowledge of the stream biofilm metabolism.

These were a high flood and human riparian removal in Riera Major, and a drastic dry period in La Solana.

In October 1994, a high flood occurred in Riera Major which washed and homogenized the sandy substrate. This event was "utilized" to study the evolution of the heterotrophic metabolism in the sand at different depths from the same starting point. After the flood, the ectoenzyme activity of β-glucosidase, β-xylosidase and phosphatase, respiratory activity, bacterial density, chlorophyll-a and benthic organic matter (ash free dry weight, AFDW) were analysed in the surface (0-2 cm in depth) and subsurface (7-10 cm in depth) zones in order to investigate whether both zones differ in terms of organic matter utilization and the relative effect of environmental conditions. An intensive sampling was performed (every fifteeen days) during the period October 1994-December 1994 and samples were taken monthly from December 1994 to August 1995. Nearly one year was considered appropriate for this study to examine any difference in the time pattern between the surface and subsurface zone as well as the different autochthonous versus allochthonous organic matter input throughout the year.

In mid-February 1995 there was a human disturbance in the Riera Major stream which consisted in the total removal of the riparian vegetation in the very stretch where the annual study had been performed. The effect of this disturbance on the benthic communities was investigated in terms of algal biomass, bacterial density and activity (ectoenzymatic, photosynthetic, respiratory) of the epilithic biofilm. Samples were taken monthly during the period March-June 1995 and every fifteen days during the period July-August 1995.

In the La Solana stream, water flow can be very low and even non-existent during the summer months. This occurred during June-July 1994 when the stream was totally dry. The recover of the biofilm (cyanobacterial crust) metabolism (ectoenzymatic, photosynthetic and respiratory activities) after the drought period was analysed through a rewetting experiment performed in the laboratory.

A wider study of the ectoenzymatic activities in stream biofilms was performed during 1995 (over the four seasons) for the two undisturbed Mediterranean streams (Riera Major and La Solana) by measuring the enzyme kinetics (Michaelis-Menten approach) of the three enzymes studied. This provided data about the enzyme affinity for the substrate (Km, apparent Michaelis constant) and the turnover time of substrate hydrolysis (Tt), which are a better approach to the real hydrolytic activity than the potential activity measurements performed in the seasonal studies.

As a comparison to European streams a short study was carried out in the Breitenbach, a first-order stream in Hesse (West Germany) (Fig. 7) during October-November 1995. This stream is located very close to the Limnologische Flußstation des Max-Planck-Instituts für Limnologie (Limnological River Station of the Max Planck Institute of Limnology) in Schlitz, where this research was performed. The epilithic (by using the artificial clay tiles) ectoenzyme activities of β-glucosidase, β-xylosidase, phosphatase and leucine-aminopeptidase were analysed by the kinetic approach (Michaelis-Menten kinetics), which permits the calculation of Vmax (maximal reaction velocity), Km and Tt. By knowing these parameters an insight into

enzyme affinity and efficiency of substrate utilization can be obtained. Since this study was performed in the autumn, the effect of the fallen leaves was considerable.

Although the relative importance of autochthonous versus allochthonous organic matter utilization by the heterotrophs was investigated throughout the annual study in the three Mediterranean streams, a special study of the effect of growing algae on the ectoenzymatic activities of the microbial community of the biofilms was performed, since a bacterial-algal link was suspected. For this purpose two colonization experiments were carried out in the forest stream (Riera Major) by using the artificial substrates (clay tiles) during two periods (spring and summer) which differ in light availability. During the period June-July 1994 (canopy cover, low light availability) colonization by algae and bacteria was followed by an intensive sampling (12 sampling times) in which ectoenzymatic activities (β-glucosidase and β-xylosidase), respiratory activity, photosynthetic activity, bacterial and algal density and chlorophyll-a were measured. The biofilm formation on the clay tiles was observed under the scanning electron microscope (SEM). The second experiment was performed during March-May 1995 (no canopy cover, high light availability); the sampling procedure was similar to the summer experiment but more complex and complete since light and dark conditions were considered. The dark conditions were achieved by placing the substrates inside a dark tube immersed in the stream.

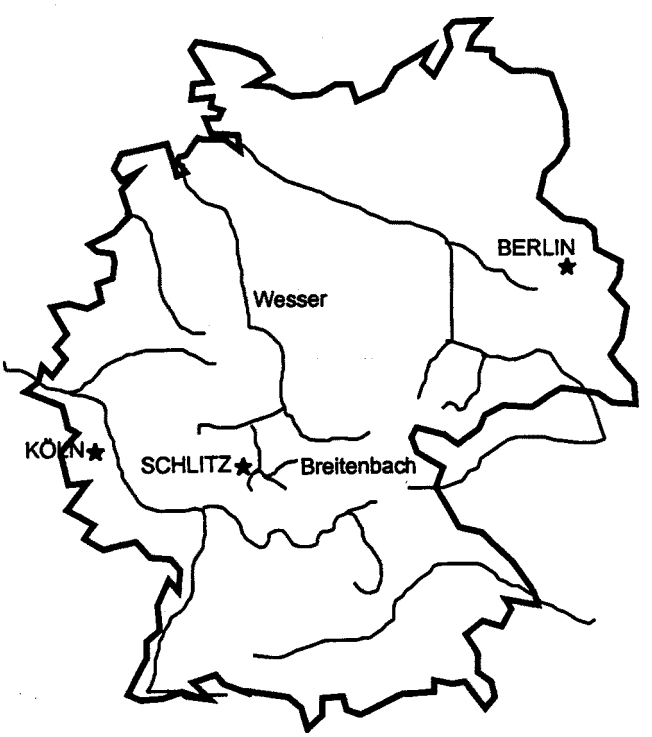


Fig. 7. Location of the Breitenbach first-order stream in Germany.

Finally, data were analysed together to describe any general trend in the ectoenzymatic activities on Mediterranean stream biofilms. The results were also compared to the activities measured in other streams from other regions. The relationship between the bacteria/algae biomass ratio for each biofilm studied and the respective ectoenzymatic activity was also analysed.

2. METHODS

Introduction

The main technique used in this study is the measurement of the ectoenzymatic activities in different stream biofilms: epilithic (growing on artificial substrates), epipsammic (growing on sand) and thick mats (stromatolitic cyanobacterial crust). In this chapter the development of this method in stream biofilms is widely described. The other techniques related to biofilm metabolism applied in this study such as respiratory activity (electron transport system, ETS) and photosynthetic activity ($H^{14}CO_3$ incorporation) are also described. The algal and bacterial density and biomass analysis are also described, as well as the analysis of the physico-chemical parameters in stream water. Limitations and solved and unsolved problems when applying these techniques to stream biofilms are also noted. The particular sampling, sample processing and number of replicates for the different biofilm samples is explained in each chapter.

2.1. The measurement of ectoenzymatic activities in stream biofilms by methylumbelliferyl (MUF)-substrates

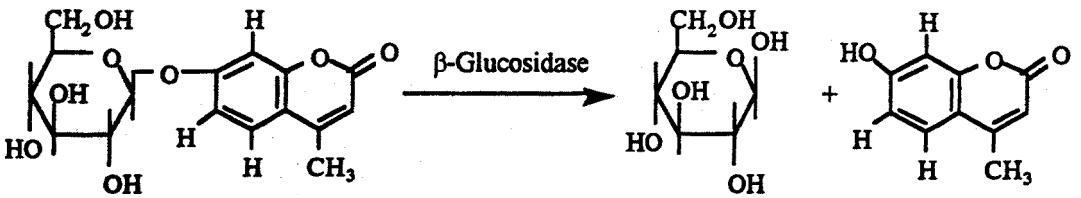
Introduction

The measurement of ectoenzymatic activities by utilizing MUF-substrate analogues was first developed by Hoppe (1983) within the marine environment. The technique for measuring such enzymatic activities is based on an incubation of the natural sample with an artificial substrate which mimics the natural substrate by having the very link that the enzyme is able to hydrolyse. This link connects an organic molecule with an artificial molecule (e.g. methylumbelliferone, MUF) which emits fluorescent light when it is free of any linkage (Fig. 1). The measurement of this fluorescence after the incubation time is directly related to the artificial substrate molecules cleaved by the natural enzyme activity. In the first studies of extracellular enzyme activities, chromogenic artificial substrates were used (e.g. Sinsabaugh and Linkins 1988) measuring the absorbance of the coloured compound at the end of the incubation. Nowadays, in aquatic environments fluorogenic substrates are preferred because of their great sensitivity (Chróst and Krambeck 1986, Münster et al. 1989). This feature permits a short incubation time, avoiding the problems of possible nutrient depletion and/or changes in the bacterial community, as well as detecting even very low activities. The fluoresceindiacetate (FDA) is also a fluorogenic model substrate which is hydrolyzed non-specifically by hydrolytic enzymes and has been used as a general measure of the hydrolytic enzyme activity (Meyer-Reyl and Köster 1992).

The validity of using artificial MUF-substrates in natural waters was demonstrated by Hoppe (1983), Somville (1984) and Chróst (1990) who found a competitive inhibition of ectoenzymatic activity (increase in hydrolyzation time and apparent Km) when adding natural substrate analogues (cellobiose for MUF-β-D-glucoside and phosphogluconic acid or glucose-6-phosphate for MUF-phosphate). A high especifity of the polysaccharidic enzymes and aminopeptidase and phosphatase activities for the MUF-substrates was clearly demonstrated by Bögershausen and Marxsen (1995).

Several constraints on the use of MUF-substrates are worth to be noted. First of all, MUF-substrates are not absolutely specific (S.E. Jones, pers. comm.), and Sala (1995) has observed that several polysaccharidic enzymes are present when a specific substrate is added. Moreover, the utilization of artificial substrates for measuring enzymatic activity may overestimate the real activity because of the simplicity of their molecules (dimeric substrate), in contrast to the natural substrates (oligomeric or polymeric) found in the natural environment (King 1986, Chróst and Rai 1993).

A better approximation to real enzymatic activity could be achieved by the use of radiolabelled substrates which involve only a small transformation of the natural substrate. They are highly sensitive and need very low incubation periods (Chróst 1990). However, few substrates are commercially available and the technique is much more expensive.



MUF-β-D-glucoside D-glucose MUF

Fig. 1. Diagram of the reaction between 4-methylumbelliferyl-β-D-glucoside and β-D-glucosidase obtaining D-glucose and MUF.

Incubation procedure

- Sample preparation

The stream biofilms collected at the different sampling sites - artificial substrates (clay tiles 0.64 cm^2 surface area), stream sediment (ca. 2 ml in volume) and/or natural stream biofilm (stromatolitic cyanobacterial crust, ca. 1.1 cm^2 surface area) - were placed in the field in the incubation vials (sterile glass vials) with stream water and kept cold (on ice) and dark during transport. The fresh biofilms were incubated two to three hours after sampling. Control-killed samples were prepared as samples in 40% formalin for 30 min before the assay.

The utilization of the artificial substrates for collecting epilithic biofilms and the collection of the natural sandy biofilms and the natural thick cyanobacterial crust permited the assay of the microbial activities without any removal of the biofilm, which is important when studying these structured and complex communities (Blenkinsopp and Costerton 1991, Nivens et al. 1995).

- Preparation of the MUF-substrates

The artificial MUF-substrates (100 mg) (Table 1) were diluted with 50 ml of autoclaved distilled water and kept in the freezer (-20°C) in dark sterilized vials till use. Prior to dilution, 2 to 5 ml of hydroximethylether was added to the solid MUF-substrate to facilitate its dissolution in water (Hoppe 1983), and left for 10-12 hours cold (4°C) and in the dark (to minimize photo-degradation). The MUF-β-D-glucoside was also sonicated for 2 min to help its solubilization in water.

On each sampling date, the artificial substrates were thawed and diluted to a final concentration of 0.3 mM with filter-sterilized river water (Gelman, 0.2 μm). The 0.3 mM substrate concentration was chosen as substrate-saturated conditions (see below, *Substrate concentration*) for all the standard assays of potential ectoenzymatic activity measurements.

Alkaline (EC 3.1.3.1) and acid (3.1.3.2) phosphatases were not differentiated. Acid phosphatases usually have pH optima between 4 and 6, whereas pH optima for alkaline

phosphatases are above 7 (between 9-10). Mainly alkaline phosphatase will be active in the Mediterranean streams (pH around 8) while both types of phosphatase could be active in the Breitenbach (pH 7.1).

TABLE 1. Artificial MUF-substrates used in this study for the measurement of β-glucosidase, β-xylosidase and phosphatase activity (the Enzyme Commission number, EC is specified). The analogue natural substrates are also shown.

Ectoenzyme	Natural substrate	Artificial substrate
β-D-glucosidase (EC 3.2.1.21)	Cellobiose	MUF-β-D-glucoside
β-D-xylosidase (EC 3.2.1.37)	Xylobiose	MUF-β-D-xyloside
Phosphatase (EC 3.1.3.1-2)	Phosphomonoesters	MUF-phosphate

For the experiments performed in the Breitenbach (chapter 7) the Leucine-aminopeptidase activity (EC 3.4.11.1) was also measured by using the fluorogenic substrate Leucine-MCA (L-leucine-4-methyl-coumarinyl-7-amide from Calbiochem). The assay for the measurement of this activity was similar to that for the activities using the MUF substrate-analogues. The fluorescent compound MCA is released after the enzyme activity. The specific procedure is explained in chapter 7.

- *Preparation of the buffer.*

Glycine 0.05 M, pH 10.4 (for 100 ml):

80.35 ml NaOH 0.2 M (0.8g in 100ml)
19.64 ml Glycine 0.2 M (1.8765g in 25ml)

The buffer was prepared with filter-sterilized stream water (Gelman, 0.2 μm). The final pH was measured with a pH meter.

- *Incubation*

See the procedure summarized in Fig. 2.

2 ml of each MUF substrate (0.3 mM) was added to each fresh sample (previously eliminating the stream water), control-killed samples (previously eliminating the formalin, two replicates) and blank vials (two replicates).

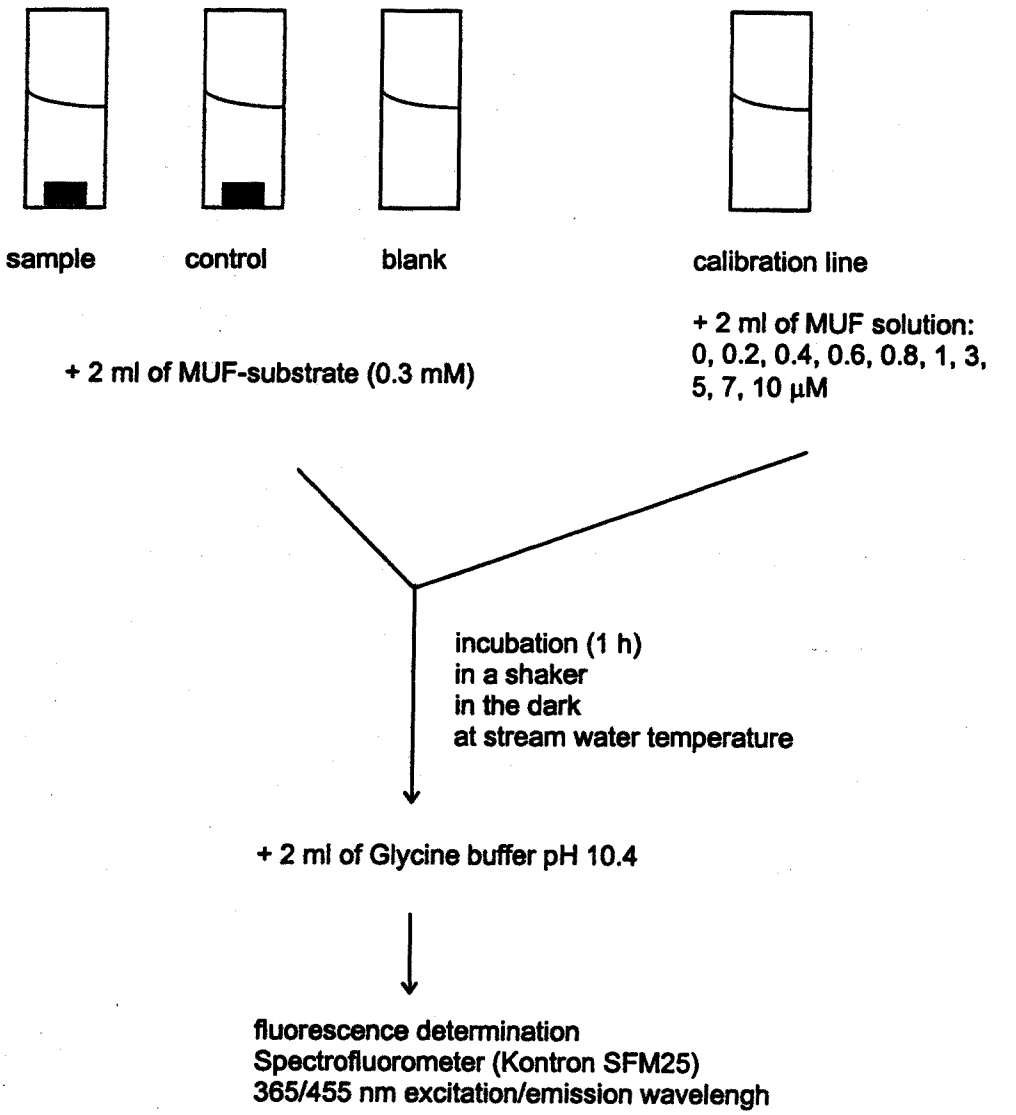


Fig. 2. Incubation procedure for ectoenzymatic measurements in stream biofilms

The incubations were performed in the dark under continuous shaking to avoid diffusion limitation of substrates due to the formation of boundary layers (Scholz and Boon 1993) for 1h (see below, *Incubation time*) at ambient stream water temperature (see below, *Incubation temperature*). At the end of the incubation 2 ml of 0.05 M Glycine buffer pH 10.4 was added to stop the reaction and convert the MUF into the most fluorescent anionic form (Chróst and Krambeck 1986). The adjustment of the pH is important for these enzymatic analyses (e.g. Scholtz and Marxsen 1996) especially in acidic environments such as polyhumic lakes (Münster et al. 1989). The incubations should preferably be carried out at the pH of the natural waters (Chróst and Krambeck 1986). In our experiments, pH was very stable thanks to the highly

buffered Mediterranean waters. The addition of buffer solution was found also to be an efficient method for preventing further enzyme activity without the need to add formalin to the samples. However, in the colonization experiment performed in Riera Major (chapter 8.1) 100 µl of 40% formalin was added at the end of the assay.

Fluorescence was measured at 455 nm under an excitation wavelength of 365 nm (the wavelength of maximal absorption of MUF, Somville 1984) (Kontron, SFM25). Quantification was achieved by calibrating the spectrofluorimeter with standard alkaline solutions of MUF (0-10 mM) performed with filter-sterilized river water and calculating the calibration line. The standard solutions of MUF were incubated together with the fresh samples. The intensity of fluorescence of the blanks was subtracted from all samples to correct for nonenzymatic hydrolysis of the substrate or fluorescent substances in the water solution. Activity in formaldehyde-killed controls was subtracted from activities in all samples to correct for abiotic activity.

Ectoenzymatic activity was expressed as nmol of MUF released per hour and cm^2 of biofilm. In some experiments the specific ectoenzymatic activity (on a cell basis) was also calculated. However, a careful use of this parameter since the fraction of the bacterial community which actually contributed to the metabolic activity was unknown (Karner et al. 1992).

Substrate concentration

To measure potential ectoenzymatic activities, incubation must be performed with substrate saturation conditions. Thus, prior to the beginning of the experiments the kinetic behaviour of the enzyme activity was analysed in the streams studied in order to decide the MUF-substrate concentration to be added in each assay.

MUF-substrates were thawed and diluted with filter-sterilized river water (Gelman, 0.2 µm) to 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 mM for MUF-β-D-glucoside and to 0.05, 0.1, 0.3, 0.5 for MUF-β-D-xyloside and MUF-phosphate. Triplicate biofilm samples and formaldehyde-killed controls for each sample type (Riera Major sand and tiles, La Solana cyanobacterial crust and river Ter tiles) were incubated with each substrate concentration in the dark under continuous shaking for 1h at stream temperature (as described in *Incubation procedure*, Incubation). Blanks for each substrate concentration were also incubated.

The saturation curves obtained when plotting the MUF-substrate concentration against ectoenzymatic activity followed Michaelis-Menten kinetics (Fig. 3). A non-linear regression analysis was performed for each curve using the Enzfitter program (Leatherbarrow 1987) in order to calculate the apparent Michaelis constant (Km). The Km values were in the range 0.03-0.21 mM for β-glucosidase, 0.08-0.25 mM for β-xylosidase and 0.02-0.13 mM for phosphatase activities. To ensure saturation conditions a 0.3 mM substrate concentration was chosen for all the standard assays. From Malcolm (1983) 83% of Vmax reaction velocity is attained when the substrate concentration is five times the Km.

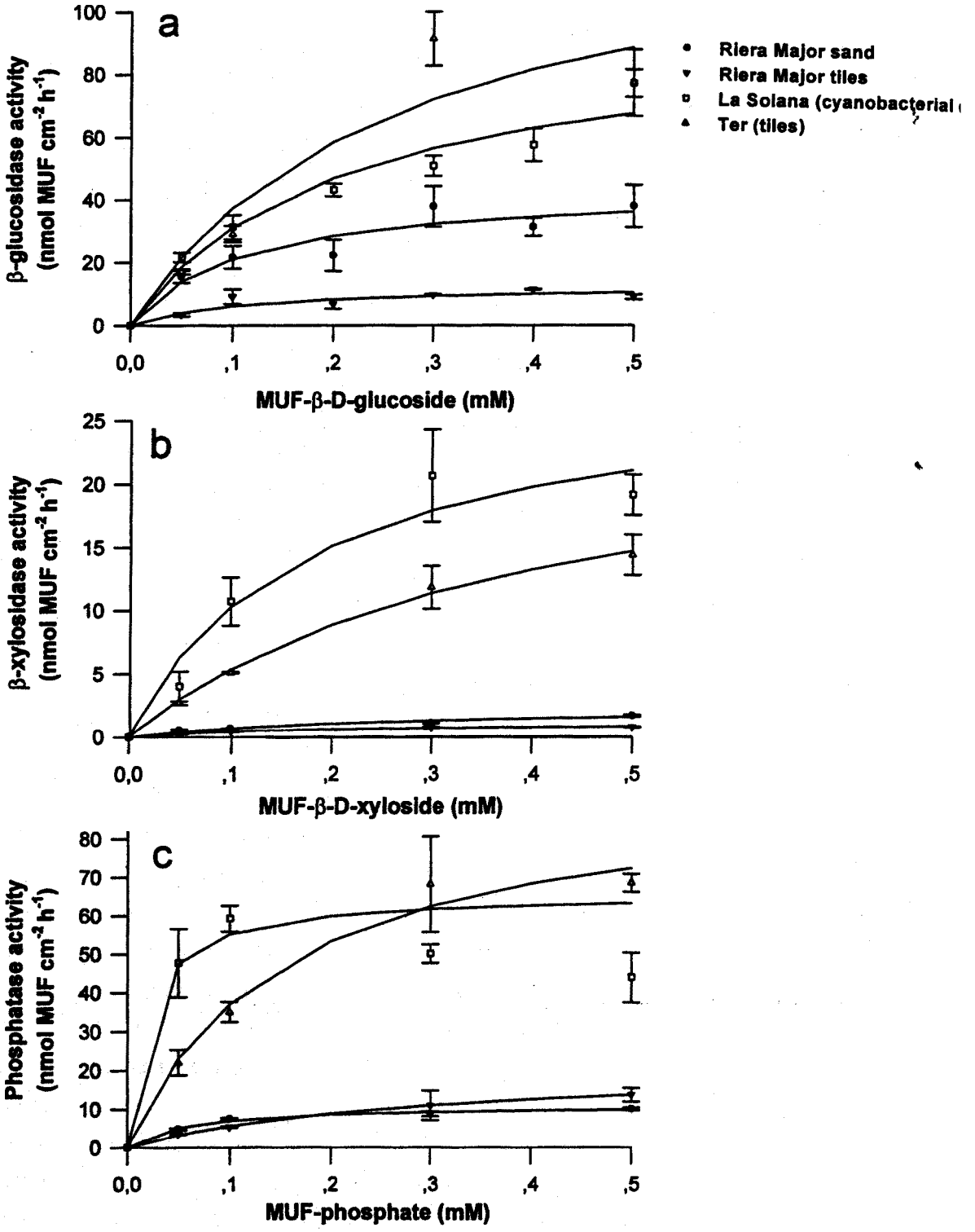


Fig. 3. Substrate saturation curves of the β-glucosidase activity (a), β-xylosidase activity (b) and phosphatase activity (c) in Riera Major epipsammic (sand) and epilithic (tiles) biofilms, La Solana cyanobacterial crust and the river Ter epilithon (tiles) with MUF substrate increased in November-December 1993 and March 1994 for the river Ter. Values are means ± standard error (vertical bars), n=3. Lines indicate the Michaelis-Menten kinetics obtained by non-linear regression analysis.

However, the 0.3 mM substrate concentration was chosen as a compromise between limited solubility of high concentrations of the MUF-substrates in water and zero order uptake kinetics. Higher MUF-substrate concentrations would express significant fluorescence at the same wavelength as the MUF (Boon 1989), interfering with the results. A similar saturation concentration was noted for MUF-substrates in lake water (Chróst and Krambeck 1986) and epilithic biofilms (Jones and Lock 1989, Chapell and Goulder 1992).

The choice for measuring extracellular enzyme activities at substrate saturating conditions is generally accepted as a measurement of potential activity since it can be measured over a short time without problems of contamination, low activity, non-saturation of enzyme or product inhibition (Chróst and Krambeck 1986). In addition, when performing the assays at saturation concentrations, competition between the MUF-substrates and any naturally occurring substrates is minimised (e.g. Freeman et al. 1995). However, several authors have measured the activities at very low substrate concentrations to resemble the natural substrate concentration in aquatic environments (e.g. Somville 1984).

Incubation time

Three replicates for each stream biofilm were assayed for β-glucosidase activity according to the standard incubation procedure described. Incubations were carried out over 15, 30, 60, 90, 120 and 180 minutes.

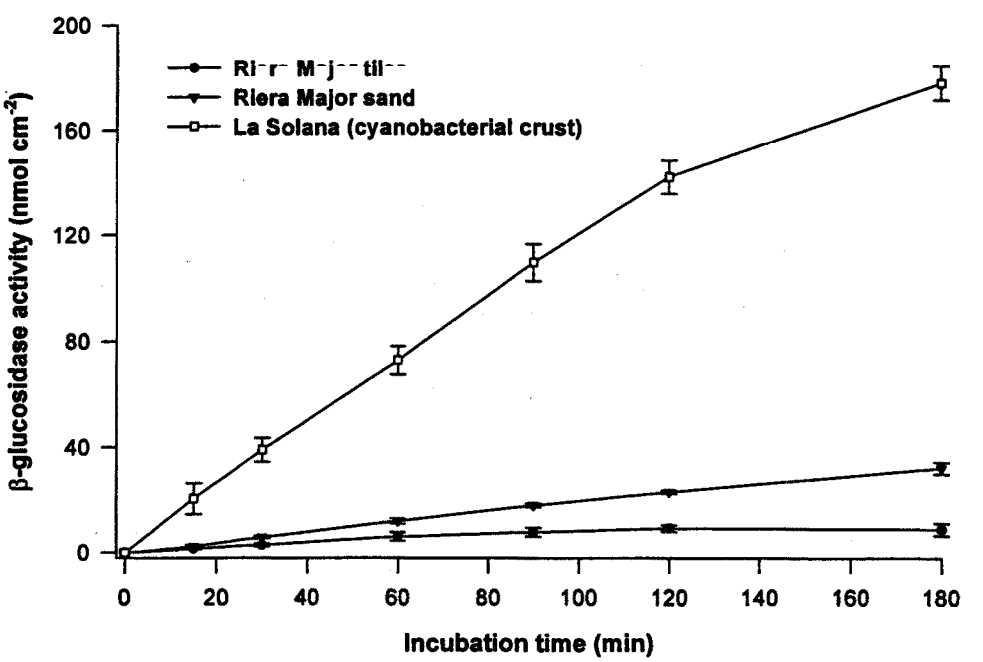


Fig. 4. Effect of increasing incubation period on β-glucosidase activity for Riera Major epipsammic and epilithic biofilms and La Solana cyanobacterial crust.

The increase in fluorescence was linear with time for at least 90 minutes (Fig. 4). A 1h incubation period was chosen for all the standard assays in order to ensure that all activities were measured over the linear portion. The adoption of this short incubation period avoided potential interference with the assays through microbial growth (S.E. Jones, pers. com.).

After the performance of all the experiments even lower incubation period (20-30 minutes) can be suggested for the phosphatase assays since this enzyme is especially active in the Mediterranean stream biofilms studied.

Incubation temperature

There is an effect of temperature on the ectoenzymatic activities in aquatic environments (e.g. Münster et al. 1992, Scholz and Marxsen 1996, Sala 1995). Some authors perfom the ectoenzyme incubations at optimal temperature conditions to eliminate their effect on the measurements. However, in this study, incubations were always performed at natural stream water temperature to be as close as possible to the natural conditions of the biofilm. This is a relevant factor because one of the objectives was to determine whether seasonality was an important factor of variation.

2.2. Respiratory activity

ETS activity was assayed by measuring the reduction of the electron transport acceptor INT (2-3 tetrazolium chloride) to INT-formazan (iodonitrotetrazolium formazan) (Blenkinsopp and Lock 1990). Biofilm samples and controls (samples in 40% formalin for 30 min before the assay) were incubated in 0.02% INT solution in filter-sterilized stream water. The incubation procedure is summarized in Fig. 1. Incubations were performed in a shaker at ambient stream temperature for 10-12 h in the dark. These long incubations were performed over the night of the sampling date itself. The incubation time recommended for measuring the respiratory activity in stream biofilms ranges from 8 to 15 h (Blenkinsopp and Lock 1990).

At the end of the incubation period, the supernatant liquid was poured away and 8 ml of cold methanol was added to extract the INT-formazan. The vials were then placed for a minimum of 1h at 4°C in the dark. To ensure total extraction, samples were sonicated (2 min) in a sonication bath (Selecta) full of ice and water to avoid an increase in temperature during sonication, which could degrade the INT-formazan. The dark conditions must be applied during the incubation and extraction to avoid photodegradation of both INT and INT-formazan. The extracts were filtered (GF/F filters, Whatman) and absorbance at 480nm was measured spectrophotometrically (Perkin-Elmer, Lamdda 2 UV/VIS Spectrophotometer). A stock solution of 30 $\mu g\ ml^{-1}$ INT-formazan (Aldrich) in methanol was used to prepare a

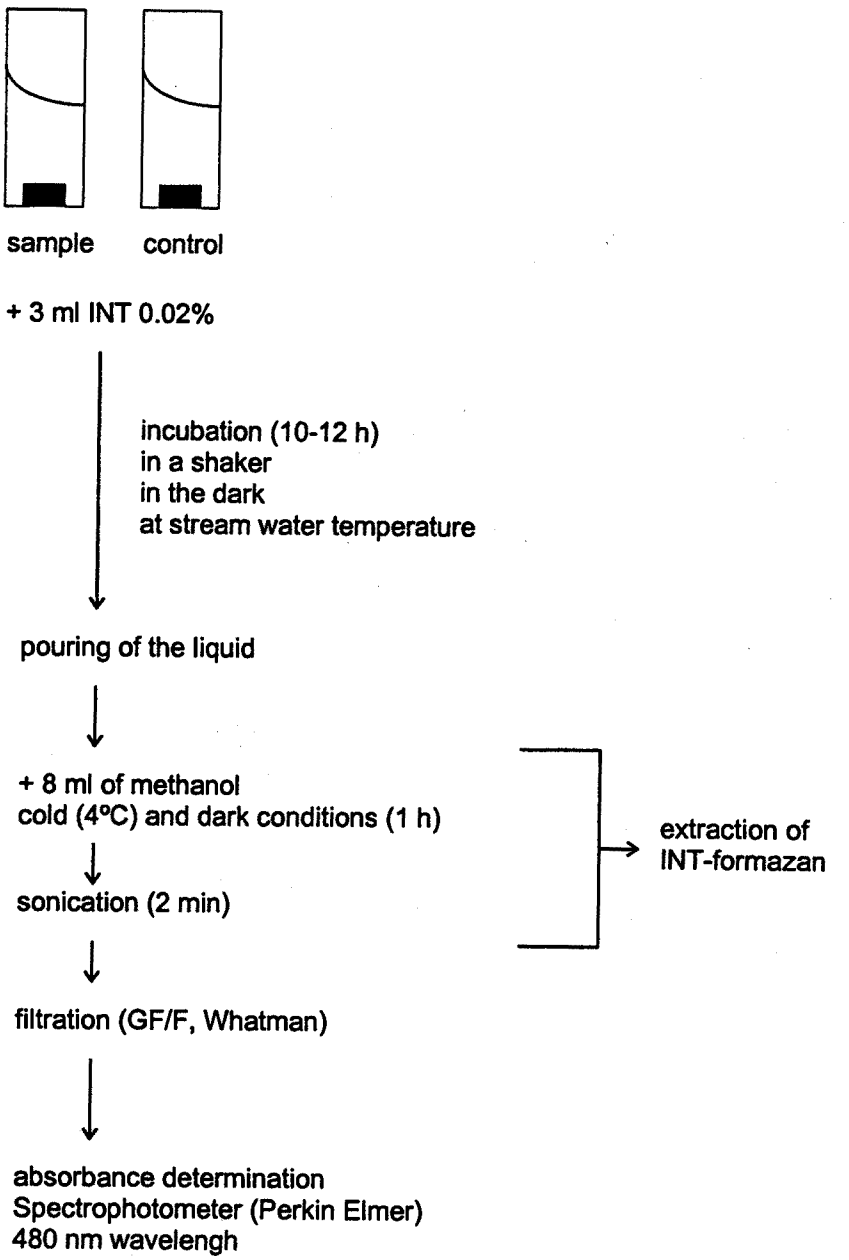


Fig. 1. Diagram for the protocol of the measurements of respiratory activity (ETS) in stream biofilms

standard curve (0, 5, 10, 15, 20, 30 μg ml^{-1}). The results were expressed as μg of formazan per cm^2 per hour. In chapter 8 ETS activity was also expressed as μg of formazan per cell per hour, by summing the bacterial and algal cells growing on the artificial substrates. Although expressing the respiratory activity in a cell basis provides a calibration of the results by the cell

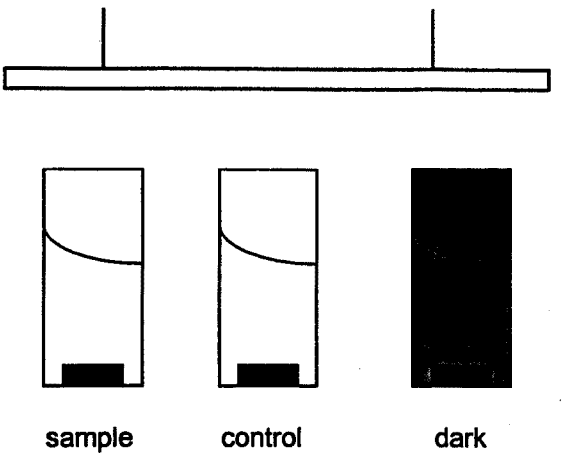
density, this calculation is a simplification since other heterotrophic organisms of the biofilm such as protozoa and fungi, could be also responsible for the respiratory activity.

2.3. Photosynthetic activity

Primary production was measured as $H^{14}CO_3$ incorporation. This method has been developed in phytoplankton but has also been used in stream benthic communities (e.g. Hill and Boston 1991). The ^{14}C uptake method gives similar results to the method of measuring changes in dissolved oxygen but it has a greater sensitivity at low production levels (Hunding and Hargrave 1973, Stevenson 1996).

The samples collected in the field were placed in glass vials with stream water and transported in cold, dark conditions to the laboratory when the incubation was performed (3-4 hours after sampling). The incubation procedure is summarized in Fig. 1. Samples were incubated under a battery of fluorescent tubes (150 μmol photons m^{-2} s^{-1}, a saturating irradiance for these communities, Guasch and Sabater 1995). Quenching and nonphotosynthetic uptake of carbon were determined in controls (samples with 40% formaldehyde 30 minutes before the assay) and darkened tubes (wrapped with aluminium foil), respectively, and were subtracted from photosynthetic uptake. Prior to the beginning of the incubation, samples were exposed for 10 min to experimental irradiance for the recovery of the photosynthetic system after transport from the field. 10 ml of a solution containing 1 μCi of $NaH^{14}CO_3$ (acetic acid sodium salt, 1-^{14}C, from Dupont) diluted with filter-sterilized stream water (0.2 μm, Gelman) was injected into each tube. Samples were incubated in a shaker for two hours at ambient stream temperature. At the end of the incubation period, samples were gently rinsed, sonicated in distilled water (5 ml) for 2 min (sonication bath, Selecta) and 15 ml of the scintillation liquid (Biogreen 1 from Scharlau, which gives fluorescence light to the β radiations), was added. Radioactivity was measured in a Packard tri-carb 1500 liquid scintillation analyser (5 min per vial).

This rather long incubation time was necessary to detect possibly low photosynthetic activities, especially in the colonization experiments (chapter 8). The possible excretion of labeled organic carbon molecules during incubation was controled using epilithic biofilms from Riera Major in summer (from the open stretch and when the maximum photosynthetic activity was detected) by adding 100 μl of hydrochloric acid 0.6 N at the end of the incubation time and placing the vials under the fume for two days to eliminate all unincorporated ^{14}C. Afterwards the scintillation liquid was added and the radioactivity measured in the scintillation analyser. On average, only 0.45% (SD=0.058, n=15) of the ^{14}C incorporated was excreted during the incubation time.



+ 1 μCi $NaH^{14}CO_3$
(10 ml sol. 0.1 μCi ml^{-1})

↓ incubation (2 h)
in a shaker
at stream water temperature

pouring of the liquid
gently washing the substrates with distilled water
(liquid waste to a special radioactive container)

↓

+ 5 ml of distilled water

↓

Sonication (2 min)

↓

+ 15 ml of scintillation liquid

↓

Radioactivity measurement
(Packard tri-carb 1500)
5 min per vial

Fig. 1. Diagram for the protocol of the measurement of ^{14}C incorporation in stream biofilms.

The calculation of the incorporation of inorganic carbon is based on the direct relationship between the C incorporated in relation to the total C available and the ^{14}C incorporated in relation to the ^{14}C available expressed as following:

$$\frac{C-incorporated}{C-available}=\frac{^{14}C-incorporated}{^{14}C-available}$$

For the calculation of the photosynthetic activity the concentration of inorganic carbon (C-available) must be known and therefore it was analyzed for each streamwater and sampling time as described in section 2.6 (physico-chemical parameters).

The formula applied for the measurement of C incorporation was as follows:

$$C-incorporation=\frac{1.06^{a}.\left(DPM_{sample}-DPM_{control}\right).\left(0.01DIC^{b}\right)}{DPM_{ini}^{c}}.\frac{15ml}{5ml.S.H}$$

where:

a= correction factor for algae for the preference of the unlabeled inorganic carbon over the radioactive

b= dissolved inorganic carbon expressed in $\mu g\ L^{-1}$ and therefore multiplied by the factor 0.01 L (=10 ml of solution incubated) to give the total (unlabeled) inorganic carbon available

c= initial DPM of the solution injected in the incubation

S= surface of the incubated biofilm sample in cm^2

H= incubation time in hours

The initial DPM (DPM_{ini}) can be calculated by using the transformation of the added µCi to DPM ($2.22\ 10^6$ DPM per µCi), resulting in a value of 22000 initial DPM. However, to check for any error or imprecise dilution of the radioactive material, the radioactivity of the initial solution was measured. Therefore at each sampling date, 0.1 ml of the $H^{14}CO_3$ solution with 0.1 ml of phenylalanine (to fix the inorganic carbon) was incubated, the scintillation liquid was added (15 ml) and the radioactivity measured. The resulting DPM obtained by this procedure multiplied by 100 (since 0.1 ml was taken from the solution of $0.1\mu Ciml^{-1}$) was used as the initial DPM for the calculations of inorganic carbon incorporation.

Carbon incorporation was calculated separately for the light and the dark incubated samples to test for the importance of dark incorporation in the biofilms studied. The activity was expressed as $\mu g\ C\ cm^{-2}\ h^{-1}$.

2.4. Bacterial density and biomass

Stream biofilm samples for bacterial counting were placed in sterile glass vials and fixed with 2% formalin in the field. Samples were preserved in the dark till counting.

The protocol for preparation of samples for bacterial counting is summarized in Fig. 1. First of all each fixed sample was sonicated for 90 s in a sonication bath (Selecta, 40 W power, 40 kHz ultrasounds frequency). A 90 second sonication time was found to be enough to remove all material from the stream biofilm (grown on artificial substrates) since no differences were found between an empty substrate (clay tile 0.64 cm^2, 1cm high) and a colonized tile which had been sonicated for 90 s which appeared totally clean under the SEM (Fig. 2). Although a longer period might be necessary for thicker natural biofilms such as those found in La Solana stream (cyanobacterial crust, chapter 4), such a longer sonication time would break the bacteria (C. Freeman, pers. comm.). For those thick biofilms, small pieces were used and two 90 s periods (90s plus 90s) were performed. To ensure the total extraction of bacteria from the cyanobacterial crust, bacterial density was also estimated in totally crushed cyanobacterial crust, the values being in the same range as those obtained by sonication (90 s plus 90 s) but the counting was much more difficult due to the high presence of particles (carbonates). The 90 s sonication time was also applied for sand samples being similar to the sonication time reported for counting bacteria on sediments (Epstein and Rossel 1995).

Once suspended samples were obtained, they were appropriately diluted. Subsamples for dilution were taken immediately after the sonication time except for the sand samples. For the preparation of sand samples for bacterial enumeration the accumulation of detrital material had to be avoided to diminish the masking of bacteria with sand grains when counting under the microscope (Schallenberg et al. 1989, Epstein and Rossel 1995). For this reason, the sonicated sand samples were shaken and left for several minutes (2-3) for sedimentation of the larger particles and the supernatant was collected. These 2-3 minutes were observed not to decrease bacterial density of the Riera Major sand samples.

A dilution factor from 10 to 40 was applied to the supernatant from sand samples, while for epilithic biofilms and the cyanobacterial crust a dilution factor of 5-10 was applied. After dilution, samples were stained for 5 min with 2 $\mu g\ ml^{-1}$ of 4,6-diamidino-2 phenylindole (DAPI, Sigma) and filtered through 0.2 μm irgalan black stained polycarbonate filters (Hobbie et al. 1977, Porter and Feig 1980, Herbert 1990). Sand samples were stained with DAPI for 6-7 min, which provided better stained preparations in such detritic samples. Bacteria were counted using a fluorescence microscope (Reichert-Jung, Polyvar) under 1250 magnification. Usually 20 fields were counted per filter for a total 400-1500 organisms.

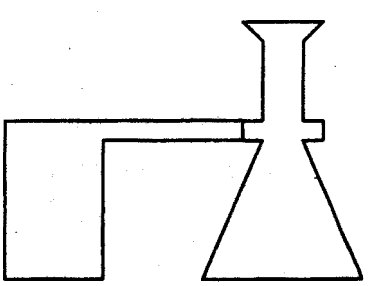
preserved sample in 2% formalin
(10 ml of stream water + 0.5 ml of
40% formalin)

sonication:
90 s epilithic and sandy biofilms
90 s + 90 s cyanobacterial crust

sand samples

shaking
sedimentation of larger particles (2-3 m
take the supernatant for dilution

dilution:
1:10-1:40 sand samples
1:5-1:10 epilithic biofilms, cyanobacterial crust



5 ml of the diluted sample
+ 0.1 ml DAPI (from a solution of 0.1 mg ml^{-1})
5 min in the filtration column (6 min for sand samples)

filtration

sample preparation

observation under the
fluorescent microscope

Fig. 1. Protocol diagram for sample preparation for bacterial enumeration.

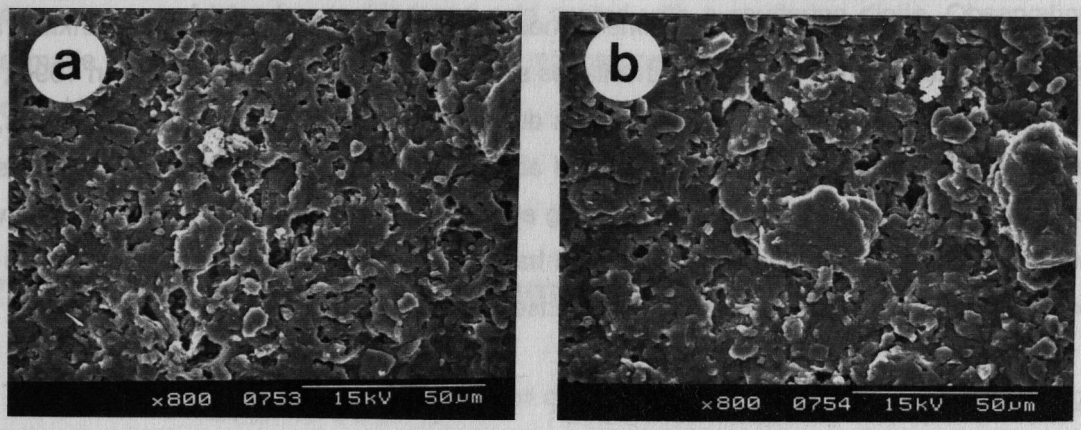


Fig. 2. SEM photographs of an empty tile (a) and a 90 s sonicated tile after being colonized in the stream for eight weeks (b).

Bacterial numbers in all the studied biofilms ranged between 10^9-10^{10} cells cm^{-2} being in general higher than values reported in the literature. Although possible environmental differences are discussed in each chapter, this higher bacterial density, especially for sand samples, could be a result of better detachment and a lower masking effect than when the current protocol (developed for planktonic environments) is applied, as has been observed by Epstein and Rossel (1995) and Schalenberg et al. (1989) when developing a special protocol for sediment bacteria enumeration.

Bacterial shapes and sizes were distinguished and biovolume was calculated by approximating the different shapes to an analogous geometric formula. Bacterial biovolume was calculated for each sample considering cocci to be spherical, filament forms to be cylindrical, and rod-shaped to be cylindrical with hemispherical edges. Bacterial biomass was calculated from biovolume and density of each bacterial shape observed using a conversion factor of 2.2 10^{-13} gC μm^{-3} (Bratbak and Dundas 1984, Kemp 1990), which lies in the middle of the values reported in the literature (Psenner 1990). The mean cell volume at each sample time was also calculated.

When applying this direct counting method we must know that all bacteria including non-active cells, are counted. Inactive cells (dead or dormant) may be present especially in sandy biofilms (Bott and Kaplan 1985, Hendricks 1993). Even though active bacteria can be distinguished under the fluorescent microscope after an incubation with cyano-tetrazolium chloride (Rodriguez et al. 1992) or with fluorescein diacetate (Lundgren 1981, Chrzanowski et al. 1988), their visualization would be difficult with high detritus-content samples. Furthermore, the direct counting metod with DAPI stain is rather generalized in aquatic studies permitting a comparison with other environments. Although counting bacteria under fluorescence microscope gives a good approach to bacterial density, the extremely low size of some aquatic bacteria (0.2 µm diameter) makes this counting extremely delicate, sometimes depending on the quality of the microscope and the bias due to the personal eye (Kepner and Pratt 1994). We must make as objective as possible our rules when counting. Nowadays, image analysis by

computer and a video-camara installed over the fluorescent microscope permit more precise results. The technique of flow cytometry has been used recently in phytoplankton and bacterioplankton offering numerous advantages such as precision and accuracy (Karl 1994), but seems not to be practical for benthic bacteria due to the presence of filaments and chains, as well as detritus material also being dyed and absorbed to bacteria. No significant relationship was found between direct fluorescent counting and flow cytometry flux counting performed with sand samples. A total washing and extraction has to be applied to soil samples to analyse the bacterial population by flow cytometry (Christensen et al. 1995).

2.5. Algal density and biomass

Chlorophyll-a in the biofilms was measured after extraction in 90% acetone for 12 h in the dark at 4°C. To ensure complete extraction of chlorophyll, samples were sonicated for 4 min at the end of the extraction period. For La Solana cyanobacterial crust (chapter 4) a second extraction with acetone was necessary. Chlorophyll-a concentration was determined spectrophotometrically (Perkin-Elmer, Lambda2 UV/VIS spectrophotometer) after filtration (GF/F Whatman) of the extract, following Jeffrey and Humphrey (1975).

Algal density was estimated in the epilithic biofilms (growing on artificial substrates) for the two colonization experiments (chapter 8). After sonication of the samples (2 min) in a sonication bath, the suspended material was sedimented using a phytoplankton cuvette following the technique of Utermöhl (1958). An appropriate dilution (from 2 to 8 times) was applied to the most concentrated samples. For each sample 45 fields were counted (200-700 cells) under a 60x magnification on an inverted microscope.

Algal composition was also determined in each stream biofilm (epipsammic, epilithic, cyanobacterial crust) after the sonication of the samples (2 min) and observation under the light microscope at 400 magnification.

2.6. Scanning Electron Microscope observations

SEM observations of the biofilms growing on the artificial substrates (clay tiles) were made to certify the appropriate sonication time and to describe community structure. For this purpose, separate samples were fixed in the field with 2.5% gluteraldehyde in phosphate buffer pH 7.5 and stored in the dark until SEM observations were made.

For the observation of the bacterial community (usually covered by algae) tiles were sonicated (90 s) and the material was collected with a filter (Gelman, 0.2 μm pore size). The filter was prepared for SEM observations.

To preserve the soft structures, samples were first frozen with nitrogen slush (-210°C), under vacuum, and later freeze-dried and gold coated with a sputtering diode. Observations were made using a Hitachi S-2300 electron microscope under 15 KV (Serveis Científics i Tècnics, UB).

2.7. Physical and chemical parameters

Temperature, pH, dissolved oxygen and conductivity were measured in the field using a pH meter (WTW), an oxygen meter (WTW) and a conductivity meter (WTW) with electrodes (Table 1). Incident light (PAR) was measured with a LiCor quantum (Li-192SB) in the air and immediately above the stream substrates (using an underwater sensor).

TABLE 1. Field and laboratory techniques for the measurements of the physical and chemical parameters in the studied streams.

Field measurements	**Tecnique**
pH	pH meter (WTW)
Dissolved oxygen	oxygen meter (WTW)
Conductivity	conductivity meter (WTW)
Temperature	WTW meters
Incident light	LiCor quantum sensor (Li-192SB)
Underwater light	LiCor quantum sensor (Li-192SB)
Laboratory analyses (nutrient content)	**Technique**
Nitrate	ion chromatography
Ammonia	spectrophotometry (indophenol blue method)
Soluble reactive phosphorus (SRP)	spectrophotometry (ascorbic acid method)
Dissolved organic carbon (DOC)	total organic carbon analyzer
Dissolved inorganic carbon (DIC)	total organic carbon analyzer

Water samples were filtered through precombusted Whatman GF/F filters and analysed for inorganic nutrients (nitrate, ammonium and soluble reactive phosphorus, SRP), dissolved organic carbon (DOC) and dissolved inorganic carbon (DIC) (Table 1). Three samples per sample analysed were used. Nutrients were analysed according to APHA (1989). Nitrate was analysed by ion chromatography (Kontron) with an IC-Pack anion column (4.5 x 50 mm) and an ultraviolet detector. Ammonium and soluble reactive phosphorus were analysed spectrophotometrically (indophenol blue method and ascorbic acid method, respectively) following the procedure of Grasshoff et al (1983). The DIN:SRP ratio was calculated as the

molar ratio of dissolved inorganic nitrogen to soluble reactive phosphorus. DIC and DOC were measured using a total organic carbon analyser (TOC-5000, Shimadzu) that performed combustion, followed by phosphoric acid titration. In chapter 8 (section 8.1) alkalinity was measured by a titration with sufuric acid 0.02 N using a mixt indicator (methyl red and bromocresol blue, switching at pH 4.3), and DIC was calculated from measures of alkalinity, temperature, pH and conductivity using the computer program WATEQX (Van Gaans 1989).

Water velocity was measured in the field by recording the increase in conductivity with time after a slug-injection of a concentrated chloride solution to the stream water, approximately 50 m upstream from the sampling point (Gordon et al. 1993). Discharge was calculated by mass balance from integration of the concentration hydrograph (Triska et al. 1989).

3. RIERA MAJOR: A FOREST MEDITERRANEAN STREAM

Study site

Riera Major is an undisturbed, second-order stream, tributary of the river Ter, located in the north of Catalonia (90 Km N of Barcelona, NE Spain) (41°56' N, 2°25' E) (see Fig. 6 in chapter 1) at the altitude range of 960-460 m above sea level. It is an oligotrophic stream of moderate alkalinity that drains a siliceous watershed (Martí and Sabater 1996). The stream is 6 Km in length and drains a siliceous watershed (granodiorite) of ca. 16 km^2 of surface area. Mean discharge during 1993-94 was ca. 60 L s^{-1}. Riparian vegetation is made up mainly of alder (*Alnus glutinosa* (L.) Gaertn.), which furnishes a dense canopy from spring to late autumn, therefore providing minimum incident light in summer (10-30 μmol photons m^{-2} s^{-1}). On average, 60% of the stream bottom is covered by boulders and stones while 40% is covered by sand. In winter, diatoms develop upon *Hildenbrandia rivularis* Liebm. (Rhodophyceae), which permanently colonizes the river bed. In spring, patches of Cyanobacteria (mainly *Oscillatoria* spp. and *Phormidium autumnale* (Ag.) Gom.) and filamentous Chlorophyceae (*Cladophora glomerata* (Linn.) Kütz) replace the diatoms (Guasch and Sabater 1994).

Study site in Riera Major.

3.1. Role of epipsammic and epilithic biofilms in organic matter utilization in a forest stream

Abstract

Sand and rocks (cobbles) were the two substrate types available for microbial colonization in Riera Major. The respective role of epilithic biofilms (growing on artificial substrates, clay tiles) and epipsammic biofilms (growing on the surficial sandy substrate, distinguishing the mid-channel to the stream-edge zone) in the degradation of organic matter was evaluated during a hydrologic (annual) cycle. Several structural and functional characteristics were different in the two types of biofilms. Algae were well developed on tiles, but occasionally found on sand. Bacteria were more diverse, and of higher biomass and biovolume in the epilithon. Seasonal variations in heterotrophic metabolism and biomass were observed only for the epipsammon. Higher β-glucosidase and β-xylosidase activities were measured in the epipsammon than in the epilithon, indicating that degradation of cellulosic and hemicellulosic molecules was greater on sand than on tiles. Two mechanisms appeared to regulate ectoenzymatic activities: organic matter accumulation in the epipsammon, and autotrophic excretion in the epilithon. The organic matter accumulated on sand could enhance the heterotrophic metabolism there. Sand, especially in the stream-edge zone, showed the highest enzymatic activities in the stream indicating its relevance in degrading a greater amount of macromolecules than the epilithon.

Introduction

Ectoenzymes have been widely analysed in aquatic ecosystems (e.g. Hoppe 1983, Somville 1984, Chróst et al. 1989, Boschker et al. 1995, see chapter 1 for revision), and their role in organic matter cycling is well-stablished (chapter 1). Enzymatic activity has been detected in the stream water column (Chapell and Goulder 1995), but it is much more significant in streambed substrates (Marxsen and Witzel 1991). Both the biofilms covering the surface area of the sediment grains (e.g. Leichtfried 1985, Bretschko and Leightfried 1988) and the biofilms covering the streambed rocks and stones (e.g. Geesey et al. 1978) are important for the metabolism and the organic matter retention of the river system (Bretschko 1995). Biofilm metabolism in the epipsammon (growing on sand) and the epilithon (growing on rocks) may be affected by substrate mobility and the availability of light and nutrients (Lock 1993). In the sediment, grain size determines the surface area available for colonization by biofilms (Bott and Kaplan 1985, Marxsen and Witzel 1990). The different characteristics of each substrate may result in differences in organic matter use.

The aim of this study was to assess the respective role of epilithic biofilm and epipsammic biofilm in the degradation of organic carbon, in a stream where the two substrates are well represented. Both epilithon and epipsammon are well developed in Riera Major, an oligotrophic forest stream. The Mediterranean climate results in marked seasonal variations of temperature, light, and nutrients (Guasch and Sabater 1994, Martí and Sabater 1996), which

may affect ectoenzymatic activities (Sinsabaugh and Linkins 1988, Jones and Lock 1989). It was examined the relative contribution of the epilithic and the epipsammic biofilms to the breakdown of the stream organic matter by measuring the activity of ectoenzymes (β-glucosidase, β-xylosidase, and phosphatase). Artificial substrates (clay tiles) were used for the study of the epilithic biofilm while sand was collected from the mid-channel of the stream-bed and the edge of the stream-bed zones for the study of the epipsammon. The study was performed over one year in order to consider variations in water quality, light, temperature, and discharge which may affect organic matter utilization.

Materials and methods

Sampling

Field samples of epipsammic and epilithic biofilms were taken at monthly intervals from January 1994 to February 1995, as well as physico-chemical parameters. In October 1994 there was a flood, increasing the discharge of the stream to 600 L s^{-1} (i.e. 10-15 times the previous average discharge). In November 1994, after the flood, only sand samples were collected, and the stream-edge zone was not considered.

All measurements of ectoenzymatic, respiratory, and photosynthetic activity were performed in the laboratory, two to three hours after sampling.

Epipsammic biofilms: sand sampling. Sediments in the stream-edge zone and in the mid-channel zone were different in grain size and organic matter accumulation. Therefore, sand samples (2 cm sediment depth), were obtained both from the mid-channel and from the stream-edge zones using a PVC sand-corer sampler (5 cm diameter). The mid-channel zone was established in riffle areas in the middle of the stream bed. Stream-edge sand was collected from submerged areas near the stream bank. Water velocity was up to ten times higher in the mid-channel than in the stream-edge zone. Several corer sand samples (10-12) were obtained from both the mid-channel and the stream-edge zones in a stream stretch of 15 m long, 4 m wide and 14 cm deep, on average, and 2 % slope. Sand samples for the metabolic activities and biomass were randomly collected from the corer sand samples. Sand samples for bacterial counts were collected in sterile glass vials and fixed with 2% formalin. Sand samples for measurements of enzyme activity, chlorophyll-a, electron transport system activity, and organic matter were placed in sterile glass vials with stream water, and were maintained cold (on ice) in the dark until arrival in the laboratory. For all parameters measured, sand samples were approximately 2 ml in volume.

Epilithic biofilms: artificial substrates. Artificial substrates were used to study the epilithic biofilm. Small, unglazed clay tiles (0.64 cm^2 of surface area and 1cm height) were glued using colourless silicone onto flat surfaces of natural boulders, in the way they were completely

covering regular surfaces of the stream stones (Fig. 1), and immersed in a stream riffle stretch (the same stretch where the sand samples were collected) to allow colonization.

Fig. 1. Picture of a stream boulder covered by the artificial substrates glued on it, after two months colonization.

Previous observations of the bacterial and algal community from stream colonized tiles, its resembling to the natural epilithic community, and the results from a colonization study (Sabater and Romaní 1996) showed the optimal colonization period in the stream (in terms of diversity and biomass) to be 6 to 8 weeks. In laboratory studies a mature biofilm was achieved in three weeks (Hamilton 1987) while in natural conditions a eight week colonization time has been necessary (Jones and Lock 1993). Furthermore, the suitability of using the artificial substrates was also checked by counting bacteria and algal cell density in natural stones and compared to 6 to 8 week colonized tiles. Although cell density was higher for the artificial clay tiles (which may be a result of counting only the colonized surface area while for the natural stones all the surface, over side, and under side were considered), values for the two substrates were in the same range (Table 1). Therefore, tiles with biofilms of this age (six-to-eight weeks) were used for the analyses of the epilithic biofilms. The tiles were collected in the field, separating them from the boulders with a spatule, gently rinsed of coarse debris and placed in sterile glass vials with stream water. Samples were maintained cold (on ice), in the dark, during

transport. Tiles for bacterial counts were collected in sterile glass vials and fixed with 2% formalin. Tiles for SEM observations were fixed in the field with 2.5% gluteraldehyde in phosphate buffer pH 7.5 and stored in the dark until SEM observations were made.

TABLE 1. Bacterial and algal cell density in colonized clay tiles (6 to 8 week old) and natural stones. Mean values and standard deviation (in parenthesis) are shown.

	Tiles (n=9)	**Natural stones** (n=6)
Bacterial cell density (10^{10} cm^{-2})	2.14 (0.36)	1.32 (0.28)
Algal cell density (10^{5} cm^{-2})	4.11 (1.39)	0.65 (0.11)

Physico-chemical parameters. At each sampling date, temperature, pH, dissolved oxygen, conductivity and incident light were measured in the field. Filtered (precombusted Whatman GF/F filters) water samples (three replicates for each analysis) were taken in order to analyse inorganic nutrients (nitrate, ammonium, and soluble reactive phosphorus), as well as dissolved organic carbon (DOC) and dissolved inorganic carbon (DIC), following the procedures described in chapter 2 (section 2.7).

Bacterial and algal biomass, organic matter, and SEM observations

Bacterial density (DAPI stain, epifluorescence microscopy) was estimated in triplicate both on tiles and sand (from the mid-channel and the stream-edge zones) samples. Bacterial shapes and sizes were distinguished and biovolume was calculated and tranformed to bacterial biomass (conversion factor of 2.2 10^{-13} gC μm^{-3}). The community structure on tiles was also observed under the SEM. Chlorophyll-a (acetone extraction) was measured in triplicate in tiles and sand (from the mid-channel and the stream-edge zones) samples. The quotient of the optical densities at 430/665 nm was also calculated (Margalef 1983). All measurements were determined following the procedures described in chapter 2.

Organic matter on sand samples (from the mid-channel and the stream-edge zones) was measured as ash free dry weight (AFDW), after 4 h at 450°C, and was expressed as mg cm^{-2} of grain surface area (see below for calculation of sand surface area).

Metabolism measurements

Extracellular β-D-glucosidase, β-D-xylosidase, and phosphatase potential activities were determined in tiles and sand samples (from the mid-channel and the stream-edge zones) (five replicates) and controls (substrate killed samples) (two replicates). Two blanks of filter-sterilized stream water were also incubated for each enzyme. The Electron Transport System (ETS) activity was measured using five replicates for each sample type (tiles, mid-channel sand, and stream-edge sand) and two controls. Primary production was measured in tiles and sand

(from mid-channel and stream-edge zone) using five replicates, two control and two dark-incubated samples. All measurements were determined following the procedures described in chapter 2.

Estimation of sand surface area

Biomass and activity measurements performed in sand samples were normalized to cm^2 of sand surface area. Specific sand surface was calculated following Prenant (1960) and Fenchel (1969) as described in Marxsen and Witzel (1991). First, sand samples (from the mid-channel and stream-edge zones) were dried and grain sizes measured by granulometry (Table 2).

TABLE 2. Particle size distribution in mid-channel and stream-edge sand from Riera Major stream.

Particle diameter (μm)	Mid-channel sand Weight (%)	Stream-edge sand Weight (%)
2000	24.53	5.70
1000	41.52	19.87
500	26.07	45.80
315	4.30	16.50
250	0.63	2.58
125	1.70	6.33
100	0.26	0.61
63	0.50	1.02
<63	0.50	1.59

Secondly, the number of sand grains retained in each sieve was calculated by the formula:

$$\log n = -0.14225 - \frac{5\log d_1 + \log d_2}{2} + \log p$$

where: n= number of grains retained in the sieve 2

d_1 and d_2= sizes of the sieves 1 and 2 in cm, $d_1 > d_2$

p= dry weight of the sand obtained between the sieves 1 and 2 (retained in sieve 2)

and the constant -0.14225 was obtained from the following calculation:

$$\log \frac{6}{\pi . c^3 . \sigma}$$

where: c= shape coeficient, c=1 for espherical

σ= density, σ= 2.65 for quartz grains

The average radius of the grains found in each sieve will be:

$$r = \sqrt{\frac{d_1 d_2}{4}}$$

For each sieve we know then the number of grains retained (n) and the average radius of the grains (r), and therefore we can calculate the total grain surface area for the sand retained in each sieve (considering sand grains as esphericals) by the formula:

Sand surface area (in cm^2) for one sieve= $4.\pi.r^2.n$

The total sand surface area for the sand analysed will be obtained by the summ of the grain surface area calculated for the sand retained in each sieve size.

The conversion factor between dry weight of sand and total grain surface area was obtained for mid-channel and stream-edge sand, being 1.4798 $cm^2\ g^{-1}$ for mid-channel sand, and 2.8285 $cm^2\ g^{-1}$ for stream-edge sand. All the samples used for the biomass and metabolic measurements were dry weighted (after 2 days at 110°C) and the respective conversion factor was applied to express the results per cm^2 of grain surface area.

Data analyses

Differences between biofilm activities in mid-channel sand and stream-edge sand, and between mid-channel sand and tiles, were analyzed using an analysis of variance (ANOVA, two factor with replicates). Possible differences between mid-channel sand and stream-edge sand on a given sampling date were explored using a two-tailed *t*-test. Correlation analysis of environmental and biological variables was performed using the product-moment Pearson coefficient. The relationships of activity and biomass measurements to the environmental parameters were also analyzed using non-linear regression (logarithmic, quadratic, power, exponential and inverse) and were compared to a linear regression. Moving averages-correlation was calculated between β-glucosidase or β-xylosidase activity and levels of chlorophyll-a observed the previous month. Monthly averages of these variables were recalculated from daily values obtained by trapezoidal interpolation. The recalculation of monthly averages was necessary in order to obtain the same time interval between samples.

Results

Physical and chemical parameters

The physical and chemical characteristics of Riera Major are summarized in Table 3. The minimum temperatures (3.9-5.7 °C) and highest concentrations of dissolved oxygen (11.4-12.9 mg L^{-1}) were reached in winter. Water in this period had a high content of dissolved inorganic nitrogen (270.7-510.53 µg L^{-1}) and low soluble reactive phosphorus (2.1-3.5 µg L^{-1}) (maximum DIN:SRP ratio). Light irradiance was maximum in late winter and spring (313.5 µmol photons m^{-2} s^{-1}), and decreased in summer because of the development of the riparian canopy. The maximum water temperature (16.2 °C) and conductivity (244 µS cm^{-1}) were recorded in summer, coinciding with the lowest discharge (15 L s^{-1}) of the whole period. Ammonium concentration was also very low in summer (3.7 µg L^{-1}). DOC reached maximum values in autumn (4.2 mg L^{-1}), coinciding with the period of maximum riparian leaf fall.

TABLE 3. Physical and chemical characteristics of Riera Major stream. Values are seasonal means for the study period (1994-1995). The minimum (min.), maximum (max.), annual mean and standard deviation of the mean are also shown. DIN:SRP is the molar ratio of dissolved inorganic nitrogen to soluble reactive phosphorus.

	Winter 1994 (n=2)	Spring 1994 (n=2)	Summer 1994 (n=2)	Autumn 1994 (n=3)	Winter 1995 (n=2)	min.	max.	mean (SD)
Temperature (°C)	5.67	9.40	16.20	9.70	3.90	1.90	17	8.76 (4.3)
Light (µmol $m^{-2}s^{-1}$)	52	313.5	10.28	56.75	177.15	7.25	612	116.01 (174)
Discharge (L s^{-1})		35	15	70	55	15	90	50 (24)
Cond. (µS cm^{-1})	182.67	216	244	160.33	170.8	126	254	190.9 (39)
pH	8.28	8.09	7.42	7.74	7.69	7.01	8.48	7.87 (0.4)
Oxygen (mg L^{-1})	11.37	10.32	8.30	10.00	12.95	7.70	13.1	10.60 (1.6)
NO_3-N (µg L^{-1})	244.71	202.89	372.68	779.60	463.55	29.0	1711	446.04 (431)
NH_4-N (µg L^{-1})	25.99	13.41	3.74	25.58	46.98	4.34	69.4	23.36 (17)
SRP (µg L^{-1})	2.18	8.80	6.07	10.71	3.49	1.41	12.1	6.65 (3.8)
DIN:SRP	336.77	58.18	193.62	185.56	677.49	35.9	1190	280.8 (309)
DOC (mg L^{-1})	1.43	2.87	1.33	4.20	3.98	1.10	5.88	2.89 (1.5)
DIC (mg L^{-1})	29.58	25.26	27.96	16.03	18.71	11.2	32.2	22.83 (6.6)

Epipsammic biomass and activities. The β-glucosidase, β-xylosidase, and respiratory (ETS) activities were not significantly different in the mid-channel sand than in the stream-edge sand (ANOVA, Table 4) (Fig. 2a, b, Fig. 3a). Only in March, the three activities were significantly

higher in the mid-channel sand than in the stream-edge sand (two-tailed *t*-test, p=0.0001 for β-glucosidase, p=0.0074 for β-xylosidase, p=0.0047 for ETS). However, phosphatase activity (Fig 2c) was significantly higher in mid-channel sand than in stream-edge sand for the whole study period (ANOVA, Table 4).

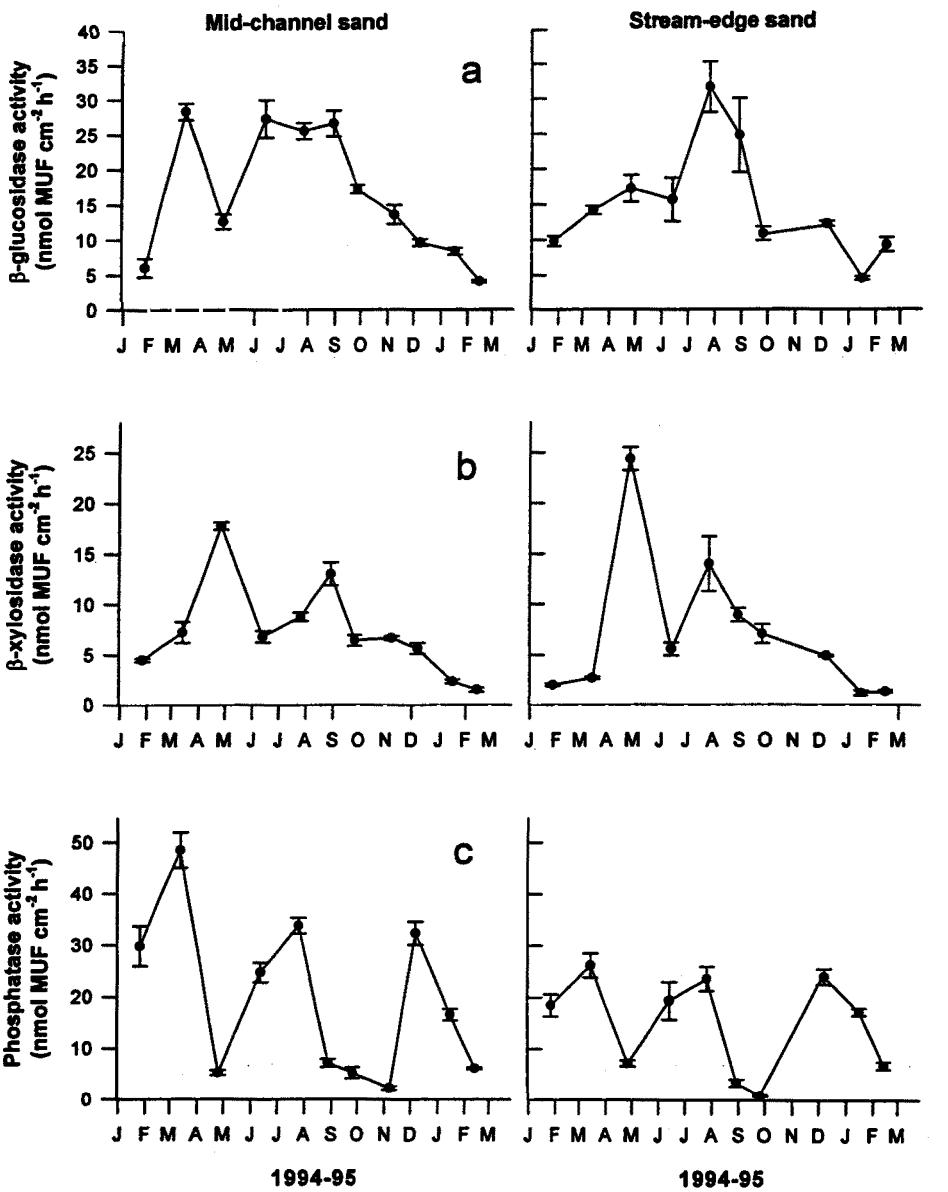


Fig. 2. Temporal variation of epipsammic activities and biomass in Riera Major stream, January 1994-February 1995: a) β-glucosidase activity, b) β-xylosidase activity, c) Phosphatase activity. Means ± standard errors (vertical lines) are shown, n=5.

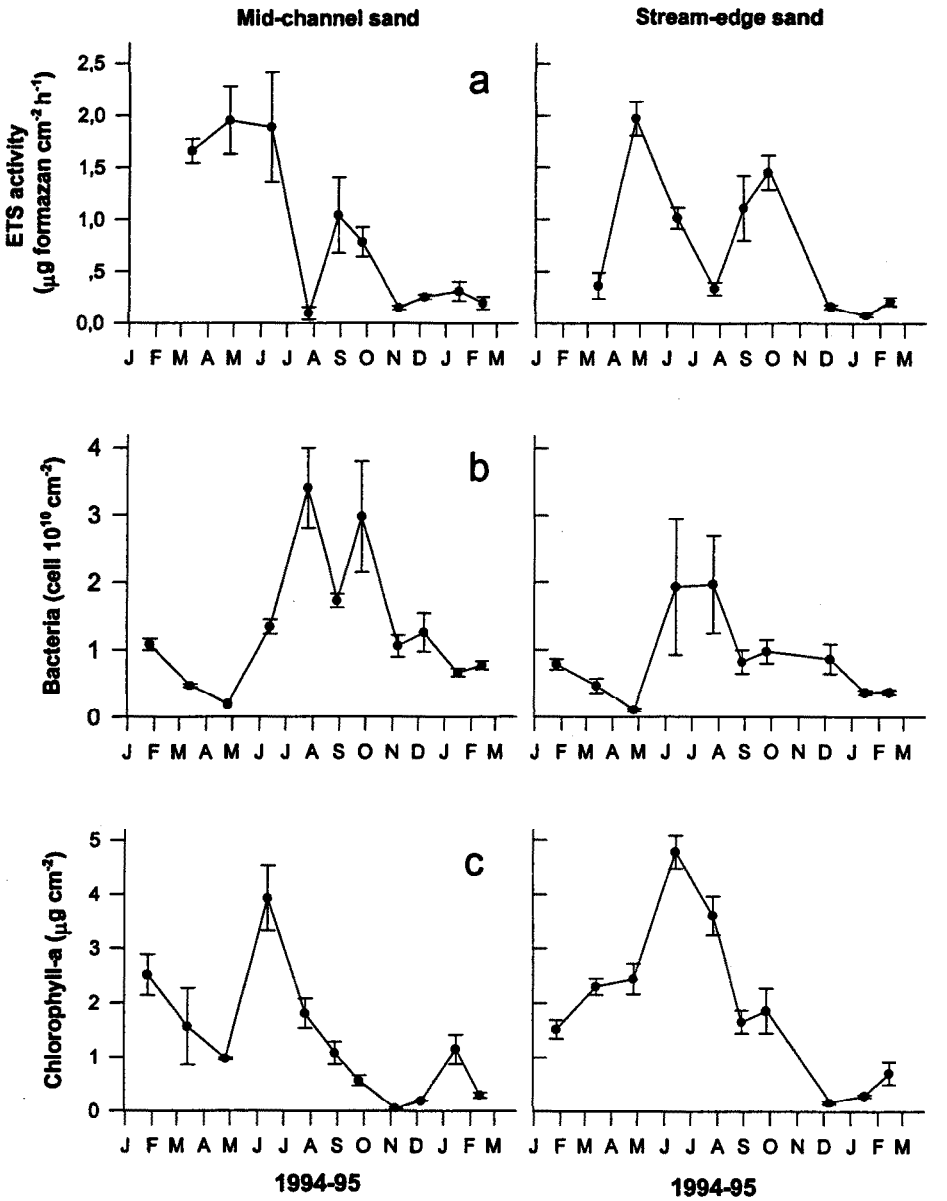


Fig. 3. Temporal variation of epipsammic activities and biomass in Riera Major stream, January 1994-February 1995: a) Respiratory (ETS) activity, b) Bacterial cell density, c) Chlorophyll-a density. Means ± standard errors (vertical lines) are shown, n=5 in a, n=3 in b and c.

Bacteria cell density ranged from 0.1-3.4 10^{10} cell cm^{-2} (Fig. 3b). Although the ANOVA revealed that there were significant differences between the mid-channel and the stream-edge sand (Table 4), the major variability was explained by the seasonal variation (53.4% of the total variance while the differences in habitat explained only 6.6% of the total variance). Significant higher bacterial density in the mid-channel than in the stream-edge sand was found in August 1994, January and February 1995 (two-tailed *t*-test, $p<0.03$).

TABLE 4. Annual averages of ectoenzymatic and respiratory activities and biomass on Riera Major biofilms from the mid-channel sand and stream-edge sand. Differences between the two habitats are expressed by the probability of the F-Fisher after the ANOVA analysis. The star (*) indicates the significant higher values in mid-channel or stream-edge sand.

	Mid-channel sand			**Stream-edge sand**			**F-Fisher**
	Mean	SD	n	Mean	SD	n	prob.
β-glucosidase (nmol cm^{-2} h^{-1})	16.39	9.33	55	15.59	10.75	50	0.34
β-xylosidase (nmol cm^{-2} h^{-1})	7.38	4.68	55	7.20	7.21	50	0.55
Phosphatase (nmol cm^{-2} h^{-1})	19.93*	15.3	55	14.74	9.64	50	$2\ 10^{-10}$
ETS (μg cm^{-2} h^{-1})	0.83	0.79	30	0.74	0.68	27	0.09
Chlorophyll-a (μg cm^{-2})	1.29	1.20	33	2.02*	1.69	30	0.0004
Organic matter (mg cm^{-2})	5.17	2.58	11	8.77	7.42	10	0.39
Bacteria (cell 10^{10} cm^{-2})	1.36*	1.08	33	0.87	0.85	30	0.0035
Bact. biomass (mgC cm^{-2})	0.16*	0.14	33	0.096	0.079	30	0.0006
Bact. biovolume (μm^3 $cell^{-1}$)	0.053	0.009	33	0.057	0.016	30	0.10

A high diversity of bacterial morphotypes were observed under the fluorescent microscope (Fig. 4). Cyanobacteria chains and filaments, sometimes covered with sheaths, and occasionally diatom cells (*Achnanthes* sp., *Cocconeis* sp., *Navicula* sp., *Diatoma* sp., *Amphora* sp.) were also observed in sand samples under the fluorescence microscope.

More than 90% of epipsammic bacteria were small cocci (0.2-0.4 μm diameter) and coccobacilli (0.5-0.8 μm long) (Fig. 5). In April, both in mid-channel and stream-edge sand there were a higher proportion of filaments (Fig. 5).

Bacterial biomass was at a maximum in summer (Table 5), and was significantly greater in mid-channel sand compared to stream-edge sand (ANOVA, Table 4). Bacterial biovolume ranged from 0.03-0.12 μm^3 $cell^{-1}$ (Table 5), and showed no significant differences between mid-channel and stream-edge sand (ANOVA, Table 4).

Accumulation of organic matter (Table 5) was not significantly different in the mid-channel sand (5.2 mg AFDW cm^{-2} in annual average) than in the stream-edge sand (8.8 mg AFDW cm^{-2} in annual average) (ANOVA, Table 4).

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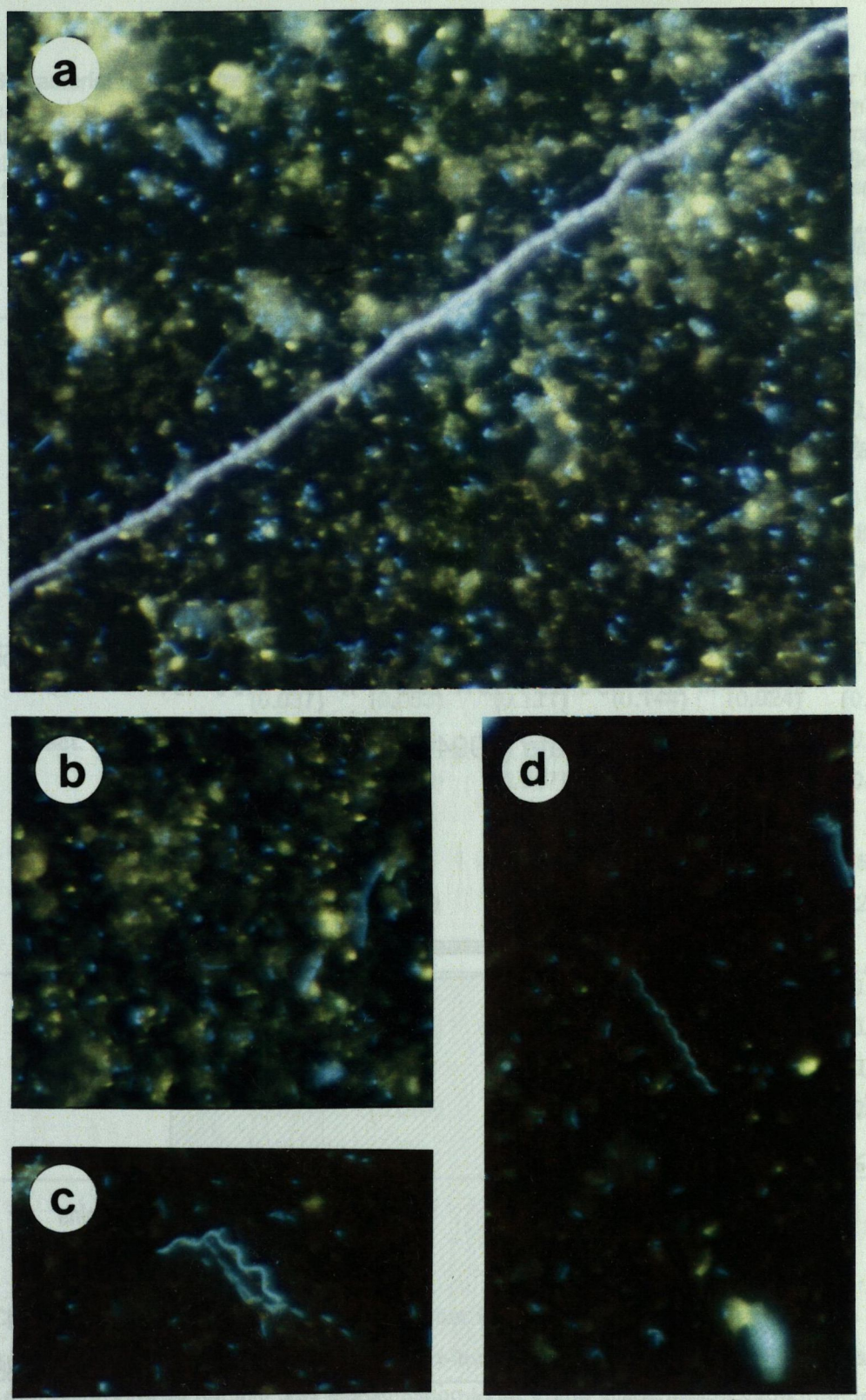

Fig. 4. Photographs under the fluorescent microcope of DAPI stained bacterial preparations from Riera Major sand. (a) A high diversity in the small bacteria morphotypes is observed (cocci and coccobacilli). A cyanobacterial filament is crossing the field. (From mid-channel sand sample collected in September 1994). (b) A chain of rod-shaped bacteria and a filament is observed at the bottom of the photograph. (From stream-edge sand sample collected in February 1995). (c) and (d) Spirochaeta shapes are also observed (From tiles collected in December 1994).

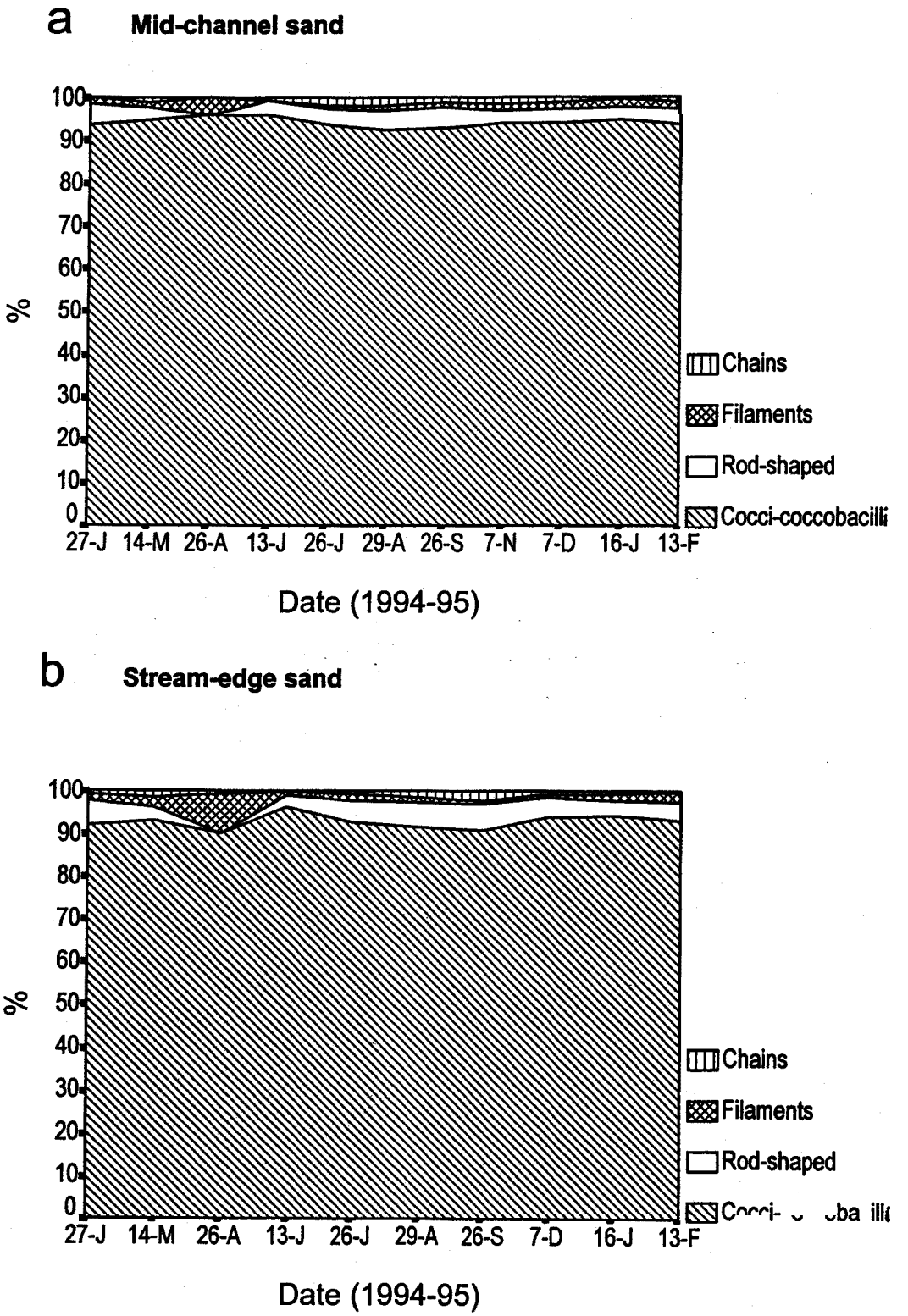

Fig. 5. Relative abundance (%) of the different bacterial morphotypes in Riera Major mid-channel sand (a) and stream-edge sand (b) observed during the study period (January 1994-February 1995).

TABLE 5. Bacterial biomass (mgC cm^{-2}), mean bacterial cell volume (μm^3) on Riera Major biofilms from stream-edge sand, mid-channel sand and tiles, and organic matter (mg AFDW cm^{-2}) from sand. Values are seasonal means, standard deviations (in brackets), and number of samples for the calculation of the seasonal mean (n) are shown for bacterial biomass and biovolume. Seasonal means of organic matter are calculated from monthly averages and the range is indicated. The annual means and standard deviation are also shown.

		Winter 1994	Spring 1994	Summer 1994	Autumn 1994	Winter 1995	Mean (SD)
Stream-edge sand	**Bact. biomass**	0.077	0.093	0.158	0.076	0.043	0.096
		(0.037)	(0.119)	(0.103)	(0.038)	(0.003)	(0.079)
	Bact. biovolume	*0.055*	*0.068*	*0.054*	*0.053*	*0.053*	*0.057*
		(0.011)	*(0.034)*	*(0.006)*	*(0.007)*	*(0.006)*	*(0.016)*
	(n)	6	6	6	6	6	30
	Organic matter	6.29	12.39	15.24	7.55	2.39	8.77
		1.3-17.4	10.5-15.4	1.8-26	2.6-27	1.7-2.9	(7.42)
	(n)	2	2	2	2	2	10
Mid-channel sand	**Bact. biomass**	0.084	0.073	0.322	0.169	0.083	0.164
		(0.037)	(0.053)	(0.121)	(0.144)	(0.024)	(0.141)
	Bact. biovolume	*0.050*	*0.049*	*0.058*	*0.055*	*0.052*	*0.053*
		(0.005)	*(0.009)*	*(0.007)*	*(0.009)*	*(0.010)*	*(0.009)*
	(n)	6	6	6	9	6	33
	Organic matter	8.93	6.68	4.20	3.66	3.11	5.17
		2.1-16.9	5.9-10	2.9-5.1	2.3-4.5	1.7-3.8	(2.58)
	(n)	2	2	2	3	2	11
Tiles	**Bact. biomass**	0.390	0.567	0.208	0.296	0.277	0.350
		(0.146)	(0.358)	(0.070)	(0.131)	(0.055)	(0.215)
	Bact. biovolume	*0.054*	*0.104*	*0.050*	*0.061*	*0.065*	*0.067*
		(0.011)	*(0.067)*	*(0.015)*	*(0.017)*	*(0.009)*	*(0.036)*
	(n)	6	6	6	6	6	30

Chlorophyll-a density was significantly higher in the stream-edge sand than in the mid-channel sand on May and August 1994 (two tailed *t*-test, $p<0.04$) (Fig. 3c). The ANOVA analysis revealed that chlorophyll-a density was significantly higher in the stream-edge sand than in the mid-channel sand (Table 4) but the variability was mainly explained by the seasonal changes (73.1% of total variability while the habitat differences explained only 4.4% of variability).

No significant photosynthetic activity was measured in sand samples. In most of the incubations a high quenching effect has been detected since similar values were obtained from control (killed) samples than from light incubated samples. Therefore, although $H^{14}CO_3$

incorporation could occur in sand, values would be under the detection limit for the technique used.

TABLE 6. Significant coefficients of determination from the regression analyses between enzymatic and respiratory activity measurements and physical and chemical parameters, biomass, and organic matter in mid-channel sand, stream-edge sand, and tiles. The regression curve (Regres.) that better fits the relationship: linear (+ or -), exponential (exp., + or -), logarithmic (log.), power (+ or -), inverse or quadratic (quadr.) is shown. The adjusted square correlation (Adj. r^2) and level of significance of F-Fisher after the ANOVA analysis are also indicated. The term "past chl." means the levels of chlorophyll-a observed the previous month.

	Stream-edge sand			**Mid-channel sand**			**Tiles**		
	Regres	*Adj.r^2*	*signif F*	*Regres*	*Adj.r^2*	*signif F*	*Regres*	*Adj.r^2*	*signif F*
β-glucosidase									
Temp.	power+	0.81	<0.001	linear+	0.54	0.010			
Oxygen	inverse	0.65	0.005	exp.-	0.36	0.048			
Ammonium	log.	0.82	<0.001	log.	0.36	0.049			
OM	linear+	0.47	0.028						
Past Chl.	linear+	0.89	<0.0001	linear+	0.51	0.006	linear-	0.30	0.037
β-xylosidase									
Temp.	power+	0.57	0.012	power+	0.47	0.019			
Oxygen	exp.-	0.40	0.049	exp.-	0.36	0.049			
Ammonium	exp.-	0.47	0.029	power-	0.39	0.039			
OM	linear+	0.63	0.011						
Chl-a							inverse	0.60	0.014
Phosphatase									
Light							linear+	0.41	0.045
ETS									
OM	quadr.	0.72	0.021	linear+	0.74	0.001			
Chl-a	power+	0.49	0.034						
Light							linear+	0.64	0.009
Bacteria									
OM	linear+	0.52	0.011						
Chl.	linear+	0.41	0.026						
Chlorophyll-a									
OM	linear+	0.51	0.012	linear+	0.47	0.011			
pH							linear+	0.47	0.016

Significant relationships between the epipsammic activities and biomass to the physico-chemical parameters are summarized in Table 6. β-glucosidase and β-xylosidase activities in mid-channel and stream-edge sand were significantly related to temperature, oxygen, and

ammonium (Table 6). In stream-edge sand, both activities were also positively related to organic matter content (Table 6). β-glucosidase activity was significantly correlated with the chlorophyll-a levels observed the previous month (herafter referred to as "past chlorophyll") (Table 6).

Bacterial density in stream-edge sand was significantly correlated with chlorophyll-a and organic matter content (Table 6). Chlorophyll-a present in sand biofilms was correlated with organic matter content (Table 6).

Epilithic biomass and activities. β-glucosidase activity was ca. 4 nmol cm^{-2} h^{-1} throughout the study period, with a peak in July (Fig. 6a). β-xylosidase activity was also relatively constant, with a maximum in May (Fig. 6b). However, phosphatase activity was variable over the study period (Fig. 6c). ETS activity was highest in March and decreased during the year (Fig 6d).

Bacterial density ranged from 1.7-3.4 10^{10} cell cm^{-2} (Fig. 6e). 80-85% of epilithic bacteria were small cocci (0.2-0.4 μm diameter) and coccobacilli (0.5-0.8 μm long) (Fig. 7). Rod-shaped bacteria (1.5-2.5 μm long) were found as free cells and filaments. In the epilithon, a higher diversity in bacterial morphotypes than in the epipsammon was observed (Fig. 4). The diversity of the bacterial community was rather constant over the study period except the increase of chains abundance to ca. 14% and the decrease of rod-shaped bacteria in April (Fig. 7). Bacterial biomass was 0.35 mgC cm^{-2} in annual average, being maximum in spring (Table 5). Bacterial biovolume ranged from 0.04-0.18 μm^3 $cell^{-1}$, the highest being also recorded in spring (Table 5).

Chlorophyll-a was at a maximum in early spring 1994 and 1995 (Fig. 6f), and was positively correlated with water pH (Table 6). The average photosynthetic activity was 2.7 mg cm^{-2} h^{-1} and the maximum was recorded in January 1995 (Fig. 6g).

The appearance of the bacterial and algal community which composed the epilithic biofilms (grown on the tiles) was observed under the scanning electron microscope (Fig. 8). The algal community of the epilithic biofilms (observed under optical microscopy) was mainly composed by diatoms of the genera *Gomphonema* sp. (usually stalked), *Achnanthes* sp., *Navicula* sp. and in less density the genera *Cocconeis*, *Cymbella*, *Fragilaria*, *Diatoma*, *Synedra*, *Nitzschia*, *Roicosphenia*, *Amphora*. Filamentous cyanobacteria (*Homeothrix* sp., *Lyngbya* sp., *Phormidium* sp., *Oscillatoria* sp., *Chamaesiphon* sp.) and pleurocapsales (*Pleurocapsa* sp., *Dermocarpa* sp.) usually found as brown-black spots on the tiles, were also observed. In several tiles the red algae (Rhodophyceae) *Hyldrenbrandia rivularis* (liebm.) appeared. A wide description of the algal community of Riera Major epilithon can be found in Guasch (1995).

The significant relationships found between the activities and biomass to the physico-chemical parameters are shown in Table 6. Phosphatase and ETS activities in the epilithic biofilms were significantly correlated with light, while β-glucosidase activity was negatively correlated with "past chlorophyll" (Table 6). β-xylosidase activity showed a negative relationship with chlorophyll-a (Table 6).

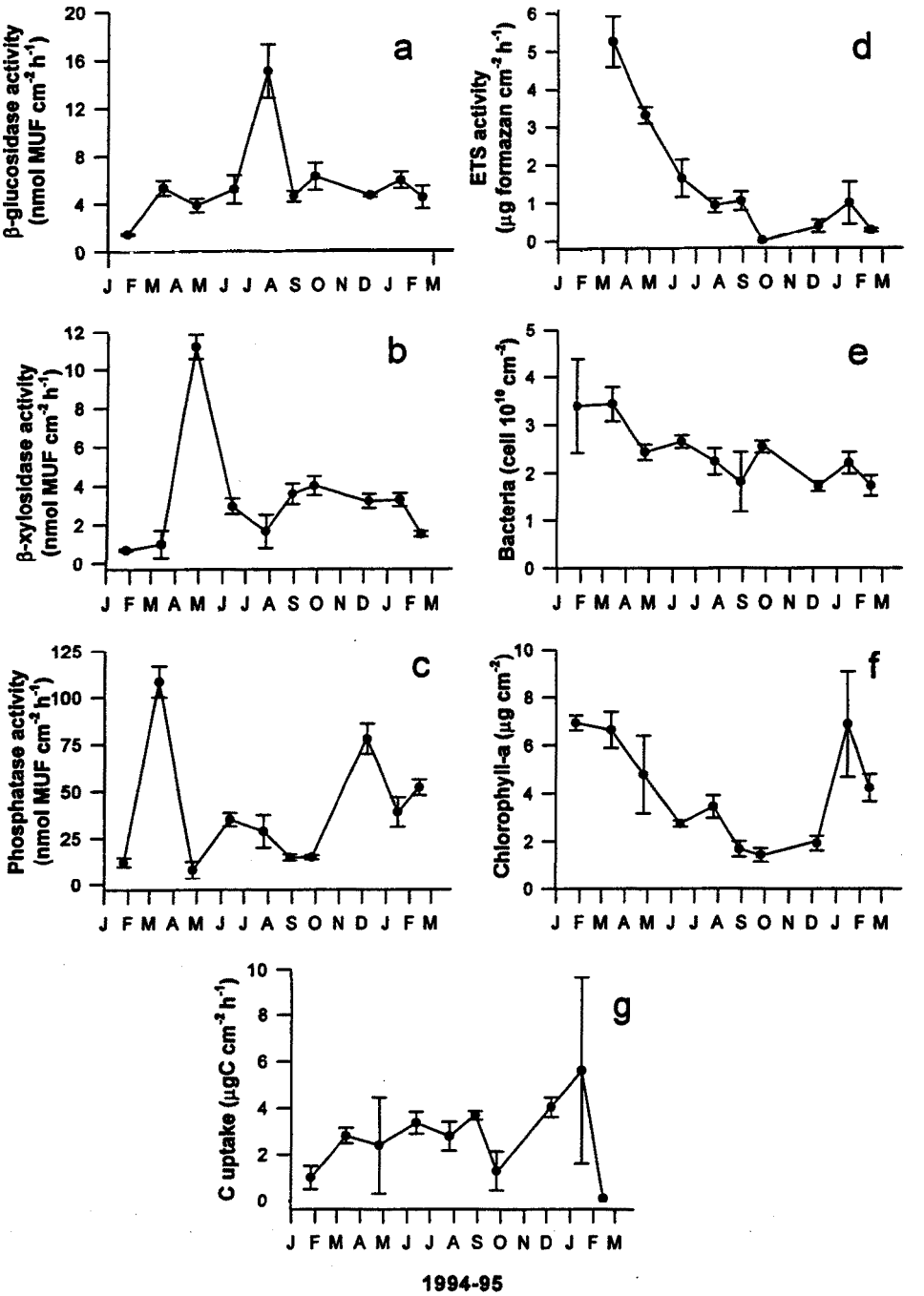

Fig. 6. Temporal variation of epilithic activities and biomass in Riera Major stream, January 1994-February 1995: a) β-glucosidase activity, b) β-xylosidase activity, c) Phosphatase activity, d) Respiratory (ETS) activity, e) Bacterial cell density, f) Chlorophyll-a density, g) $H^{14}CO_3$ incorparation. Means ± standard errors (vertical lines) are shown, n=5 in a, b, c, d, and g, n=3 in e and f.

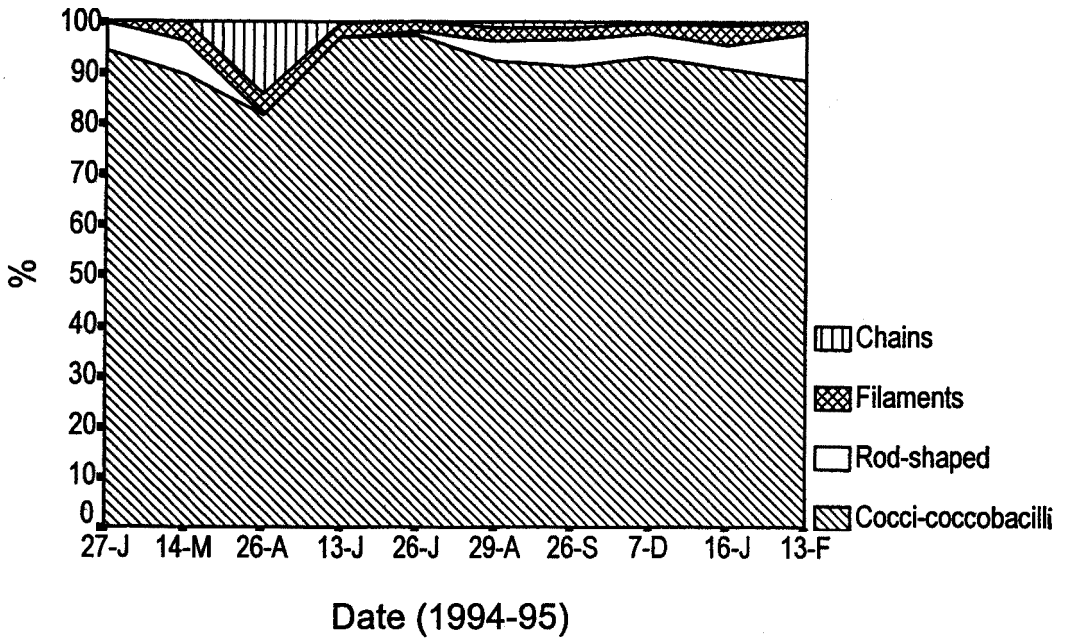

Fig. 7. Relative abundance (%) of the different bacterial morphotypes in Riera Major tiles observed during the study period (January 1994-February 1995).

Epilithic vs epipsammic. Highly significant differences were found between biofilm activities and biomass in the tiles when compared to the mid-channel sand (Table 7). The β-glucosidase and β-xylosidase activities were much more higher in the mid-channel sand than in tiles, while the phosphatase activity, chlorophyll-a density, bacterial density, and bacteria biovolume and biomass were higher in the tiles. ETS activity was slightly higher in tiles than in mid-channel sand.

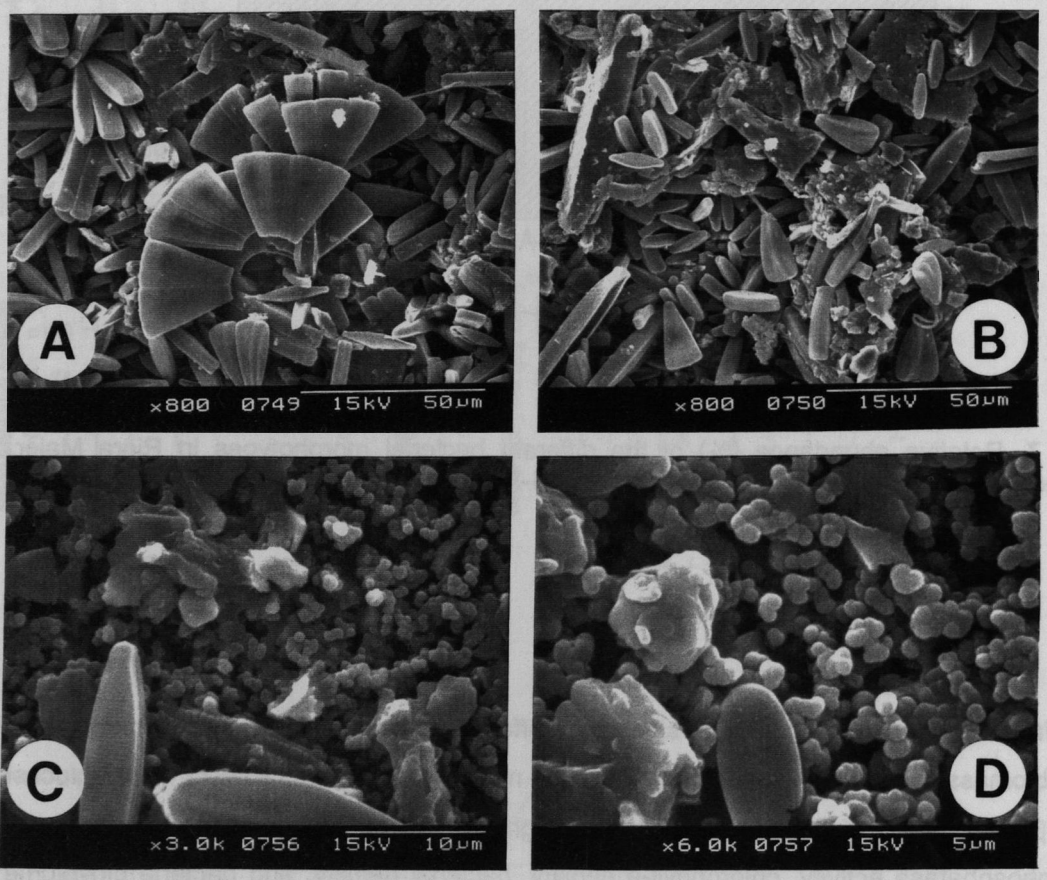

Fig. 8. SEM photographs of the biofilms grown on the artificial substrates (tiles) in Riera Major, after six-to-eight weeks of colonization in the stream. a) Mainly diatoms covered the colonized tiles. Cells of *Meridion circulare* (Greville) Agardh can be observed at the center of the photograph, b) The genera *Achnanthes* and *Gomphonema* were highly abundant in the epilithon. A stalked *Gomphonema* sp. can be seen in the center of the photograph. c) and d) View of the bacteria found on epilithic biofilms after sonication (90 s) of the tiles, collection of the material (Gelman filters, 0.2 µm pore size) and SEM preparation (section 2.6). Mainly small coccus and coccobacilli were observed.

TABLE 7. Annual averages of ectoenzymatic and respiratory activities and biomass in Riera Major biofilms from the mid-channel sand and tiles. Differences between the two habitats are expressed by the probability of the F-Fisher after the ANOVA analysis. The star (*) indicates the significant higher values in epipsammic or epilithic biofilms.

	Mid-channel sand			Tiles			F-Fisher
	Mean	SD	n	Mean	SD	n	prob.
β-glucosidase (nmol cm^{-2} h^{-1})	16.39*	9.33	55	6.12	4.93	50	$7\ 10^{-27}$
β-xylosidase (nmol cm^{-2} h^{-1})	7.38*	4.68	55	3.29	3.04	50	$2\ 10^{-28}$
Phosphatase (nmol cm^{-2} h^{-1})	19.93	15.3	55	37.49*	31.4	50	$2\ 10^{-9}$
ETS (μg cm^{-2} h^{-1})	0.83	0.79	30	1.56*	1.80	27	0.001
Chlorophyll-a (μg cm^{-2})	1.29	1.20	33	4.07*	2.50	30	$3\ 10^{-10}$
Bacteria (cell 10^{10} cm^{-2})	1.36	1.08	33	2.41*	0.84	30	$4\ 10^{-7}$
Bact. biomass (mgC cm^{-2})	0.16	0.14	33	0.35*	0.21	30	$4\ 10^{-10}$
Bact. biovolume (μm^3 $cell^{-1}$)	0.053	0.009	33	0.067*	0.036	30	10^{-6}

Discussion

The structural and functional characteristics of the microbenthic community were different in epilithic and epipsammic biofilms of Riera Major. Apart from a higher algal biomass composing the epilithic biofilm, a higher algal activity is suspected for these biofilm than for the sandy biofilm. There were no live algae attached to sand grains when observed under fluorescence microscopy, meaning that the algae present in the sand were mostly of drifting origin. This was underlied by the correlation between chlorophyll-a and organic matter (Table 6) and the no significant photosynthetic activity measured in the epipsammon. However, epilithic primary production is responsible for variations in the water pH of the stream (Guasch 1995) (Table 6). Moreover, bacteria in the epilithon were at a more constant density (Fig. 6), with greater diversity in morphotypes and with significantly higher biomass and cell volume than epipsammic (Table 5 and Table 7). The differences in bacterial cell biovolume are an ecological indicator of the specific features of the two habitats. Cell size is related to many properties of organisms, such as growth rate, abundance, and diversity, as well as to environmental properties (e.g. temperature, nutrient availability) (Peters 1983, Chrzanowski et al. 1988, Tulonen et al. 1994, Carlson and Ducklow 1996) and food chain effects (e.g. predation) (Sommaruga and Psenner 1995). The higher surface:volume ratio of smaller bacteria may facilitate a higher nutrient uptake capacity due to the higher surface area in contact with the surrounding environment (Ferguson and Rublee 1976, Button 1994). This property is important in sand where diffusion of nutrients through the sand grains is probably more difficult than in the epilithon (Novitsky 1983). On the other hand, the higher cell size in the epilithon may be a response to nutrient quality such as the utilization of algal exudates, since higher growth

efficiency has been described for bacteria when using algal DOC (Sundh and Bell 1992). In planktonic environments bacterial cell size have been commonly related to predation pressure (e.g. Gonzalez et al. 1990), but a minimal effect of benthic flagellates on bacteria has been suggested for freshwater sediments (Epstein and Shiaris 1992, Gasol 1993, Starink et al. 1996).

Seasonal variations in the heterotrophic metabolism and biomass were characteristic of the epipsammon (Fig. 2 and Fig. 3), and were indicated by the relationship of β-glucosidase and β-xylosidase to the environmental variables of the stream (Table 6). However, heterotrophic variables measured in the epilithon were not related to the physical and chemical parameters of the stream and did not follow a clear seasonal pattern (Fig. 6). This is in contrast to what one would expect in a Mediterranean stream (Sabater et al. 1995) and to the noted effect of environmental variables (especially temperature) on biofilm metabolism (Sinsabaugh and Linkins 1988, Chapell and Goulder 1994a). Although a longer study should be planned to test the lack of seasonal variation in the epilithon, the presence of a developed polysaccharide matrix in the epilithon with the capacity to accumulate high quality organic matter could confer a certain degree of independence on the heterotrophic community of the biofilm to changes in water quality (Ford and Lock 1985, Freeman and Lock 1995). This may obscure possible relationships with environmental parameters in the epilithon.

The significantly higher β-glucosidase and β-xylosidase activities in sand (Table 7) indicated that the degradation of organic material of cellulosic and hemicellulosic origin (Deshpande and Eriksson 1988, Lachke 1988) is more significant in the sandy biofilms than in the epilithon. Even though high heterotrophic activity is expected in sediments from shallow oligotrophic streams (Hedin 1990), the epipsammic biofilm in Riera Major appears to be particularly active when compared to ectoenzymatic activities in other stream sediments (Marxsen and Witzel 1990) or marine and freshwater sediments (Poremba 1995, Sala 1995), and similar to those on estuarine sediments (Sinsabaugh and Findlay 1995). However, enzymatic activities measured in the epilithon are similar to those reported in other streams (Sinsabaugh et al. 1991a, Jones and Lock 1993, Scholz and Boon 1993).

β-glucosidase and β-xylosidase activities per cell were calculated under the assumption that most of the bacterial cells were active. Although being aware that this is not a realistic approach since a proportion of bacteria might be inactive, this provides a "normalization" of the data and therefore a better comparison of the capacity and efficiency of the microorganisms to degrade polysaccharides in each biofilm can be done. β-glucosidase and β-xylosidase activities per cell were much higher in sandy biofilms, especially in the stream-edge zone, than in the tiles (Fig. 9). The lower ectoenzymatic activities per cell observed in the epilithon may be related to the release of easily assimilable organic compounds by the epilithic algae, which in turn may repress ectoenzymatic activities (Chróst and Overbeck 1990). This regulatory effect of autotrophy on the heterotrophic activity of the epilithon is expressed by the negative relationship between enzymatic activities and chlorophyll-a content (Table 6). On the other

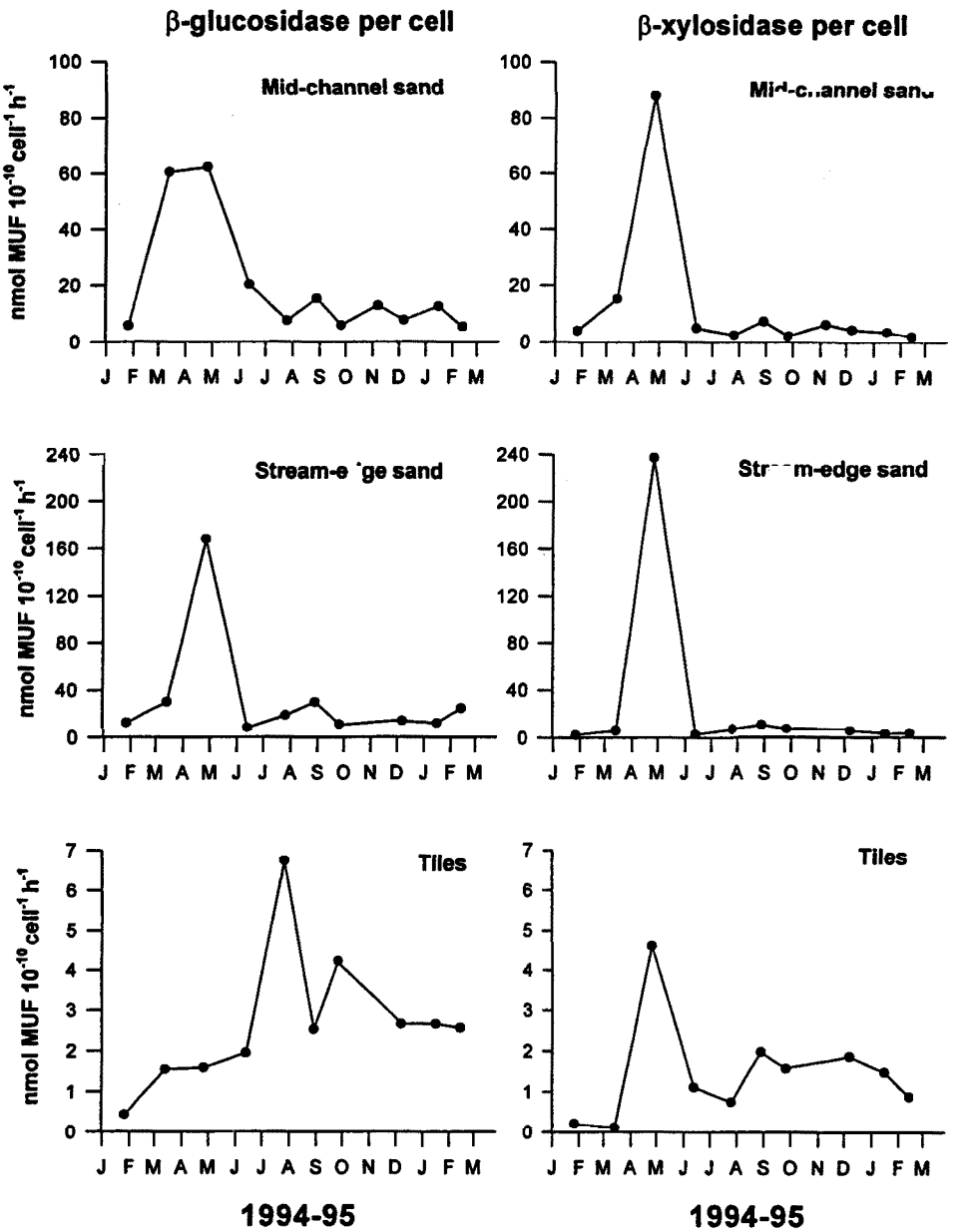

Fig. 9. Temporal variation of β-glucosidase and β-xylosidase activities per cell in mid-channel sand, stream-edge sand and tiles in Riera Major stream, January 1994-February 1995.

hand, regulation of these enzymes in the epipsammon is related to the accumulation of organic matter. The one-month delay of β-glucosidase activity with respect to chlorophyll-a (significant correlation between β-glucosidase and "past chlorophyll", Table 6) may indicate that initial accumulation and degradation processes happen before this enzyme acts (Atlas and Bartha 1987) and bacteria are able to utilize cell lysis products (Haack and McFeters 1982a). Cell lysis

would increase release of polysaccharides and induce β-glucosidase activity (Chróst et al. 1989). Thus, the high β-glucosidase activity in August 1994 might respond to a peak of chlorophyll-a in July 1994 (Fig. 2 and Fig. 3). A similar delay in β-glucosidase activity with respect to maximum chlorophyll-a has been observed in eutrophic lakes after spring phytoplankton blooms (Chróst and Overbeck 1990, Middelboe et al. 1995). Ectoenzyme regulation by organic matter accumulation in the epipsammon is further suggested by the positive correlation of β-glucosidase and β-xylosidase activities with the organic matter content (Table 6).

Higher phosphatase activity was measured in tiles compared to sand (Table 7) possibly because of the higher algal biomass and therefore a higher contribution of algal phosphatase than in sand (Cotner and Wetzel 1992, Sinsabaugh et al. 1992). However, the lower phosphatase activity in the epipsammon is related to the phosphorus sorption capacity and organic matter accumulation in sand (Klotz 1985, Sinsabaugh et al. 1993) which increase phosphorus availability. Therefore, the significantly lower phosphatase activity in the stream-edge sand compared to the mid-channel sand may be related to higher organic matter accumulation and lower grain size which enhances the phosphorus sorption capacity and phosphorus availability of biofilms (Meyer 1979, Ellis and Stanford 1988).

The comparison of the capacity of the biofilm to degrade polymeric organic matter between the three stream habitats showed that the epipsammic biofilm is much more efficient in the utilization of polyssacharidic compounds than the epilithic biofilm and the mid-channel epipsammic biofilm has a higher capacity to degrade phosphomonoesteres than the stream-edge epipsammic biofilm.

Going further to the organic matter degradation capacity of the different stream benthic substrates, an approach to the epilithic, the mid-channel sandy and the stream-edge sandy heterotrophic activities per streambed surface area has been made (Table 8). Sand measurements were expressed per unit of streambed surface area by integrating the sand of 2 cm depth cores (19.6 cm^2 streambed area). For the epilithon, the cm^2 of the tiles were considered cm^2 of rocky streambed surface area. Although a wider study on the microbial activities in a sand depth profile, as well as the consideration of other components (such as the underside of the rocks) would still be necessary, by these results a first approach on the importance of each substrate at the surficial streambed zone (i.e. the area in direct contact to the stream channel flowing water) can be obtained.

When expressed per unit of streambed surface area, stream-edge sand showed higher enzymatic activities than mid-channel sand (Table 8). Even though higher enzymatic activities in the stream-edge sand are related to their smaller grain size (Marxsen and Witzel 1990), this implies that a high enzymatic cleavage of macromolecules can occur in the stream-edge zone.

TABLE 8. Activities and biomass in stream-edge sand, mid-channel sand and tiles expressed per unit of streambed surface area (cm^2).

	SANDY STREAMBED				ROCKY STREAMBED	
	stream-edge zone		mid-channel zone		epilithon (tiles)	
	Mean (n=10)	SD	Mean (n=11)	SD	Mean (n=10)	SD
β-glucosidase (nmol cm^{-2} h^{-1})	121.52	60.66	59.75	31.84	5.68	3.55
β-xylosidase (nmol cm^{-2} h^{-1})	57.89	54.78	26.99	16.07	2.39	1.31
Phosphatase (nmol cm^{-2} h^{-1})	118.31	70.73	70.49	53.73	39.11	32.35
ETS (μg cm^{-2} h^{-1})	4.57	3.35	3.10	2.47	1.55	1.70
Chlorophyll-a (μg cm^{-2})	16.30	12.64	4.71	4.01	4.06	2.16
Bacteria (cell 10^{10} cm^{-2})	6.96	4.82	4.97	3.50	2.41	0.61

Differences were even greater when activities in sand were compared to the rocky streambed in terms of streambed surface area (Table 8). In particular, β-glucosidase and β-xylosidase activities were up to ten times higher in the sandy streambed (Table 8). The organic matter accumulated on sand substrates may enhance the heterotrophic processes in this habitat (Hudson et al. 1992). These results emphasize that in a low-order, forest stream the sand habitat is functionally important, since it is potentially able to degrade a higher volume of macromolecules (especially polysaccharides) than the rocky streambed.